ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2014 July 21; 20(27): 8751-9216

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

A peer-reviewed, online, open-access journal of gastroenterology and hepatology

Editorial Board

2014-2017

The *World Journal of Gastroenterology* Editorial Board consists of 1353 members, representing a team of worldwide experts in gastroenterology and hepatology. They are from 68 countries, including Albania (1), Algeria (1), Argentina (7), Australia (31), Austria (9), Belgium (10), Brazil (20), Brunei Darussalam (1), Bulgaria (2), Cambodia (1), Canada (25), Chile (4), China (161), Croatia (1), Cuba (1), Czech (6), Denmark (2), Egypt (9), Estonia (2), Finland (6), France (17), Germany (56), Greece (31), Guatemala (1), Hungary (14), Iceland (1), India (33), Indonesia (2), Iran (10), Ireland (9), Israel (18), Italy (195), Japan (151), Jordan (1), Kuwait (1), Lebanon (7), Lithuania (1), Malaysia (1), Mexico (10), Morocco (1), Netherlands (5), New Zealand (4), Nigeria (3), Norway (6), Pakistan (6), Poland (12), Portugal (8), Puerto Rico (1), Qatar (1), Romania (10), Russia (3), Saudi Arabia (2), Singapore (7), Slovenia (2), South Korea (64), Spain (51), Sri Lanka (1), Sudan (1), Sweden (12), Switzerland (5), Thailand (7), Trinidad and Tobago (1), Tunisia (2), Turkey (56), United Kingdom (47), United States (173), Venezuela (1), and Vietnam (1).

Austria

Felix Aigner, *Innsbruck*
Gabriela A Berlakovich, *Vienna*
Herwig R Cerwenka, *Graz*
Peter Ferenci, *Wien*
Alfred Gangl, *Vienna*
Kurt Lenz, *Linz*
Markus Peck-Radosavljevic, *Vienna*
Markus Raderer, *Vienna*
Stefan Riss, *Vienna*

Belgium

Michael George Adler, *Brussels*
Benedicte Y De Winter, *Antwerp*
Mark De Ridder, *Jette*
Olivier Detry, *Liege*
Denis Dufrane Dufrane, *Brussels*
Nikos Kotzampassakis, *Liège*
Geert KMM Robaeys, *Genk*
Xavier Sagaert, *Leuven*
Peter Starkel, *Brussels*
Eddie Wisse, *Keerbergen*

Brazil

SMP Balzan, *Santa Cruz do Sul*
JLF Caboclo, *Sao jose do rio preto*
Fábio Guilherme Campos, *Sao Paulo*
Claudia RL Cardoso, *Rio de Janeiro*
Roberto J Carvalho-Filho, *Sao Paulo*
Carla Daltro, *Salvador*
José Sebastiao dos Santos, *Ribeirao Preto*
Eduardo LR Mello, *Rio de Janeiro*
Sthela Maria Murad-Regadas, *Fortaleza*
Claudia PMS Oliveira, *Sao Paulo*
Júlio C Pereira-Lima, *Porto Alegre*
Marcos V Perini, *Sao Paulo*
Vietla Satyanarayana Rao, *Fortaleza*
Raquel Rocha, *Salvador*
AC Simoes e Silva, *Belo Horizonte*
Mauricio F Silva, *Porto Alefre*
Aytan Miranda Sipahi, *Sao Paulo*
Rosa Leonôra Salerno Soares, *Niterói*
Cristiane Valle Tovo, *Porto Alegre*
Eduardo Garcia Vilela, *Belo Horizonte*

Brunei Darussalam

Vui Heng Chong, *Bandar Seri Begawan*

Bulgaria

Tanya Kirilova Kadiyska, *Sofia*
Mihaela Petrova, *Sofia*

Cambodia

Francois Rouet, *Phnom Penh*

Canada

Brian Bressler, *Vancouver*
Frank J Burczynski, *Winnipeg*
Wangxue Chen, *Ottawa*
Francesco Crea, *Vancouver*
Mirko Diksic, *Montreal*
Jane A Foster, *Hamilton*
Hugh J Freeman, *Vancouver*
Shahrokh M Ghobadloo, *Ottawa*
Yuewen Gong, *Winnipeg*
Philip H Gordon, *Quebec*
Rakesh Kumar, *Edmonton*
Wolfgang A Kunze, *Hamilton*
Patrick Labonte, *Laval*
Zhikang Peng, *Winnipeg*
Jayadev Raju, *Ottawa*
Maitreyi Raman, *Calgary*
Giada Sebastiani, *Montreal*
Maida J Sewitch, *Montreal*
Eldon A Shaffer, *Alberta*
Christopher W Teshima, *Edmonton*
Jean Sévigny, *Québec*
Pingchang Yang, *Hamilton*
Pingchang Yang, *Hamilton*
Eric M Yoshida, *Vancouver*
Bin Zheng, *Edmonton*

Chile

Marcelo A Beltran, *La Serena*
Flavio Nervi, *Santiago*
Adolfo Parra-Blanco, *Santiago*
Alejandro Soza, *Santiago*

China

Zhao-Xiang Bian, *Hong Kong*
San-Jun Cai, *Shanghai*
Guang-Wen Cao, *Shanghai*
Long Chen, *Nanjing*
Ru-Fu Chen, *Guangzhou*
George G Chen, *Hong Kong*
Li-Bo Chen, *Wuhan*
Jia-Xu Chen, *Beijing*
Hong-Song Chen, *Beijing*
Lin Chen, *Beijing*
Yang-Chao Chen, *Hong Kong*
Zhen Chen, *Shanghai*
Ying-Sheng Cheng, *Shanghai*
Kent-Man Chu, *Hong Kong*
Zhi-Jun Dai, *Xi'an*
Jing-Yu Deng, *Tianjin*
Yi-Qi Du, *Shanghai*
Zhi Du, *Tianjin*
Hani El-Nezami, *Hong Kong*
Bao-Ying Fei, *Hangzhou*
Chang-Ming Gao, *Nanjing*
Jian-Ping Gong, *Chongqing*
Zuo-Jiong Gong, *Wuhan*
Jing-Shan Gong, *Shenzhen*
Guo-Li Gu, *Beijing*
Yong-Song Guan, *Chengdu*
Mao-Lin Guo, *Luoyang*
Jun-Ming Guo, *Ningbo*
Yan-Mei Guo, *Shanghai*
Xiao-Zhong Guo, *Shenyang*
Guo-Hong Han, *Xi'an*
Ming-Liang He, *Hong Kong*
Peng Hou, *Xi'an*
Zhao-Hui Huang, *Wuxi*
Feng Ji, *Hangzhou*
Simon Law, *Hong Kong*
Yu-Yuan Li, *Guangzhou*
Meng-Sen Li, *Haikou*
Shu-De Li, *Shanghai*
Zong-Fang Li, *Xi'an*
Qing-Quan Li, *Shanghai*
Kang Li, *Lasa*
Han Liang, *Tianjin*
Xing'e Liu, *Hangzhou*
Zheng-Wen Liu, *Xi'an*
Xiao-Fang Liu, *Yantai*
Bin Liu, *Tianjin*
Quan-Da Liu, *Beijing*
Hai-Feng Liu, *Beijing*
Fei Liu, *Shanghai*
Ai-Guo Lu, *Shanghai*
He-Sheng Luo, *Wuhan*
Xiao-Peng Ma, *Shanghai*
Yong Meng, *Shantou*
Ke-Jun Nan, *Xi'an*
Siew Chien Ng, *Hong Kong*
Simon SM Ng, *Hong Kong*
Zhao-Shan Niu, *Qingdao*
Bo-Rong Pan, *Xi'an*
Di Qu, *Shanghai*
Rui-Hua Shi, *Nanjing*
Bao-Min Shi, *Shanghai*
Xiao-Dong Sun, *Hangzhou*
Si-Yu Sun, *Shenyang*
Guang-Hong Tan, *Haikou*
Wen-Fu Tang, *Chengdu*
Anthony YB Teoh, *Hong Kong*
Wei-Dong Tong, *Chongqing*
Eric Tse, *Hong Kong*
Hong Tu, *Shanghai*
Rong Tu, *Haikou*
Jian-She Wang, *Shanghai*
Kai Wang, *Jinan*
Xiao-Ping Wang, *Xianyang*
Dao-Rong Wang, *Yangzhou*
De-Sheng Wang, *Xi'an*
Chun-You Wang, *Wuhan*
Ge Wang, *Chongqing*
Xi-Shan Wang, *Harbin*
Wei-hong Wang, *Beijing*
Zhen-Ning Wang, *Shenyang*
Wai Man Raymond Wong, *Hong Kong*
Chun-Ming Wong, *Hong Kong*
Jian Wu, *Shanghai*
Sheng-Li Wu, *Xi'an*
Wu-Jun Wu, *Xi'an*
Bing Xia, *Wuhan*
Qing Xia, *Chengdu*
Yan Xin, *Shenyang*
Dong-Ping Xu, *Beijing*
Jian-Min Xu, *Shanghai*
Wei Xu, *Changchun*
Ming Yan, *Jinan*
Xin-Min Yan, *Kunming*
Yi-Qun Yan, *Shanghai*
Feng Yang, *Shanghai*
Yong-Ping Yang, *Beijing*
He-Rui Yao, *Guangzhou*
Thomas Yau, *Hong Kong*
Winnie Yeo, *Hong Kong*
Jing You, *Kunming*
Jian-Qing Yu, *Wuhan*
Ying-Yan Yu, *Shanghai*
Wei-Zheng Zeng, *Chengdu*
Zong-Ming Zhang, *Beijing*

Dian-Liang Zhang, *Qingdao*
Ya-Ping Zhang, *Shijiazhuang*
You-Cheng Zhang, *Lanzhou*
Jian-Zhong Zhang, *Beijing*
Ji-Yuan Zhang, *Beijing*
Hai-Tao Zhao, *Beijing*
Jian Zhao, *Shanghai*
Jian-Hong Zhong, *Nanning*
Ying-Qiang Zhong, *Guangzhou*
Ping-Hong Zhou, *Shanghai*
Yan-Ming Zhou, *Xiamen*
Tong Zhou, *Nanchong*
Li-Ming Zhou, *Chengdu*
Guo-Xiong Zhou, *Nantong*
Feng-Shang Zhu, *Shanghai*
Jiang-Fan Zhu, *Shanghai*
Zhao-Hui Zhu, *Beijing*

Croatia

Tajana Filipec Kanizaj, *Zagreb*

Cuba

Damian Casadesus, *Havana*

Czech

Jan Bures, *Hradec Kralove*
Marcela Kopacova, *Hradec Kralove*
Otto Kucera, *Hradec Kralove*
Marek Minarik, *Prague*
Pavel Soucek, *Prague*
Miroslav Zavoral, *Prague*

Denmark

Vibeke Andersen, *Odense*
E Michael Danielsen, *Copenhagen*

Egypt

Mohamed MM Abdel-Latif, *Assiut*
Hussein Atta, *Cairo*
Ashraf Elbahrawy, *Cairo*
Mortada Hassan El-Shabrawi, *Cairo*
Mona El Said El-Raziky, *Cairo*
Elrashdy M Redwan, *New Borg Alrab*
Zeinab Nabil Ahmed Said, *Cairo*
Ragaa HM Salama, *Assiut*
Maha Maher Shehata, *Mansoura*

Estonia

Margus Lember, *Tartu*
Tamara Vorobjova, *Tartu*

Finland

Marko Kalliomäki, *Turku*
Thomas Kietzmann, *Oulu*
Kaija-Leena Kolho, *Helsinki*
Eija Korkeila, *Turku*
Heikki Makisalo, *Helsinki*
Tanja Pessi, *Tampere*

France

Armando Abergel Clermont, *Ferrand*
Elie K Chouillard, *Polssy*
Pierre Cordelier, *Toulouse*
Pascal P Crenn, *Garches*
Catherine Daniel, *Lille*
Fanny Daniel, *Paris*
Cedric Dray, *Toulouse*
Benoit Foligne, *Lille*
Jean-Noel Freund, *Strasbourg*
Nathalie Janel, *Paris*
Majid Khatib, *Bordeaux*
Jacques Marescaux, *Strasbourg*
Jean-Claude Marie, *Paris*
Hang Nguyen, *Clermont-Ferrand*
Hugo Perazzo, *Paris*
Alain L Servin, *Chatenay-Malabry*
Chang Xian Zhang, *Lyon*

Germany

Stavros A Antoniou, *Monchengladbach*
Erwin Biecker, *Siegburg*
Hubert E Blum, *Freiburg*
Thomas Bock, *Berlin*
Katja Breitkopf-Heinlein, *Mannheim*
Elke Cario, *Essen*
Güralp Onur Ceyhan, *Munich*
Angel Cid-Arregui, *Heidelberg*
Michael Clemens Roggendorf, *München*
Christoph F Dietrich, *Bad Mergentheim*
Valentin Fuhrmann, *Hamburg*
Nikolaus Gassler, *Aachen*
Andreas Geier, *Wuerzburg*
Markus Gerhard, *Munich*
Anton Gillessen, *Muenster*
Thorsten Oliver Goetze, *Offenbach*
Daniel Nils Gotthardt, *Heidelberg*
Robert Grützmann, *Dresden*
Thilo Hackert, *Heidelberg*
Joerg Haier, *Muenster*
Claus Hellerbrand, *Regensburg*
Harald Peter Hoensch, *Darmstadt*
Jens Hoeppner, *Freiburg*
Richard Hummel, *Muenster*
Jakob Robert Izbicki, *Hamburg*
Gernot Maximilian Kaiser, *Essen*
Matthias Kapischke, *Hamburg*
Michael Keese, *Frankfurt*
Andrej Khandoga, *Munich*
Jorg Kleeff, *Munich*
Alfred Koenigsrainer, *Tuebingen*
Peter Christopher Konturek, *Saalfeld*
Michael Linnebacher, *Rostock*
Stefan Maier, *Kaufbeuren*
Oliver Mann, *Hamburg*
Marc E Martignoni, *Munic*
Thomas Minor, *Bonn*
Oliver Moeschler, *Osnabrueck*
Jonas Mudter, *Eutin*
Sebastian Mueller, *Heidelberg*
Matthias Ocker, *Berlin*
Andreas Ommer, *Essen*
Albrecht Piiper, *Frankfurt*
Esther Raskopf, *Bonn*
Christoph Reichel, *Bad Brückenau*
Elke Roeb, *Giessen*
Udo Rolle, *Frankfurt*
Karl-Herbert Schafer, *Zweibrücken*
Andreas G Schreyer, *Regensburg*
Manuel A Silva, *Penzberg*
Georgios C Sotiropoulos, *Essen*
Ulrike S Stein, *Berlin*
Dirk Uhlmann, *Leipzig*
Michael Weiss, *Halle*
Hong-Lei Weng, *Mannheim*
Karsten Wursthorn, *Hamburg*

Greece

Alexandra Alexopoulou, *Athens*
Nikolaos Antonakopoulos, *Athens*
Stelios F Assimakopoulos, *Patras*
Grigoris Chatzimavroudis, *Thessaloniki*
Evangelos Cholongitas, *Thessaloniki*
Gregory Christodoulidis, *Larisa*
George N Dalekos, *Larissa*
Maria Gazouli, *Athens*
Urania Georgopoulou, *Athens*
Eleni Gigi, *Thessaloniki*
Stavros Gourgiotis, *Athens*
Leontios J Hadjileontiadis, *Thessaloniki*
Thomas Hyphantis, *Ioannina*
Ioannis Kanellos, *Thessaloniki*
Stylianos Karatapanis, *Rhodes*
Michael Koutsilieris, *Athens*
Spiros D Ladas, *Athens*
Theodoros K Liakakos, *Athens*
Emanuel K Manesis, *Athens*
Spilios Manolakopoulos, *Athens*
Gerassimos John Mantzaris, *Athens*
Athanasios D Marinis, *Piraeus*
Nikolaos Ioannis Nikiteas, *Athens*
Konstantinos X Papamichael, *Athens*
George Sgourakis, *Athens*
Konstantinos C Thomopoulos, *Patras*
Konstantinos Triantafyllou, *Athens*
Christos Triantos, *Patras*
Georgios Zacharakis, *Athens*
Petros Zezos, *Alexandroupolis*
Demosthenes E Ziogas, *Ioannina*

Guatemala

Carlos Maria Parellada, *Guatemala*

Hungary

Mihaly Boros, *Szeged*
Tamás Decsi, *Pécs*
Gyula Farkas, *Szeged*
Andrea Furka, *Debrecen*
Y vette Mandi, *Szeged*
Peter L Lakatos, *Budapest*
Pal Miheller, *Budapest*
Tamás Molnar, *Szeged*
Attila Olah, *Gyor*
Maria Papp, *Debrecen*
Zoltan Rakonczay, *Szeged*

Ferenc Sipos, *Budapest*
Miklós Tanyi, *Debrecen*
Tibor Wittmann, *Szeged*

Iceland

Tryggvi Bjorn Stefánsson, *Reykjavík*

India

Brij B Agarwal, *New Delhi*
Deepak N Amarapurkar, *Mumbai*
Shams ul Bari, *Srinagar*
Sriparna Basu, *Varanasi*
Runu Chakravarty, *Kolkata*
Devendra C Desai, *Mumbai*
Nutan D Desai, *Mumbai*
Suneela Sunil Dhaneshwar, *Pune*
Radha K Dhiman, *Chandigarh*
Pankaj Garg, *Mohali*
Uday C Ghoshal, *Lucknow*
Kalpesh Jani, *Vadodara*
Premashis Kar, *New Delhi*
Jyotdeep Kaur, *Chandigarh*
Rakesh Kochhar, *Chandigarh*
Pradyumna K Mishra, *Mumbai*
Asish K Mukhopadhyay, *Kolkata*
Imtiyaz Murtaza, *Srinagar*
P Nagarajan, *New Delhi*
Samiran Nundy, *Delhi*
Gopal Pande, *Hyderabad*
Benjamin Perakath, *Vellore*
Arun Prasad, *New Delhi*
D Nageshwar Reddy, *Hyderabad*
Lekha Saha, *Chandigarh*
Sundeep Singh Saluja, *New Delhi*
Mahesh Prakash Sharma, *New Delhi*
Sadiq Saleem Sikora, *Bangalore*
Sarman Singh, *New Delhi*
Rajeev Sinha, *Jhansi*
Rupjyoti Talukdar, *Hyderabad*
Rakesh Kumar Tandon, *New Delhi*
Narayanan Thirumoorthy, *Coimbatore*

Indonesia

David Handojo Muljono, *Jakarta*
Andi Utama, *Jakarta*

Iran

Arezoo Aghakhani, *Tehran*
Seyed Mohsen Dehghani, *Shiraz*
Ahad Eshraghian, *Shiraz*
Hossein Khedmat, *Tehran*
Sadegh Massarrat, *Tehran*
Marjan Mohammadi, *Tehran*
Roja Rahimi, *Tehran*
Farzaneh Sabahi, *Tehran*
Majid Sadeghizadeh, *Tehran*
Farideh Siavoshi, *Tehran*

Ireland

Gary Alan Bass, *Dublin*
David J Brayden, *Dublin*
Ronan A Cahill, *Dublin*
Glen A Doherty, *Dublin*
Liam J Fanning, *Cork*
Barry Philip McMahon, *Dublin*
RossMcManus, *Dublin*
Dervla O'Malley, *Cork*
Sinead M Smith, *Dublin*

Israel

Dan Carter, *Ramat Gan*
Jorge-Shmuel Delgado, *Metar*
Eli Magen, *Ashdod*
Nitsan Maharshak, *Tel Aviv*
Shaul Mordechai, *Beer Sheva*
Menachem Moshkowitz, *Tel Aviv*
William Bahij Nseir, *Nazareth*
Shimon Reif, *Jerusalem*
Ram Reifen, *Rehovot*
Ariella Bar-Gil Shitrit, *Jerusalem*
Noam Shussman, *Jerusalem*
Igor Sukhotnik, *Haifa*
Nir Wasserberg, *Petach Tiqwa*
Jacob Yahav, *Rehovot*
Doron Levi Zamir, *Gedera*
Shira Zelber-Sagi, *Haifa*
Romy Zemel, *Petach-Tikva*

Italy

Ludovico Abenavoli, *Catanzaro*
Luigi Elio Adinolfi, *Naples*
Carlo Virginio Agostoni, *Milan*
Anna Alisi, *Rome*
Piero Luigi Almasio, *Palermo*
Donato Francesco Altomare, *Bari*
Amedeo Amedei, *Florence*
Pietro Andreone, *Bologna*
Imerio Angriman, *Padova*
Vito Annese, *Florence*
Paolo Aurello, *Rome*
Salavtore Auricchio, *Naples*
Gian Luca Baiocchi, *Brescia*
Gianpaolo Balzano, *Milan*
Antonio Basoli, *Rome*
Gabrio Bassotti, *San Sisto*
Mauro Bernardi, *Bologna*
Alberto Biondi, *Rome*
Ennio Biscaldi, *Genova*
Massimo Bolognesi, *Padua*
Luigi Bonavina, *Milano*
Aldo Bove, *Chieti*
Raffaele Bruno, *Pavia*
Luigi Brusciano, *Napoli*
Giuseppe Cabibbo, *Palermo*
Carlo Calabrese, *Bologna*
Daniele Calistri, *Meldola*
Vincenza Calvaruso, *Palermo*
Lorenzo Camellini, *Reggio Emilia*
Marco Candela, *Bologna*
Raffaele Capasso, *Naples*
Lucia Carulli, *Modena*
Renato David Caviglia, *Rome*
Luigina Cellini, *Chieti*
Giuseppe Chiarioni, *Verona*
Claudio Chiesa, *Rome*
Michele Cicala, *Roma*
Rachele Ciccocioppo, *Pavia*
Sandro Contini, *Parma*
Gaetano Corso, *Foggia*
Renato Costi, *Parma*
Alessandro Cucchetti, *Bologna*
Rosario Cuomo, *Napoli*
Giuseppe Currò, *Messina*
Paola De Nardi, *Milano*
Giovanni D De Palma, *Naples*
Raffaele De Palma, *Napoli*
Giuseppina De Petro, *Brescia*
Valli De Re, *Aviano*
Paolo De Simone, *Pisa*
Giuliana Decorti, *Trieste*
Emanuele Miraglia del Giudice, *Napoli*
Isidoro Di Carlo, *Catania*
Matteo Nicola Dario Di Minno, *Naples*
Massimo Donadelli, *Verona*
Mirko D'Onofrio, *Verona*
Maria Pina Dore, *Sassari*
Luca Elli, *Milano*
Massimiliano Fabozzi, *Aosta*
Massimo Falconi, *Ancona*
Ezio Falletto, *Turin*
Silvia Fargion, *Milan*
Matteo Fassan, *Verona*
Gianfranco Delle Fave, *Roma*
Alessandro Federico, *Naples*
Francesco Feo, *Sassari*
Davide Festi, *Bologna*
Natale Figura, *Siena*
Vincenzo Formica, *Rome*
Mirella Fraquelli, *Milan*
Marzio Frazzoni, *Modena*
Walter Fries, *Messina*
Gennaro Galizia, *Naples*
Andrea Galli, *Florence*
Matteo Garcovich, *Rome*
Eugenio Gaudio, *Rome*
Paola Ghiorzo, *Genoa*
Edoardo G Giannini, *Genova*
Luca Gianotti, *Monza*
Maria Cecilia Giron, *Padova*
Alberto Grassi, *Rimini*
Gabriele Grassi, *Trieste*
Francesco Greco, *Bergamo*
Luigi Greco, *Naples*
Antonio Grieco, *Rome*
Fabio Grizzi, *Rozzano*
Laurino Grossi, *Pescara*
Salvatore Gruttadauria, *Palermo*
Simone Guglielmetti, *Milan*
Tiberiu Hershcovici, *Jerusalem*
Calogero Iacono, *Verona*
Enzo Ierardi, *Bari*
Amedeo Indriolo, *Bergamo*
Raffaele Iorio, *Naples*
Paola Iovino, *Salerno*
Angelo A Izzo, *Naples*
Loreta Kondili, *Rome*
Filippo La Torre, *Rome*
Giuseppe La Torre, *Rome*
Giovanni Latella, *L'Aquila*
Salvatore Leonardi, *Catania*
Massimo Libra, *Catania*
Anna Licata, *Palermo*
C armela Loguercio, *Naples*
Amedeo Lonardo, *Modena*
Carmelo Luigiano, *Catania*
Francesco Luzza, *Catanzaro*
Giovanni Maconi, *Milano*
Antonio Macrì, *Messina*
Mariano Malaguarnera, *Catania*

Francesco Manguso, *Napoli*
Tommaso Maria Manzia, *Rome*
Daniele Marrelli, *Siena*
Gabriele Masselli, *Rome*
Sara Massironi, *Milan*
Giuseppe Mazzarella, *Avellino*
Michele Milella, *Rome*
Giovanni Milito, *Rome*
Antonella d'Arminio Monforte, *Milan*
Fabrizio Montecucco, *Genoa*
Giovanni Monteleone, *Rome*
Mario Morino, *Torino*
Vincenzo La Mura, *Milan*
Gerardo Nardone, *Naples*
Riccardo Nascimbeni, *Brescia*
Gabriella Nesi, *Florence*
Giuseppe Nigri, *Rome*
Erica Novo, *Turin*
Veronica Ojetti, *Rome*
Michele Orditura, *Naples*
Fabio Pace, *Seriate*
Lucia Pacifico, *Rome*
Omero Alessandro Paoluzi, *Rome*
Valerio Pazienza, *San Giovanni Rotondo*
Rinaldo Pellicano, *Turin*
Adriano M Pellicelli, *Rome*
Nadia Peparini, *Ciampino*
Mario Pescatori, *Rome*
Antonio Picardi, *Rome*
Alberto Pilotto, *Padova*
Alberto Piperno, *Monza*
Anna Chiara Piscaglia, *Rome*
Maurizio Pompili, *Rome*
Francesca Romana Ponziani, *Rome*
Cosimo Prantera, *Rome*
Girolamo Ranieri, *Bari*
Carlo Ratto, *Tome*
Barbara Renga, *Perugia*
Alessandro Repici, *Rozzano*
Maria Elena Riccioni, *Rome*
Lucia Ricci-Vitiani, *Rome*
Luciana Rigoli, *Messina*
Mario Rizzetto, *Torino*
Ballarin Roberto, *Modena*
Roberto G Romanelli, *Florence*
Claudio Romano, *Messina*
Luca Roncucci, *Modena*
Cesare Ruffolo, *Treviso*
L ucia Sacchetti, *Napoli*
Rodolfo Sacco, *Pisa*
Lapo Sali, *Florence*
Romina Salpini, *Rome*
Giulio Aniello, *Santoro Treviso*
Armando Santoro, *Rozzano*
Edoardo Savarino, *Padua*
Marco Senzolo, *Padua*
Annalucia Serafino, *Rome*
Giuseppe S Sica, *Rome*
Pierpaolo Sileri, *Rome*
Cosimo Sperti, *Padua*
Vincenzo Stanghellini, *Bologna*
Cristina Stasi, *Florence*
Gabriele Stocco, *Trieste*
Roberto Tarquini, *Florence*
Mario Testini, *Bari*
Guido Torzilli, *Milan*
Guido Alberto Massimo, *Tiberio Brescia*
Giuseppe Toffoli, *Aviano*
Alberto Tommasini, *Trieste*
Francesco Tonelli, *Florence*
Cesare Tosetti Porretta, *Terme*
Lucio Trevisani, *Cona*
Guglielmo M Trovato, *Catania*
Mariapia Vairetti, *Pavia*
Luca Vittorio Valenti, *Milano*
Mariateresa T Ventura, *Bari*
Giuseppe Verlato, *Verona*
Alessandro Vitale, *Padova*
Marco Vivarelli, *Ancona*
Giovanni Li Volti, *Catania*
Giuseppe Zanotti, *Padua*
Vincenzo Zara, *Lecce*
Gianguglielmo Zehender, *Milan*
Anna Linda Zignego, *Florence*
Rocco Antonio Zoccali, *Messina*
Angelo Zullo, *Rome*

Japan

Yasushi Adachi, *Sapporo*
Takafumi Ando, *Nagoya*
Masahiro Arai, *Tokyo*
Makoto Arai, *Chiba*
Takaaki Arigami, *Kagoshima*
Itaru Endo,*Yokohama*
Munechika Enjoji, *Fukuoka*
Shunji Fujimori, *Tokyo*
Yasuhiro Fujino, *Akashi*
Toshiyoshi Fujiwara, *Okayama*
Yosuke Fukunaga, *Tokyo*
Toshio Fukusato, *Tokyo*
Takahisa Furuta, *Hamamatsu*
Osamu Handa, *Kyoto*
Naoki Hashimoto, *Osaka*
Yoichi Hiasa, *Toon*
Masatsugu Hiraki, *Saga*
Satoshi Hirano, *Sapporo*
Keiji Hirata, *Fukuoka*
Toru Hiyama, *Higashihiroshima*
Akira Hokama, *Nishihara*
Shu Hoteya, *Tokyo*
Masao Ichinose, *Wakayama*
Tatsuya Ide, *Kurume*
Masahiro Iizuka, *Akita*
Toshiro Iizuka, *Tokyo*
Kenichi Ikejima, *Tokyo*
Tetsuya Ikemoto, *Tokushima*
Hiroyuki Imaeda, *Saitama*
Atsushi Imagawa, *Kan-onji*
Hiroo Imazu, *Tokyo*
Akio Inui, *Kagoshima*
Shuji Isaji, *Tsu*
Toru Ishikawa, *Niigata*
Toshiyuki Ishiwata, *Tokyo*
Soichi Itaba, *Kitakyushu*
Yoshiaki Iwasaki, *Okayama*
Tatehiro Kagawa, *Isehara*
Satoru Kakizaki, *Maebashi*
Naomi Kakushima, *Shizuoka*
Terumi Kamisawa, *Tokyo*
Akihide Kamiya, *Isehara*
Osamu Kanauchi, *Tokyo*
Tatsuo Kanda, *Chiba*
Shin Kariya, *Okayama*
Shigeyuki Kawa, *Matsumoto*
Takumi Kawaguchi, *Kurume*
Takashi Kawai, *Tokyo*
Soo Ryang Kim, *Kobe*
Shinsuke Kiriyama, *Gunma*
Tsuneo Kitamura, *Urayasu*
Masayuki Kitano, *Osakasayama*
Hirotoshi Kobayashi, *Tokyo*
Hironori Koga, *Kurume*
Takashi Kojima, *Sapporo*
Satoshi Kokura, *Kyoto*
Shuhei Komatsu, *Kyoto*
Tadashi Kondo, *Tokyo*
Yasuteru Kondo, *Sendai*
Yasuhiro Kuramitsu, *Yamaguchi*
Yukinori Kurokawa, *Osaka*
Shin Maeda, *Yokohama*
Koutarou Maeda, *Toyoake*
Hitoshi Maruyama, *Chiba*
Atsushi Masamune, *Sendai*
Hiroyuki Matsubayashi, *Suntogun*
Akihisa Matsuda, *Inzai*
Hirofumi Matsui, *Tsukuba*
Akira Matsumori, *Kyoto*
Yoichi Matsuo, *Nagoya*
Y Matsuzaki, *Ami*
Toshihiro Mitaka, *Sapporo*
Kouichi Miura, *Akita*
Shinichi Miyagawa, *Matumoto*
Eiji Miyoshi, *Suita*
Toru Mizuguchi, *Sapporo*
Nobumasa Mizuno, *Nagoya*
Zenichi Morise, *Nagoya*
Tomohiko Moriyama, *Fukuoka*
Kunihiko Murase, *Tusima*
Michihiro Mutoh, *Tsukiji*
Akihito Nagahara, *Tokyo*
Hikaru Nagahara, *Tokyo*
Hidenari Nagai, *Tokyo*
Koichi Nagata, *Shimotsuke-shi*
Masaki Nagaya, *Kawasaki*
Hisato Nakajima, *Nishi-Shinbashi*
Toshifusa Nakajima, *Tokyo*
Hiroshi Nakano, *Kawasaki*
Hiroshi Nakase, *Kyoto*
Toshiyuki Nakayama, *Nagasaki*
Takahiro Nakazawa, *Nagoya*
Shoji Natsugoe, *Kagoshima City*
Tsutomu Nishida, *Suita*
Shuji Nomoto, *Naogya*
Sachiyo Nomura, *Tokyo*
Takeshi Ogura, *Takatsukishi*
Nobuhiro Ohkohchi, *Tsukuba*
Toshifumi Ohkusa, *Kashiwa*
Hirohide Ohnishi, *Akita*
Teruo Okano, *Tokyo*
Satoshi Osawa, *Hamamatsu*
Motoyuki Otsuka, *Tokyo*
Michitaka Ozaki, *Sapporo*
Satoru Saito, *Yokohama*
Chouhei Sakakura, *Kyoto*
Naoaki Sakata, *Sendai*
Ken Sato, *Maebashi*
Toshiro Sato, *Tokyo*
Tomoyuki Shibata, *Toyoake*
H Shimada, *Tokyo*
Tomohiko Shimatani, *Kure*
Yukihiro Shimizu, *Nanto*
Tadashi Shimoyama, *Hirosaki*
Masayuki Sho, *Nara*
Ikuo Shoji, *Kobe*
Atsushi Sofuni, *Tokyo*
Takeshi Suda, *Niigata*
M Sugimoto, *Hamamatsu*
Ken Sugimoto, *Hamamatsu*
Haruhiko Sugimura, *Hamamatsu*
Shoichiro Sumi, *Kyoto*
Hidekazu Suzuki, *Tokyo*
Masahiro Tajika, *Nagoya*
Hitoshi Takagi, *Takasaki*
Toru Takahashi, *Niigata*

Yoshihisa Takahashi, *Tokyo*
Shinsuke Takeno, *Fukuoka*
Akihiro Tamori, *Osaka*
Kyosuke Tanaka, *Tsu*
Shinji Tanaka, *Hiroshima*
Atsushi Tanaka, *Tokyo*
Yasuhito Tanaka, *Nagoya*
Shinji Tanaka, *Tokyo*
Minoru Tomizawa, *Yotsukaido City*
Kyoko Tsukiyama-Kohara, *Kagoshima*
Takuya Watanabe, *Niigata*
Kazuhiro Watanabe, *Sendai*
Satoshi Yamagiwa, *Niigata*
Takayuki Yamamoto, *Yokkaichi*
Hiroshi Yamamoto, *Otsu*
Kosho Yamanouchi, *Nagasaki*
Ichiro Yasuda, *Gifu*
Yutaka Yata, *Maebashi-city*
Shin-ichi Yokota, *Sapporo*
Norimasa Yoshida, *Kyoto*
Hiroshi Yoshida, *Tama-City*
Hitoshi Yoshiji, *Kashihara*
Kazuhiko Yoshimatsu, *Tokyo*
Kentaro Yoshioka, *Toyoake*
Nobuhiro Zaima, *Nara*

Jordan

Khaled Ali Jadallah, *Irbid*

Kuwait

Islam Khan, *Kuwait*

Lebanon

Bassam N Abboud, *Beirut*
Kassem A Barada, *Beirut*
Marwan Ghosn, *Beirut*
Iyad A Issa, *Beirut*
Fadi H Mourad, *Beirut*
AIa Sharara, *Beirut*
Rita Slim, *Beirut*

Lithuania

Antanas Mickevicius, *Kaunas*

Malaysia

Huck Joo Tan, *Petaling Jaya*

Mexico

Richard A Awad, *Mexico City*
Carlos R Camara-Lemarroy, *Monterrey*
Norberto C Chavez-Tapia, *Mexico City*
Wolfgang Gaertner, *Mexico City*
Diego Garcia-Compean, *Monterrey*
Arturo Panduro, *Guadalajara*
OT Teramoto-Matsubara, *Mexico City*
Felix Tellez-Avila, *Mexico City*
Omar Vergara-Fernandez, *Mexico City*
Saúl Villa-Trevino, *Cuidad de México*

Morocco

Samir Ahboucha, *Khouribga*

Netherlands

Robert J de Knegt, *Rotterdam*
Tom Johannes Gerardus Gevers, *Nijmegen*
Menno Hoekstra, *Leiden*
BW Marcel Spanier, *Arnhem*
Karel van Erpecum, *Utrecht*

New Zealand

Leo K Cheng, *Auckland*
Andrew Stewart Day, *Christchurch*
Jonathan Barnes Koea, *Auckland*
Max Petrov, *Auckland*

Nigeria

Olufunmilayo Adenike Lesi, *Lagos*
Jesse Abiodun Otegbayo, *Ibadan*
Stella Ifeanyi Smith, *Lagos*

Norway

Trond Berg, *Oslo*
Trond Arnulf Buanes, *Krokkleiva*
Thomas de Lange, *Rud*
Magdy El-Salhy, *Stord*
Rasmus Goll, *Tromso*
Dag Arne Lihaug Hoff, *Aalesund*

Pakistan

Zaigham Abbas, *Karachi*
Usman A Ashfaq, *Faisalabad*
Muhammad Adnan Bawany, *Hyderabad*
Muhammad Idrees, *Lahore*
Saeed Sadiq Hamid, *Karachi*
Yasir Waheed, *Islamabad*

Poland

Thomas Brzozowski, *Cracow*
Magdalena Chmiela, *Lodz*
Krzysztof Jonderko, *Sosnowiec*
Anna Kasicka-Jonderko, *Sosnowiec*
Michal Kukla, *Katowice*
Tomasz Hubert Mach, *Krakow*
Agata Mulak, *Wroclaw*
Danuta Owczarek, *Kraków*
Piotr Socha, *Warsaw*
Piotr Stalke, *Gdansk*
Julian Teodor Swierczynski, *Gdansk*
Anna M Zawilak-Pawlik, *Wroclaw*

Portugal

Marie Isabelle Cremers, *Setubal*
Ceu Figueiredo, *Porto*
Ana Isabel Lopes, *LIsbon*
M Paula Macedo, *Lisboa*
Ricardo Marcos, *Porto*
Rui T Marinho, *Lisboa*
Guida Portela-Gomes, *Estoril*
Filipa F Vale, *Lisbon*

Puerto Rico

Caroline B Appleyard, *Ponce*

Qatar

Abdulbari Bener, *Doha*

Romania

Mihai Ciocirlan, *Bucharest*
Dan LucianDumitrascu, *Cluj-Napoca*
Carmen Fierbinteanu-Braticevici, *Bucharest*
Romeo G Mihaila, *Sibiu*
Lucian Negreanu, *Bucharest*
Adrian Saftoiu, *Craiova*
Andrada Seicean, *Cluj-Napoca*
Ioan Sporea, *Timisoara*
Letiția Adela Maria Streba, *Craiova*
Anca Trifan, *Iasi*

Russia

Victor Pasechnikov, *Stavropol*
Vasiliy Ivanovich Reshetnyak, *Moscow*
Vitaly Skoropad, *Obninsk*

Saudi Arabia

Abdul-Wahed N Meshikhes, *Dammam*
M Ezzedien Rabie, *Khamis Mushait*

Singapore

Brian KP Goh, *Singapore*
Richie Soong, *Singapore*
Ker-Kan Tan, *Singapore*
Kok-Yang Tan, *Singapore*
Yee-Joo Tan, *Singapore*
Mark Wong, *Singapore*
Hong Ping Xia, *Singapore*

Slovenia

Matjaz Homan, *Ljubljana*
Martina Perse, *Ljubljana*

South Korea

Sang Hoon Ahn, *Seoul*
Soon Koo Baik, *Wonju*
Soo-Cheon Chae, *Iksan*
Byung-Ho Choe, *Daegu*

Suck Chei Choi, *Iksan*
Hoon Jai Chun, *Seoul*
Yeun-Jun Chung, *Seoul*
Young-Hwa Chung, *Seoul*
Ki-Baik Hahm, *Seongnam*
Sang Young Han, *Busan*
Seok Joo Han, *Seoul*
Seung-Heon Hong, *Iksan*
Jin-Hyeok Hwang, *Seoungnam*
Jeong Won Jang, *Seoul*
Jin-Young Jang, *Seoul*
Dae-Won Jun, *Seoul*
Young Do Jung, *Kwangju*
Gyeong Hoon Kang, *Seoul*
Sung-Bum Kang, *Seoul*
Koo Jeong Kang, *Daegu*
Ki Mun Kang, *Jinju*
Chang Moo Kang, *Seodaemun-gu*
Gwang Ha Kim, *Busan*
Sang Soo Kim, *Goyang-si*
Jin Cheon Kim, *Seoul*
Tae Il Kim, *Seoul*
Jin Hong Kim, *Suwon*
Kyung Mo Kim, *Seoul*
Kyongmin Kim, *Suwon*
Hyung-Ho Kim, *Seongnam*
Seoung Hoon Kim, *Goyang*
Sang Il Kim, *Seoul*
Hyun-Soo Kim, *Wonju*
Jung Mogg Kim, *Seoul*
Dong Yi Kim, *Gwangju*
Kyun-Hwan Kim, *Seoul*
Jong-Han Kim, *Ansan*
Ja-Lok Ku, *Seoul*
Kyu Taek Lee, *Seoul*
Hae-Wan Lee, *Chuncheon*
Inchul Lee, *Seoul*
Jung Eun Lee, *Seoul*
Sang Chul Lee, *Daejeon*
Song Woo Lee, *Ansan-si*
Hyuk-Joon Lee, *Seoul*
Seong-Wook Lee, *Yongin*
Kil Yeon Lee, *Seoul*
Jong-Inn Lee, *Seoul*
Kyung A Lee, *Seoul*
Jong-Baeck Lim, *Seoul*
Eun-Yi Moon, *Seoul*
SH Noh, *Seoul*
Seung Woon Paik, *Seoul*
Won Sang Park, *Seoul*
Sung-Joo Park, *Iksan*
Kyung Sik Park, *Daegu*
Se Hoon Park, *Seoul*
Yoonkyung Park, *Gwangju*
Seung-Wan Ryu, *Daegu*
Dong Wan Seo, *Seoul*
Il Han Song, *Cheonan*
Myeong Jun Song, *Daejeon*
Yun Kyoung Yim, *Daejeon*
Dae-Yeul Yu *Daejeon*

Spain

Mariam Aguas, *Valencia*
Raul J Andrade, *Málaga*
Antonio Arroyo, *Elche*
Josep M Bordas, *Barcelona*
Lisardo Boscá, *Madrid*
Ricardo Robles Campos, *Murcia*
Jordi Camps, *Reus*
Carlos Cervera *Barcelona*
Alfonso Clemente, *Granada*
Pilar Codoner-Franch, *Valencia*
Fernando J Corrales, *Pamplona*
Fermin Sánchez de Medina, *Granada*
Alberto Herreros de Tejada, *Majadahonda*
Enrique de-Madaria, *Alicante*
JE Dominguez-Munoz, *Santiago de Compostela*
Vicente Felipo, *Valencia*
CM Fernandez-Rodriguez, *Madrid*
Carmen Frontela-Saseta, *Murcia*
Julio Galvez, *Granada*
Maria Teresa García, *Vigo*
MI Garcia-Fernandez, *Málaga*
Emilio Gonzalez-Reimers, *La Laguna*
Marcel Jimenez, *Bellaterra*
Angel Lanas, *Zaragoza*
Juan Ramón Larrubia, *Guadalajara*
Antonio Lopez-Sanroman, *Madrid*
Vicente Lorenzo-Zuniga, *Badalona*
Alfredo J Lucendo, *Tomelloso*
Vicenta Soledad Martinez-Zorzano, *Vigo*
José Manuel Martin-Villa, *Madrid*
Julio Mayol, *Madrid*
Manuel Morales-Ruiz, *Barcelona*
Alfredo Moreno-Egea, *Murcia*
Albert Pares, *Barcelona*
Maria Pellise, *Barcelona*
José Perea, *Madrid*
Miguel Angel Plaza, *Zaragoza*
María J Pozo, *Cáceres*
Enrique Quintero, *La Laguna*
Jose M Ramia, *Madrid*
Francisco Rodriguez-Frias, *Barcelona*
Silvia Ruiz-Gaspa, *Barcelona*
Xavier Serra-Aracil, *Barcelona*
Vincent Soriano, *Madrid*
Javier Suarez, *Pamplona*
Carlos Taxonera, *Madrid*
M Isabel Torres, *Jaén*
Manuel Vazquez-Carrera, *Barcelona*
Benito Velayos, *Valladolid*
Silvia Vidal, *Barcelona*

Sri Lanka

Arjuna Priyadarsin De Silva, *Colombo*

Sudan

Ishag Adam, *Khartoum*

Sweden

Roland G Andersson, *Lund*
Bergthor Björnsson, *Linkoping*
Johan Christopher Bohr, *Örebro*
Mauro D'Amato, *Stockholm*
Thomas Franzen, *Norrkoping*
Evangelos Kalaitzakis, *Lund*
Riadh Sadik, *Gothenburg*
Per Anders Sandstrom, *Linkoping*
Ervin Toth, *Malmö*
Konstantinos Tsimogiannis, *Vasteras*
Apostolos V Tsolakis, *Uppsala*

Switzerland

Gieri Cathomas, *Liestal*
Jean Louis Frossard, *Geneve*
Christian Toso, *Geneva*
Stephan Robert Vavricka, *Zurich*
Dominique Velin, *Lausanne*

Thailand

Thawatchai Akaraviputh, *Bangkok*
P Yoysungnoen Chintana, *Pathumthani*
Veerapol Kukongviriyapan, *Muang*
Vijittra Leardkamolkarn, *Bangkok*
Varut Lohsiriwat, *Bangkok*
Somchai Pinlaor, *Khaon Kaen*
D Wattanasirichaigoon, *Bangkok*

Trinidad and Tobago

B Shivananda Nayak, *Mount Hope*

Tunisia

Ibtissem Ghedira, *Sousse*
Lilia Zouiten-Mekki, *Tunis*

Turkey

Sami Akbulut, *Diyarbakir*
Inci Alican, *Istanbul*
Mustafa Altindis, *Sakarya*
Mutay Aslan, *Antalya*
Oktar Asoglu, *Istanbul*
Yasemin Hatice Balaban, *Istanbul*
Metin Basaranoglu, *Ankara*
Yusuf Bayraktar, *Ankara*
Süleyman Bayram, *Adiyaman*
Ahmet Bilici, *Istanbul*
Ahmet Sedat Boyacioglu, *Ankara*
Züleyha Akkan Cetinkaya, *Kocaeli*
Cavit Col, *Bolu*
Yasar Colak, *Istanbul*
Cagatay Erden Daphan, *Kirikkale*
Mehmet Demir, *Hatay*
Ahmet Merih Dobrucali, *Istanbul*
Gülsüm Ozlem Elpek, *Antalya*
Ayse Basak Engin, *Ankara*
Eren Ersoy, *Ankara*
Osman Ersoy, *Ankara*
Yusuf Ziya Erzin, *Istanbul*
Mukaddes Esrefoglu, *Istanbul*
Levent Filik, *Ankara*
Ozgur Harmanci, *Ankara*
Koray Hekimoglu, *Ankara*
Abdurrahman Kadayifci, *Gaziantep*
Cem Kalayci, *Istanbul*
Selin Kapan, *Istanbul*
Huseyin Kayadibi, *Adana*
Sabahattin Kaymakoglu, *Istanbul*
Metin Kement, *Istanbul*
Mevlut Kurt, *Bolu*
Resat Ozaras, *Istanbul*

Elvan Ozbek, *Adapazari*
Cengiz Ozcan, *Mersin*
Hasan Ozen, *Ankara*
Halil Ozguc, *Bursa*
Mehmet Ozturk, *Izmir*
Orhan V Ozkan, *Sakarya*
Semra Paydas, *Adana*
Ozlem Durmaz Suoglu, *Istanbul*
Ilker Tasci, *Ankara*
Müge Tecder-ünal, *Ankara*
Mesut Tez, *Ankara*
Serdar Topaloglu, *Trabzon*
Murat Toruner, *Ankara*
Gokhan Tumgor, *Adana*
Oguz Uskudar, *Adana*
Mehmet Yalniz, *Elazig*
Mehmet Yaman, *Elazig*
Veli Yazisiz, *Antalya*
Yusuf Yilmaz, *Istanbul*
Ozlem Yilmaz, *Izmir*
Oya Yucel, *Istanbul*
Ilhami Yuksel, *Ankara*

United Kingdom

Nadeem Ahmad Afzal, *Southampton*
Navneet K Ahluwalia, *Stockport*
Yeng S Ang, *Lancashire*
Ramesh P Arasaradnam, *Coventry*
Ian Leonard Phillip Beales, *Norwich*
John Beynon, *Swansea*
Barbara Braden, *Oxford*
Simon Bramhall, *Birmingham*
Geoffrey Burnstock, *London*
Ian Chau, *Sutton*
Thean Soon Chew, *London*
Helen G Coleman, *Belfast*
Anil Dhawan, *London*
Sunil Dolwani, *Cardiff*
Piers Gatenby, *London*
Anil T George, *London*
Pasquale Giordano, *London*
Paul Henderson, *Edinburgh*
Georgina Louise Hold, *Aberdeen*
Stefan Hubscher, *Birmingham*
Robin D Hughes, *London*
Nusrat Husain, *Manchester*
Matt W Johnson, *Luton*
Konrad Koss, *Macclesfield*
Anastasios Koulaouzidis, *Edinburgh*
Simon Lal, *Salford*
John S Leeds, *Aberdeen*
Hongxiang Liu, *Cambridge*
Michael Joseph McGarvey, *London*
Michael Anthony Mendall, *London*
Alexander H Mirnezami, *Southampton*
J Bernadette Moore, *Guildford*
Claudio Nicoletti, *Norwich*
Savvas Papagrigoriadis, *London*
David Mark Pritchard, *Liverpool*
James A Ross, *Edinburgh*
Kamran Rostami, *Worcester*
Xiong Z Ruan, *London*
Dina Tiniakos, *Newcastle upon Tyne*
Frank I Tovey, *London*
Dhiraj Tripathi, *Birmingham*
Vamsi R Velchuru, *Great Yarmouth*
Nicholas T Ventham, *Edinburgh*
Diego Vergani, *London*
Jack Westwood Winter, *Glasgow*
Terence Wong, *London*
Ling Yang, *Oxford*

United States

Daniel E Abbott, *Cincinnati*
Ghassan K Abou-Alfa, *New York*
Julian Abrams, *New York*
David William Adelson, *Los Angeles*
Jonathan Steven Alexander, *Shreveport*
Tauseef Ali, *Oklahoma City*
Mohamed R Ali, *Sacramento*
Rajagopal N Aravalli, *Minneapolis*
Hassan Ashktorab, *Washington*
Shashi Bala, *Worcester*
Charles F Barish, *Raleigh*
P Patrick Basu, *New York*
Robert L Bell, *Berkeley Heights*
David Bentrem, *Chicago*
Henry J Binder, *New Haven*
Joshua Bleier, *Philadelphia*
Wojciech Blonski, *Johnson City*
Kenneth Boorom, *Corvallis*
Brian Boulay, *Chicago*
Carla W Brady, *Durham*
Kyle E Brown, *Iowa City*
Adeel AButt, *Pittsburgh*
Weibiao Cao, *Providence*
Andrea Castillo, *Cheney*
Fernando J Castro, *Weston*
Adam S Cheifetz, *Boston*
Adam S Cheifetz, *Boston*
Xiaoxin Luke Chen, *Durham*
Ramsey Cheung, *Palo Alto*
Parimal Chowdhury, *Little Rock*
Edward John Ciaccio, *New York*
Dahn L Clemens, *Omaha*
Yingzi Cong, *Galveston*
Laura Iris Cosen-Binker, *Boston*
Joseph John Cullen, *Lowa*
Mark J Czaja, *Bronx*
Mariana D Dabeva, *Bronx*
Christopher James Damman, *Seattle*
Isabelle G De Plaen, *Chicago*
Abhishek Deshpande, *Cleveland*
Punita Dhawan, *Nashville*
Hui Dong, *La Jolla*
Wael El-Rifai, *Nashville*
Sukru H Emre, *New Haven*
Paul Feuerstadt, *Hamden*
Josef E Fischer, *Boston*
Laurie N Fishman, *Boston*
Joseph Che Forbi, *Atlanta*
Temitope Foster, *Atlanta*
AmyEFoxx-Orenstein, *Scottsdale*
Daniel E Freedberg, *New York*
Shai Friedland, *Palo Alto*
Virgilio George, *Indianapolis*
Ajay Goel, *Dallas*
Oliver Grundmann, *Gainesville*
Stefano Guandalini, *Chicago*
Chakshu Gupta, *St. Joseph*
Grigoriy E Gurvits, *New York*
Xiaonan Han, *Cincinnati*
Mohamed Hassan, *Jackson*
Martin Hauer-Jensen, *Little Rock*
Koichi Hayano, *Boston*
Yingli Hee, *Atlanta*
Samuel B Ho, *San Diego*
Jason Ken Hou, *Houston*
Lifang Hou, *Chicago*
K-Qin Hu, *Orange*
Jamal A Ibdah, *Columbia*
Robert Thomas Jensen, *Bethesda*
Huanguang "Charlie" Jia, *Gainesville*
Rome Jutabha, *Los Angeles*
Andreas M Kaiser, *Los Angeles*
Avinash Kambadakone, *Boston*
David Edward Kaplan, *Philadelphia*
Randeep Kashyap, *Rochester*
Rashmi Kaul, *Tulsa*
Ali Keshavarzian, *Chicago*
Amir Maqbul Khan, *Marshall*
Nabeel Hasan Khan, *New Orleans*
Sahil Khanna, *Rochester*
Kusum K Kharbanda, *Omaha*
Hyun Sik Kim, *Pittsburgh*
Joseph Kim, *Duarte*
Jae S Kim, *Gainesville*
Miran Kim, *Providence*
Timothy R Koch, *Washington*
Burton I Korelitz, *New York*
Betsy Kren, *Minneapolis*
Shiu-Ming Kuo, *Buffalo*
Michelle Lai, *Boston*
Andreas Larentzakis, *Boston*
Edward Wolfgang Lee, *Los Angeles*
Daniel A Leffler, *Boston*
Michael Leitman, *New York*
Suthat Liangpunsakul, *Indianapolis*
Joseph K Lim, *New Haven*
Elaine Y Lin, *Bronx*
Henry C Lin, *Albuquerque*
Rohit Loomba, *La Jolla*
James David Luketich, *Pittsburgh*
Mohammad F Madhoun, *Oklahoma City*
Thomas C Mahl, *Buffalo*
Ashish Malhotra, *Bettendorf*
Pranoti Mandrekar, *Worcester*
John Marks, *Wynnewood*
Wendy M Mars, *Pittsburgh*
Julien Vahe Matricon, *San Antonio*
Craig J McClain, *Louisville*
George K Michalopoulos, *Pittsburgh*
Tamir Miloh, *Phoenix*
Ayse Leyla Mindikoglu, *Baltimore*
Huanbiao Mo, *Denton*
Klaus Monkemuller, *Birmingham*
John Morton, *Stanford*
Adnan Muhammad, *Tampa*
Michael J Nowicki, *Jackson*
Patrick I Okolo, *Baltimore*
Giusepp Orlando, *Winston Salem*
Natalia A Osna, *Omaha*
Virendra N Pandey, *Newark*
Mansour A Parsi, *Cleveland*
Michael F Picco, *Jacksonville*
Daniel S Pratt, *Boston*
Xiaofa Qin, *Newark*
Janardan K Reddy, *Chicago*
Victor E Reyes, *Galveston*
Jon Marc Rhoads, *Houston*
Giulia Roda, *New York*
Jean-Francois Armand Rossignol, *Tampa*
Paul A Rufo, *Boston*
Madhusudana Girija Sanal, *New York*
Miguel Saps, *Chicago*
Sushil Sarna, *Galveston*
Ann O Scheimann, *Baltimore*
Bernd Schnabl, *La Jolla*

World Journal of Gastroenterology

Contents Weekly Volume 20 Number 27 July 21, 2014

TOPIC HIGHLIGHT

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1353 experts in gastroenterology and hepatology from 68 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. ISI, Journal Citation Reports®, Gastroenterology and Hepatology, 2012 Impact Factor: 2.547 (34/74); Total Cites: 19145 (6/74); Current Articles: 944 (1/74); and Eigenfactor® Score: 0.06035 (6/74).

NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORIAL OFFICE
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: bpgoffice@wjgnet.com
Help desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
July 21, 2014

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8751

World J Gastroenterol 2014 July 21; 20(27): 8751-8763
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Escherichia coli-host macrophage interactions in the pathogenesis of inflammatory bowel disease

Ahmed Tawfik, Paul K Flanagan, Barry J Campbell

Ahmed Tawfik, Paul K Flanagan, Barry J Campbell, Department of Gastroenterology, Institute of Translational Medicine, University of Liverpool, Liverpool L69 3GE, United Kingdom
Author contributions: The review was proposed by Campbell BJ; Tawfik A, Flanagan PK and Campbell BJ drafted the initial manuscript; Campbell BJ reviewed and edited the draft; all authors approved the final manuscript before submission.
Supported by Award from the Ministry of Higher Education and Scientific Research/Cultural Attache - London Libyan Embassy, No. UM873-611-23962; National Institute for Health Research (NIHR) Biomedical Research Fellowship, No. BRF-2011-025; Shire Innovation Fund for Specialist Registrars; and funding from Crohn's amd Colitis United Kingdom, No. M-08-1/M-13-2; Liverpool NIHR-Biomedical Research Centre for Microbial Diseases (01CD1); and Support of the European Science Foundation, in the framework of the Research Networking Programme, The European Network for Gastrointestinal Health Research
Correspondence to: Dr. Barry J Campbell, Department of Gastroenterology, Institute of Translational Medicine, University of Liverpool, Nuffield Building, Crown Street, Liverpool, L69 3GE, United Kingdom. bjcampbl@liv.ac.uk
Telephone: +44-151-7946829 Fax: +44-151-7946825
Received: October 22, 2013 Revised: January 7, 2014
Accepted: April 1, 2014
Published online: July 21, 2014

Abstract

Multiple studies have demonstrated alterations in the intestinal microbial community (termed the microbiome) in Crohn's disease (CD) and several lines of evidence suggest these changes may have a significant role in disease pathogenesis. In active and quiescent disease, both the faecal and mucosa-associated microbiome are discordant with matched controls with reduced biodiversity, changes in dominant organisms and increased temporal variation described. Mucosa-associated adherent, invasive *Escherichia coli* (*E. coli*) (AIEC), pro-inflammatory and resistant to killing by mucosal macrophages, appear to be particularly important. AIEC possess several virulence factors which may confer pathogenic potential in CD. Type-1 pili (FimH) allow adherence to intestinal cells *via* cell-surface carcinoembryonic antigen-related cell adhesion molecules and possession of long polar fimbrae promotes translocation across the intestinal mucosa *via* microfold (M)-cells of the follicle-associated epithelium. Resistance to stress genes (*htrA*, *dsbA* and *hfq*) and tolerance of an acidic pH may contribute to survival within the phagolysosomal environment. Here we review the current understanding of the role of mucosa-associated *E. coli* in Crohn's pathogenesis, the role of the innate immune system, factors which may contribute to prolonged bacterial survival and therapeutic strategies to target intracellular *E. coli*.

Key words: Crohn's disease; Inflammatory bowel disease; *Escherichia coli*; Intra-macrophage survival and replication; Phagolysosome; Autophagy

Core tip: There is significant evidence implicating adherent, invasive mucosa-associated *Escherichia coli* (AIEC) in the pathogenesis of Crohn's disease. AIEC translocate M-cells of Peyer's patches and lymphoid follicles of the colon, and then to survive and replicate within underlying mucosal macrophages. How Crohn's AIEC resist killing and adapt to the environment within the phagolysosme to survive and grow within macrophages is still poorly understood. Here we review the current understanding of the role of AIEC in Crohn's pathogenesis, the role of the innate immune system, factors which may contribute to prolonged bacterial survival and therapeutic strategies to target intracellular AIEC.

Tawfik A, Flanagan PK, Campbell BJ. *Escherichia coli*-host macrophage interactions in the pathogenesis of inflammatory

bowel disease. *World J Gastroenterol* 2014; 20(27): 8751-8763 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8751.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8751

INTRODUCTION

Crohn's disease (CD) is a chronic relapsing inflammatory bowel disease (IBD) of multifactorial aetiology, affecting any part of the gastrointestinal tract from mouth to anus. Patients typically suffer from abdominal pain, diarrhoea and weight loss which may be associated with extra-intestinal manifestations including erythema nodosum, iritis and arthritis. The intestinal pathological findings are characterised by transmural inflammation, deep mucosal ulcers, abscesses, fissures and granuloma formation[1]. These chronic inflammatory lesions are proposed to develop due to a disrupted intestinal barrier, Paneth cell dysfunction and a disturbed innate immune response, resulting in the accumulation of antigen-presenting cells (such as dendritic cells and macrophages), lymphocytes and plasma cells within the intestinal mucosal layer[1,2]. Pathological characteristics resemble the mucosal lesions and intestinal inflammation elicited by known enteric gut pathogens such as *Shigella* and *Salmonella* spp[3].

CD is classically described to have a bimodal incidence with the highest rates seen in adolescents and young adults and a second peak in later years, although this has recently been questioned[4]. It is associated with a small increase in mortality (standardised mortality ratio 1.52) but very considerable morbidity, disrupting work, study and family life[5]. Historically approximately 80% of cases needed surgery at some time[6] but the use of immunosuppressants and biologics has increased and is associated with a reduced 5 years risk of major surgery[7]. The condition is more common in Europe and North America[8]. However, incidence is rapidly increasing worldwide particularly in developed nations adopting a western style diet, as seen in Japan[9]. Likewise, those emigrating from poor and developing nations to the West, within a few years of moving are at increased risk of developing CD presumably due to a key change in their lifestyle and environment[10].

The gut microbiota plays an essential role in the shaping of the intestinal immune response in healthy individuals[11]. There is now very strong evidence that both a reduction in the numbers of beneficial bacteria and increases in numbers of harmful bacteria living naturally in the gut are present in CD[12] although it is less clear which of these changes might be causative and which might be a consequence of inflammation. Several independent groups have consistently shown changes in both the faecal and mucosa-associated microbiome in Crohn's patients and unaffected relatives[13-15], an imbalance referred to as "dysbiosis" (Figure 1). Changes are typified by reduced biodiversity and alterations in the dominant organisms, specifically reduction in beneficial firmicutes and increase in numbers of proteobacteria [including *Escherichia coli* (*E. coli*)][14,16,17].

There is also clear evidence to suggest that a number of lifestyle factors contribute to the dysbiosis of gut microbiota observed in CD (see Figure 1). This includes key environmental triggers such as smoking[18], with cessation abrogating the observed dysbiosis[19]. Also a key risk factor in CD is a intake of a "westernised" diet, high in fat and sugar, low in fruit and vegetable fibre[20]. In a mouse model with a humanised microbiota, a switch to a high fat, high sugar diet altered the microbiome within 1 d[21]. A similar diet has also been observed to increase numbers of Proteobacteria, such as *Bilophila wadsworthia*[22] and mucosally adherent, invasive *E. coli* (AIEC)[23].

GENETIC SUSCEPTIBILITIES IN BACTERIAL RECOGNITION, AUTOPHAGY AND PHAGOCYTE-SPECIFIC GENES IN CD

The recent identification of genes associated with CD has been informative in improving our understanding of its pathogenesis, highlighting impairment of genetic components essential for innate immunity, intestinal barrier integrity and in microbial recognition and clearance[24] (see Figure 1). Following on from earlier work[25,26], Genome-wide association studies have now identified 163 IBD risk loci, 30 of which are CD specific and 110 shared between ulcerative colitis and Crohn's[27]. Identified polymorphisms in the innate immune system of Crohn's patients include genes that are linked to processes such as pathogen recognition [nucleotide-binding oligomerization domain-containing-2 (*NOD2*)/Crohn's-associated gene identified was Caspase-recruitment domain 15 (*CARD15*) and interleukin 23 receptor (*IL23R*)] and autophagy [immunity-related GTPase M (*IRGM*) and autophagy-related 16-like 1 (*ATG16L1*)], all relevant to killing of bacteria within macrophages[24-26].

The first *CARD15* encoding the NOD2 receptor[28,29]. Mutations in this gene probably account for about 15% of Crohn's causation in the West although there are geographical variations with a lesser effect in northern European countries and no apparent impact on CD causation in Japan[30]. The NOD2/CARD15 protein is part of the innate immune system and is expressed in the cytoplasm of macrophages and Paneth cells[31]. CD-associated mutations in NOD2/CARD15 affect the leucine-rich domain recognising the bacterial cell wall peptidoglycan component, muramyl dipeptide (MDP), of both Gram-positive and Gram-negative bacteria. After recognition, NOD2 activates nuclear factor kappa B and induces the production and release of proinflammatory cytokines. Crohn's-associated *NOD2/CARD15* mutations are considered to be loss of function mutations with evidence for reduced production of anti-bacterial defensins by Paneth cells and for a reduced IL-8 response to MDP by macrophages[32]. In association with *NOD2/CARD15* mutations, polymorphism in genes *SLC22A4* and *SLC22A5*,

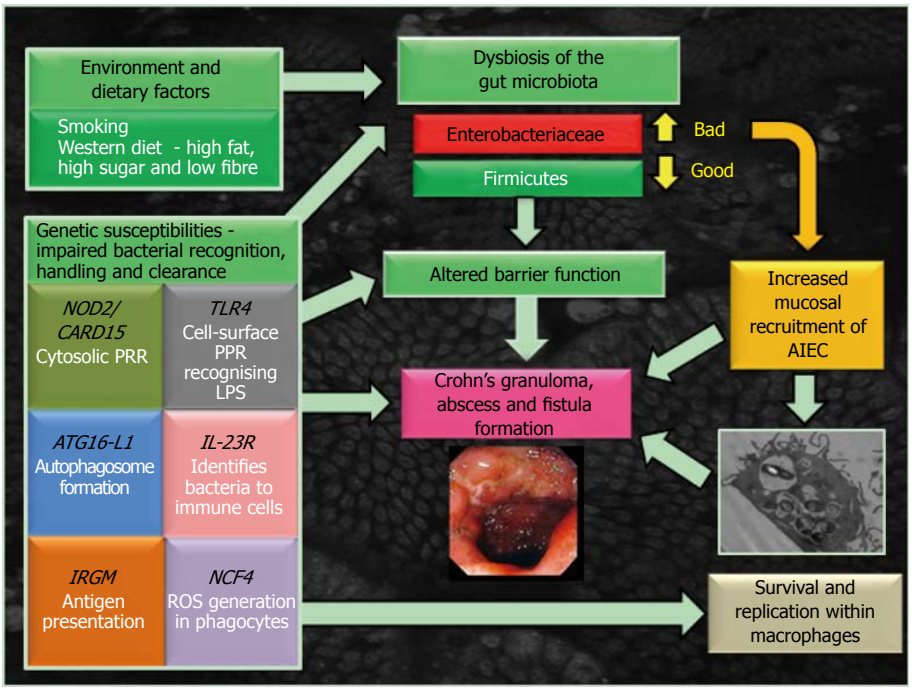


Figure 1 Model for the development of Crohn's disease. AIEC: Adherent, invasive *Escherichia coli*; ATG16L1: Autophagy-related 16-like 1; CARD15/NOD2: Caspase-recruitment domain 15/nucleotide-binding oligomerization domain-containing-2 receptor; IL-23R: Interleukin-23 receptor; IRGM: Immunity-related GTPase M; LPS: Lipopolysaccharide; *NCF4*: Neutrophil cytosolic factor-4 gene; PRR: Pathogen recognition receptor; ROS: Reactive oxygen species; TLR4: Toll-like receptor 4.

encoding the organic cation transporters OCTN1 and OCTN2 have also been identified with variants expressed in intestinal epithelial cells, T cells and macrophages[33]. In addition, a mutation in two haplotypes of *DLG5*, encoding scaffolding protein, has also been confirmed to be associated with *NOD2/CARD15* mutations in Crohn's patients[34].

Two other key genes associated with Crohn's are *ATG16L1* and *IRGM*[35-37]. Both encode proteins that play a key role in autophagy, a cellular process facilitate not only disposal of protein aggregates, DNA, lipids and damaged organelles but also an integral step in the mechanism by which macrophages degrade, kill and clear invading phagocytosed bacteria (a process also termed xenophagy), including Mycobacteria and Salmonellae[38-40].

Additional Crohn's susceptibility loci relevant to aberrant microbial recognition and handling and/or phagocyte function include toll-like receptor 4 (*TLR4*), leucine-rich repeat serine, threonine protein kinase-2 (*LRRK2*), neutrophil cytosolic factor-4 (*NCF4*) and *IL-23R*.

TLR4 is an apical cell-surface pathogen recognition receptor on intestinal epithelial cells, macrophages and dendritic cells, key in detection of lipopolysaccharide (LPS) presented on the outer-membrane surface of Gram-negative bacteria, with polymorphism of TLR4 at D299G leading to hypo-responsiveness to LPS[41]. LRRK2 has been linked to CD through the association of a single-nucleotide polymorphism on chromosome 12q12[26] and in murine studies where LRRK2-deficiency resulted in increased inflammation and significantly poorer clinical outcomes following administration of dextran sodium sulphate to induce colitis[42]. The identification of *NCF4* as a Crohn's susceptibility gene is also important[36]. *NCF4* encodes the p40-phox subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase crucial for reactive oxygen species (ROS) production by phagocytic cells in response to microbial infection, with molecular defects in NADPH oxidase already established to result in chronic granulomatous disease[43]. Key studies show that altered neutrophil recruitment, along with an abnormal production of cytokines and reduced bacterial clearance, follow either acute trauma to the rectum and ileum[44], or subcutaneous injection of heat-killed *E. coli* in Crohn's patients[45]; (see Figure 2). Whilst these studies suggest macrophages may be involved in a key step of the observed immune dysfunction in CD, it is not yet clear whether this represents an inherent defect in macrophage function.

Variants of the *IL-23R* gene have also been linked to Crohn's[46]. IL-23R is expressed by activated dendritic cells and macrophages, and IL-23 can induce production of inflammatory cytokines that may contribute to intestinal inflammation[47].

SPECIFIC BACTERIA IN THE PATHOGENESIS OF CD

There have been a number of distinctive studies that strongly favour the hypothesis that a specific bacterium plays a pivotal role in the initiation of chronic inflammation and development of CD. Early serological and culture studies suggested that *Mycobacterium avium* subspecies *paratuberculosis* (MAP), an obligate intracellular bacterium causing a chronic intestinal inflammatory disease

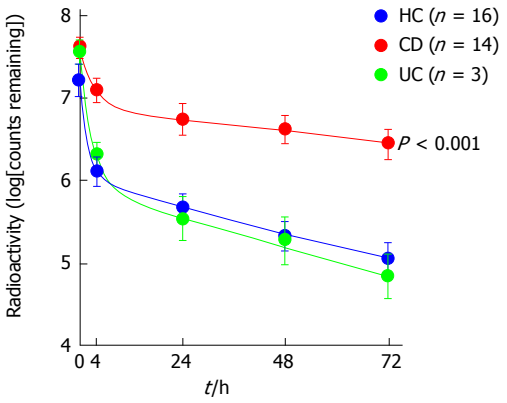


Figure 2 Patients with Crohn's disease exhibit reduced bacterial clearance of subcutaneously injected ^{32}P-labelled heat-killed *Escherichia coli* relative to healthy controls and patients with ulcerative colitis. Reproduced with permission. © 2009 Rockefeller University Press. Originally published in *Journal of Experimental Medicine* 206: 1883-1897[45]. CD: Crohn's disease; HC: Healthy controls; UC: Ulcerative colitis.

in cattle (Johne's disease), was more prevalent in Crohn's patients[48,49]. A study by Ryan and colleagues[50] also confirmed the presence of MAP DNA in granulomatous lesions of CD patients. MAP-reactive CD4 T cells have also been found in patients with Crohn's[51]. Even though, MAP has been hypothesised to be as contributing agent for Crohn's pathogenesis, there is still great controversy, and absence of conclusive evidence, to fully supporting this hypothesis[52]. Our own studies have suggested perhaps that microbial mannan (present in yeast cell walls and Mycobacterium species such as MAP) may be a key environmental factor to suppress macrophage killing of intracellular bacteria[53]. The shared susceptibility association of *NOD2* and *IL-23R* polymorphisms seen in both CD and Mycobacterial disease suggests MAP may yet be important in CD pathogenesis[54].

Faecalibacterium prausnitzii may also be important with low levels strongly associated with early disease recurrence after intestinal surgery[55]. This effect may be due to bacterial production of anti-inflammatory molecules with culture supernatant shown to reduce the severity of colitis in an animal model.

The finding of increased mucosa-associated *E. coli* in the sub-mucus niche or within the mucosa itself has proved particularly consistent in CD[12]. Early serological studies described high antibody titres against *E. coli* in Crohn's patients compared to unaffected controls[56] and this was later supported by immunohistochemical studies demonstrating *E. coli* antigens within macrophages in CD tissue[57]. Many groups, including our own, have shown an increase in mucosa-associated *E. coli* in CD, both in the ileum and in the colorectum[58-64]. We ourselves observed that aerobic culture of colonoscopic biopsies after removal of the mucus layer with dithiothreitol is often sterile in control colons whereas the colon in CD and colon cancer contains increased bacterial numbers in this sub-mucus niche, more than half of which are *E. coli*[60], even though these organisms account for less than 1% of the faecal microbiota[65]. Poor correlation between site of inflammation and presence of *E. coli*[63] and tendency to show that the same organisms can be identified from various sites within the same colon[60,66] are compatible with the organisms having a causative role in the inflammation rather than merely colonising inflamed mucosa. Evidence for a primary pathogenic role is also given by their presence within granulomas[67], the histological hallmark of CD, by their ability to induce granuloma formation *in vitro*[68] and ability for similar *E. coli* to cause granulomatous colitis in dogs[69], and potentially in cats and swine too[70].

These *E. coli* pathovars associated with CD have been designated AIEC based on their ability to adhere to, and invade into, intestinal epithelial cell-lines, induce release of pro-inflammatory cytokines, and possess an ability to survive and replicate with intestinal macrophages[71]. Phylogenetic analysis shows that most mucosa-associated *E. coli* isolated from the tissue of Crohn's patients belong to groups B2 and D[65] as per extra-intestinal isolates, whereas most commensal *E. coli* strains would belong to group A[72].

CROHN'S AIEC-HOST INTESTINAL MUCOSA INTERACTIONS

Aphthous ulcers of the "dome" or follicle-associated epithelium (FAE), overlying Peyer's patches in the distal ileum and lymphoid follicles of the colon, are likely the initial mucosal lesions occurring in Crohn's patients[73-75], and have been observed in patients using magnifying chromoendoscopy[76]. The FAE effectively forms the interface between the intestinal lymphoid system and the luminal environment. Specialized microfold (M) cells accounting for about 5% of cells in the FAE are optimized for antigen adherence and transport, and for immunological sampling of microorganisms[77]. Several invasive bacteria take advantage of the transcytotic characteristics of M cells to use them to cross the gut, including *Yersinia*, *Salmonella* and *Shigella* spp[78-80]. It was suspected that the portal of mucosal entry of AIEC was also likely through M cells[81] and own recent studies successfully modelling M cells *in vitro*, demonstrated that Crohn's AIEC could indeed translocate through M cells (up to 20-fold compared with parent Caco2 cells) and through isolated human ileal FAE[82]. Adhesion and subsequent translocation of AIEC across murine and human Peyer's patches, and across M cells *in vitro*, was observed to be dependent on possession of the *lpf* operon, encoding long polar fimbriae (Lpf) in AIEC[83]. Isolates expressing *lpf* have been found to be more prevalent in Crohn's mucosae than that of non-IBD controls[84]. *Ex vivo* studies also indicate a defective mucosal barrier to bacteria in the Peyer's patches from Crohn's patients[85,86]. It is plausible therefore that increased bacterial load at M cells is important in the development of Crohn's. A striking correlation also exists between the age-related incidence of CD and the number of Peyer's patches in the small bowel, the latter peaking in late adolescence and then

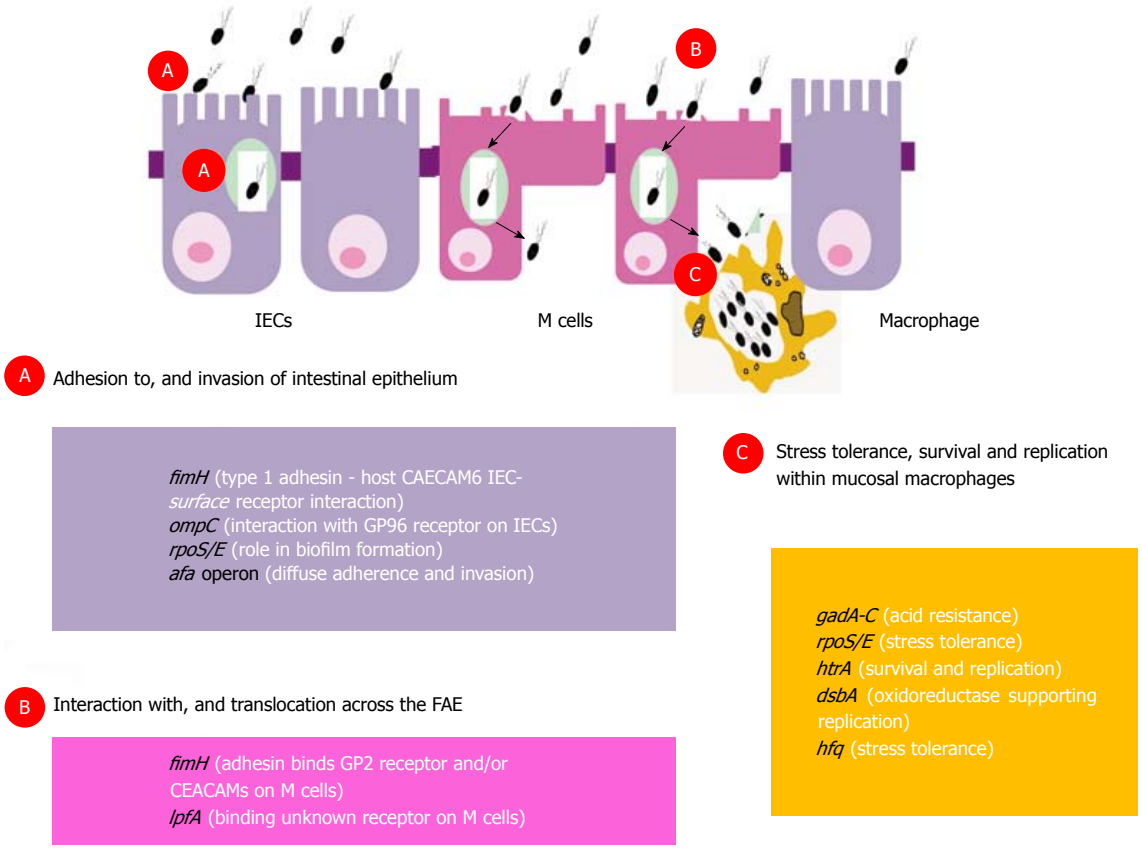


Figure 3 Crohn's mucosally associated adherent, invasive *Escherichia coli* host mucosa interactions: genotype-phenotype relationships. A: Adhesion to, and invasion of intestinal epithelium; B: Mucosal entry across the follicle-associated epithelium; C: Tolerance to stress, habituation and replication within mucosal macrophages. *afa*: Operon encoding afimbrial adhesin; CEACAM: Carcinoembryonic antigen-related cell adhesion molecule; *dsbA*: Gene encoding bacterial disulfide oxidoreductase; *fimH*: Gene encoding bacterial type-1 fimbrial adhesin; *gadA-C*: Glutamate-dependent acid resistance genes; GP2: Glycoprotein 2 receptor; GP96: Endoplasmic reticulum stress response glycoprotein 96; *hfq*: Gene encoding RNA-binding host factor essential for replication of the bacteriophage Qβ; *htrA*: Gene encoding high temperature stress protein A; IECs: Intestinal epithelial cells; *lpfA*: Gene encoding long polar fimbriae adhesin; M cells: Microfold cells; *ompC*: Gene encoding outer-membrane vesicle protein C; *rpoS/E*: Genes encoding stress tolerance sigma factors.

falling away[87].

Ileal AIEC isolates also typically express type-1 pili (FimH) on their surface supporting adherence to ileal enterocytes *via* interaction with carcinoembryonic antigen-related cell adhesion molecule-6 (CEACAM6) receptors known to be over expressed on the inflamed ileal (but not colonic) epithelium in Crohn's[88]. Highly glycosylated CEACAMs have also been proposed as M cell microbial receptors[89]. It is plausible that one or more members of the CEACAM receptor family may play an important role in regulating endocytosis of CD mucosa-associated *E. coli* into host M cells. A recent study also reported that the glycoprotein 2 (GP2), specifically expressed on the apical plasma membrane of M cells among enterocytes, is recognized by FimH[90]. By an intriguing coincidence it has also recently been found that the same GP2 protein is the epitope for the "anti-pancreatic" antibody found in CD sera[91]. In addition, Crohn's AIEC outer-membrane vesicles (OMV), also show ability to interact with enterocyte endoplasmic reticulum stress response glycoprotein 96 receptor, increased in expression on the inflamed intestinal epithelium[92]. These OMVs, in association with flagellin, also possess significant ability to evoke pro-inflammatory cytokine release[93]. Colonic mucosally associated AIEC isolates expressing afimbrial adhesin *afa* operon, more commonly associated with diarrhoeagenic diffusely adherent *E. coli*, have also been observed to be more prevalent in CD patients than in non-IBD controls[84]. The presence of the *afa* operon correlates with diffuse adherence to, and invasion of intestinal epithelial cells[84].

A summary of Crohn's AIEC genotype relevant to host intestinal mucosa interactions is summarised in Figure 3.

VIRULENCE FACTORS SUPPORTING CROHN'S AIEC SURVIVAL AND REPLICATION WITHIN HOST MACROPHAGES

AIEC isolated from Crohn's ileal and colonic biopsy tissue demonstrate ability to survive and replicate within phagolysosomes of host macrophages[94,95]; see Figure 4. However, they are not unique in this ability as other pathogens are also known to survive and replicate within macrophages, including Mycobacteria, *Salmonella, Shigella, Coxiella, Brucella, Legionella* and *Listeria* species. Key de-

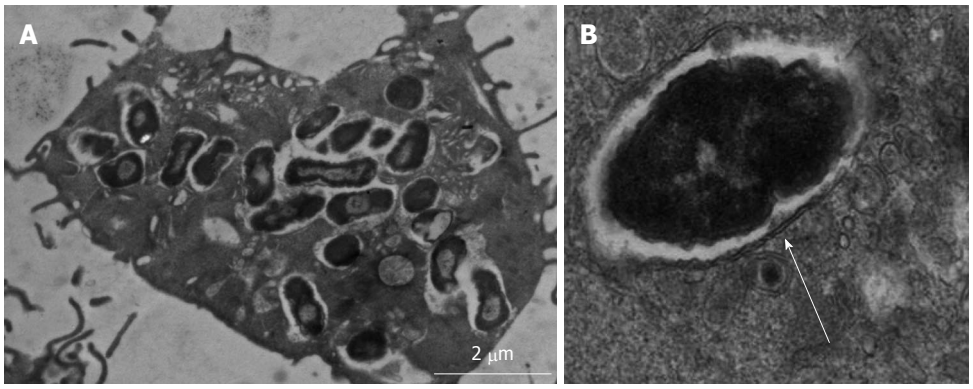


Figure 4 Transmission electron micrograph of adherent, invasive *Escherichia coli* within macrophages[1]. A: Crohn's disease colonic mucosa-associated isolate HM605 surviving and replicating within vesicles of J774-A1 murine macrophages; B: Double membrane around intra-macrophage vesicle indicates bacteria are contained within phagolysosomes (arrow). [1]Images courtesy of Dr. Carol L Roberts (University of Liverpool, United Kingdom).

fence mechanisms adopted by these pathogens support their resistance to killing within the low pH, low nutrient environment, high oxidative and nitrosative stress environment of the phagolysosome. For example, *Shigella* and *Listeria* are able to escape from the mature phagolysosome, Salmonellae can inhibit fusion of phagosome with the lysosome, whilst *Mycobacterium tuberculosis* is able to modify the intra-phagolysosome environment[96]. Key genes supporting AIEC survival and replication within macrophages have been identified (see Figure 3) using isogenic mutants of the "paradigm" ileal AIEC LF82, including *htrA* (encoding high temperature stress protein), *dsbA* (encoding an oxidoreductase) and *hfq* (encoding a RNA chaperone important in mediating bacterial adaptation to chemical stress)[97-99]. However, HtrA and DsbA are fairly ubiquitous in *E. coli*, and it is likely that other unidentified factors are needed to support AIEC survival within the stressful conditions of the phagolysosome.

Acid stress is the antimicrobial environment likely encountered by active enteric bacteria within the phagolysosome. *Salmonella* spp., *Shigella* spp. and *E. coli* have all been reported to possess a repertoire of low pH inducible systems that support resistance, tolerance and habituation during environmental acid stress. Likewise, AIEC certainly appear to be tolerant of the low pH intra-phagolysosome environment[97]. *E. coli* is notable due to its possession of four known acid resistance systems. The first system requires sigma factor RpoS and the cyclic AMP receptor protein CRP, with RpoS functioning as a major environmental stress response regulator in both *E. coli* and Salmonellae[100]. Deletion of RpoS from a Crohn's AIEC (strain O83:H1) has been observed to increase sensitivity of this clinical isolate to oxidative stress[101]. The second system requires extracellular glutamate. The components of glutamate-dependent acid response are two isoforms of glutamate decarboxylase encoded by *gadA* and *gadB*, and a glutamate-γ-aminobutyric acid antiporter encoded by *gadC*[102,103]. Murine AIEC have been observed to respond to chronic intestinal inflammation by up-regulating expression of *gadA* and *gadB*[104]. The third acid resistance system requires is arginine-dependent utilising of arginine decarboxylase (AdiA and AdiC) antiporter[100] and the fourth is lysine dependent, involving lysine decarboxylase[103]. In addition, *E. coli* also harbour specific mechanisms that enable them to resist high levels of ROS that form the oxidative and super-oxidative response to phagocytosed pathogens. These defensive resources have recently been found to be grouped particularly into two regulated sets of genes *soxRS* and *oxy*R regulons[105,106].

DEFECTIVE AUTOPHAGY AND LACK OF CLEARANCE OF AIEC

ATG16L1 and IRGM function in autophagosome formation and evidence from our own studies supports a role for autophagy as an antimicrobial mechanism downstream of toll-like receptor and NOD-like receptor signalling. Activation of NOD2 by MDP induces autophagy in antigen-presenting cells (such as dendritic cells and macrophages) in a receptor-interacting serine-threonine kinase-2 dependent manner[107]. Knock-down of *ATG16L1* and *IRGM* using siRNA approaches results in defective recognition and clearance of Crohn's mucosa-associated *E. coli* within host epithelial cells and macrophages[108]. However, deficiency in either gene did not interfere with the replication and survival ability of other non-pathogenic, environmental, commensal, or gastroenteritis-inducing *E. coli*, suggesting a specific role for autophagy in restraining AIEC. Similarly, expression of the Crohn's variant ATG16L1*300A in intestinal Caco2 epithelial cells impairs their ability to capture internalized *Salmonella* spp. within autophagosomes[109] and is also associated with abnormalities in Paneth cell granule exocytosis[110], impaired production of antimicrobial α-defensins[111], and increased production of pro-inflammatory cytokines IL-1β and IL-18 by macrophages in response to LPS[112].

STRATEGIES TO TARGET INTRA-MACROPHAGE AIEC IN CD

If AIEC have a primary pathogenic role then it follows

that targeted treatment should lead to clinical benefit. This hypothesis is supported by studies in Boxer dogs which develop a granulomatous colitis following infection with an AIEC strain[69], with subsequent clinical resolution following treatment with the 4-quinolone antibiotics, enrofloxacin[113]. However bacterial antibiotic resistance is common both in animal and human studies and is associated with poor clinical outcome[114]. Trials of antibiotics in the treatment of active CD have been disappointing to date with good evidence only for their use in the prevention of post-operative disease recurrence[115,116]. A large metanalysis recently failed to show any clear benefit for their use in maintenance of remission or in the treatment of active luminal or peri-anal disease[117]. In some trials, early open label studies were positive only for later randomised trials to fail to show clear benefit[118,119], which may, in part, be due to the development of antibiotic resistance. *In vitro,* quinolone-based antibiotics regimens to target intra-macrophage Crohn's AIEC isolates are effective[95] but again single antibiotic use likely increases the risk of drug resistance, a problem highlighted by a recent study in which multidrug resistance was seen in 61.5% of Crohn's AIEC isolates[120]. Triple antibiotic regimens are superior to ciprofloxacin mono-therapy and reduce intra-macrophage AIEC survival to 3% relative to untreated controls[95]. Unfortunately significant drug-drug interactions occur with some antibiotics and azathioprine which have limited the use of triple combinations to date. Consequently, alternative strategies are being explored including using adjuvant agents to manipulate the phagolysosomal environment to support microbial phagocytosis.

A more promising strategy may be to alter phagolysosomal pH to aid bacterial killing within macrophages. It has already been shown that AIEC are dependent on an acidic environment for survival[97] and that alkalinisation leads to reduced survival. Hydroxychloroquine, a weak base able to increase phagolysosomal pH, is known to improve killing of bacteria where intra-macrophage survival plays a key step in disease pathogenesis[119]. For example, *Coxiella burnetii* the agent of Q fever, maintains an intracellular lifestyle through adaptation to survival at an acidic pH[121,122]. *Coxiella* survival was significantly reduced *in vitro* by hydroxychloroquine treatment and this benefit translated into clinical response in a randomised trial[123,124]. Hydroxychloroquine in combination with antibiotics, is also now standard therapy for treatment of Whipple's disease, where replication of *Tropheryma whipplei* within tissue macrophages is a central part of the pathogenesis[125]. Similarly, our own recent studies have shown that dose-dependent enhancement of macrophage killing of Crohn's AIEC can be seen with hydroxychloroquine treatment and synergy with standard antibiotics is also observed[126].

Vitamin D supplementation also enhances killing of intracellular AIEC in both murine and human macrophages[127]. This may be due to enhancement of the respiratory burst but effects are likely to be multimodal with influences on several intracellular pathways. Cellular production of the antimicrobial peptides, such as cathelicidin antimicrobial peptide (CAMP) and β2 defensin, follows stimulation of toll-like receptors in the presence of vitamin D and conversely, vitamin D deficiency leads to impaired macrophage function due to defective defensin production[128]. This has significance in CD, where muramyl dipeptide stimulation in the presence of vitamin D leads to increased *CAMP* expression. Futhermore, vitamin D stimulates NOD2 expression and leads to downstream β2 defensin production[129]. Vitamin D deficiency is common in CD with up to 70% of patients affected, even in quiescent disease[130,131]. This now appears to have clinical consequence with several studies demonstrating a correlation between serum levels and disease behaviour. In a large prospective cohort study with nearly 1.5 m patient years of follow up, a validated method for predicting vitamin D levels was used to compare the incidence of CD in the lowest quartile relative to the highest quartile, finding the highest risk associated with the lowest Vitamin D levels[132]. This correlation is not limited to the relative disease risk and recent studies now show a clear correlation between disease behaviour and serum concentrations. CD activity, defined both by CDAI and CRP level, has been shown to be inversely correlated with Vitamin D levels, with greatest activity seen in those with the lowest levels[133]. Furthermore, in a retrospective study of 3217 patients, a lower likelihood of requiring surgery for Crohn's was seen with higher vitamin D levels, when using a cut off of 30 ng/mL[134]. Given these findings we might therefore expect a clinical effect from Vitamin D supplementation. This question was addressed in a randomised double-blind placebo-controlled trial in which a trend was seen towards lower relapse rates in patients treated with 1200 U/d of Vitamin D, although this did not quite reach significance[135]. However a significant reduction in risk of requiring surgery was seen for deficient patients who normalised their vitamin D levels with supplementation[134]. Overall these data suggest a clinical role for vitamin D supplementation in CD although further clinical trials are required. Whilst no data yet exists for the effect of vitamin D on AIEC-macrophage interactions *in vivo*, it appears that supplementation may hold promise as a clinical strategy for targeting Crohn's mucosa-associated *E. coli*.

Smoking has long been associated with disease activity and leads to greater treatment requirements, more stricturing disease, more peri-anal disease and shorter disease free survival[135,136]. These affects are likely to be multimodal in origin with effects seen on macrophage function, gut microbiota and vitamin D levels[137-139]. Interventional studies clearly show benefit from smoking cessation[140] and that this is an achievable therapeutic aim[141]. There are some data to support a hypothesis that this may in part be due to recovery of immune cell function but to date this has not been systematically studied in CD[142].

CONCLUSION

Based on the findings of a diversity of individual studies,

there has been accumulating evidence proving the implication of bacteria such as AIEC in the pathogenesis of CD, a chronic-relapsing IBD. AIEC have been shown to translocate M cells of Peyer's patches and lymphoid follicles of the colon, and then to survive and replicate within underlying mucosal macrophages and dendritic cells. However, the mechanism of how Crohn's AIEC resist killing process and adapt to the environment within the phagolysosme to survive and grow within macrophages without inducing cell death is still poorly understood. There is no doubt that further investigation is warranted to characterise and identify the key virulence factors relevant to AIEC phenotype, supporting current and novel, targeted treatments for future clinical benefit.

REFERENCES

1 **Baumgart DC**, Sandborn WJ. Crohn's disease. *Lancet* 2012; **380**: 1590-1605 [PMID: 22914295 DOI: 10.1016/S0140-6736(12)60026-9]

2 **Baumgart DC**, Carding SR. Inflammatory bowel disease: cause and immunobiology. *Lancet* 2007; **369**: 1627-1640 [PMID: 17499605 DOI: 10.1016/S0140-6736(07)60750-8]

3 **Campieri M**, Gionchetti P. Bacteria as the cause of ulcerative colitis. *Gut* 2001; **48**: 132-135 [PMID: 11115835 DOI: 10.1136/gut.48.1.132]

4 **del Val JH**. Old-age inflammatory bowel disease onset: a different problem? *World J Gastroenterol* 2011; **17**: 2734-2739 [PMID: 21734781 DOI: 10.3748/wjg.v17.i22.2734]

5 **Canavan C**, Abrams KR, Mayberry JF. Meta-analysis: mortality in Crohn's disease. *Aliment Pharmacol Ther* 2007; **25**: 861-870 [PMID: 17402989 DOI: 10.1111/j.1365-2036.2007.03276.x]

6 **Vermeire S**, van Assche G, Rutgeerts P. Review article: Altering the natural history of Crohn's disease--evidence for and against current therapies. *Aliment Pharmacol Ther* 2007; **25**: 3-12 [PMID: 17229216 DOI: 10.1111/j.1365-2036.2006.03134.x]

7 **Rungoe C**, Langholz E, Andersson M, Basit S, Nielsen NM, Wohlfahrt J, Jess T. Changes in medical treatment and surgery rates in inflammatory bowel disease: a nationwide cohort study 1979-2011. *Gut* 2013; Epub ahead of print [PMID: 24056767 DOI: 10.1136/gutjnl-2013-305607]

8 **Molodecky NA**, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012; **142**: 46-54.e42; quiz e30 [PMID: 22001864 DOI: 10.1053/j.gastro.2011.10.001]

9 **Shoda R**, Matsueda K, Yamato S, Umeda N. Epidemiologic analysis of Crohn disease in Japan: increased dietary intake of n-6 polyunsaturated fatty acids and animal protein relates to the increased incidence of Crohn disease in Japan. *Am J Clin Nutr* 1996; **63**: 741-745 [PMID: 8615358]

10 **Barreiro-de Acosta M**, Alvarez Castro A, Souto R, Iglesias M, Lorenzo A, Dominguez-Muñoz JE. Emigration to western industrialized countries: A risk factor for developing inflammatory bowel disease. *J Crohns Colitis* 2011; **5**: 566-569 [PMID: 22115376 DOI: 10.1016/j.crohns.2011.05.009]

11 **Chow J**, Lee SM, Shen Y, Khosravi A, Mazmanian SK. Host-bacterial symbiosis in health and disease. *Adv Immunol* 2010; **107**: 243-274 [PMID: 21034976 DOI: 10.1016/B978-0-12-381300-8.00008-3]

12 **Flanagan P**, Campbell BJ, Rhodes JM. Bacteria in the pathogenesis of inflammatory bowel disease. *Biochem Soc Trans* 2011; **39**: 1067-1072 [PMID: 21787349 DOI: 10.1042/BST0391067]

13 **Kang S**, Denman SE, Morrison M, Yu Z, Dore J, Leclerc M, McSweeney CS. Dysbiosis of fecal microbiota in Crohn's disease patients as revealed by a custom phylogenetic microarray. *Inflamm Bowel Dis* 2010; **16**: 2034-2042 [PMID: 20848492]

14 **Willing B**, Halfvarson J, Dicksved J, Rosenquist M, Järnerot G, Engstrand L, Tysk C, Jansson JK. Twin studies reveal specific imbalances in the mucosa-associated microbiota of patients with ileal Crohn's disease. *Inflamm Bowel Dis* 2009; **15**: 653-660 [PMID: 19023901 DOI: 10.1002/ibd.20783]

15 **Joossens M**, Huys G, Cnockaert M, De Preter V, Verbeke K, Rutgeerts P, Vandamme P, Vermeire S. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. *Gut* 2011; **60**: 631-637 [PMID: 21209126 DOI: 10.1136/gut.2010.223263]

16 **Manichanh C**, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, Nalin R, Jarrin C, Chardon P, Marteau P, Roca J, Dore J. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut* 2006; **55**: 205-211 [PMID: 16188921 DOI: 10.1136/gut.2005.073817]

17 **Mondot S**, Kang S, Furet JP, Aguirre de Carcer D, McSweeney C, Morrison M, Marteau P, Doré J, Leclerc M. Highlighting new phylogenetic specificities of Crohn's disease microbiota. *Inflamm Bowel Dis* 2011; **17**: 185-192 [PMID: 20722058 DOI: 10.1002/ibd.21436]

18 **Mahid SS**, Minor KS, Stevens PL, Galandiuk S. The role of smoking in Crohn's disease as defined by clinical variables. *Dig Dis Sci* 2007; **52**: 2897-2903 [PMID: 17401688 DOI: 10.1007/s10620-006-9624-0]

19 **Biedermann L**, Zeitz J, Mwinyi J, Sutter-Minder E, Rehman A, Ott SJ, Steurer-Stey C, Frei A, Frei P, Scharl M, Loessner MJ, Vavricka SR, Fried M, Schreiber S, Schuppler M, Rogler G. Smoking cessation induces profound changes in the composition of the intestinal microbiota in humans. *PLoS One* 2013; **8**: e59260 [PMID: 23516617 DOI: 10.1371/journal.pone.0059260]

20 **Chapman-Kiddell CA**, Davies PS, Gillen L, Radford-Smith GL. Role of diet in the development of inflammatory bowel disease. *Inflamm Bowel Dis* 2010; **16**: 137-151 [PMID: 19462428 DOI: 10.1002/ibd.20968]

21 **Turnbaugh PJ**, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Sci Transl Med* 2009; **1**: 6ra14 [PMID: 20368178 DOI: 10.1126/scitranslmed.3000322]

22 **Devkota S**, Wang Y, Musch MW, Leone V, Fehlner-Peach H, Nadimpalli A, Antonopoulos DA, Jabri B, Chang EB. Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice. *Nature* 2012; **487**: 104-108 [PMID: 22722865 DOI: 10.1038/nature11225]

23 **Martinez-Medina M**, Denizot J, Dreux N, Robin F, Billard E, Bonnet R, Darfeuille-Michaud A, Barnich N. Western diet induces dysbiosis with increased E coli in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. *Gut* 2014; **63**: 116-124 [PMID: 23598352 DOI: 10.1136/gutjnl-2012-304119]

24 **Lee JC**, Parkes M. Genome-wide association studies and Crohn's disease. *Brief Funct Genomics* 2011; **10**: 71-76 [PMID: 21436303 DOI: 10.1093/bfgp/elr009]

25 **Barrett JC**, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, Brant SR, Silverberg MS, Taylor KD, Barmada MM, Bitton A, Dassopoulos T, Datta LW, Green T, Griffiths AM, Kistner EO, Murtha MT, Regueiro MD, Rotter JI, Schumm LP, Steinhart AH, Targan SR, Xavier RJ; NIDDK IBD Genetics Consortium, Libioulle C, Sandor C, Lathrop M, Belaiche J, Dewit O, Gut I, Heath S, Laukens D, Mni M, Rutgeerts P, Van Gossum A, Zelenika D, Franchimont D, Hugot JP, de Vos M, Vermeire S, Louis E; Belgian-French IBD Consortium; Wellcome Trust Case Control Consortium, Cardon LR, Anderson CA, Drummond H, Nimmo E, Ahmad T, Prescott NJ, Onnie CM, Fisher SA, Marchini J, Ghori J, Bumpstead S, Gwilliam R, Tremelling M, Deloukas P, Mansfield J, Jewell D, Satsangi J, Mathew CG, Parkes M, Georges M, Daly MJ.

Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet* 2008; **40**: 955-962 [PMID: 18587394 DOI: 10.1038/ng.175]

26 **Franke A**, McGovern DP, Barrett JC, Wang K, Radford-Smith GL, Ahmad T, Lees CW, Balschun T, Lee J, Roberts R, Anderson CA, Bis JC, Bumpstead S, Ellinghaus D, Festen EM, Georges M, Green T, Haritunians T, Jostins L, Latiano A, Mathew CG, Montgomery GW, Prescott NJ, Raychaudhuri S, Rotter JI, Schumm P, Sharma Y, Simms LA, Taylor KD, Whiteman D, Wijmenga C, Baldassano RN, Barclay M, Bayless TM, Brand S, Büning C, Cohen A, Colombel JF, Cottone M, Stronati L, Denson T, De Vos M, D'Inca R, Dubinsky M, Edwards C, Florin T, Franchimont D, Gearry R, Glas J, Van Gossum A, Guthery SL, Halfvarson J, Verspaget HW, Hugot JP, Karban A, Laukens D, Lawrance I, Lemann M, Levine A, Libioulle C, Louis E, Mowat C, Newman W, Panés J, Phillips A, Proctor DD, Regueiro M, Russell R, Rutgeerts P, Sanderson J, Sans M, Seibold F, Steinhart AH, Stokkers PC, Torkvist L, Kullak-Ublick G, Wilson D, Walters T, Targan SR, Brant SR, Rioux JD, D'Amato M, Weersma RK, Kugathasan S, Griffiths AM, Mansfield JC, Vermeire S, Duerr RH, Silverberg MS, Satsangi J, Schreiber S, Cho JH, Annese V, Hakonarson H, Daly MJ, Parkes M. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet* 2010; **42**: 1118-1125 [PMID: 21102463 DOI: 10.1038/ng.717]

27 **Jostins L**, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, Lee JC, Schumm LP, Sharma Y, Anderson CA, Essers J, Mitrovic M, Ning K, Cleynen I, Theatre E, Spain SL, Raychaudhuri S, Goyette P, Wei Z, Abraham C, Achkar JP, Ahmad T, Amininejad L, Ananthakrishnan AN, Andersen V, Andrews JM, Baidoo L, Balschun T, Bampton PA, Bitton A, Boucher G, Brand S, Büning C, Cohain A, Cichon S, D'Amato M, De Jong D, Devaney KL, Dubinsky M, Edwards C, Ellinghaus D, Ferguson LR, Franchimont D, Fransen K, Gearry R, Georges M, Gieger C, Glas J, Haritunians T, Hart A, Hawkey C, Hedl M, Hu X, Karlsen TH, Kupcinskas L, Kugathasan S, Latiano A, Laukens D, Lawrance IC, Lees CW, Louis E, Mahy G, Mansfield J, Morgan AR, Mowat C, Newman W, Palmieri O, Ponsioen CY, Potocnik U, Prescott NJ, Regueiro M, Rotter JI, Russell RK, Sanderson JD, Sans M, Satsangi J, Schreiber S, Simms LA, Sventoraityte J, Targan SR, Taylor KD, Tremelling M, Verspaget HW, De Vos M, Wijmenga C, Wilson DC, Winkelmann J, Xavier RJ, Zeissig S, Zhang B, Zhang CK, Zhao H; International IBD Genetics Consortium (IIBDGC), Silverberg MS, Annese V, Hakonarson H, Brant SR, Radford-Smith G, Mathew CG, Rioux JD, Schadt EE, Daly MJ, Franke A, Parkes M, Vermeire S, Barrett JC, Cho JH. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* 2012; **491**: 119-124 [PMID: 23128233 DOI: 10.1038/nature11582]

28 **Hugot JP**, Chamaillard M, Zouali H, Lesage S, Cézard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 2001; **411**: 599-603 [PMID: 11385576 DOI: 10.1038/35079107]

29 **Ogura Y**, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nuñez G, Cho JH. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 2001; **411**: 603-606 [PMID: 11385577 DOI: 10.1038/35079114]

30 **Schreiber S**, Rosenstiel P, Albrecht M, Hampe J, Krawczak M. Genetics of Crohn disease, an archetypal inflammatory barrier disease. *Nat Rev Genet* 2005; **6**: 376-388 [PMID: 15861209 DOI: 10.1038/nrg1607]

31 **Wehkamp J**, Harder J, Weichenthal M, Schwab M, Schäffeler E, Schlee M, Herrlinger KR, Stallmach A, Noack F, Fritz P, Schröder JM, Bevins CL, Fellermann K, Stange EF. NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. *Gut* 2004; **53**: 1658-1664 [PMID: 15479689 DOI: 10.1136/gut.2003.032805]

32 **van Heel DA**, Ghosh S, Butler M, Hunt KA, Lundberg AM, Ahmad T, McGovern DP, Onnie C, Negoro K, Goldthorpe S, Foxwell BM, Mathew CG, Forbes A, Jewell DP, Playford RJ. Muramyl dipeptide and toll-like receptor sensitivity in NOD2-associated Crohn's disease. *Lancet* 2005; **365**: 1794-1796 [PMID: 15910952 DOI: 10.1016/S0140-6736(05)66582-8]

33 **Peltekova VD**, Wintle RF, Rubin LA, Amos CI, Huang Q, Gu X, Newman B, Van Oene M, Cescon D, Greenberg G, Griffiths AM, St George-Hyslop PH, Siminovitch KA. Functional variants of OCTN cation transporter genes are associated with Crohn disease. *Nat Genet* 2004; **36**: 471-475 [PMID: 15107849 DOI: 10.1038/ng1339]

34 **Stoll M**, Corneliussen B, Costello CM, Waetzig GH, Mellgard B, Koch WA, Rosenstiel P, Albrecht M, Croucher PJ, Seegert D, Nikolaus S, Hampe J, Lengauer T, Pierrou S, Foelsch UR, Mathew CG, Lagerstrom-Fermer M, Schreiber S. Genetic variation in DLG5 is associated with inflammatory bowel disease. *Nat Genet* 2004; **36**: 476-480 [PMID: 15107852 DOI: 10.1038/ng1345]

35 **Hampe J**, Franke A, Rosenstiel P, Till A, Teuber M, Huse K, Albrecht M, Mayr G, De La Vega FM, Briggs J, Günther S, Prescott NJ, Onnie CM, Häsler R, Sipos B, Fölsch UR, Lengauer T, Platzer M, Mathew CG, Krawczak M, Schreiber S. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet* 2007; **39**: 207-211 [PMID: 17200669 DOI: 10.1038/ng1954]

36 **Rioux JD**, Xavier RJ, Taylor KD, Silverberg MS, Goyette P, Huett A, Green T, Kuballa P, Barmada MM, Datta LW, Shugart YY, Griffiths AM, Targan SR, Ippoliti AF, Bernard EJ, Mei L, Nicolae DL, Regueiro M, Schumm LP, Steinhart AH, Rotter JI, Duerr RH, Cho JH, Daly MJ, Brant SR. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet* 2007; **39**: 596-604 [PMID: 17435756 DOI: 10.1038/ng2032]

37 **Parkes M**, Barrett JC, Prescott NJ, Tremelling M, Anderson CA, Fisher SA, Roberts RG, Nimmo ER, Cummings FR, Soars D, Drummond H, Lees CW, Khawaja SA, Bagnall R, Burke DA, Todhunter CE, Ahmad T, Onnie CM, McArdle W, Strachan D, Bethel G, Bryan C, Lewis CM, Deloukas P, Forbes A, Sanderson J, Jewell DP, Satsangi J, Mansfield JC, Cardon L, Mathew CG. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. *Nat Genet* 2007; **39**: 830-832 [PMID: 17554261 DOI: 10.1038/ng2061]

38 **Gutierrez MG**, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell* 2004; **119**: 753-766 [PMID: 15607973 DOI: 10.1016/j.cell.2004.11.038]

39 **Sanjuan MA**, Dillon CP, Tait SW, Moshiach S, Dorsey F, Connell S, Komatsu M, Tanaka K, Cleveland JL, Withoff S, Green DR. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature* 2007; **450**: 1253-1257 [PMID: 18097414 DOI: 10.1038/nature06421]

40 **Birmingham CL**, Brumell JH. Autophagy recognizes intracellular Salmonella enterica serovar Typhimurium in damaged vacuoles. *Autophagy* 2006; **2**: 156-158 [PMID: 16874057]

41 **Ouburg S**, Mallant-Hent R, Crusius JB, van Bodegraven AA, Mulder CJ, Linskens R, Peña AS, Morré SA. The toll-like receptor 4 (TLR4) Asp299Gly polymorphism is associated with colonic localisation of Crohn's disease without a major role for the Saccharomyces cerevisiae mannan-LBP-CD14-TLR4 pathway. *Gut* 2005; **54**: 439-440 [PMID: 15710998 DOI:

10.1136/gut.2004.051383]
42 **Liu Z**, Lee J, Krummey S, Lu W, Cai H, Lenardo MJ. The kinase LRRK2 is a regulator of the transcription factor NFAT that modulates the severity of inflammatory bowel disease. *Nat Immunol* 2011; **12**: 1063-1070 [PMID: 21983832 DOI: 10.1038/ni.2113]
43 **Volpp BD**, Nauseef WM, Clark RA. Two cytosolic neutrophil oxidase components absent in autosomal chronic granulomatous disease. *Science* 1988; **242**: 1295-1297 [PMID: 2848318 DOI: 10.1126/science.2848318]
44 **Marks DJ**, Harbord MW, MacAllister R, Rahman FZ, Young J, Al-Lazikani B, Lees W, Novelli M, Bloom S, Segal AW. Defective acute inflammation in Crohn's disease: a clinical investigation. *Lancet* 2006; **367**: 668-678 [PMID: 16503465 DOI: 10.1016/S0140-6736(06)68265-2]
45 **Smith AM**, Rahman FZ, Hayee B, Graham SJ, Marks DJ, Sewell GW, Palmer CD, Wilde J, Foxwell BM, Gloger IS, Sweeting T, Marsh M, Walker AP, Bloom SL, Segal AW. Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease. *J Exp Med* 2009; **206**: 1883-1897 [PMID: 19652016 DOI: 10.1084/jem.20091233]
46 **Duerr RH**, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ, Steinhart AH, Abraham C, Regueiro M, Griffiths A, Dassopoulos T, Bitton A, Yang H, Targan S, Datta LW, Kistner EO, Schumm LP, Lee AT, Gregersen PK, Barmada MM, Rotter JI, Nicolae DL, Cho JH. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 2006; **314**: 1461-1463 [PMID: 17068223 DOI: 10.1126/science.1135245]
47 **McGovern D**, Powrie F. The IL23 axis plays a key role in the pathogenesis of IBD. *Gut* 2007; **56**: 1333-1336 [PMID: 17872562 DOI: 10.1136/gut.2006.115402]
48 **Feller M**, Huwiler K, Stephan R, Altpeter E, Shang A, Furrer H, Pfyffer GE, Jemmi T, Baumgartner A, Egger M. Mycobacterium avium subspecies paratuberculosis and Crohn's disease: a systematic review and meta-analysis. *Lancet Infect Dis* 2007; **7**: 607-613 [PMID: 17714674 DOI: 10.1016/S1473-3099(07)70211-6]
49 **Naser SA**, Ghobrial G, Romero C, Valentine JF. Culture of Mycobacterium avium subspecies paratuberculosis from the blood of patients with Crohn's disease. *Lancet* 2004; **364**: 1039-1044 [PMID: 15380962 DOI: 10.1016/S0140-6736(04)17058-X]
50 **Ryan P**, Bennett MW, Aarons S, Lee G, Collins JK, O'Sullivan GC, O'Connell J, Shanahan F. PCR detection of Mycobacterium paratuberculosis in Crohn's disease granulomas isolated by laser capture microdissection. *Gut* 2002; **51**: 665-670 [PMID: 12377804 DOI: 10.1136/gut.51.5.665]
51 **Olsen I**, Tollefsen S, Aagaard C, Reitan LJ, Bannantine JP, Andersen P, Sollid LM, Lundin KE. Isolation of Mycobacterium avium subspecies paratuberculosis reactive CD4 T cells from intestinal biopsies of Crohn's disease patients. *PLoS One* 2009; **4**: e5641 [PMID: 19479064 DOI: 10.1371/journal.pone.0005641]
52 **Mendoza JL**, San-Pedro A, Culebras E, Cíes R, Taxonera C, Lana R, Urcelay E, de la Torre F, Picazo JJ, Díaz-Rubio M. High prevalence of viable Mycobacterium avium subspecies paratuberculosis in Crohn's disease. *World J Gastroenterol* 2010; **16**: 4558-4563 [PMID: 20857526 DOI: 10.3748/wjg.v16.i36.4558]
53 **Mpofu CM**, Campbell BJ, Subramanian S, Marshall-Clarke S, Hart CA, Cross A, Roberts CL, McGoldrick A, Edwards SW, Rhodes JM. Microbial mannan inhibits bacterial killing by macrophages: a possible pathogenic mechanism for Crohn's disease. *Gastroenterology* 2007; **133**: 1487-1498 [PMID: 17919633 DOI: 10.1053/j.gastro.2007.08.004]
54 **Zhang F**, Liu H, Chen S, Low H, Sun L, Cui Y, Chu T, Li Y, Fu X, Yu Y, Yu G, Shi B, Tian H, Liu D, Yu X, Li J, Lu N, Bao F, Yuan C, Liu J, Liu H, Zhang L, Sun Y, Chen M, Yang Q, Yang H, Yang R, Zhang L, Wang Q, Liu H, Zuo F, Zhang H, Khor CC, Hibberd ML, Yang S, Liu J, Zhang X. Identification of two new loci at IL23R and RAB32 that influence susceptibility to leprosy. *Nat Genet* 2011; **43**: 1247-1251 [PMID: 22019778 DOI: 10.1038/ng.973]
55 **Sokol H**, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux JJ, Blugeon S, Bridonneau C, Furet JP, Corthier G, Grangette C, Vasquez N, Pochart P, Trugnan G, Thomas G, Blottière HM, Doré J, Marteau P, Seksik P, Langella P. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci USA* 2008; **105**: 16731-16736 [PMID: 18936492 DOI: 10.1073/pnas.0804812105]
56 **Tabaqchali S**, O'Donoghue DP, Bettelheim KA. Escherichia coli antibodies in patients with inflammatory bowel disease. *Gut* 1978; **19**: 108-113 [PMID: 344155 DOI: 10.1136/gut.19.2.108]
57 **Liu Y**, van Kruiningen HJ, West AB, Cartun RW, Cortot A, Colombel JF. Immunocytochemical evidence of Listeria, Escherichia coli, and Streptococcus antigens in Crohn's disease. *Gastroenterology* 1995; **108**: 1396-1404 [PMID: 7729631 DOI: 10.1016/0016-5085(95)90687-8]
58 **Darfeuille-Michaud A**, Neut C, Barnich N, Lederman E, Di Martino P, Desreumaux P, Gambiez L, Joly B, Cortot A, Colombel JF. Presence of adherent Escherichia coli strains in ileal mucosa of patients with Crohn's disease. *Gastroenterology* 1998; **115**: 1405-1413 [PMID: 9834268 DOI: 10.1016/S0016-5085(98)70019-8]
59 **Darfeuille-Michaud A**, Boudeau J, Bulois P, Neut C, Glasser AL, Barnich N, Bringer MA, Swidsinski A, Beaugerie L, Colombel JF. High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. *Gastroenterology* 2004; **127**: 412-421 [PMID: 15300573 DOI: 10.1053/j.gastro.2004.04.061]
60 **Martin HM**, Campbell BJ, Hart CA, Mpofu C, Nayar M, Singh R, Englyst H, Williams HF, Rhodes JM. Enhanced Escherichia coli adherence and invasion in Crohn's disease and colon cancer. *Gastroenterology* 2004; **127**: 80-93 [PMID: 15236175 DOI: 10.1053/j.gastro.2004.03.054]
61 **Mylonaki M**, Rayment NB, Rampton DS, Hudspith BN, Brostoff J. Molecular characterization of rectal mucosa-associated bacterial flora in inflammatory bowel disease. *Inflamm Bowel Dis* 2005; **11**: 481-487 [PMID: 15867588 DOI: 10.1097/01.MIB.0000159663.62651.4f]
62 **Sasaki M**, Sitaraman SV, Babbin BA, Gerner-Smidt P, Ribot EM, Garrett N, Alpern JA, Akyildiz A, Theiss AL, Nusrat A, Klapproth JM. Invasive Escherichia coli are a feature of Crohn's disease. *Lab Invest* 2007; **87**: 1042-1054 [PMID: 17660846 DOI: 10.1038/labinvest.3700661]
63 **Kotlowski R**, Bernstein CN, Sepehri S, Krause DO. High prevalence of Escherichia coli belonging to the B2+D phylogenetic group in inflammatory bowel disease. *Gut* 2007; **56**: 669-675 [PMID: 17028128 DOI: 10.1136/gut.2006.099796]
64 **Martinez-Medina M**, Aldeguer X, Lopez-Siles M, González-Huix F, López-Oliu C, Dahbi G, Blanco JE, Blanco J, Garcia-Gil LJ, Darfeuille-Michaud A. Molecular diversity of Escherichia coli in the human gut: new ecological evidence supporting the role of adherent-invasive E. coli (AIEC) in Crohn's disease. *Inflamm Bowel Dis* 2009; **15**: 872-882 [PMID: 19235912 DOI: 10.1002/ibd.20860]
65 **Eckburg PB**, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the human intestinal microbial flora. *Science* 2005; **308**: 1635-1638 [PMID: 15831718 DOI: 10.1126/science.1110591]
66 **Swidsinski A**, Khilkin M, Kerjaschki D, Schreiber S, Ortner M, Weber J, Lochs H. Association between intraepithelial Escherichia coli and colorectal cancer. *Gastroenterology* 1998; **115**: 281-286 [PMID: 9679033 DOI: 10.1016/S0016-5085(98)70194-5]
67 **Ryan P**, Kelly RG, Lee G, Collins JK, O'Sullivan GC, O'Connell J, Shanahan F. Bacterial DNA within granulomas of pa-

tients with Crohn's disease--detection by laser capture microdissection and PCR. *Am J Gastroenterol* 2004; **99**: 1539-1543 [PMID: 15307874 DOI: 10.1111/j.1572-0241.2004.40103.x]
68 **Meconi S**, Vercellone A, Levillain F, Payré B, Al Saati T, Capilla F, Desreumaux P, Darfeuille-Michaud A, Altare F. Adherent-invasive Escherichia coli isolated from Crohn's disease patients induce granulomas in vitro. *Cell Microbiol* 2007; **9**: 1252-1261 [PMID: 17223928 DOI: 10.1111/j.1462-5822.2006.00868.x]
69 **Simpson KW**, Dogan B, Rishniw M, Goldstein RE, Klaessig S, McDonough PL, German AJ, Yates RM, Russell DG, Johnson SE, Berg DE, Harel J, Bruant G, McDonough SP, Schukken YH. Adherent and invasive Escherichia coli is associated with granulomatous colitis in boxer dogs. *Infect Immun* 2006; **74**: 4778-4792 [PMID: 16861666 DOI: 10.1128/IAI.00067-06]
70 **Martinez-Medina M**, Garcia-Gil J, Barnich N, Wieler LH, Ewers C. Adherent-invasive Escherichia coli phenotype displayed by intestinal pathogenic E. coli strains from cats, dogs, and swine. *Appl Environ Microbiol* 2011; **77**: 5813-5817 [PMID: 21705530 DOI: 10.1128/AEM.02614-10]
71 **Darfeuille-Michaud A**. Adherent-invasive Escherichia coli: a putative new E. coli pathotype associated with Crohn's disease. *Int J Med Microbiol* 2002; **292**: 185-193 [PMID: 12398209 DOI: 10.1078/1438-4221-00201]
72 **Boyd EF**, Hartl DL. Chromosomal regions specific to pathogenic isolates of Escherichia coli have a phylogenetically clustered distribution. *J Bacteriol* 1998; **180**: 1159-1165 [PMID: 9495754]
73 **Morson BC**. The early histological lesion of Crohn's disease. *Proc R Soc Med* 1972; **65**: 71-72 [PMID: 5015484]
74 **Rickert RR**, Carter HW. The "early" ulcerative lesion of Crohn's disease: correlative light- and scanning electron-microscopic studies. *J Clin Gastroenterol* 1980; **2**: 11-19 [PMID: 7347352 DOI: 10.1097/00004836-198003000-00003]
75 **Fujimura Y**, Kamoi R, Iida M. Pathogenesis of aphthoid ulcers in Crohn's disease: correlative findings by magnifying colonoscopy, electron microscopy, and immunohistochemistry. *Gut* 1996; **38**: 724-732 [PMID: 8707119 DOI: 10.1136/gut.38.5.724]
76 **Shikuwa S**, Isomoto H, Mizuta Y, Suematsu T, Ito M, Kohno S. Magnifying videoendoscopic findings of Peyer's patches in the terminal ileum of Crohn's disease. *Gut* 2007; **56**: 894-895 [PMID: 17519501 DOI: 10.1136/gut.2007.120717]
77 **Kraehenbuhl JP**, Neutra MR. Epithelial M cells: differentiation and function. *Annu Rev Cell Dev Biol* 2000; **16**: 301-332 [PMID: 11031239 DOI: 10.1146/annurev.cellbio.16.1.301]
78 **Marra A**, Isberg RR. Invasin-dependent and invasin-independent pathways for translocation of Yersinia pseudotuberculosis across the Peyer's patch intestinal epithelium. *Infect Immun* 1997; **65**: 3412-3421 [PMID: 9234806]
79 **Jones BD**, Ghori N, Falkow S. Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *J Exp Med* 1994; **180**: 15-23 [PMID: 8006579 DOI: 10.1084/jem.180.1.15]
80 **Sansonetti PJ**, Arondel J, Cantey JR, Prévost MC, Huerre M. Infection of rabbit Peyer's patches by Shigella flexneri: effect of adhesive or invasive bacterial phenotypes on follicle-associated epithelium. *Infect Immun* 1996; **64**: 2752-2764 [PMID: 8698505]
81 **Gullberg E**, Söderholm JD. Peyer's patches and M cells as potential sites of the inflammatory onset in Crohn's disease. *Ann N Y Acad Sci* 2006; **1072**: 218-232 [PMID: 17057202 DOI: 10.1196/annals.1326.028]
82 **Roberts CL**, Keita AV, Duncan SH, O'Kennedy N, Söderholm JD, Rhodes JM, Campbell BJ. Translocation of Crohn's disease Escherichia coli across M-cells: contrasting effects of soluble plant fibres and emulsifiers. *Gut* 2010; **59**: 1331-1339 [PMID: 20813719 DOI: 10.1136/gut.2009.195370]
83 **Chassaing B**, Rolhion N, de Vallée A, Salim SY, Prorok-Hamon M, Neut C, Campbell BJ, Söderholm JD, Hugot JP, Colombel JF, Darfeuille-Michaud A. Crohn disease--associated adherent-invasive E. coli bacteria target mouse and human Peyer's patches via long polar fimbriae. *J Clin Invest* 2011; **121**: 966-975 [PMID: 21339647 DOI: 10.1172/JCI44632]
84 **Prorok-Hamon M**, Friswell MK, Alswied A, Roberts CL, Song F, Flanagan PK, Knight P, Codling C, Marchesi JR, Winstanley C, Hall N, Rhodes JM, Campbell BJ. Colonic mucosa-associated diffusely adherent afaC+ Escherichia coli expressing lpfA and pks are increased in inflammatory bowel disease and colon cancer. *Gut* 2014; **63**: 761-770 [PMID: 23846483 DOI: 10.1136/gutjnl-2013-304739]
85 **Keita AV**, Salim SY, Jiang T, Yang PC, Franzén L, Söderkvist P, Magnusson KE, Söderholm JD. Increased uptake of non-pathogenic E. coli via the follicle-associated epithelium in longstanding ileal Crohn's disease. *J Pathol* 2008; **215**: 135-144 [PMID: 18348161 DOI: 10.1002/path.2337]
86 **Salim SY**, Silva MA, Keita AV, Larsson M, Andersson P, Magnusson KE, Perdue MH, Söderholm JD. CD83+CCR7-dendritic cells accumulate in the subepithelial dome and internalize translocated Escherichia coli HB101 in the Peyer's patches of ileal Crohn's disease. *Am J Pathol* 2009; **174**: 82-90 [PMID: 19095953 DOI: 10.2353/ajpath.2009.080273]
87 **Van Kruiningen HJ**, West AB, Freda BJ, Holmes KA. Distribution of Peyer's patches in the distal ileum. *Inflamm Bowel Dis* 2002; **8**: 180-185 [PMID: 11979138 DOI: 10.1097/00054725-200205000-00004]
88 **Barnich N**, Darfeuille-Michaud A. Abnormal CEACAM6 expression in Crohn disease patients favors gut colonization and inflammation by adherent-invasive E. coli. *Virulence* 2010; **1**: 281-282 [PMID: 21178454 DOI: 10.4161/viru.1.4.11510]
89 **Baranov V**, Hammarström S. Carcinoembryonic antigen (CEA) and CEA-related cell adhesion molecule 1 (CEACAM1), apically expressed on human colonic M cells, are potential receptors for microbial adhesion. *Histochem Cell Biol* 2004; **121**: 83-89 [PMID: 14758482 DOI: 10.1007/s00418-003-0613-5]
90 **Hase K**, Kawano K, Nochi T, Pontes GS, Fukuda S, Ebisawa M, Kadokura K, Tobe T, Fujimura Y, Kawano S, Yabashi A, Waguri S, Nakato G, Kimura S, Murakami T, Iimura M, Hamura K, Fukuoka S, Lowe AW, Itoh K, Kiyono H, Ohno H. Uptake through glycoprotein 2 of FimH(+) bacteria by M cells initiates mucosal immune response. *Nature* 2009; **462**: 226-230 [PMID: 19907495 DOI: 10.1038/nature08529]
91 **Roggenbuck D**, Hausdorf G, Martinez-Gamboa L, Reinhold D, Büttner T, Jungblut PR, Porstmann T, Laass MW, Henker J, Büning C, Feist E, Conrad K. Identification of GP2, the major zymogen granule membrane glycoprotein, as the autoantigen of pancreatic antibodies in Crohn's disease. *Gut* 2009; **58**: 1620-1628 [PMID: 19549613 DOI: 10.1136/gut.2008.162495]
92 **Rolhion N**, Barnich N, Bringer MA, Glasser AL, Ranc J, Hébuterne X, Hofman P, Darfeuille-Michaud A. Abnormally expressed ER stress response chaperone Gp96 in CD favours adherent-invasive Escherichia coli invasion. *Gut* 2010; **59**: 1355-1362 [PMID: 20587550 DOI: 10.1136/gut.2010.207456]
93 **Subramanian S**, Rhodes JM, Hart CA, Tam B, Roberts CL, Smith SL, Corkill JE, Winstanley C, Virji M, Campbell BJ. Characterization of epithelial IL-8 response to inflammatory bowel disease mucosal E. coli and its inhibition by mesalamine. *Inflamm Bowel Dis* 2008; **14**: 162-175 [PMID: 17941093 DOI: 10.1002/ibd.20296]
94 **Bringer MA**, Glasser AL, Tung CH, Méresse S, Darfeuille-Michaud A. The Crohn's disease-associated adherent-invasive Escherichia coli strain LF82 replicates in mature phagolysosomes within J774 macrophages. *Cell Microbiol* 2006; **8**: 471-484 [PMID: 16469058 DOI: 10.1111/j.1462-5822.2005.00639.x]
95 **Subramanian S**, Roberts CL, Hart CA, Martin HM, Edwards SW, Rhodes JM, Campbell BJ. Replication of Colonic Crohn's Disease Mucosal Escherichia coli Isolates within Macrophages and Their Susceptibility to Antibiotics. *Antimicrob Agents Chemother* 2008; **52**: 427-434 [PMID: 18070962 DOI:

10.1128/AAC.00375-07]
96 **Amano A**, Nakagawa I, Yoshimori T. Autophagy in innate immunity against intracellular bacteria. *J Biochem* 2006; **140**: 161-166 [PMID: 16954534 DOI: 10.1093/jb/mvj162]
97 **Bringer MA**, Barnich N, Glasser AL, Bardot O, Darfeuille-Michaud A. HtrA stress protein is involved in intramacrophagic replication of adherent and invasive Escherichia coli strain LF82 isolated from a patient with Crohn's disease. *Infect Immun* 2005; **73**: 712-721 [PMID: 15664909 DOI: 10.1128/IAI.73.2.712-721.2005]
98 **Bringer MA**, Rolhion N, Glasser AL, Darfeuille-Michaud A. The oxidoreductase DsbA plays a key role in the ability of the Crohn's disease-associated adherent-invasive Escherichia coli strain LF82 to resist macrophage killing. *J Bacteriol* 2007; **189**: 4860-4871 [PMID: 17449627 DOI: 10.1128/JB.00233-07]
99 **Simonsen KT**, Nielsen G, Bjerrum JV, Kruse T, Kallipolitis BH, Møller-Jensen J. A role for the RNA chaperone Hfq in controlling adherent-invasive Escherichia coli colonization and virulence. *PLoS One* 2011; **6**: e16387 [PMID: 21298102 DOI: 10.1371/journal.pone.0016387]
100 **Castanie-Cornet MP**, Penfound TA, Smith D, Elliott JF, Foster JW. Control of acid resistance in Escherichia coli. *J Bacteriol* 1999; **181**: 3525-3535 [PMID: 10348866]
101 **Allen CA**, Niesel DW, Torres AG. The effects of low-shear stress on Adherent-invasive Escherichia coli. *Environ Microbiol* 2008; **10**: 1512-1525 [PMID: 18312396 DOI: 10.1111/j.1462-2920.2008.01567.x]
102 **Foster JW**. Escherichia coli acid resistance: tales of an amateur acidophile. *Nat Rev Microbiol* 2004; **2**: 898-907 [PMID: 15494746 DOI: 10.1038/nrmicro102]
103 **Iyer R**, Williams C, Miller C. Arginine-agmatine antiporter in extreme acid resistance in Escherichia coli. *J Bacteriol* 2003; **185**: 6556-6561 [PMID: 14594828 DOI: 10.1128/JB.185.22.6556-6561.2003]
104 **Tchaptchet S**, Fan TJ, Goeser L, Schoenborn A, Gulati AS, Sartor RB, Hansen JJ. Inflammation-induced acid tolerance genes gadAB in luminal commensal Escherichia coli attenuate experimental colitis. *Infect Immun* 2013; **81**: 3662-3671 [PMID: 23876805 DOI: 10.1128/IAI.00355-13]
105 **Pomposiello PJ**, Bennik MH, Demple B. Genome-wide transcriptional profiling of the Escherichia coli responses to superoxide stress and sodium salicylate. *J Bacteriol* 2001; **183**: 3890-3902 [PMID: 11395452 DOI: 10.1128/JB.183.13.3890-3902.2001]
106 **Imlay JA**. Cellular defenses against superoxide and hydrogen peroxide. *Annu Rev Biochem* 2008; **77**: 755-776 [PMID: 18173371 DOI: 10.1146/annurev.biochem.77.061606.161055]
107 **Cooney R**, Baker J, Brain O, Danis B, Pichulik T, Allan P, Ferguson DJ, Campbell BJ, Jewell D, Simmons A. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med* 2010; **16**: 90-97 [PMID: 19966812 DOI: 10.1038/nm.2069]
108 **Lapaquette P**, Glasser AL, Huett A, Xavier RJ, Darfeuille-Michaud A. Crohn's disease-associated adherent-invasive E. coli are selectively favoured by impaired autophagy to replicate intracellularly. *Cell Microbiol* 2010; **12**: 99-113 [PMID: 19747213 DOI: 10.1111/j.1462-5822.2009.01381.x]
109 **Kuballa P**, Huett A, Rioux JD, Daly MJ, Xavier RJ. Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant. *PLoS One* 2008; **3**: e3391 [PMID: 18852889 DOI: 10.1371/journal.pone.0003391]
110 **Cadwell K**, Liu JY, Brown SL, Miyoshi H, Loh J, Lennerz JK, Kishi C, Kc W, Carrero JA, Hunt S, Stone CD, Brunt EM, Xavier RJ, Sleckman BP, Li E, Mizushima N, Stappenbeck TS, Virgin HW. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature* 2008; **456**: 259-263 [PMID: 18849966 DOI: 10.1038/nature07416]
111 **Wehkamp J**, Salzman NH, Porter E, Nuding S, Weichenthal M, Petras RE, Shen B, Schaeffeler E, Schwab M, Linzmeier R, Feathers RW, Chu H, Lima H, Fellermann K, Ganz T, Stange EF, Bevins CL. Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc Natl Acad Sci USA* 2005; **102**: 18129-18134 [PMID: 16330776 DOI: 10.1073/pnas.0505256102]
112 **Saitoh T**, Fujita N, Jang MH, Uematsu S, Yang BG, Satoh T, Omori H, Noda T, Yamamoto N, Komatsu M, Tanaka K, Kawai T, Tsujimura T, Takeuchi O, Yoshimori T, Akira S. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature* 2008; **456**: 264-268 [PMID: 18849965 DOI: 10.1038/nature07383]
113 **Mansfield CS**, James FE, Craven M, Davies DR, O'Hara AJ, Nicholls PK, Dogan B, MacDonough SP, Simpson KW. Remission of histiocytic ulcerative colitis in Boxer dogs correlates with eradication of invasive intramucosal Escherichia coli. *J Vet Intern Med* 2009; **23**: 964-969 [PMID: 19678891 DOI: 10.1111/j.1939-1676.2009.0363.x]
114 **Craven M**, Dogan B, Schukken A, Volkman M, Chandler A, McDonough PL, Simpson KW. Antimicrobial resistance impacts clinical outcome of granulomatous colitis in boxer dogs. *J Vet Intern Med* 2010; **24**: 819-824 [PMID: 20492483 DOI: 10.1111/j.1939-1676.2010.0527.x]
115 **D'Haens GR**, Vermeire S, Van Assche G, Noman M, Aerden I, Van Olmen G, Rutgeerts P. Therapy of metronidazole with azathioprine to prevent postoperative recurrence of Crohn's disease: a controlled randomized trial. *Gastroenterology* 2008; **135**: 1123-1129 [PMID: 18727929 DOI: 10.1053/j.gastro.2008.07.010]
116 **Rutgeerts P**, Van Assche G, Vermeire S, D'Haens G, Baert F, Noman M, Aerden I, De Hertogh G, Geboes K, Hiele M, D'Hoore A, Penninckx F. Ornidazole for prophylaxis of postoperative Crohn's disease recurrence: a randomized, double-blind, placebo-controlled trial. *Gastroenterology* 2005; **128**: 856-861 [PMID: 15825069 DOI: 10.1053/j.gastro.2005.01.010]
117 **Khan KJ**, Ullman TA, Ford AC, Abreu MT, Abadir A, Marshall JK, Talley NJ, Moayyedi P. Antibiotic therapy in inflammatory bowel disease: a systematic review and meta-analysis. *Am J Gastroenterol* 2011; **106**: 661-673 [PMID: 21407187 DOI: 10.1038/ajg.2011.72]
118 **Leiper K**, Martin K, Ellis A, Watson AJ, Morris AI, Rhodes JM. Clinical trial: randomized study of clarithromycin versus placebo in active Crohn's disease. *Aliment Pharmacol Ther* 2008; **27**: 1233-1239 [PMID: 18315579 DOI: 10.1111/j.1365-2036.2008.03661.x]
119 **Leiper K**, Morris AI, Rhodes JM. Open label trial of oral clarithromycin in active Crohn's disease. *Aliment Pharmacol Ther* 2000; **14**: 801-806 [PMID: 10848665 DOI: 10.1046/j.1365-2036.2000.00753.x]
120 **Dogan B**, Scherl E, Bosworth B, Yantiss R, Altier C, McDonough PL, Jiang ZD, Dupont HL, Garneau P, Harel J, Rishniw M, Simpson KW. Multidrug resistance is common in Escherichia coli associated with ileal Crohn's disease. *Inflamm Bowel Dis* 2013; **19**: 141-150 [PMID: 22508665 DOI: 10.1002/ibd.22971]
121 **Rolain JM**, Colson P, Raoult D. Recycling of chloroquine and its hydroxyl analogue to face bacterial, fungal and viral infections in the 21st century. *Int J Antimicrob Agents* 2007; **30**: 297-308 [PMID: 17629679 DOI: 10.1016/j.ijantimicag.2007.05.015]
122 **Maurin M**, Benoliel AM, Bongrand P, Raoult D. Phagolysosomes of Coxiella burnetii-infected cell lines maintain an acidic pH during persistent infection. *Infect Immun* 1992; **60**: 5013-5016 [PMID: 1452331]
123 **Maurin M**, Benoliel AM, Bongrand P, Raoult D. Phagolysosomal alkalinization and the bactericidal effect of antibiotics: the Coxiella burnetii paradigm. *J Infect Dis* 1992; **166**: 1097-1102 [PMID: 1402021 DOI: 10.1093/infdis/166.5.1097]
124 **Raoult D**, Houpikian P, Tissot Dupont H, Riss JM, Arditi-Djiane J, Brouqui P. Treatment of Q fever endocarditis: com-

parison of 2 regimens containing doxycycline and ofloxacin or hydroxychloroquine. *Arch Intern Med* 1999; **159**: 167-173 [PMID: 9927100 DOI: 10.1001/archinte.159.2.167]

125 **Boulos A**, Rolain JM, Raoult D. Antibiotic susceptibility of Tropheryma whipplei in MRC5 cells. *Antimicrob Agents Chemother* 2004; **48**: 747-752 [PMID: 14982759 DOI: 10.1128/AAC.48.3.747-752.2004]

126 **Flanagan PK**, Campbell BJ, Rhodes JM. Hydroxychloroquine inhibits intra-macrophage replication of Crohn's disease E. coli and enhances the antimicrobial effect of antibiotics doxycycline and ciprofloxacin. *Gut* 2011; **60** (Suppl 3): A210

127 **Flanagan PK**, Campbell BJ, Rhodes JM. Vitamin D enhances macrophage function and improves killing of Crohn's associated E. coli. *J Crohns Colitis* 2012; **7** (Supp 1): S20 [DOI: 10.1016/S1873-9946(13)60049-5]

128 **Hewison M**. Vitamin D and the immune system: new perspectives on an old theme. *Endocrinol Metab Clin North Am* 2010; **39**: 365-379, table of contents [PMID: 20511058 DOI: 10.1016/j.ecl.2010.02.010]

129 **Wang TT**, Dabbas B, Laperriere D, Bitton AJ, Soualhine H, Tavera-Mendoza LE, Dionne S, Servant MJ, Bitton A, Seidman EG, Mader S, Behr MA, White JH. Direct and indirect induction by 1,25-dihydroxyvitamin D3 of the NOD2/CARD15-defensin beta2 innate immune pathway defective in Crohn disease. *J Biol Chem* 2010; **285**: 2227-2231 [PMID: 19948723 DOI: 10.1074/jbc.C109.071225]

130 **Farraye FA**, Nimitphong H, Stucchi A, Dendrinos K, Boulanger AB, Vijjeswarapu A, Tanennbaum A, Biancuzzo R, Chen TC, Holick MF. Use of a novel vitamin D bioavailability test demonstrates that vitamin D absorption is decreased in patients with quiescent Crohn's disease. *Inflamm Bowel Dis* 2011; **17**: 2116-2121 [PMID: 21910173 DOI: 10.1002/ibd.21595]

131 **Suibhne TN**, Cox G, Healy M, O'Morain C, O'Sullivan M. Vitamin D deficiency in Crohn's disease: prevalence, risk factors and supplement use in an outpatient setting. *J Crohns Colitis* 2012; **6**: 182-188 [PMID: 22325172 DOI: 10.1016/j.crohns.2011.08.002]

132 **Ananthakrishnan AN**, Khalili H, Higuchi LM, Bao Y, Korzenik JR, Giovannucci EL, Richter JM, Fuchs CS, Chan AT. Higher predicted vitamin D status is associated with reduced risk of Crohn's disease. *Gastroenterology* 2012; **142**: 482-489 [PMID: 22155183 DOI: 10.1053/j.gastro.2011.11.040]

133 **Jørgensen SP**, Hvas CL, Agnholt J, Christensen LA, Heickendorff L, Dahlerup JF. Active Crohn's disease is associated with low vitamin D levels. *J Crohns Colitis* 2013; **7**: e407-e413 [PMID: 23403039 DOI: 10.1016/j.crohns.2013.01.012]

134 **Ananthakrishnan AN**, Cagan A, Gainer VS, Cai T, Cheng SC, Savova G, Chen P, Szolovits P, Xia Z, De Jager PL, Shaw SY, Churchill S, Karlson EW, Kohane I, Plenge RM, Murphy SN, Liao KP. Normalization of plasma 25-hydroxy vitamin D is associated with reduced risk of surgery in Crohn's disease. *Inflamm Bowel Dis* 2013; **19**: 1921-1927 [PMID: 23751398 DOI: 10.1097/MIB.0b013e3182902ad9]

135 **Jørgensen SP**, Agnholt J, Glerup H, Lyhne S, Villadsen GE, Hvas CL, Bartels LE, Kelsen J, Christensen LA, Dahlerup JF. Clinical trial: vitamin D3 treatment in Crohn's disease - a randomized double-blind placebo-controlled study. *Aliment Pharmacol Ther* 2010; **32**: 377-383 [PMID: 20491740 DOI: 10.1111/j.1365-2036.2010.04355.x]

136 **Nunes T**, Etchevers MJ, Domènech E, García-Sánchez V, Ber Y, Peñalva M, Merino O, Nos P, Garcia-Planella E, Casbas AG, Esteve M, Taxonera Samsó C, Montoro Huguet M, Gisbert JP, Martín Arranz MD, García-Sepulcre MF, Barreiro-de Acosta M, Beltrán B, Alcaide Suárez N, Saro Gismera C, Cabriada JL, Cañas-Ventura A, Gomollón F, Panés J. Smoking does influence disease behaviour and impacts the need for therapy in Crohn's disease in the biologic era. *Aliment Pharmacol Ther* 2013; **38**: 752-760 [PMID: 23980933 DOI: 10.1111/apt.12440]

137 **Tobin MV**, Logan RF, Langman MJ, McConnell RB, Gilmore IT. Cigarette smoking and inflammatory bowel disease. *Gastroenterology* 1987; **93**: 316-321 [PMID: 3596168]

138 **Benjamin JL**, Hedin CR, Koutsoumpas A, Ng SC, McCarthy NE, Prescott NJ, Pessoa-Lopes P, Mathew CG, Sanderson J, Hart AL, Kamm MA, Knight SC, Forbes A, Stagg AJ, Lindsay JO, Whelan K. Smokers with active Crohn's disease have a clinically relevant dysbiosis of the gastrointestinal microbiota. *Inflamm Bowel Dis* 2012; **18**: 1092-1100 [PMID: 22102318 DOI: 10.1002/ibd.21864]

139 **King TE**, Savici D, Campbell PA. Phagocytosis and killing of Listeria monocytogenes by alveolar macrophages: smokers versus nonsmokers. *J Infect Dis* 1988; **158**: 1309-1316 [PMID: 3143765 DOI: 10.1093/infdis/158.6.1309]

140 **Cosnes J**, Beaugerie L, Carbonnel F, Gendre JP. Smoking cessation and the course of Crohn's disease: an intervention study. *Gastroenterology* 2001; **120**: 1093-1099 [PMID: 11266373 DOI: 10.1053/gast.2001.23231]

141 **Nunes T**, Etchevers MJ, Merino O, Gallego S, García-Sánchez V, Marín-Jiménez I, Menchén L, Barreiro-de Acosta M, Bastida G, García S, Gento E, Ginard D, Martí E, Gomollón F, Arroyo M, Monfort D, García-Planella E, Gonzalez B, Loras C, Agustí C, Figueroa C, Sans M. High smoking cessation rate in Crohn's disease patients after physician advice--the TABACROHN Study. *J Crohns Colitis* 2013; **7**: 202-207 [PMID: 22626507 DOI: 10.1016/j.crohns.2012.04.011]

142 **Kotani N**, Kushikata T, Hashimoto H, Sessler DI, Muraoka M, Matsuki A. Recovery of intraoperative microbicidal and inflammatory functions of alveolar immune cells after a tobacco smoke-free period. *Anesthesiology* 2001; **94**: 999-1006 [PMID: 11465626 DOI: 10.1097/00000542-200106000-00013]

P- Reviewers: D'Elios MM, Ghigo E
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8764

World J Gastroenterol 2014 July 21; 20(27): 8764-8782
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Neurological disorders and inflammatory bowel diseases

Giovanni Casella, Gian Eugenio Tontini, Gabrio Bassotti, Luca Pastorelli, Vincenzo Villanacci, Luisa Spina, Vittorio Baldini, Maurizio Vecchi

Giovanni Casella, Vittorio Baldini, Medical Department, Desio Hospital, 20033 Desio, Italy
Gian Eugenio Tontini, Luca Pastorelli, Luisa Spina, Maurizio Vecchi, Gastroenterology and Gastrointestinal Endoscopy Unit, IRCCS Policlinico San Donato, 20097 San Donato Milanese, Italy
Gabrio Bassotti, Gastroenterology Section, Department of Medicine, University of Perugia Medical School, 06123 Perugia, Italy
Luca Pastorelli, Maurizio Vecchi, Department of Biomedical Sciences for the Health, University of Milan, 20146 Milan, Italy
Vincenzo Villanacci, Institute of Pathology, Spedali Civili, 25123 Brescia, Italy
Author contributions: All authors designed and wrote the current review article.
Correspondence to: Maurizio Vecchi, MD, Gastroenterology and Gastrointestinal Endoscopy Unit, IRCCS Policlinico San Donato, via Morandi 30, 20097 San Donato Milanese, Italy. maurizio.vecchi@unimi.it
Telephone: +39-2-52774655 Fax: +39-2-52774655
Received: October 28, 2013 Revised: February 14, 2014
Accepted: April 15, 2014
Published online: July 21, 2014

Abstract

Extraintestinal manifestations occur in about one-third of patients living with inflammatory bowel disease (IBD) and may precede the onset of gastrointestinal symptoms by many years. Neurologic disorders associated with IBD are not frequent, being reported in 3% of patients, but they often represent an important cause of morbidity and a relevant diagnostic issue. In addition, the increasing use of immunosuppressant and biological therapies for IBD may also play a pivotal role in the development of neurological disorders of different type and pathogenesis. Hence, we provide a complete and profound review of the main features of neurological complications associated with IBD, with particular reference to those related to drugs and with a specific focus on their clinical presentation and possible pathophysiological mechanisms.

Key words: Inflammatory bowel diseases; Crohn's disease; Ulcerative colitis; Extraintestinal manifestations; Neurological disorders; Multiple sclerosis; Progressive multifocal encephalopathy; Demyelinating neuropathies; Cerebrovascular diseases; Side effects

Core tip: Extraintestinal manifestations occur in about one-third of patients with inflammatory bowel disease (IBD) and may precede the onset of gastrointestinal symptoms by many years. Neurological disorders are uncommon in IBD but they can represent an important cause of morbidity and relevant diagnostic issue. Furthermore, the use of immunosuppressant and biological therapies for IBD may also play a pivotal role in the development of neurological disorders. Hence, we review the main features of neurological complications associated with IBD, with particular reference to those related to drugs, thereby focusing on their clinical presentation and possible pathophysiological mechanisms.

Casella G, Tontini GE, Bassotti G, Pastorelli L, Villanacci V, Spina L, Baldini V, Vecchi M. Neurological disorders and inflammatory bowel diseases. *World J Gastroenterol* 2014; 20(27): 8764-8782 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8764.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8764

INTRODUCTION

Inflammatory bowel diseases (IBDs) are common causes of gastrointestinal morbidity in western countries. Extraintestinal manifestations are frequent in the course of IBD and, in some cases, may be the first manifestation of IBD, sometimes preceding the onset of gastrointestinal symptoms by many years[1].

Among the many extraintestinal manifestations af-

fecting various organs, neurological disorders of different type and pathogenesis have been documented[2]. Overall, the occurrence of neurological disorders during the course of IBD is uncommon[3], but they may represent an important cause of morbidity. Neurological complications appear to be more common in men and they usually appear after IBD diagnosis, rarely coinciding with exacerbations of the underlying bowel disease[3]. Only a few systematic studies have investigated their frequency in patients with IBD and the results have been frequently inconsistent due to differences in the methods used for case finding and outcome evaluation[4].

One of the largest studies, performed for this purpose on 638 patients with IBD [either ulcerative colitis (UC) or Crohn's disease (CD)], found neurological disorders in 3%[3], with another study[5] indicating a possible increase in the prevalence of demyelinating diseases, particularly of multiple sclerosis (MS). The increasing use of immunosuppressant and biological therapies may also influence the probability of IBD-associated neurological disorders because these agents, although rarely used, may cause central nervous system (CNS) white matter lesions[6], opportunistic infections[7] with clinical symptoms similar to MS[2], or JC virus (JCV)-mediated progressive multifocal leukoencephalopathy (PML)[7].

On the basis of these considerations, it is obvious that a physician treating IBD/patients should be able to recognize unexplained neurological symptoms, consider their association with IBD, and address a proper diagnostic and therapeutic work-up, possibly with the collaboration of a consultant neurologist.

In this review, we went through the different neurological complications associated with IBD, with particular reference to those related to drugs, with a specific focus on their clinical presentation and their possible pathophysiological mechanisms[8]. We divided the review into the following specific sections: (1) side effects of medications; (2) cerebrovascular diseases; (3) immune-mediated neurological disorders; and (4) miscellaneous.

SIDE EFFECTS OF MEDICATIONS

Biologics

Anti-tumor necrosis factor (TNF)-α drugs such as infliximab, adalimubab, certolizumab, etanercept, onercept[9] and anti-α4 integrin such as natalizumab and MLN020[10], generally referred to as biologic drugs, have all been tested in the treatment of IBD, but etanercept, onercept and MLN020 have not been registered for clinical use, and thus have only had a limited exposure. Although their use has been occasionally associated with the induction or exacerbation of several neurological diseases in IBD patients[11], the diagnosis of a possible causal relationship is usually made on the time-correlation between the use of the drug and the appearance of the neurological manifestation[12]. In a screening study by the Food and Drug Administration on Adverse Event Reporting System (FAERS), Deepak *et al*[13] reported 772 distinct neurological adverse effects secondary to TNF-α inhibitor exposure over a 10-year period. Thus, particular attention should be placed when an IBD patient on biologic therapy develops neurological symptoms, looking for a cause-effect relationship[14]. Conversely, in patients with neurological diseases before the start of biologic therapy, a neurological consultation must be performed and possible alternative therapies, including surgery, should be considered.

PML

Because of its severity and high mortality rate, PML is the most feared neurological complication for patients treated with biologics. Although its occurrence was originally observed in patients treated with natalizumab in combination with β1α interferon for MS, this disease has also been reported in IBD patients treated with natalizumab only and also in very few cases undergoing anti-TNF therapy[7]. PML is a demyelinating disease caused by the reactivation of JCV, a virus with specific tropism for glial cells in the brain[15]. JCV may be reactivated from sites of latency in lymphoid tissues in conditions characterized by reduced immune surveillance[16]. The explanation for the appearance of PML in natalizumab-treated patients is apparently related to both the action of JCV and to a probable increased release of infected lymphocytes from bone marrow determined by the binding of α4β1 integrin with the drug[17].

Yousry *et al*[18], in a review of about 3000 patients treated with natalizumab for MS, CD or rheumatoid arthritis, did not find any case of PML, thus suggesting a risk of PML of < 1 per 1000 patients treated for a mean time of 17.9 mo. The drug, at this moment, is not approved for IBD in Europe, while its use is confined to patients showing no response to anti-TNF agents in the United States. Clinicians should consider PML in the presence of visual defects (45% of all cases) and/or mental impairment (38% of all cases) such as dementia, confusion and personality changes. Indeed, cognitive impairment and behavioral changes frequently are the earliest clinical manifestations of PML[14]. A motor weakness may also be present.

The diagnosis is confirmed by magnetic resonance imaging (MRI), which reveals white matter lesions with typical T2 and T1 signals[18]. Cerebrospinal fluid (CSF) examination is usually normal but polymerase chain reaction (PCR) amplification of the JCV DNA is an important diagnostic tool. It is debated whether patients with IBD, similarly to what happens for MS patients treated with natalizumab, should undergo serial testing for anti-JCV antibodies before and during treatment either with natalizumab or with anti-TNF[18]. Indeed, patients on natalizumab can be risk stratified for the development of PML based on JCV antibody status, history of immunosuppressive drug and duration of natalizumab treatment. Singh *et al*[14] described that all cases of natalizumab-induced PML occurred in patients who were JCV antibody positive. The seroprevalence of JCV-specific IgG in healthy blood donors is estimated to be 50% by 30 years of age and this percentage in-

creases to 60% by 70 years of age[14]. The risk increases with duration of natalizumab therapy, particularly after 24 mo, but some cases have been reported after only 6 mo of therapy[14].

Discontinuation of natalizumab is recommended at the first suspicion of PML[14], and plasmapheresis is the recommended therapy to remove natalizumab, accelerate desaturation of the targeted α4-integrin receptors and restore leukocyte transmigration[14]. When immunosuppression is rapidly reverted in cases of natalizumab-associated PML, an exuberant immune response may occur: this condition has been termed immune reconstitution inflammatory syndrome[14]. The response targeting JCV is evident 2-6 wk later in the CNS and it often results in paradoxical worsening of PML symptoms[14]. High dose corticosteroids are recommended if clinical and radiographic worsening are noted several weeks after immune restoration. Despite these treatments, the clinical outcome of natalizumab-induced PML patients is poor with a reported mortality of 60% in patients with 6 mo follow-up[14].

Posterior reversible encephalopathy syndrome and similar diseases

Zamvar *et al*[19] described a posterior reversible encephalopathy syndrome in a 14-year-old boy affected by CD following infliximab infusion with generalized tonic-clonic seizures and visual disturbances probably caused by occipital lobe involvement. The patient recovered after drug discontinuation by which time he returned to normal.

Brigo *et al*[20] also described a 74-year-old man with CD, without a prior history of seizures, presenting with a seizure after the second infusion of infliximab and caused by a reversible encephalopathy syndrome, an acute form of encephalopathy characterized by headache, seizures and area of increased T2 signal in the posterior quadrants of the brain on MRI[20]. A direct correlation between seizures and infliximab treatment is likely in these cases because a sharp clinical improvement occurred 7 d after infliximab discontinuation.

Faivre *et al*[21] described a 64-year-old woman with CD developing encephalitis associated with acute neuropathy after two infusions of infliximab. The patient had acute anterograde memory deficiency associated with epileptic episodes without infectious, vascular, tumor or toxic causes[21]. MRI showed bilateral hippocampal hypersignals suggestive of limbic encephalitis. The clinical symptoms disappeared after infliximab withdrawal and seizures never relapsed even after discontinuation of antiepileptic drugs.

CNS vasculitis

The most common autoimmune manifestation associated with anti-TNF therapy is the development of antinuclear antibodies and anti ds-DNA autoantibodies without an associated clinical syndrome[22]. A systemic lupus erythematosus (SLE)/lupus-like syndrome occurs in some of these patients and it has been named anti-TNF-induced lupus (ATIL). This syndrome[23] usually shows a high prevalence of anti-dsDNA antibodies (> 90%) and a low prevalence of anti-histone antibodies (57%) in contrast to what is usually seen in drug-related lupus syndromes. As in patients with spontaneous lupus, patients with ATIL may develop vasculitis. This was the case in a 53-year-old woman with ileo-colonic CD in whom adalimubab therapy was complicated by the development of SLE with CNS vasculitis. The patient showed headache, drowsiness, visual defect in her right eye associated with pleural, peritoneal and pericardial effusion 4 mo after initiation of adalimubab therapy at a dose of 40 mg subcutaneously every other week. Brain MRI showed features suggestive of cerebral vasculitis.

Ramos-Casals *et al*[24] found 233 cases of autoimmune diseases possibly induced by TNF-targeted therapies with a prevalence of vasculitis and lupus respectively of 48% and 39%. Among the 92 patients with ATIL, CNS vasculitis was observed only in two patients (1 treated with infliximab for CD and 1 treated with etanercept for rheumatoid arthritis). In the case reported by Vannucchi *et al*[22], the autoantibodies disappeared and the clinical picture returned to normal 6 mo after anti-TNF withdrawal.

MS

The development or exacerbations of MS[25] or CNS demyelination are well-described neurological complications of TNF-α antagonist therapy. TNF-α antagonist might be effective for inflammatory neurological disorders such as MS because elevated levels of TNF-α have been demonstrated in serum or CSF of patients with MS[26].

Microglia and macrophages in the CNS secrete TNF-α with a direct role in the pathogenesis and demyelination of MS[27]. There are two forms of TNF-α: a trans-membrane protein (tmTNF) and a soluble form (sTNF); both interact with two distinct receptors, TNFR1 and TNFR2[28]. In the first stages of MS, TNF-α is involved in demyelination, while in later stages it is fundamental for remyelination[29]. In a double-blind placebo-controlled phase II human study of lenercept, a recombinant TNFR1 fusion protein, more lenercept-treated patients experienced exacerbations compared to placebo patients and these exacerbations occurred early, leading to early study termination[30].

There are several case reports of the development of MS[31] or CNS demyelination during treatment with TNF-α antagonist such as infliximab or adalimubab. The mechanisms of induction or exacerbation of MS and/or CNS demyelination are unknown. One possible hypothesis is that TNF-α has anti-inflammatory effects that may contribute to "off" signals in MS. The "on/off" balance of TNF-mediated signals is relevant to MS and the removal of TNF-α might potentiate the disease[30]. Anti TNF-α drugs, particularly infliximab, do not appear to cross the blood-brain barrier and neutralize local TNF-α-mediated tissue injury. Nevertheless, infliximab causes enhanced permeability of the barrier increasing the activation of myelin-specific peripheral autoreactive T cells[32]. TNFR2 function is important for the enhance-

ment of remyelination and the use of TNF-α antagonists may inhibit the tmTNF-TNFR2 axis[33].

The Mayo Clinic reported one case of MS in 500 CD patients treated with infliximab and another patient has been reported among 651 IBD patients treated with infliximab in the Danish Crohn Colitis database; three further cases of MS were reported in Edinburgh's experience of 620 patients treated with IFX[34]. It is unclear whether these demyelinating events are coincidental or causally associated with the use of TNF-α antagonists because the interval between the administration of anti-TNF-α agents and the appearance of symptoms varies greatly. Most researchers have reported that the average time between the beginning of treatment and the onset of neurological symptoms is about 5 mo[35].

Demyelinating neuropathies

The proposed pathogenesis of anti-TNFα-associated neuropathies encompasses both T cells and humoral immune attacks against peripheral nerve myelin, vasculitis-induced nerve ischemia and inhibition of signaling support for axons[11]. Most of these neuropathies improve over a period of several months after withdrawal of the drug, with or without additional immunomodulating treatment[11].

Guillan-Barré syndrome and its variant Miller-Fisher syndrome

Guillan-Barré syndrome (GBS)[36] and Miller-Fisher syndrome[37] are two types of demyelinating peripheral neuropathies reported during treatment with TNF-α antagonists. GBS is a post-infectious, immune-mediated disease, generally presenting as an acute inflammatory demyelinating polyneuropathy[36] characterized by ascending paralysis with rapid, progressive, symmetric limb weakness and areflexia.

The annual incidence is 1.5 cases/100000 and the mortality rate is about 5%. Approximately 10% of patients are still severely disabled at 1 year after diagnosis. Miller-Fisher syndrome is a rare variant of it and its main manifestation is descending paralysis affecting the eye muscles with the triad ophthalmoplegia, ataxia and areflexia[37]. It is possible that TNF-α antibodies unmask latent infections or cause an increased susceptibility to infections triggering or worsening the autoimmune demyelinating processes[11]. Two-thirds of GBS cases are associated with bacterial or viral infections such as *Campylobacter jejuni*, *Mycoplasma pneumoniae*, *Haemophilus influenzae*, cytomegalovirus (CMV), Epstein-Barr virus (EBV) and varicella zoster virus[37].

It is important to remember that GBS may be an independent extraintestinal manifestation of IBD induced by vasculitis, malnutrition or vitamin deficiencies[37]. Drug withdrawal is always suggested in the management of these patients and, in troublesome cases, cyclophosphamide or intravenous immunoglobulin is needed[37]. Vadikolias *et al*[38] reported a case of a 40-year-old man affected by CD developing autoimmune demyelinating acute paraplegia 4 mo after starting infliximab therapy. Some authors suggest that search subclinical demyelinating processes before initiating anti-TNF-α therapy, particularly in young patients[5], should become a recommended standard practice. Shin *et al*[36] described 15 cases of GBS identified from the postmarketing database of anti-TNF (9 patients on infliximab, 5 on etanercept, and 1 on adalimubab). The symptoms were reported between 6 wk and 2 years after the start of these therapies[36]. Thirteen patients have been subjected to regular follow-up and 12 showed a partial or complete resolution after adequate therapy for GBS[36]. Deepak *et al*[13], in the FAERS about neurological events in patients treated with TNF-α inhibitors, reported 153 cases out of a total of 772 with neurological manifestations related to anti-TNF-α therapy.

Lewis-Summer syndrome

Nancey *et al*[39] reported two cases of Lewis-Summer syndrome (LSS), also called multifocal acquired demyelinating sensory and motor neuropathy, related to the use of infliximab. LSS is a rare, dysimmune, multifocal peripheral nerve disorder described for the first time in 1982 by Lewis *et al*[40], and characterized by asymmetric multifocal and sensory involvement of the nerve roots and trunks of the upper and lower limbs. The disease should be suspected in the presence of distal, asymmetric weakness affecting the upper or lower limbs with initial sensory impairment followed by motor involvement[39]. An electromyographic study showing persistent multifocal conduction blocks allows the diagnosis[39]. LSS involves only peripheral nerves without any damage to central myelin[39].

Multifocal motor neuropathy with conduction block

Barber *et al*[41] reported one case of multifocal motor neuropathy with conduction block (MMNCB) following treatment with infliximab. This is an asymmetric motor neuropathy with diagnostic features including the presence of multifocal partial motor conduction block and the presence of blood anti-GM1 antibodies in 50% of cases. No more than 10 cases of MMNCB associated with anti-TNF therapy have been reported in the international literature. The rate of progression of demyelinating neuropathies is highly variable ranging from a few days to many years and the recovery is not always certain after drug withdrawal[41].

Following suspicion of peripheral neuropathies related to biologic therapy, a consultation with a neurologist should always be suggested for a differential diagnosis between small fiber polyneuropathies and an axonal sensory-motor neuropathies (SM-PNs) characterized by areflexia, sensory ataxia, minor cutaneous sensory deficiency (distal dysesthesia)[41] and variable degree of motor dysfunction.

Ischemia and inhibition of signaling support for axonal transport are the mechanisms proposed for secondary axonal loss. It is conceivable that the adverse effect

of TNF-α antagonists on peripheral nerves is cumulative and, therefore, the severity of neuropathy is proportional to the total dose of the drug received[3]. These data emphasize the importance of long-term vigilance during the course of the treatment with TNF-α antagonists. In most cases, drug withdrawal resolves the complication[3].

Chronic inflammatory demyelinating polyneuropathy

Some cases of Chronic inflammatory demyelinating polyneuropathy (CIDP) have been reported during anti-TNF therapy[42,43], appearing 4-17 mo after initiation of infliximab, with the possible presence of GM2 antibodies in the serum. CIDP is characterized by weakness in the proximal and distal extremity muscle groups associated with bilateral foot drop and a stocking-glove pattern mostly localized in the lower extremities[42]. Electrodiagnostic studies reveal progressive acquired demyelinating sensory and motor peripheral polyneuropathy. Withdrawal of the offending agent does not always reverse the immune process and chronic immunotherapy may be needed to control the inflammatory process and improve clinical outcome[42].

Infections of the nervous system

B-lymphocyte depletion in immunocompromised patients causes meningitis by encapsulated bacterial pathogens, while T-lymphocyte depletion or impaired macrophage function cause the development of infections by intracellular pathogens such as fungi, particularly *Aspergillus* and *Nocardia*, viruses such as herpes simplex virus (HSV), JCV, CMV, human herpes virus 6 and parasites such as *Toxoplasma gondii* (*T. gondii*)[44].

In general, although anti-TNF agents may increase the risk of infections, even at neurological levels, and particularly for intracellular organisms, several observations indicate that the risk of opportunistic infections is greatly increased when patients are treated with more than one immunosuppressant drug. Thus, the main recommendation originating from these observations is to limit multiple immune suppression to the shortest time possible.

Patients with infections of the nervous system may present with many clinical manifestations including meningeal signs, mass lesions, encephalopathy, seizures and stroke-like presentation[44].

CNS fungal infections

CNS infections by *Aspergillus* are usually characterized by mass lesions such as brain abscesses or by cerebral infarction, and more rarely by meningitis[44]. *Cryptococcus neoformans* may show as subacute meningitis with fever and headache without neck stiffness. Mass cerebral lesions usually have a subacute or chronic presentation while meningitis and encephalitis have a more acute presentation. In patients with meningeal signs and/or encephalopathy, a lumbar puncture should be performed with strain culture or serology of CSF[44]. MRI should always be performed in patients with a strong clinical suspicion of encephalopathy. MRI should be performed and tissue biopsy sampling may be always considered[44] in patients with cerebral mass lesions, for a differential diagnosis between tuberculosis, lymphoma and toxoplasmosis. Lumbar puncture should also be always performed in these cases because a positive EBV PCR in the CSF suggests the presence of CNS lymphoma.

Meningococcal meningoencephalitis

Majumder and Kumar[45] described a case of a 51-year-old woman affected by CD showing meningococcal meningitis during treatment with certolizumab in clinical remission for 6 mo. Meningococcal vaccination is safe and it should always be considered in high-risk IBD patients, particularly in those subjected to biologic therapy[46].

Listeria infection

In 2000 Morelli *et al*[47] described for the first time a *Listeria* infection complicating infliximab therapy in a CD patient. *Listeria monocytogenes* (*L. monocytogenes*) is a Gram-positive, rod-shaped, facultative intracellular organism. About 1%-5% of all healthy adults are asymptomatic carriers of *L. monocytogenes*. In the United States, 2500 cases/year are reported and the mortality rate is 15%-30%. Mortality of *Listeria* meningoencephalitis infection, associated with sepsis, is particularly high in pregnant women, neonates and immunocompromised subjects, where it may reach 33% of cases[47]. Listeriosis is a foodborne infection caused by ingestion of soft cheeses, unpasteurized milk, unwashed vegetables, ready-to-eat foods such as hot dogs and cold cuts, and it may be more frequent in populations eating raw food such as those living in Africa and Asia. It is important to wash the hands and to scrub fruits and vegetables[47]. TNF-α, produced by monocytes, macrophages, lymphocytes, and fibroblasts, has a crucial role against *L. monocytogenes* as well as all other intracellular organisms. In fact, TNF-α is crucial in host resistance against intracellular organisms by mediating local inflammation to control infection[47]. Generally, blood culture becomes positive for Gram-positive bacilli after 32 h and lumbar puncture reveals a high percentage of polymorphonuclear leukocytes. Ampicillin and gentamicin is the treatment of choice for listeriosis[47]. On the basis of the above considerations, it is important that patients receiving anti-TNF therapy observe safe food practices and refrain from the above-mentioned food products.

Campylobacter fetus infection

Umehara *et al*[48] described *Campylobacter fetus* (*C. fetus*) meningitis in a CD patient on long-term steroid therapy after only one infusion of infliximab. *C. fetus* is a Gram-negative, mo(b)tile, bacterial species with a typical S-shaped rod morphology and it is particularly present in immunocompromised, pregnant women and neonates in whom it may cause endocarditis, thrombophlebitis, pneumonia, pleurisy and arthritis[49]. The bacterium lives in the intestinal flora of cattle and sheep where it causes

spontaneous abortions[50].

Toxoplasmosis

Young *et al*[51] described a case of cerebral toxoplasmosis during infliximab therapy associated with low doses of prednisone, methotrexate and leflunomide in a 36-year-old woman affected by severe rheumatoid arthritis. The most important neurological symptoms were diffuse headache, slurred speech, weakness in the left arm, and a grand mal seizure. MRI imaging showed two lesions within the right hemisphere and the diagnosis of *T. gondii* infection was obtained by brain biopsy[51]. *T. gondii* is an obligate intracellular parasite infecting up to a third of the world's population[51]. *T. gondii* infection is acquired by ingestion of food or water contaminated with oocysts shed by cats, or by eating undercooked or raw meat containing tissue cysts[52]. Primary infection is usually subclinical but, in some patients, cervical lymphadenopathy, ocular disease, encephalitis, myocarditis and pneumonitis may occur[52]. The disease may be life-threatening in immunocompromised patients, and CNS involvement has been described in AIDS patients[52], and patients receiving corticosteroid[52] and anti-TNF-α[52] therapy. Lassoued *et al*[53] described two cases of chorioretinitis related to *T. gondii* during anti-TNF therapy, with malaise, low-grade fever and visual defects as major complaints[52]. TNF-α has an important role in the protection against *T. gondii* infection, playing a synergic role with interferon γ[54]. Response to treatment for toxoplasmosis occurs early and, in patients with compatible MRI of the brain, empirical treatment should be started[51]; early response to treatment usually confirms toxoplasmosis diagnosis.

Nocardiosis

Wendling *et al*[55] described cerebral nocardiosis during adalimubab and methotrexate therapy for rheumatoid arthritis. A 63-year-old Caucasian man showed the appearance of subcutaneous nodules in the trunk with histological diagnosis of pyogenic granulomas, pulmonary nodules upon chest radiography, and neurological signs such as headache, vertigo, cerebellar dysarthria after 8 mo combined therapy[55]. Brain computed tomography (CT) and MRI showed two lesions with edema and a mass effect in the right parietal region and cerebellum. Surgical biopsies revealed a pyogenic abscess and the presence of *Nocardia farcinica*. Nocardiosis is caused by an opportunistic, aerobic, Gram-positive, filamentous bacterium of the order Actinomycetales. The most common species are *Nocardia asteroides*, *Nocardia brasiliensis*, and *Nocardia otitidis caviatum*. This bacterium has a long incubation period[55]. Modes of contamination include inhalation and direct inoculation through the skin[55]. Systemic nocardiosis is defined by the presence of two or more foci of infection[55]; the lung is the most common primary site of systemic nocardiosis (60%-80% of cases) and cerebral or other locations may occur in 20%-40% of cases. CNS involvement is responsible for the worst prognosis with a 75% rate of mortality, particularly in lupus patients[53]. TNF plays a role in the clearance of *Nocardia* in animal models[55]. However, nocardiosis is rare during anti-TNF-α therapy and only eight cases[55] have been reported among 300000 patients treated with anti-TNF agents in the United States. Anti-TNF-α may accelerate and disseminate previously undiagnosed nocardiosis, particularly when therapy comprises corticosteroids and methotrexate.

Herpes simplex infection

Herpes simplex encephalitis (HSE) has been reported in patients receiving TNF-α antagonist therapy[56]. Also, TNF-α inhibitor therapy appears to be associated with an increased risk of herpes zoster[57]. An increase in the risk of severe HSV infection is related to the use of TNF-α inhibitors because TNF is an important element of the innate immune response to HSV-1 encephalitis, as reported in animal models[58]. About 95% of all cases of HSE are attributed to HSV-1 and, only occasionally, HSV-2 has been described[59]. Bradford *et al*[56] has identified three adults affected by HSE during monoclonal antibody TNF-α inhibitors. The patients clinically showed altered mental status, such depression or reduced affective response, slow mental processing, memory disturbances, fever, meningismus and headache. In patients receiving TNF-α the clinical manifestations may be atypical. Brain MRI shows characteristic temporal lobe involvement, CSF PCR positive for HSV DNA, and the clinical picture significantly improves after acyclovir therapy[56]. More than 90% of adult patients with HSE have temporal lobe abnormalities on brain MRI and a positive HSV PCR at clinical presentation[60]. Weil *et al*[61] reported that these diagnostic tests might be initially negative in 5%-27% of patients if they are performed early in the disease course. Bradford *et al*[56] reported that two of three patients initially had normal brain MRI and negative results for CSF HSV PCR. Recently published guidelines[62] emphasize the need to repeat HSV PCR in 3-7 d if the results are initially negative and the clinical course is highly suggestive for HSE. Bradford *et al*[56] suggest that in patients on anti TNF-α therapy and a clinical presentation suggestive for HSE, empirical acyclovir treatment should be performed until the result of a second PCR. The mortality of HSE at 1 year is 14%-22% and most survivors have residual neurological and cognitive deficits[63].

Lu *et al*[64] reported a case of Bell's palsy caused by HSV in a 43-year-old woman on adalimumab therapy (40 mg biweekly) for CD for 3 years. The patient showed small painful erythematous ulcers on her oral mucosa and lips, fever and right-sided facial palsy suggestive of Bell's palsy secondary to HSV infection. Her symptoms disappeared after 7 d treatment with three 1-g tablets/d valacyclovir, associated with adalimubab withdrawal, but symptoms recurred after rechallenge with adalimubab. Bell's palsy is an idiopathic peripheral facial nerve paralysis and reactivation of HSV may play a major role

through inflammation of the facial nerve[65].

EBV infection

Nozaki *et al*[66] reported one case of Epstein-Barr encephalitis during TNF-α antagonist therapy but the patient also had concurrent HIV infection. Several studies have investigated the possibility of EBV reactivation in patients treated with infliximab but no significant increased risk emerged[67]. Lavagna *et al*[68], in a study of 60 patients with CD treated with infliximab, did not observe any EBV viremia or any clinical manifestations of EBV infection during and after treatment. Serum EBV DNA was never found in a series of EBV-IgG-positive patients treated with TNF-α blockers[69].

Cerebral tuberculosis

Tissot *et al*[70] described a 40-year-old man with CD treated with infliximab monotherapy for 16 mo complicated by the appearance of neurological symptoms such as blurred vision, and motor and sensitive deficiency of the right lower limb. Clinical examination showed a papular erythematous skin lesion localized on the left lateral cervical region and multiple cervical nodes. Cerebral MRI revealed multiple ring-enhancing lesions suggestive of tuberculoma, and chest-abdomen CT showed an upper lobes alveolar syndrome and multiple abdominal lymph nodes suggestive of miliary tuberculosis. This diagnosis was confirmed by skin lesion biopsy with evidence of giant cell granuloma, and PCR for *Mycobacterium tuberculosis* in cultures of bronchial washing lavage. The patient was subjected to classical anti-tuberculosis treatment, with progressive disappearance of all cerebral lesions and complete resolution of neurological symptoms. Therapy for cerebral tuberculosis should comprise four drugs (isoniazid, rifampicin, pyrazinamide and ethambutol) for 2 mo followed by isoniazid and rifampicin for at least 10 mo[70]. Andrisani *et al*[71], in their experience of 92 IBD patients who were candidates for anti-TNF therapy, suggested screening for high-risk latent tuberculosis reactivation during therapy by means of Quantiferon TB-Gold (QFT-G) and tuberculin skin test (TST) because both are useful for identification of high-risk patients.

Ocular nervous disorders

Anterior optic neuropathy[72] and retrobulbar demyelinating optic neuropathy[73] have also been reported as possible complications of infliximab therapy. The clinician should be alert to anterior optic neuropathy when patients, during anti-TNF therapy, develop bilateral simultaneous sudden visual loss with decreased visual acuity, without pain related to eye movements, and swollen optic discs with bilateral inferior arcuate defects upon ophthalmology evaluation. Retrobulbar (posterior) optic neuropathy shows the same symptoms of anterior optic neuropathy, perhaps with monolateral visual loss, and only an ophthalmologist may obtain the diagnosis with specific tests such as visual field and flash visual evoked potentials[73]. Risk factors such as stroke, arterial hypertension, diabetes, atherosclerosis and hypercholesterolemia should always be ruled out[74]. Anterior optic neuropathy generally appears early within the first three infusions of infliximab[74]. In the study of Tissot *et al*[70], three patients with infliximab-related anterior optic neuropathy were described; one had impaired visual loss after rechallenge with infliximab infusion, confirming the association between infliximab and optic nerve damage. To date, four cases of toxic, infliximab-related anterior optic neuropathy and 10 cases of retrobulbar optic neuritis have been described[75]. No patients with toxic anterior optic neuropathy improved after pulsed intravenous infusion of methylprednisolone while all 10 patients with retrobulbar optic neuritis did improve[73]. At this moment, more data are needed to evaluate the exact pathogenic mechanism and dose relationships between these ocular manifestations and infliximab[74]. Deepak *et al*[13], in a FAERS Study about neurological manifestations in patient on anti TNF-α therapy, reported 105 cases of optic neuritis (13.6%) in 772 patients with neurological manifestations during anti TNF-α therapy.

Steroids

Repeated or prolonged exposure to steroids may cause myopathy that needs to be differentiated from predominant involvement of large motor fibers[75]. Steroid therapy may also produce a psychotic condition[76]. Corticosteroid therapy increases the risk of infection in a dose-dependent fashion[77] and corticosteroid-treated patients with intestinal disease have a relative risk of lethal and nonlethal infections of 1.4 (95%CI: 1.1-1.7, $P = 0.02$). Doses of prednisone > 20 mg/d are associated with a twofold increase in overall relative risk of lethal or nonlethal infectious complications compared with controls ($P < 0.004$). *L. monocytogenes* sepsis and meningitis have been described in adult and pediatric patients treated with high doses of steroids with or without azathioprine[78]. The long-term use of steroids may also predispose to the development of *C. fetus* meningitis[78].

Sulfasalazine

Sulfasalazine is still used for both CD and UC because of its low cost/effectiveness ratio. However, its use is mostly hampered by the frequent occurrence of side effects and neurological complications have also been reported.

Severe neurotoxicity leading to drug withdrawal has been reported in < 5% of patients[79]. The neurological toxicity of the drug appears mainly to be related to folate deficiency[80]; a typical adverse result of chronic sulfasalazine intake through different mechanisms: oxidative damage to red cells leading to hemolysis[81]; inhibition of jejunal hydrolysis of pteroylpolyglutamates blocking absorption of dietary folates; and competition with the three enzymes (dehydrofolate reductase, serine transhydroxymethylase and methylene tetrahydrofolate reductase) mainly involved in folate metabolism. Patients with IBD are predisposed to hyperhomocysteinemia[80],

which is considered a risk factor for cardio-cerebrovascular events, and part of this condition might be related to the folate-depleting role of sulfasalazine.

Mechanisms other than folate deficiency appear to be present in patients treated with sulfasalazine who develop neurological disorders but the pathophysiological aspects are currently unknown[81]. Liedorp *et al*[82] described axonal polyneuropathy occurring after 2 years treatment with sulfasalazine without blood folic acid deficiency. Mut *et al*[83] noted a reversible encephalopathy after only 3 wk of sulfasalazine therapy, and the clinical symptoms and MRI lesions resolved completely after drug discontinuation. Gold *et al*[84] have suggested that the drug might also be implicated in the occurrence of MS.

Methotrexate

Methotrexate is an immunosuppressant with anti-folate activity[85]. It is highly ionized with low lipid solubility and it does not readily cross the blood-brain barrier. Methotrexate is a cell-cycle-specific agent that inhibits the enzyme dihydrofolate reductase, preventing the conversion of folic acid to tetrahydrofolic acid and thus inhibiting cell replication[85]. Methotrexate also causes a relative excess of homocysteine determining small-vessel vasculopathy[85]. The risk of neurotoxicity increases with higher doses.

The association between low oral weekly doses of methotrexate and the development of posterior reversible encephalopathy syndrome (PRES) has been described in only a few cases. PRES often shows nonspecific symptoms such as headache, seizures, visual disturbances including cortical blindness, altered mental status, and even coma. Seizures are present in up to 88%, visual disturbances in 60%, and headache with altered mental function in > 50% of patients. In these studies, oral methotrexate had been taken for 3-7 years before the onset of symptoms. The patient described by Hart *et al*[85] was exposed to a total dose of 1560 mg over 4 years. Thus, the association of neurotoxicity with long-term use of methotrexate suggests a cumulative toxic effect on the blood-brain barrier. Neuroimaging, particularly MRI, is essential to obtain a diagnosis: classical lesions are symmetrical and located in the subcortical and cortical areas of the posterior circulation. The frontal lobe, brainstem, basal ganglia, thalamus, and even the spinal cord may also be involved.

Methotrexate-associated neurotoxicity is often termed as leukoencephalopathy (LEP), frequently presenting as transient seizures[85]. Also, headache, confusion and disorientation may be present[86]. LEP is a structural alteration of cerebral white matter in which myelin suffers the most damage. The basic pathophysiological mechanisms leading to methotrexate-induced LEP are unknown but they are multifactorial and include adenosine accumulation, homocysteine elevation, and its excitatory effect on N-methyl-D-aspartate receptor and alteration in biopterin metabolism[85]. The white matter changes are strictly localized to the cerebellum and this selective site may be explained by the involvement of Purkinje cell axons[81]. Only a few methotrexate-induced white matter changes during oral treatment[87] have been reported and most of them appeared to be related to folic acid deficiency. Chronic folic acid supplementation is thus mandatory in patients undergoing methotrexate treatment to avoid, if possible, these neurological side effects.

Metronidazole

Metronidazole is a commonly used antibiotic in the treatment of IBD, particularly in patients affected by CD[88] with perianal involvement. Peripheral neuropathy is a well-documented side effect of the drug, reported in 21-39% of CD patients treated with metronidazole, especially in patients receiving > 1.5 g/d of the drug for > 30 d[89]. Thus, monitoring the neurological state of the patient during metronidazole therapy is strongly encouraged[88]. Both demyelinating and nondemyelinating neuropathies can be observed[89]. Metronidazole-induced neuropathies are characterized by sensory manifestations with occasional ataxic features and generally are transient and resolve completely on discontinuation of medication[14,89]. Peripheral neuropathies are one of the most frequent neurological complications described in IBD patients[78]. The incidence of spontaneous peripheral neuropathy in IBD patients varies from 0.9%[2] to 3.6%[11]. These conditions are also described in other sections of this paper as side effects of biological therapies and in autoimmune nervous system disorders. Indeed, polyneuropathies in IBD may result from multiple interactions between immune-mediated disorders, nutritional imbalances, malabsorption, weight loss, vitamin deficiencies and drug-induced changes[89]. The most important symptoms of peripheral neuropathy are paresthesia and increased threshold for temperature detection (the last sign is indicative of early neuropathy)[88]. Axonal polyneuropathy usually is characterized by sensory loss and dysesthesia in a glove-and-stocking distribution, and decreased or absent ankle jerks with infrequent motor involvement[84]. Small fiber nondemyelinating sensory neuropathy is characterized by subjective numbness and tingling in the absence of demonstrable abnormalities on electromyography and nerve conduction studies[72], and may be misdiagnosed as fibromyalgia upon typically normal electrophysiological testing[14]. IBD patients with restricted sensory involvement are usually younger than those with concomitant involvement of motor and sensory large fibers[90]. Only some of these patients (particularly patients with CD) have been previously treated with metronidazole, and the disease progressed also after drug discontinuation, suggesting its contributory but not causative role[76]. The physician should suspect peripheral neuropathy in cases of sensitive disturbances of the upper and lower extremities, ataxia and/or impairment of walking[84].

Chatzkel *et al*[91] described a 15-year-old girl affected by CD with ataxia and dysmetria 7 d after initiation of

treatment with metronidazole. Cranial MRI revealed bilateral symmetric T2/FLAIR hyperintense lesions of the dentate nuclei without contrast enhancement or restricted diffusion, and the lesions disappeared completely after drug discontinuation[91].

Cyclosporine A

Cyclosporine A is a cyclic polypeptide that interferes with the transcription of cytokines, causing the blocking of activation and maturation of various cell types involved in cell-mediated immunity[92]. This drug has been mostly used in severe refractory UC. Neurotoxicity is one of the major adverse events of this treatment, involving up to 25% of treated patients and including seizures, tremors, paresthesia, ataxia, motor deficits, aphasia, altered consciousness, and various degrees of visual and oculomotor disturbances[92]. The pathogenesis of these neurological side effects is poorly understood. Cyclosporine A may rarely cause accelerated hypertension leading to progressive reversible encephalopathy syndrome[14]; this condition is more frequent in patients with low total serum cholesterol[14].

Irreversible bilateral optic neuropathy has also been described[92] and two possible mechanisms have been proposed: direct toxicity to peripheral nerves, or thromboembolism leading to ischemic optic neuropathy[93].

Cerebellar atrophy[93] caused by cyclosporine A therapy performed despite hypomagnesemia may have its first manifestation in nystagmus. However, cyclosporine-A-induced neurotoxicity has also been described in the absence of known risk factors such as hypocholesterolemia, hypomagnesemia, previous seizure disorders and arterial hypertension[94]. The differences in neurological side effects between oral and intravenous cyclosporine A is another matter of uncertainty[95]. Cyclosporine A is insoluble in water and intravenous formulations are prepared in a polyoxyethilated (POE) castor oil and ethyl alcohol solution[96]. In *in vitro* experiments, 0.1% POE castor oil determines axonal swelling and degeneration while 0.001% POE castor oil may induce demyelination. It has thus been suggested that residues of ethylene or its polymerization products might at least contribute to the neurotoxicity of intravenous cyclosporine A *in vivo*. However, the Cosmetic Ingredient Review Expert Panel has concluded that these cosmetic ingredients (POE castor oil and its derivatives) are safe in practical use[96] and no serious neurotoxicity may be attributable to them.

Azathioprine

This drug has no specific neurotoxicity but it represents a predisposing factor to infection, particularly associated with its prolonged use. Spinal epidural abscess has been described as a complication of azathioprine therapy[97]. Spinal epidural abscess is a rare but known neurological complication of CD[97]; predisposing conditions are immunosuppressive therapy and the presence of intra-abdominal and/or retroperitoneal fistulas[97]. A high index of suspicion should be raised if the patient shows back pain during or immediately after a flare of CD, with or without neurological signs, because this condition is an alarm sign for the presence of inflamed paravertebral and spinal structures[89]. Spinal epidural abscess represents a neurosurgical emergency in the presence of unresponsive back pain or progressive neurological deterioration such as bowel and/or bladder dysfunction. In patients with spinal epidural abscess associated with bowel fistulas and psoas abscesses determined by CD, a combined and highly specialized medical and surgical approach is needed to prevent recurrence[98]. Prolonged antibiotic use is recommended also if cultures are negative.

Murai *et al*[99] reported a myelo-radiculitis determined by *Cryp. neoformans* in a UC patient on immunosuppressive therapy with azathioprine. Robineau *et al*[100] reported a case of HSE related to azathioprine therapy in a 28-year-old woman treated for 4 years[100]. The diagnosis was based on the presence of photophobia, headache, asthenia and nausea associated with nuchal rigidity; lumbar puncture revealed a marked increase of lymphocytes (98%) in cerebral fluid with a detection of HSV-1 DNA determined by PCR. Intravenous acyclovir administration (15 mg/kg every 8 h) for 3 wk completely resolved this complication.

CEREBROVASCULAR DISEASES

In general, the risk of both arterial and venous thrombosis[101], as well as of thromboembolic events, is significantly increased in IBD patients. As a consequence, thromboembolic complications have been reported in various organs, including the brain. Indeed, cerebrovascular disorders have been documented in 0.12%-4% of all IBD patients, and probably they represent the most frequently reported neurological complications[102]. Obviously, cerebrovascular or cardiovascular events and their sequelae may be of particular severity, especially if one considers the usually young age of IBD patients. The relative risk of stroke is higher in young patients, especially women and patients with CD[14]. Thus, a large number of studies addressing the possible underlying causes of this predisposition in IBD and proposing possible prophylactic and therapeutic strategies have been published in recent decades. As a whole, these studies suggest that active disease, even at an outpatient level, appears to be the most important predisposing factor, through many pathogenic factors activated by the ongoing inflammation. Indeed, the intimate inter-relationship between inflammation and coagulation has become clear in recent years with disease clinical and subclinical activity[103] shown to be associated with hypercoagulability related to various factors such as qualitative and quantitative abnormalities of platelets[104] and coagulation factors[94], decreased anticoagulant activity[100], hypofibrinolysis[105], malabsorption and hypercatabolism leading to vitamin B6 deficiency[106], endothelial changes[102] leading also to reduced activation of protein C, dehydration, and corticosteroid therapy[107].

The abundance of the findings showing an association between clinical activity and risk of thrombotic events has led to the introduction of antithrombotic

prophylaxis in the therapeutic guidelines of hospitalized patients with severe relapse.

However, although active disease is particularly associated with an increased risk of these complications, some cases of vascular accidents have been described during remission[101], suggesting that IBD represents a risk factor for thrombosis. The search for a possible genetic association between IBD and carriage of factor V Leiden, G20210A prothrombin and methylene tetrahydrofolate reductase mutations has provided negative results[102], thus suggesting that other, probably acquired although not related to inflammation, factors may indeed play a more important role. One of these factors might be hyperhomocysteinemia[80,81], which may derive from a lack of attention to nutritional status, although in many cases the presence of subclinical inflammation cannot be ruled out. It is important to consider a higher risk in postoperative state associated with the development of arterio-arterial embolism, cardioembolism, and *in situ* cerebral thrombosis[14].

Arterial thromboembolism

An increased risk of these complications is observed in patients with active UC and particularly in those with total colitis[108]; even if active disease is associated with an increased risk, some cases have been described during remission[100]. Men and women are equally affected. The cerebrovascular involvement appears to be more frequent among younger IBD patients, as reported by Houissa *et al*[101] who described four cases of arterial thrombosis in IBD and three of these were younger than 25 years. Intestinal inflammation may lead to increased risk for thrombosis through several pathways: by activating the coagulation cascade; decreasing anticoagulant activity; and inducing hypofibrinolysis, malabsorption and hypercatabolism with vitamin deficiencies that may lead to hyperhomocysteinemia - a well known risk factor for thrombosis[101]. Also dehydration, immobility, sepsis, surgery and corticosteroid therapy may determine cerebral thrombosis in IBD patients[101].

The neurological presentation of cerebral arterial thrombosis may vary from headache (95%), to mono- or bilateral paresis (43%), general or focal seizures (47%), or dysphasia (37%)[104]. The clinical sequelae of cerebral vascular thrombosis can be devastating, especially in young patients with active and complicated IBD, leading to high mortality and disability in about 60% of cases[101]. Conventional CT or MRI identifies the exact site of cerebral affected areas. At present, no guidelines are available for the treatment of cerebral thrombosis and stroke in IBD[108]. Low molecular weight heparin (LMWH) is the most common drug used for the prophylaxis and treatment of vascular thromboembolism.

Given the heightened risk of thromboembolism in patients with IBD, prophylaxis with LMWH is recommended in hospitalized IBD patients, considering exacerbation of the disease[14]. Long-term use of anticoagulant therapy in the treatment of arterial ischemic cerebral lesions is limited, although the presence of a hypercoagulable condition should always be considered an indication for lifelong anticoagulation with warfarin[107]. Thrombolysis with recombinant tissue plasminogen activator, urokinase or streptokinase should also be considered in early cerebral arterial ischemic conditions (within 3 h from development of clinical symptoms) and this procedure, in expert hands, may be considered safe and effective[109]. In selected cases, thrombectomy should also be considered[110]. Rapid evaluation and appropriate multidisciplinary consultation are required for optimal diagnosis and management.

Venous and sinus thrombosis

Cerebral venous and sinus thrombosis is a rare condition and accounts for about 1% of all strokes[111]. Cerebral venous and sinus thrombosis that concurrently develops with UC is rare[111] and they may be associated with abnormalities in the coagulation system[100]. Cerebral venous thrombosis appears to be more common in UC than in CD patients[112], and is more commonly localized in the superior sagittal sinus and lateral sinuses[113], although cortical venous thromboses have also been reported[113].

All patients with UC and cerebral venous thrombosis reported in literature are young, mostly men, without other risk factors[102]. Most patients have a pancolitis suggesting a role for increased endotoxemia and dehydration[102] as culprits for vascular thrombosis. The most frequent symptom is headache occurring in 75%-96% of patients[114]. The headache is often severe and diffuse and it usually precedes the appearance of neurological signs. A combination of focal defects, headache, seizures and altered consciousness is suggestive of cerebral venous thrombosis[114], although the presenting features are variable and the condition should be considered in any IBD patients with neurological symptoms, particularly during an active phase. Cerebral infarction is a dangerous complication and it appears when the thrombosis extends from the superior sagittal sinus to the superficial cerebral veins and their tributaries[114].

MRI studies in combination with MR venography are sensitive in identifying venous sinus occlusion[114]. The use of local endovascular thrombolytic agents may restore the flow more frequently and rapidly than heparin alone; however, there is no evidence of the superiority of this method and the risk of hemorrhage is high[114]. Warfarin is usually continued for at least 6 mo after a first episode of cerebral venous thrombosis, or longer in the presence of persisting predisposing factors[114]. The theoretical risk of intestinal bleeding due to anticoagulant therapy does not appear significant in practice[114]. Given the increased risk of thromboembolism in patients with IBD, aggressive mechanical and pharmacological deep vein thrombosis prophylaxis with LMWH is recommended in hospitalized patients[14].

IMMUNE-MEDIATED NEUROLOGICAL DISORDERS

MS

This topic has been already treated in this paper as a possible complication of biological therapies[25]. However, a possible spontaneous association between MS and IBD has been suspected for decades[115]. Indeed, the estimated prevalence of MS in the general population is about 0.1%, while in IBD patients the prevalence of MS has been reported at up to 0.5%[8,114,115], suggesting a 1.5-5-fold increase in the risk of having MS in IBD patients[30]. In evaluating these data, however, we should bear in mind that all studies involved a limited number of patients, thus yielding low statistical power. Also, results of the studies are greatly influenced by the methods used to look for an associated disease. Indeed, Geissler *et al*[116] observed hyperintensity of the white matter on brain MRI in almost half of the patients with IBD free of neurological symptoms compared to only 16% of healthy age-matched controls. MS has been reported to develop either before or after the clinical onset of IBD[116]. The nature of a possible pathogenic link between IBD and MS has not been identified, but a disturbance in functional T-cell subsets with aberrant proinflammatory activity of T helper 17 subsets has been suggested[2]. Animal studies[117] also suggest a link between the demyelinating lesions suggestive for MS or acute disseminated encephalomyelitis and the prothrombotic state characteristic of UC. Astrocytosis and exstensive perivenular loss of myelin have been described in rhesus monkeys suffering from colitis and cerebral venous thrombosis, and it is possible to speculate that the demyelinating lesions could have been the result of perivenular edema secondary to venous blockade[117]. Indeed, cerebral lesions in the monkey are identical to those observed in confluent leukoencephalitis and perivascular myelosis of the cerebral type; a demyelinating disease of monkeys[118]. Whatever the mechanisms of the underlying possible association between MS and IBD, brain MRI followed by a neurological consultation for further diagnostic work-up should be organized[2] as soon as a patient with a clinical history of UC or CD presents with an unexplained neurological symptom suggestive of MS, such as paresthesia in both arms, fingers, legs and toes, hyperesthesia of the fingertips and hyper-reflexia. Occasional, but clinically important, observations are those reporting that a demyelinating disease may be precipitated or aggravated by the use of infliximab for IBD[119].

Cerebral vasculitis

This topic has already been treated in the chapter on complications of biological therapies[22]. Cerebral vasculitis has been reported in association with UC[120] and can be considered a further cause of stroke. The association between UC and Takayasu's disease, particularly in Japanese patients with an HLA-B52, DR2 haplotype, is strong[121]. The pathogenetic process may be related to common immune-mediated mechanisms such as T-lymphocyte mediated cytotoxicity or immune complex deposition[121], or to a genetic susceptibility determined by patient's HLA status. The association of UC with perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCAs) or atypical ANCAs[122] suggests a common autoimmune etiology, but the existence of different antigenic recognitions by these antibodies in the two diseases is well established. Furthermore, UC-associated ANCAs lack antigenic specificity for proteinase-3 (PR3) or myeloperoxidase (MPO) and do not have the potential for the development of systemic vasculitis or for neutrophil activation[122]. Nevertheless, a p-ANCA (specific for MPO) positive UC patient affected by ischemic lesions in the white matter of the brain has been reported[122].

The clinical manifestations of cerebral vasculitis are hemiparesis, hemianopsia, personality changes, headache, aphasia, seizures, coma and progressive dementia[8]. These clinical manifestations may occur independently of the activity of the underlying bowel disease and, in some cases, they may appear even before the onset of IBD[123]. Cerebral MRI is always abnormal[8]. Nearly half of the reported patients have neurologic signs and symptoms developing during steroid therapy[8].

Necrotizing angiitis may show clinical manifestations similar to the acute hemorrhagic variant of acute disseminated encephalomyelitis (ADEM)[117]. ADEM is suggested to result from a transient autoimmune response directed against myelin or other autoantigens via molecular mimicry[117] caused by a defective epithelial barrier function in UC, leading to uncontrolled uptake of luminal antigens and stimulation of pathologic immune and inflammatory reactions[8]. The presence of lethargy should always suggest a diagnosis of ADEM.

Autoimmune myelopathy

Lossos *et al*[3] reported nine UC patients with neurological disorders and six of these had peripheral nerve disorders considered as acute inflammatory demyelinating polyradiculoneuropathy[3]. This study was limited by the absence of detailed information about CSF features and response to therapy.

Myelopathy, which may present as a slowly progressive systemic spastic paraparesis in the absence of a spinal sensory level, has been associated with UC in some case reports[3]; a link with human T-lymphotropic type 1-associated myelopathy has been proposed[124]. This syndrome may develop without spinal MRI abnormalities[3]; an immune-mediated inflammatory origin has been suggested but a possible association with the use of medications or nutritional deficiencies cannot be ruled out[3]. It is possible to consider this myelopathy or transverse myelitis as a part of a more widespread CNS disorder like MS or a vasculitis process as suggested by Ray *et al*[125].

Also, *Campylobacter jejuni* is linked to exacerbations of IBD and it may contribute to the development of autoimmune inflammatory demyelinating polyneuropathy[121].

It is noteworthy that in the patients with generalized peripheral neuropathy, a demyelinating pattern is present in 30%[125].

Myasthenia gravis

Myasthenia gravis (MG) is a typical immune-mediated disease in which T-lymphocyte function is abnormal, the thymus is enlarged, and circulating acetylcholine receptor antibodies are found[126]. MG is often associated with other autoimmune disorders such as alopecia, lichen planus, vitiligo and SLE[127]; diseases that are also observed in association with IBD. Tsuchiya *et al*[127] described an association between thymic abnormalities and IBD, with the presence of acetylcholine receptor antibodies[128]. Also, the lack of age-related involution of the thymus observed in MG has also been reported in UC[127]. T cells obtained from the thymus of patients with MG and UC have reduced ratios of suppressor ($CD8^+$) to helper ($CD4^+$) T cells compared with control subjects[128].

Diplopia and ptosis of the upper eyelid in IBD patients may be an initial manifestation of MG[129]. For the possible pathogenic association between the two diseases, intriguing observations on their therapeutic management have been reported: Finnie *et al*[126] reported a case of a patient with both MG and CD complicated by perianal disease whose bowel disease improved after thymectomy for severe uncontrolled MG. In contrast, Gower-Rousseau *et al*[130] described a patient with MG and UC in whom MG symptoms improved after proctocolectomy. Foroozan *et al*[129] reported a 21-year-old man with UC and binocular diplopia and ptosis due to MG; both ocular and gastrointestinal symptoms improved after plasmapheresis, azathioprine, prednisone and mestinon[3].

Autoimmune sensorineural hearing loss

Sensorineural hearing loss is probably an immunological manifestation of IBD[131]. The clinical manifestations of the disease are often bilateral and progressive[132]. The hearing level is unstable with periods of deterioration alternating to partial or complete clinical remission[132]. In general, the tendency is for gradual evolution towards permanent hearing loss[132]. Vestibular dysfunction symptoms such as disequilibrium and postural instability may accompany auditory symptoms and these symptoms may have a sudden onset[133].

Hearing loss generally occurs between 2 mo and 17 years after the diagnosis of UC. Hearing loss may appear during both active and remission stages of the disease and it does not have a parallel evolution. Hearing loss, if not treated, is recurrent until leading to complete deafness[133]. A more strict collaboration with ear, nose and throat specialists should be encouraged to research this condition of autoimmune inner ear. Kumar *et al*[134] noted, in a controlled audiometry study, a significant sensorineural hearing loss in UC patients compared with controls. A subclinical sensorineural hearing loss may also be present in CD patients[135].

The clinical response to steroid and immunosuppressive therapy suggests that an autoimmune process causes inner ear impairment[136]. This condition is most frequently bilateral but may also be unilateral. Aggressive treatment should be started as early as possible. At present[134], it is impossible to have detailed information about the beginning and the time-course of hearing loss because this condition is underdiagnosed. Karmody *et al*[133] reported that patients with hearing loss performed their first medical evaluation usually 3 years after the first clinical manifestation.

MISCELLANEOUS

Peripheral neuropathies

Polyneuropathies have previously been dealt as frequent side effects of metronidazole therapy but they may also occur as spontaneous extraintestinal manifestations of IBD. Gondim *et al*[89] identified 33 patients (18 with CD and 15 with UC) affected by polyneuropathies. Male sex was highly predominant (78% in CD and 75% in UC). Neurological symptoms appeared long after the diagnosis of IBD. In 33% of CD patients and 40% of UC patients, the polyneuropathy was correlated with disease activity.

In CD patients, demyelinating neuropathy was present in five patients while a nondemyelinating neuropathy was present in the other 13: small-fiber polyneuropathy (SF-PN) in two and large-fiber axonal neuropathy (LF-AN) in 11. Four patients with UC showed peripheral demyelinating neuropathies. Eleven UC patients showed nondemyelinating neuropathies: four with SF-PN and seven with LF-PN. The diagnosis of SF-PN was obtained by means of skin biopsy[89].

Oliveira *et al*[4] studied 82 IBD patients (31 with CD and 51 with UC). Five CD patients (4 women) (16.1%) had SF-PN and the first symptom was sensory abnormality. Weakness was mild and mostly located in the distal legs. Neurological examination showed decreased or absent ankle jerks, and decreased distal vibration and pinprick. Ten UC patients (19.6%) had mild axonal sensory motor polyneuropathies (SM-PN). Blood B12 levels were < 200 pmol/L in two of these 10 patients, between 200-300 pmol/L in a further two, two patients had diabetes, one was affected by hypothyroidism, and positive blood rheumatoid factor was present in two[4]. Fourteen percent of UC patients were taking steroid therapy[4].

Oliveira *et al*[4] concluded that SM-PN in UC patients was more common in women, in older individuals, in patients developing the disease later in life and in subjects with a body mass index < 18.5. The authors[4] also noted that the association with autoimmune diseases such as diabetes mellitus, hypothyroidism and positive rheumatoid factor was more frequent in UC patients with SF-PN than in CD patients.

Sassi *et al*[137], in a study of 102 consecutive patients with IBD, reported nine patients (8.8%) with peripheral neuropathies. Bernstein *et al*[138], in a large study of administrative healthcare data from the Manitoba County

between 1984 and 2003 on 8072 patients with IBD (3879 with UC and 4193 with CD), reported peripheral neuropathies in 2.4% of UC patients and 2.34 of CD patients compared to 1.35% in the general population. Peripheral neuropathies usually do not respond to treatment of the underlying IBD[14].

Cranial nerve palsies

Cranial nerve palsies can be observed in patients with IBD. The Melkersson-Rosenthal syndrome[139] is defined by recurrent facial nerve palsy, fissuring of the tongue, and noncaseating tissue granulomas, and it has been described in association with CD[140]. The long intracranial course of the sixth nerve predisposes it to injury by a variety of abnormalities[139]. Karajeh *et al*[140] described a 27-year-old female smoker with a 12-d history of diplopia on right lateral gaze associated with retro-orbital pain before the clinical diagnosis of CD. This typical clinical presentation suggests a vascular sixth nerve palsy with a sudden onset of unilateral abduction deficit accompanied by retro-orbital pain and diplopia[140]. It is hypothesized that microvascular ischemic demyelination of a portion of the nerve is the most likely cause of this clinical condition[140]. This area of ischemic demyelination subsequently undergoes remyelination with clinical recovery[140]. Complete recovery within 2-3 mo is generally observed[140].

Optical neuropathy, as outlined above, has a clinical presentation with a bilateral optic disc swelling and it is a rare condition prevalently associated with CD[141]. Romero Aroca *et al*[142] reported a 27-year-old woman affected by UC and optic neuritis that resolved after mesalamine administration. Optic neuropathy may be attributed to peripapillary inflammation, optic disc ischemia, or intracranial hypertension[142].

A local vasculitis process or a general hypercoagulability condition can determine optic nerve ischemia. Modern imaging techniques usually allow one to exclude dural venous sinus thrombosis[113]; a serious cerebrovascular complication of IBD described in another chapter of this paper. Another clinical condition is the severe erosive arthritis of the craniocervical junction that should always be considered in IBD patients with persistent neck pain because a late diagnosis may determine severe neurological defects[143].

Epilepsy

The association between IBD and epilepsy is uncertain[1]. Epileptic seizure in IBD patients may be related to structural or metabolic causes[3]. Seizures may be generalized tonic-clonic complex, simple, partial or even multiple. A MEDLINE search[8] using "epilepsy and ulcerative colitis" as keywords found only five case reports dating back to the early 1970s. According to the literature, epilepsy appears more frequently associated with CD than with UC[1].

Muscle disorders

Granulomatous myositis and myopathies are associated with both CD and UC patients[144]; these manifestations usually appear during exacerbations of IBD[145]. Orbital myositis is a nonspecific, localized orbital inflammatory process in which one or more extraocular muscles are involved[145]. Clinically, orbital myositis is characterized by acute pain exacerbated by eye movements; diplopia, swelling of the eyelid, conjunctival injection, and exophthalmos may also be present[145]. The diagnosis is based on clinical history and imaging[145]. This disease responds to steroid therapy[146]. Orbital myositis is rare in IBD[146]; sarcoidosis, Wegener's granulomatosis, rheumatoid arthritis and Lyme disease should be considered in the differential diagnosis[146].

Nonspecific orbital inflammation includes histological forms that are more difficult to distinguish such as an idiopathic granulomatous and idiopathic sclerosing pseudotumor[147]. Nonspecific orbital inflammation appears to arise from an immune reaction in the orbit secondary to a neighboring zone of inflammation or a distant autoimmune reaction[148]. It has been associated with CD, diabetes, rheumatoid arthritis and Graves' disease[148].

MRI is the method of choice to study the orbital region in orbital myositis because it is able to show the typical diffuse enlargement of extraocular muscles with blurred margins and to rule out other lesions such as tumor/pseudotumor infiltration, apical extension, cavernous sinus involvement and intracranial disease[146]. Orbital myositis most commonly affects the superior recti, the medial recti and oblique muscles.

Classic migraine

The prevalence of migraine in patients with IBD remains unknown[149]. Migraine is associated with systemic endothelial dysfunction[150], which is also proposed as a possible pathogenic factor in IBD[151]. Oliveira *et al*[4] found that headache is the most common neurological complaint reported both in CD and UC patients, in 54.8% and 56.9% of patients, respectively. In most patients headache is not disabling and it is often associated with IBD relapse and treatment[4]. Ford *et al*[149], in a study performed on about 100 IBD patients (77% women and 23% men, 66% with CD and 27% with UC) by an ID-Migraine questionnaire[148], noted a 30% prevalence of migraine in IBD patients. Migraine was more prevalent in CD (36%) than in UC (14.8%). In UC patients, the prevalence of migraine in women did not approach that of the general population (12.5% *vs* 18.2%), whereas the prevalence in men greatly exceeded that of the general population (18% *vs* 6.5%)[152].

Currently, migraine is underdiagnosed in IBD patients, although it causes limited ability to work, study and perform routine activities in a high percentage of IBD patients[146]. It is important to consider that migraine with aura may be an independent risk factor for ischemic stroke in women[153] because they have a 13.7-fold increased risk for silent infarction in the posterior territory and 2.1-fold increased risk for deep white matter lesions[154].

Sleep disturbances, depression and anxiety, chronic fatigue syndrome

Sleep disturbances are recognized reactions to inflamma-

tion[155] and may represent the first response to acute inflammation[156], although they may persist during clinical remission.

Depression and anxiety occur in IBD and they may involve sleep disturbances and asthenia[156]. Assessment of depression and anxiety in IBD is mandatory because these conditions may contribute to the subjective perception of poor quality of life[157]. Anxiety may be associated with more intense disease activity[158].

Fatigue in IBD may be considered as a consequence of the disease and its treatment[157]. Iron deficiency may cause fatigue and sleep disorders in patients with CD[157]. Patients can express fatigue even when bowel disease is inactive[159]. Minderhoud *et al*[160] showed that the fatigue score remains high during disease remission compared with normal control subjects. Lipton *et al*[152], in a study of French IBD patients, found that the scores obtained on the Multidimensional Fatigue Inventory in IBD patients were similar to those affected by cancer. Fatigue may be considered a part of the core symptoms of depression[161] and the use of antidepressants may improve chronic fatigue syndrome. In summary, although chronic fatigue syndrome is frequent in IBD patients, its precise pathogenesis is not clear and this most probably reflects a multifactorial nature of the syndrome.

Restless legs syndrome

Restless legs syndrome (RLS) is a CNS disorder characterized by a compelling urge to move the legs at rest; it contributes to sleep disturbances and impaired quality of life. RLS may be primary (idiopathic and familial) or secondary to many disorders such as pregnancy, end-stage renal failure, iron deficiency anemia, rheumatoid arthritis, diabetes, Parkinson's disease, fibromyalgia, IBD[162], gastric resection, chronic liver disease, and irritable bowel syndrome[163]. The diagnosis of RLS must be made according to the following four criteria established by the International RLS Study Group[164]: (1) an urge to move the legs, usually accompanied or caused by uncomfortable and unpleasant sensations in the legs; (2) the urge to move or unpleasant sensations begin or worsen during periods of rest or inactivity; (3) the urge to move or unpleasant sensations are partially relieved by movement at least as long as the activity continues; and (4) the urge to move or unpleasant sensations are worse in the evening or night than during the day or only occur in the evening or night.

Weinstock *et al*[162] reported a prevalence of 30% for RLS in patients with CD and noted that it appeared during or after the onset of CD symptoms, suggesting a link between CD and RLS. This association might at least partly explain the presence of both fatigue and sleep disturbances in CD patients[161]. Patients with iron deficiency anemia are at particularly high risk of developing RLS[165] because low brain iron concentration may play a role in altered dopamine levels, providing a unifying condition for most cases of the syndrome[166]. In fact, inflammatory conditions such as CD cause an increased secretion of proinflammatory cytokines [*i.e.*, interleukin (IL)-6][167] which in turn causes increased hepcidin production[163], leading to iron deficiency in the CNS as a cause of RLS[168]. Small intestinal bacterial overgrowth, which may be observed in CD patients may also cause RLS by an inflammatory state supported by IL-6 and hepcidin as in MS patients[169]; in these cases, antibiotic therapy might be beneficial[169].

Wernicke encephalopathy

Wernicke encephalopathy is a neurological complication determined by vitamin B1 deficiency[170]. Hahn *et al*[170] have reported vitamin B1 deficiency in a young patient with CD receiving total parenteral nutrition without vitamin replacement. Larnaout *et al*[171] reported a CD patient with Wernicke encephalopathy under normal enteral nutrition. This patient died and autopsy revealed the following pathological cerebral lesions: hemorrhagic necrosis around the third and fourth ventricles with vascular proliferation and pericapillary hemorrhages; numerous small hemorrhagic infarctions in the central part of the corpus callosum; marked spongiosis, predominantly in the left cerebellar white matter; slight thickening of leptomeninges with some mononuclear cells; and absence of vascular thrombosis or inflammatory perivascular cuffing in the brain and spinal cord.

Vitamin B12 deficiency

Vitamin B12 deficiency due to terminal ileal disease or surgical resection in CD may cause subacute myelopathy combined with degeneration characterized by bilateral spastic paresis, loss of pressure and vibration sensation due to degeneration of the posterior and lateral columns of the spinal cord[172].

CONCLUSION

Neurological complications of IBD, either related to drug therapy or spontaneously associated with the disease, are relatively frequent and may contribute to a high degree of morbidity and permanent damage. They are also frequently difficult to recognize and diagnose, due to their frequently unclear clinical expression. For these reasons, knowledge of the different presentations as well as of differential diagnosis and therapeutic possibilities is important for the gastroenterologist dealing with IBD patients. This paper is thus aimed at providing interested physicians with an in-depth review of the main features of neurological complications of IBD.

REFERENCES

1 **Greenstein AJ**, Janowitz HD, Sachar DB. The extra-intestinal complications of Crohn's disease and ulcerative colitis: a study of 700 patients. *Medicine* (Baltimore) 1976; **55**: 401-412 [PMID: 957999]

2 **de Lau LM**, de Vries JM, van der Woude CJ, Kuipers EJ, Siepman DA, Sillevis Smitt PA, Hintzen RQ. Acute CNS white matter lesions in patients with inflammatory bowel disease. *Inflamm Bowel Dis* 2009; **15**: 576-580 [PMID: 19023898 DOI: 10.1002/ibd.20792]

3 **Lossos A**, River Y, Eliakim A, Steiner I. Neurologic aspects of inflammatory bowel disease. *Neurology* 1995; **45**: 416-421 [PMID: 7898687]
4 **Oliveira GR**, Teles BC, Brasil EF, Souza MH, Furtado LE, de Castro-Costa CM, Rola FH, Braga LL, Gondim Fde A. Peripheral neuropathy and neurological disorders in an unselected Brazilian population-based cohort of IBD patients. *Inflamm Bowel Dis* 2008; **14**: 389-395 [PMID: 17924556]
5 **Gupta G**, Gelfand JM, Lewis JD. Increased risk for demyelinating diseases in patients with inflammatory bowel disease. *Gastroenterology* 2005; **129**: 819-826 [PMID: 16143121]
6 **Mohan N**, Edwards ET, Cupps TR, Oliverio PJ, Sandberg G, Crayton H, Richert JR, Siegel JN. Demyelination occurring during anti-tumor necrosis factor alpha therapy for inflammatory arthritides. *Arthritis Rheum* 2001; **44**: 2862-2869 [PMID: 11762947]
7 **Van Assche G**, Van Ranst M, Sciot R, Dubois B, Vermeire S, Noman M, Verbeeck J, Geboes K, Robberecht W, Rutgeerts P. Progressive multifocal leukoencephalopathy after natalizumab therapy for Crohn's disease. *N Engl J Med* 2005; **353**: 362-368 [PMID: 15947080]
8 **Scheid R**, Teich N. Neurologic manifestations of ulcerative colitis. *Eur J Neurol* 2007; **14**: 483-493 [PMID: 17437605]
9 **Rutgeerts P**, Sandborn WJ, Feagan BG, Reinisch W, Olson A, Johanns J, Travers S, Rachmilewitz D, Hanauer SB, Lichtenstein GR, de Villiers WJ, Present D, Sands BE, Colombel JF. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med* 2005; **353**: 2462-2476 [PMID: 16339095]
10 **Feagan BG**, Greenberg GR, Wild G, Fedorak RN, Paré P, McDonald JW, Dubé R, Cohen A, Steinhart AH, Landau S, Aguzzi RA, Fox IH, Vandervoort MK. Treatment of ulcerative colitis with a humanized antibody to the alpha4beta7 integrin. *N Engl J Med* 2005; **352**: 2499-2507 [PMID: 15958805]
11 **Stübgen JP**. Tumor necrosis factor-alpha antagonists and neuropathy. *Muscle Nerve* 2008; **37**: 281-292 [PMID: 18041052]
12 **Zois CD**, Katsanos KH, Kosmidou M, Tsianos EV. Neurologic manifestations in inflammatory bowel diseases: current knowledge and novel insights. *J Crohns Colitis* 2010; **4**: 115-124 [PMID: 21122494 DOI: 10.1016/j.crohns.2009.10.005]
13 **Deepak P**, Stobaugh DJ, Sherid M, Sifuentes H, Ehrenpreis ED. Neurological events with tumour necrosis factor alpha inhibitors reported to the Food and Drug Administration Adverse Event Reporting System. *Aliment Pharmacol Ther* 2013; **38**: 388-396 [PMID: 23802849]
14 **Singh S**, Kumar N, Loftus EV, Kane SV. Neurologic complications in patients with inflammatory bowel disease: increasing relevance in the era of biologics. *Inflamm Bowel Dis* 2013; **19**: 864-872 [PMID: 22552994 DOI: 10.1002/ibd.23011]
15 **Ghosh S**, Goldin E, Gordon FH, Malchow HA, Rask-Madsen J, Rutgeerts P, Vyhnálek P, Zádorová Z, Palmer T, Donoghue S. Natalizumab for active Crohn's disease. *N Engl J Med* 2003; **348**: 24-32 [PMID: 12510039]
16 **Berger JR**, Koralnik IJ. Progressive multifocal leukoencephalopathy and natalizumab--unforeseen consequences. *N Engl J Med* 2005; **353**: 414-416 [PMID: 15947082]
17 **Langer-Gould A**, Atlas SW, Green AJ, Bollen AW, Pelletier D. Progressive multifocal leukoencephalopathy in a patient treated with natalizumab. *N Engl J Med* 2005; **353**: 375-381 [PMID: 15947078]
18 **Yousry TA**, Major EO, Ryschkewitsch C, Fahle G, Fischer S, Hou J, Curfman B, Miszkiel K, Mueller-Lenke N, Sanchez E, Barkhof F, Radue EW, Jäger HR, Clifford DB. Evaluation of patients treated with natalizumab for progressive multifocal leukoencephalopathy. *N Engl J Med* 2006; **354**: 924-933 [PMID: 16510746]
19 **Zamvar V**, Puntis JW. Re: "Posterior reversible encephalopathy syndrome following infliximab infusion". *J Pediatr Gastroenterol Nutr* 2010; **50**: 353 [PMID: 20190611 DOI: 10.1097/MPG.0b013e3181a9195d]
20 **Brigo F**, Bongiovanni LG, Cerini R, Manganotti P, Storti M, Fiaschi A. Infliximab-related seizures: a first case study. *Epileptic Disord* 2011; **13**: 214-217 [PMID: 21636356 DOI: 10.1684/epo.2011.0439]
21 **Faivre A**, Franques J, De Paula AM, Gutierrez M, Bret S, Aubert S, Attarian S, Pouget J. Acute motor and sensory axonal neuropathy and concomitant encephalopathy during tumor necrosis factor-alpha antagonist therapy. *J Neurol Sci* 2010; **291**: 103-106 [PMID: 20116808 DOI: 10.1016/J.jns.2010.01.004. Epub]
22 **Vannucchi V**, Grazzini M, Pieralli F, Giannotta M, Biagioni C, Nozzoli C. Adalimumab-induced lupus erythematosus with central nervous system involvement in a patient with Crohn's disease. *J Gastrointestin Liver Dis* 2011; **20**: 201-203 [PMID: 21725519]
23 **Williams EL**, Gadola S, Edwards CJ. Anti-TNF-induced lupus. *Rheumatology* (Oxford) 2009; **48**: 716-720 [PMID: 19416947 DOI: 10.1093/rheumatology/kep080]
24 **Ramos-Casals M**, Brito-Zerón P, Muñoz S, Soria N, Galiana D, Bertolaccini L, Cuadrado MJ, Khamashta MA. Autoimmune diseases induced by TNF-targeted therapies: analysis of 233 cases. *Medicine* (Baltimore) 2007; **86**: 242-251 [PMID: 17632266]
25 **Lozeron P**, Denier C, Lacroix C, Adams D. Long-term course of demyelinating neuropathies occurring during tumor necrosis factor-alpha-blocker therapy. *Arch Neurol* 2009; **66**: 490-497 [PMID: 19364934 DOI: 10.1001/archneurol]
26 **Lin J**, Ziring D, Desai S, Kim S, Wong M, Korin Y, Braun J, Reed E, Gjertson D, Singh RR. TNFalpha blockade in human diseases: an overview of efficacy and safety. *Clin Immunol* 2008; **126**: 13-30 [PMID: 17916445]
27 **Kassiotis G**, Kollias G. Uncoupling the proinflammatory from the immunosuppressive properties of tumor necrosis factor (TNF) at the p55 TNF receptor level: implications for pathogenesis and therapy of autoimmune demyelination. *J Exp Med* 2001; **193**: 427-434 [PMID: 11181695]
28 **Titelbaum DS**, Degenhardt A, Kinkel RP. Anti-tumor necrosis factor alpha-associated multiple sclerosis. *AJNR Am J Neuroradiol* 2005; **26**: 1548-1550 [PMID: 15956528]
29 **Andreadou E**, Kemanetzoglou E, Brokalaki Ch, Evangelopoulos ME, Kilidireas C, Rombos A, Stamboulis E. Demyelinating Disease following Anti-TNFa Treatment: A Causal or Coincidental Association? Report of Four Cases and Review of the Literature. *Case Rep Neurol Med* 2013; **2013**: 671935 [PMID: 23762678 DOI: 10.1155/2013/671935]
30 TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group. *Neurology* 1999; **53**: 457-465 [PMID: 10449104 DOI: 10.1212/WNL.53.3.457]
31 **Pfueller CF**, Seipelt E, Zipp F, Paul F. Multiple sclerosis following etanercept treatment for ankylosing spondylitis. *Scand J Rheumatol* 2008; **37**: 397-399 [PMID: 18720264 DOI: 10.1080/03009740802136164]
32 **Robinson WH**, Genovese MC, Moreland LW. Demyelinating and neurologic events reported in association with tumor necrosis factor alpha antagonism: by what mechanisms could tumor necrosis factor alpha antagonists improve rheumatoid arthritis but exacerbate multiple sclerosis? *Arthritis Rheum* 2001; **44**: 1977-1983 [PMID: 11592357]
33 **Fromont A**, De Seze J, Fleury MC, Maillefert JF, Moreau T. Inflammatory demyelinating events following treatment with anti-tumor necrosis factor. *Cytokine* 2009; **45**: 55-57 [PMID: 19109035 DOI: 10.1016/j.cyto.2008.11.002]
34 **Lees CW**, Ali AI, Thompson AI, Ho GT, Forsythe RO, Marquez L, Cochrane CJ, Aitken S, Fennell J, Rogers P, Shand AG, Penman ID, Palmer KR, Wilson DC, Arnott ID, Satsangi J. The safety profile of anti-tumour necrosis factor therapy in inflammatory bowel disease in clinical practice:

analysis of 620 patient-years follow-up. *Aliment Pharmacol Ther* 2009; **29**: 286-297 [PMID: 19132970 DOI: 10.1111/J.1365-2036.2008.03882.x]
35 **Magnano MD**, Robinson WH, Genovese MC. Demyelination and inhibition of tumor necrosis factor (TNF). *Clin Exp Rheumatol* 2004; **22**: S134-S140 [PMID: 15552527]
36 **Shin IS**, Baer AN, Kwon HJ, Papadopoulos EJ, Siegel JN. Guillain-Barré and Miller Fisher syndromes occurring with tumor necrosis factor alpha antagonist therapy. *Arthritis Rheum* 2006; **54**: 1429-1434 [PMID: 16645971]
37 **Cesarini M**, Angelucci E, Foglietta T, Vernia P. Guillain-Barrè syndrome after treatment with human anti-tumor necrosis factorα (adalimumab) in a Crohn's disease patient: case report and literature review. *J Crohns Colitis* 2011; **5**: 619-622 [PMID: 22115384 DOI: 10.1016/j.crohns.2011.06.010]
38 **Vadikolias K**, Kouklakis G, Heliopoulos I, Argyropoulou P, Papanas N, Tzilonidou M, Prassopoulos P, Piperidou H. Acute paraplegia after the initiation of anti-tumour necrosis factor-alpha therapy for Crohn's disease. *Eur J Gastroenterol Hepatol* 2007; **19**: 159-162 [PMID: 17273002]
39 **Nancey S**, Bouhour F, Boschetti G, Magnier C, Gonneau PM, Souquet JC, Kaiserlian D, Flourie B. Lewis and Sumner syndrome following infliximab treatment in Crohn's disease: a report of 2 cases. *Inflamm Bowel Dis* 2010; **16**: 1450-1453 [PMID: 20128005 DOI: 10.1002/ibd.21202]
40 **Lewis RA**, Sumner AJ, Brown MJ, Asbury AK. Multifocal demyelinating neuropathy with persistent conduction block. *Neurology* 1982; **32**: 958-964 [PMID: 7202168]
41 **Barber CE**, Lee P, Steinhart AH, Lazarou J. Multifocal motor neuropathy with conduction block following treatment with infliximab. *J Rheumatol* 2010; **37**: 1778-1780 [PMID: 20675855 DOI: 10.3899/jrheum.100072]
42 **Alshekhlee A**, Basiri K, Miles JD, Ahmad SA, Katirji B. Chronic inflammatory demyelinating polyneuropathy associated with tumor necrosis factor-alpha antagonists. *Muscle Nerve* 2010; **41**: 723-727 [PMID: 20405504 DOI: 10.1002/mus.21584]
43 **Richez C**, Blanco P, Lagueny A, Schaeverbeke T, Dehais J. Neuropathy resembling CIDP in patients receiving tumor necrosis factor-alpha blockers. *Neurology* 2005; **64**: 1468-1470 [PMID: 15851749]
44 **Viget N**, Vernier-Massouille G, Salmon-Ceron D, Yazdanpanah Y, Colombel JF. Opportunistic infections in patients with inflammatory bowel disease: prevention and diagnosis. *Gut* 2008; **57**: 549-558 [PMID: 18178610 DOI: 10.1136/gut.2006.114660]
45 **Majumder S**, Kumar A. Meningococcal meningoencephalitis after certolizumab pegol treatment in a patient with Crohn's disease. *J Crohns Colitis* 2013; **7**: e19 [PMID: 22742971 DOI: 10.1016/j.crohns.2012.06-012]
46 **Dezfoli S**, Melmed GY. Vaccination issues in patients with inflammatory bowel disease receiving immunosuppression. *Gastroenterol Hepatol* (N Y) 2012; **8**: 504-512 [PMID: 23293563]
47 **Morelli J**, Wilson FA. Does administration of infliximab increase susceptibility to listeriosis? *Am J Gastroenterol* 2000; **95**: 841-842 [PMID: 10710107]
48 **Umehara Y**, Kudo M, Kawasaki M. Campylobacter fetus meningitis in a patient with Crohn's disease. *Inflamm Bowel Dis* 2009; **15**: 645-646 [PMID: 18798576 DOI: 10.1002/ibd.20686]
49 **Francioli P**, Herzstein J, Grob JP, Vallotton JJ, Mombelli G, Glauser MP. Campylobacter fetus subspecies fetus bacteremia. *Arch Intern Med* 1985; **145**: 289-292 [PMID: 3977489]
50 **Sauerwein RW**, Bisseling J, Horrevorts AM. Septic abortion associated with Campylobacter fetus subspecies fetus infection: case report and review of the literature. *Infection* 1993; **21**: 331-333 [PMID: 8300253]
51 **Young JD**, McGwire BS. Infliximab and reactivation of cerebral toxoplasmosis. *N Engl J Med* 2005; **353**: 1530-1531; discussion 1530-1531 [PMID: 16207863]
52 **Azevedo VF**, Pietrovski CF, de Almeida Santos M. [Acute toxoplasmosis infection in a patient with ankylosing spondylitis treated with adalimumab: a case report]. *Reumatismo* 2010; **62**: 283-285 [PMID: 21253622]
53 **Lassoued S**, Zabraniecki L, Marin F, Billey T. Toxoplasmic chorioretinitis and antitumor necrosis factor treatment in rheumatoid arthritis. *Semin Arthritis Rheum* 2007; **36**: 262-263 [PMID: 17067660]
54 **Chang HR**, Grau GE, Pechère JC. Role of TNF and IL-1 in infections with Toxoplasma gondii. *Immunology* 1990; **69**: 33-37 [PMID: 2107144]
55 **Wendling D**, Murad M, Mathieu S, Berger E, Rumbach L. Systemic nocardiosis in a case of rheumatoid arthritis treated with tumor necrosis factor blockers. *J Rheumatol* 2008; **35**: 539-542 [PMID: 18322977]
56 **Bradford RD**, Pettit AC, Wright PW, Mulligan MJ, Moreland LW, McLain DA, Gnann JW, Bloch KC. Herpes simplex encephalitis during treatment with tumor necrosis factor-alpha inhibitors. *Clin Infect Dis* 2009; **49**: 924-927 [PMID: 19681709 DOI: 10.1086/605498]
57 **Strangfeld A**, Listing J, Herzer P, Liebhaber A, Rockwitz K, Richter C, Zink A. Risk of herpes zoster in patients with rheumatoid arthritis treated with anti-TNF-alpha agents. *JAMA* 2009; **301**: 737-744 [PMID: 19224750 DOI: 10.1001/jama.2009.146]
58 **Sergerie Y**, Rivest S, Boivin G. Tumor necrosis factor-alpha and interleukin-1 beta play a critical role in the resistance against lethal herpes simplex virus encephalitis. *J Infect Dis* 2007; **196**: 853-860 [PMID: 17703415]
59 **Omland LH**, Vestergaard BF, Wandall JH. Herpes simplex virus type 2 infections of the central nervous system: A retrospective study of 49 patients. *Scand J Infect Dis* 2008; **40**: 59-62 [PMID: 17852910]
60 **Domingues RB**, Fink MC, Tsanaclis AM, de Castro CC, Cerri GG, Mayo MS, Lakeman FD. Diagnosis of herpes simplex encephalitis by magnetic resonance imaging and polymerase chain reaction assay of cerebrospinal fluid. *J Neurol Sci* 1998; **157**: 148-153 [PMID: 9619637]
61 **Weil AA**, Glaser CA, Amad Z, Forghani B. Patients with suspected herpes simplex encephalitis: rethinking an initial negative polymerase chain reaction result. *Clin Infect Dis* 2002; **34**: 1154-1157 [PMID: 11915008]
62 **Tunkel AR**, Glaser CA, Bloch KC, Sejvar JJ, Marra CM, Roos KL, Hartman BJ, Kaplan SL, Scheld WM, Whitley RJ. The management of encephalitis: clinical practice guidelines by the Infectious Diseases Society of America. *Clin Infect Dis* 2008; **47**: 303-327 [PMID: 18582201 DOI: 10.1086/589747]
63 **Hjalmarsson A**, Blomqvist P, Sköldenberg B. Herpes simplex encephalitis in Sweden, 1990-2001: incidence, morbidity, and mortality. *Clin Infect Dis* 2007; **45**: 875-880 [PMID: 17806053]
64 **Lu LX**, Marshall JK. Bell's palsy in a patient receiving adalimumab for Crohn's disease. *J Crohns Colitis* 2013; **7**: e115 [PMID: 22868065 DOI: 10.1016/j.crohns.2012.07.017]
65 **Kennedy PG**. Herpes simplex virus type 1 and Bell's palsy-a current assessment of the controversy. *J Neurovirol* 2010; **16**: 1-5 [PMID: 20113184 DOI: 10.3109/1355028090552446]
66 **Nozaki K**, Silver RM, Stickler DE, Abou-Fayssal NG, Giglio P, Kamen DL, Daniel R, Judson MA. Neurological deficits during treatment with tumor necrosis factor-alpha antagonists. *Am J Med Sci* 2011; **342**: 352-355 [PMID: 21876428 DOI: 10.1097/maj.0b013e31822b7bb8]
67 **Torre-Cisneros J**, Del Castillo M, Castón JJ, Castro MC, Pérez V, Collantes E. Infliximab does not activate replication of lymphotropic herpesviruses in patients with refractory rheumatoid arthritis. *Rheumatology* (Oxford) 2005; **44**: 1132-1135 [PMID: 15927999]
68 **Lavagna A**, Bergallo M, Daperno M, Sostegni R, Costa C, Leto R, Crocellà L, Molinaro G, Rocca R, Cavallo R, Pera A. Infliximab and the risk of latent viruses reactivation in

active Crohn's disease. *Inflamm Bowel Dis* 2007; **13**: 896-902 [PMID: 17345605]
69 **McKeown E**, Pope JE, Leaf S. Epstein-Barr Virus (EBV) Prevalence and the Risk of Reactivation in Patients with Inflammatory Arthritis Using Anti-TNF Agents and in those who are Biologic Naive. *Open Rheumatol J* 2009; **3**: 30-34 [PMID: 19639039 DOI: 10.2174/1874312900903010030]
70 **Tissot C**, Couraud S, Meng L, Girard P, Avrillon V, Gérinière L, Perrot E, Souquet PJ. Life-threatening disseminated tuberculosis as a complication of treatment by infliximab for Crohn's disease: report of two cases, including cerebral tuberculomas and miliary tuberculosis. *J Crohns Colitis* 2012; **6**: 946-949 [PMID: 22749231 DOI: 10.1016/j.crohns.2012.02.018]
71 **Andrisani G**, Armuzzi A, Papa A, Marzo M, Felice C, Pugliese D, De Vitis I, Rapaccini GL, Guidi L. Comparison of Quantiferon-TB Gold versus tuberculin skin test for tuberculosis screening in inflammatory bowel disease patients. *J Gastrointestin Liver Dis* 2013; **22**: 21-25 [PMID: 23539386]
72 **ten Tusscher MP**, Jacobs PJ, Busch MJ, de Graaf L, Diemont WL. Bilateral anterior toxic optic neuropathy and the use of infliximab. *BMJ* 2003; **326**: 579 [PMID: 12637403]
73 **Simsek I**, Erdem H, Pay S, Sobaci G, Dinc A. Optic neuritis occurring with anti-tumour necrosis factor alpha therapy. *Ann Rheum Dis* 2007; **66**: 1255-1258 [PMID: 17456525]
74 **Chan JW**, Castellanos A. Infliximab and anterior optic neuropathy: case report and review of the literature. *Graefes Arch Clin Exp Ophthalmol* 2010; **248**: 283-287 [PMID: 19916016 DOI: 10.1007/500417-009-1227-y]
75 **Owczarek J**, Jasińska M, Orszulak-Michalak D. Drug-induced myopathies. An overview of the possible mechanisms. *Pharmacol Rep* 2005; **57**: 23-34 [PMID: 15849374]
76 **Rutgeerts PJ**. Review article: the limitations of corticosteroid therapy in Crohn's disease. *Aliment Pharmacol Ther* 2001; **15**: 1515-1525 [PMID: 11563990]
77 **Stuck AE**, Minder CE, Frey FJ. Risk of infectious complications in patients taking glucocorticosteroids. *Rev Infect Dis* 1989; **11**: 954-963 [PMID: 2690289]
78 **Sobrevilla LA**, Tedeschilg JF, Kantrowitz W. Listeria monocytogenes meningitis as a complication of steroid therapy. *BMQ* 1962; **13**: 62-66 [PMID: 13914712]
79 **Watkinson G**. Sulphasalazine: a review of 40 years' experience. *Drugs* 1986; **32** Suppl 1: 1-11 [PMID: 2877847]
80 **Cattaneo M**, Vecchi M, Zighetti ML, Saibeni S, Martinelli I, Omodei P, Mannucci PM, de Franchis R. High prevalence of hyperchomocysteinemia in patients with inflammatory bowel disease: a pathogenic link with thromboembolic complications? *Thromb Haemost* 1998; **80**: 542-545 [PMID: 9798965]
81 **Oldenburg B**, Van Tuyl BA, van der Griend R, Fijnheer R, van Berge Henegouwen GP. Risk factors for thromboembolic complications in inflammatory bowel disease: the role of hyperhomocysteinaemia. *Dig Dis Sci* 2005; **50**: 235-240 [PMID: 15745078]
82 **Liedorp M**, Voskuyl AE, Van Oosten BW. Axonal neuropathy with prolonged sulphasalazine use. *Clin Exp Rheumatol* 2008; **26**: 671-672 [PMID: 18799104]
83 **Mut SE**, Kutlu G, Ucler S, Erdal A, Inan LE. Reversible encephalopathy due to sulfasalazine. *Clin Neuropharmacol* 2008; **31**: 368-371 [PMID: 19050416 DOI: 10.1097/WNF.0b013e31817f125d]
84 **Gold R**, Kappos L, Becker T. Development of multiple sclerosis in patient on long-term sulfasalazine. *Lancet* 1990; **335**: 409-410 [PMID: 1968133]
85 **Hart C**, Kinney MO, McCarron MO. Posterior reversible encephalopathy syndrome and oral methotrexate. *Clin Neurol Neurosurg* 2012; **114**: 725-727 [PMID: 22225972 DOI: 10.1016/j.clineuro.2011.12.008]
86 **Salkade PR**, Lim TA. Methotrexate-induced acute toxic leukoencephalopathy. *J Cancer Res Ther* 2012; **8**: 292-296 [PMID: 22842379]
87 **Koppen H**, Wessels JA, Ewals JA, Treurniet FE. Reversible leukoencephalopathy after oral methotrexate. *J Rheumatol* 2012; **39**: 1906-1907 [PMID: 22942312 DOI: 10.3899/jrheum]
88 **Duffy LF**, Daum F, Fisher SE, Selman J, Vishnubhakat SM, Aiges HW, Markowitz JF, Silverberg M. Peripheral neuropathy in Crohn's disease patients treated with metronidazole. *Gastroenterology* 1985; **88**: 681-684 [PMID: 2981752]
89 **Gondim FA**, Brannagan TH, Sander HW, Chin RL, Latov N. Peripheral neuropathy in patients with inflammatory bowel disease. *Brain* 2005; **128**: 867-879 [PMID: 15705608]
90 **Ståhlberg D**, Bárány F, Einarsson K, Ursing B, Elmqvist D, Persson A. Neurophysiologic studies of patients with Crohn's disease on long-term treatment with metronidazole. *Scand J Gastroenterol* 1991; **26**: 219-224 [PMID: 1849313]
91 **Chatzkel JA**, Vossough A. Metronidazole-induced cerebellar toxicity. *Pediatr Radiol* 2010; **40**: 1453 [PMID: 19915830 DOI: 10.1007/500247-009-1453-9]
92 **Berden JH**, Hoitsma AJ, Merx JL, Keyser A. Severe central-nervous-system toxicity associated with cyclosporin. *Lancet* 1985; **1**: 219-220 [PMID: 2857289]
93 **Porges Y**, Blumen S, Fireman Z, Sternberg A, Zamir D. Cyclosporine-induced optic neuropathy, ophthalmoplegia, and nystagmus in a patient with Crohn disease. *Am J Ophthalmol* 1998; **126**: 607-609 [PMID: 9780114]
94 **Thompson CB**, June CH, Sullivan KM, Thomas ED. Association between cyclosporin neurotoxicity and hypomagnesaemia. *Lancet* 1984; **2**: 1116-1120 [PMID: 6150182]
95 **Rosencrantz R**, Moon A, Raynes H, Spivak W. Cyclosporine-Induced neurotoxicity during treatment of Crohn's disease: lack of correlation with previously reported risk factors. *Am J Gastroenterol* 2001; **96**: 2778-2782 [PMID: 11569712]
96 Final report on the safety assessment of Ricinus Communis (Castor) Seed Oil, Hydrogenated Castor Oil, Glyceryl Ricinoleate, Glyceryl Ricinoleate SE, Ricinoleic Acid, Potassium Ricinoleate, Sodium Ricinoleate, Zinc Ricinoleate, Cetyl Ricinoleate, Ethyl Ricinoleate, Glycol Ricinoleate, Isopropyl Ricinoleate, Methyl Ricinoleate, and Octyldodecyl Ricinoleate. *Int J Toxicol* 2007; **26** Suppl 3: 31-77 [PMID: 18080873 DOI: 10.1080/1091581071663150]
97 **Gelfenbeyn M**, Goodkin R, Kliot M. Sterile recurrent spinal epidural abscess in a patient with Crohn's disease: a case report. *Surg Neurol* 2006; **65**: 178-184; discussion 184 [PMID: 16427419]
98 **Wallace JR**, Luchi M. Crohn's disease complicated by meningitis and spinal epidural abscess. *Clin Infect Dis* 1995; **20**: 1428-1429 [PMID: 7620039]
99 **Murai H**, Tokunaga H, Kubo I, Kawajiri M, Iwaki T, Taniwaki T, Kira J. Myeloradiculitis caused by Cryptococcus neoformans infection in a patient with ulcerative colitis: a neuropathological study. *J Neurol Sci* 2006; **247**: 236-238 [PMID: 16815466]
100 **Robineau O**, Enrico J, Lemaire X, Poissy J, Legout L, Senneville E, Colombel JF, Yazdanpanah Y. Herpes simplex virus meningoencephalitis in a patient with Crohn's disease on azathioprine therapy. *Am J Gastroenterol* 2010; **105**: 240-241 [PMID: 20054331 DOI: 10.1038/ajg.2009.544]
101 **Houissa F**, Salem M, Bouzaidi S, Rejeb MB, Mekki H, Debbeche R, Moussa A, Trabelsi S, Said Y, Najjar T. Cerebral thrombosis in inflammatory bowel disease: a report of four cases. *J Crohns Colitis* 2011; **5**: 249-252 [PMID: 21575890 DOI: 10.1016/j.crohns.2010.12.010]
102 **Miehsler W**, Reinisch W, Valic E, Osterode W, Tillinger W, Feichtenschlager T, Grisar J, Machold K, Scholz S, Vogelsang H, Novacek G. Is inflammatory bowel disease an independent and disease specific risk factor for thromboembolism? *Gut* 2004; **53**: 542-548 [PMID: 15016749]
103 **Koenigs KP**, McPhedran P, Spiro HM. Thrombosis in inflammatory bowel disease. *J Clin Gastroenterol* 1987; **9**: 627-631 [PMID: 3443729]
104 **Saibeni S**, Spina L, Vecchi M. Exploring the relationships between inflammatory response and coagulation cascade

in inflammatory bowel disease. *Eur Rev Med Pharmacol Sci* 2004 ; **8**: 205-208 [PMID: 15638231]
105 **Saibeni S**, Bottasso B, Spina L, Bajetta M, Danese S, Gasbarrini A, de Franchis R, Vecchi M. Assessment of thrombin-activatable fibrinolysis inhibitor (TAFI) plasma levels in inflammatory bowel diseases. *Am J Gastroenterol* 2004; **99**: 1966-1970 [PMID: 15447757]
106 **Saibeni S**, Cattaneo M, Vecchi M, Zighetti ML, Lecchi A, Lombardi R, Meucci G, Spina L, de Franchis R. Low vitamin B(6) plasma levels, a risk factor for thrombosis, in inflammatory bowel disease: role of inflammation and correlation with acute phase reactants. *Am J Gastroenterol* 2003; **98**: 112-117 [PMID: 12526945]
107 **Faioni EM**, Ferrero S, Fontana G, Gianelli U, Ciulla MM, Vecchi M, Saibeni S, Biguzzi E, Cordani N, Franchi F, Bosari S, Cattaneo M. Expression of endothelial protein C receptor and thrombomodulin in the intestinal tissue of patients with inflammatory bowel disease. *Crit Care Med* 2004; **32**: S266-S270 [PMID: 15118529]
108 **Joshi D**, Dickel T, Aga R, Smith-Laing G. Stroke in inflammatory bowel disease: a report of two cases and review of the literature. *Thromb J* 2008; **6**: 2 [PMID: 18358077 DOI: 10.1186/1477.9560-6-2]
109 **Koutroubakis IE**. Therapy insight: Vascular complications in patients with inflammatory bowel disease. *Nat Clin Pract Gastroenterol Hepatol* 2005; **2**: 266-272 [PMID: 16265230]
110 **Markowitz RL**, Ment LR, Gryboski JD. Cerebral thromboembolic disease in pediatric and adult inflammatory bowel disease: case report and review of the literature. *J Pediatr Gastroenterol Nutr* 1989; **8**: 413-420 [PMID: 2651640]
111 **Musio F**, Older SA, Jenkins T, Gregorie EM. Case report: cerebral venous thrombosis as a manifestation of acute ulcerative colitis. *Am J Med Sci* 1993; **305**: 28-35 [PMID: 8416679]
112 **Spina L**, Saibeni S, Battaglioli T, Peyvandi F, de Franchis R, Vecchi M. Thrombosis in inflammatory bowel diseases: role of inherited thrombophilia. *Am J Gastroenterol* 2005; **100**: 2036-2041 [PMID: 16128949]
113 **Saadatnia M**, Fatehi F, Basiri K, Mousavi SA, Mehr GK. Cerebral venous sinus thrombosis risk factors. *Int J Stroke* 2009; **4**: 111-123 [PMID: 19383052 DOI: 10.1111/j.1747-4949.2009.00260.x]
114 **Stam J**. Thrombosis of the cerebral veins and sinuses. *N Engl J Med* 2005; **352**: 1791-1798 [PMID: 15858188]
115 **Minuk GY**, Lewkonia RM. Possible familial association of multiple sclerosis and inflammatory bowel disease. *N Engl J Med* 1986; **314**: 586 [PMID: 3945303]
116 **Geissler A**, Andus T, Roth M, Kullmann F, Caesar I, Held P, Gross V, Feuerbach S, Schölmerich J. Focal white-matter lesions in brain of patients with inflammatory bowel disease. *Lancet* 1995; **345**: 897-898 [PMID: 7707814]
117 **Menge T**, Hemmer B, Nessler S, Wiendl H, Neuhaus O, Hartung HP, Kieseier BC, Stüve O. Acute disseminated encephalomyelitis: an update. *Arch Neurol* 2005; **62**: 1673-1680 [PMID: 16286539]
118 **Garg RK**. Acute disseminated encephalomyelitis. *Postgrad Med J* 2003; **79**: 11-17 [PMID: 12566545]
119 **Thomas CW**, Weinshenker BG, Sandborn WJ. Demyelination during anti-tumor necrosis factor alpha therapy with infliximab for Crohn's disease. *Inflamm Bowel Dis* 2004; **10**: 28-31 [PMID: 15058523]
120 **Nelson J**, Barron MM, Riggs JE, Gutmann L, Schochet SS. Cerebral vasculitis and ulcerative colitis. *Neurology* 1986; **36**: 719-721 [PMID: 3703273]
121 **Masuda H**, Ishii U, Aoki N, Nakayama H, Sato F, Karube H, Suzuki S, Kondo T. Ulcerative colitis associated with Takayasu's disease in two patients who received proctocolectomy. *J Gastroenterol* 2002; **37**: 297-302 [PMID: 11993515]
122 **Bartůnková J**, Tesar V, Sedivá A. Diagnostic and pathogenetic role of antineutrophil cytoplasmic autoantibodies. *Clin Immunol* 2003; **106**: 73-82 [PMID: 12672398]
123 **Danese S**, Semeraro S, Papa A, Roberto I, Scaldaferri F, Fedeli G, Gasbarrini G, Gasbarrini A. Extraintestinal manifestations in inflammatory bowel disease. *World J Gastroenterol* 2005; **11**: 7227-7236 [PMID: 16437620]
124 **Suzuki I**, Watanabe N, Suzuki J, Yamaguchi E, Munakata M, Fujita M. [A case of bronchiectasis accompanied by ulcerative colitis (UC) and HTLV-1 associated myelopathy (HAM)]. *Nihon Kyobu Shikkan Gakkai Zasshi* 1994; **32**: 358-363 [PMID: 8041047]
125 **Ray DW**, Bridger J, Hawnaur J, Waldek S, Bernstein RM, Dornan TL. Transverse myelitis as the presentation of Jo-1 antibody syndrome (myositis and fibrosing alveolitis) in long-standing ulcerative colitis. *Br J Rheumatol* 1993; **32**: 1105-1108 [PMID: 8252324]
126 **Finnie IA**, Shields R, Sutton R, Donnelly R, Morris AI. Crohn's disease and myasthenia gravis: a possible role for thymectomy. *Gut* 1994; **35**: 278-279 [PMID: 8307484]
127 **Tsuchiya M**, Asakura H, Yoshimatsu H. Thymic abnormalities and autoimmune diseases. *Keio J Med* 1989; **38**: 383-402 [PMID: 2698424]
128 **Mizuno Y**, Muraoka M, Shimabukuro K, Toda K, Miyagawa K, Yoshimatsu H, Tsuchiya M. Inflammatory bowel diseases and thymus disorder: reactivity of thymocytes with monoclonal antibodies. *Bull Tokyo Dent Coll* 1990; **31**: 137-141 [PMID: 2131166]
129 **Foroozan R**, Sambursky R. Ocular myasthenia gravis and inflammatory bowel disease: a case report and literature review. *Br J Ophthalmol* 2003; **87**: 1186-1187 [PMID: 12928296]
130 **Gower-Rousseau C**, Reumaux D, Bellard M, Delecourt L, Ribet M, Colombel JF. Remission of myasthenia gravis after proctocolectomy in a patient with ulcerative colitis. *Am J Gastroenterol* 1993; **88**: 1136-1138 [PMID: 8317426]
131 **Akbayir N**, Caliş AB, Alkim C, Sökmen HM, Erdem L, Ozbal A, Bölükbaş F, Akbayir N. Sensorineural hearing loss in patients with inflammatory bowel disease: a subclinical extraintestinal manifestation. *Dig Dis Sci* 2005; **50**: 1938-1945 [PMID: 16187200]
132 **Herdman RC**, Hirari M, Ramsden RT. Autoimmune inner ear disease and ulcerative colitis. *J Laryngol Otol* 1991; **105**: 330-331 [PMID: 2026952]
133 **Karmody CS**, Valdez TA, Desai U, Blevins NH. Sensorineural hearing loss in patients with inflammatory bowel disease. *Am J Otolaryngol* 2009; **30**: 166-170 [PMID: 19410121 DOI: 10.1016/j.amjoto.2008.04.009]
134 **Kumar BN**, Smith MS, Walsh RM, Green JR. Sensorineural hearing loss in ulcerative colitis. *Clin Otolaryngol Allied Sci* 2000; **25**: 143-145 [PMID: 10816220]
135 **Prescinotto R**, Zanini RV, Rapoport PB, Anadão CA, de Mendonça RR, Petrilli RB. Autoimmune hearing loss and ulcerative colitis. *Braz J Otorhinolaryngol* 2006; **72**: 572 [PMID: 17143440]
136 **Morís G**, Milla A, Ribacoba R, González C. Acute deafness as an extraintestinal manifestation of ulcerative colitis. *Eur J Intern Med* 2005; **16**: 440-442 [PMID: 16198906]
137 **Sassi SB**, Kallel L, Ben Romdhane S, Boubaker J, Filali A, Hentati F. Peripheral neuropathy in inflammatory bowel disease patients: a prospective cohort study. *Scand J Gastroenterol* 2009; **44**: 1268-1269 [PMID: 19681018 DOI: 10.1080/00365520903199871]
138 **Bernstein CN**, Wajda A, Blanchard JF. The clustering of other chronic inflammatory diseases in inflammatory bowel disease: a population-based study. *Gastroenterology* 2005; **129**: 827-836 [PMID: 16143122]
139 **Lloyd DA**, Payton KB, Guenther L, Frydman W. Melkersson-Rosenthal syndrome and Crohn's disease: one disease or two? Report of a case and discussion of the literature. *J Clin Gastroenterol* 1994; **18**: 213-217 [PMID: 8034917]
140 **Karajeh MA**, Hadjivassiliou M, Hurlstone P, Grundman MJ. Neurological sequelae of Crohn's disease: opportunity to study common etiological pathways. *J Gastroenterol Hepa-*

tol 2004; **19**: 1093-1094 [PMID: 15304135]
141 **Walker JC**, Selva D, Pietris G, Crompton JL. Optic disc swelling in Crohn's disease. *Aust N Z J Ophthalmol* 1998; **26**: 329-332 [PMID: 9843262]
142 **Romero Aroca P**, Salvat Serra M, Perena Soriano F, Martínez Salcedo I. [Anterior optic neuritis do to ulcerative colitis]. *Arch Soc Esp Oftalmol* 2001; **76**: 189-191 [PMID: 11283786]
143 **Ryken T**, Menezes A. Inflammatory bowel disease and the craniocervical junction. *Neurosurg Focus* 1999; **6**: e10 [PMID: 16972747]
144 **Druschky A**, Heckmann J, Engelhardt A. [Myositis--a rare complication of Crohn disease]. *Fortschr Neurol Psychiatr* 1996; **64**: 422-424 [PMID: 9036102]
145 **Qureshi JA**, Staugaitis SM, Calabrese LH. Neutrophilic myositis: an extra-intestinal manifestation of ulcerative colitis. *J Clin Rheumatol* 2002; **8**: 85-88 [PMID: 17041328]
146 **Ramalho J**, Castillo M. Imaging of orbital myositis in Crohn's disease. *Clin Imaging* 2008; **32**: 227-229 [PMID: 18502352 DOI: 10.1016/j.clinimag.2007.09.012]
147 **Gordon LK**. Orbital inflammatory disease: a diagnostic and therapeutic challenge. *Eye* (Lond) 2006; **20**: 1196-1206 [PMID: 17019419]
148 **Taylor SR**, McCluskey P, Lightman S. The ocular manifestations of inflammatory bowel disease. *Curr Opin Ophthalmol* 2006; **17**: 538-544 [PMID: 17065922]
149 **Ford S**, Finkel AG, Isaacs KL. Migraine in patients with inflammatory bowel disorders. *J Clin Gastroenterol* 2009; **43**: 499 [PMID: 19247199 DOI: 10.1097/MCG.0b013e318188be85]
150 **Vanmolkot FH**, Van Bortel LM, de Hoon JN. Altered arterial function in migraine of recent onset. *Neurology* 2007; **68**: 1563-1570 [PMID: 17460157]
151 **Chidlow JH**, Shukla D, Grisham MB, Kevil CG. Pathogenic angiogenesis in IBD and experimental colitis: new ideas and therapeutic avenues. *Am J Physiol Gastrointest Liver Physiol* 2007; **293**: G5-G18 [PMID: 17463183]
152 **Lipton RB**, Dodick D, Sadovsky R, Kolodner K, Endicott J, Hettiarachchi J, Harrison W. A self-administered screener for migraine in primary care: The ID Migraine validation study. *Neurology* 2003; **61**: 375-382 [PMID: 12913201]
153 **Kurth T**, Gaziano JM, Cook NR, Logroscino G, Diener HC, Buring JE. Migraine and risk of cardiovascular disease in women. *JAMA* 2006; **296**: 283-291 [PMID: 16849661]
154 **Kruit MC**, van Buchem MA, Hofman PA, Bakkers JT, Terwindt GM, Ferrari MD, Launer LJ. Migraine as a risk factor for subclinical brain lesions. *JAMA* 2004; **291**: 427-434 [PMID: 14747499]
155 **Ranjbaran Z**, Keefer L, Farhadi A, Stepanski E, Sedghi S, Keshavarzian A. Impact of sleep disturbances in inflammatory bowel disease. *J Gastroenterol Hepatol* 2007; **22**: 1748-1753 [PMID: 17914945]
156 **Keefer L**, Stepanski EJ, Ranjbaran Z, Benson LM, Keshavarzian A. An initial report of sleep disturbance in inactive inflammatory bowel disease. *J Clin Sleep Med* 2006; **2**: 409-416 [PMID: 17557469]
157 **Banovic I**, Gilibert D, Cosnes J. Crohn's disease and fatigue: constancy and co-variations of activity of the disease, depression, anxiety and subjective quality of life. *Psychol Health Med* 2010; **15**: 394-405 [PMID: 20677078 DOI: 10.1080/13548501003759155]
158 **Vidal A**, Gómez-Gil E, Sans M, Portella MJ, Salamero M, Piqué JM, Panés J. Health-related quality of life in inflammatory bowel disease patients: the role of psychopathology and personality. *Inflamm Bowel Dis* 2008; **14**: 977-983 [PMID: 18275078 DOI: 10.1002/ibd.20388]
159 **Porcelli P**, Leoci C, Guerra V. A prospective study of the relationship between disease activity and psychologic distress in patients with inflammatory bowel disease. *Scand J Gastroenterol* 1996; **31**: 792-796 [PMID: 8858749]
160 **Minderhoud IM**, Oldenburg B, Wismeijer JA, van Berge Henegouwen GP, Smout AJ. IBS-like symptoms in patients with inflammatory bowel disease in remission; relationships with quality of life and coping behavior. *Dig Dis Sci* 2004; **49**: 469-474 [PMID: 15139501]
161 **Avellaneda Fernández A**, Pérez Martín A, Izquierdo Martínez M, Arruti Bustillo M, Barbado Hernández FJ, de la Cruz Labrado J, Díaz-Delgado Peñas R, Gutiérrez Rivas E, Palacín Delgado C, Rivera Redondo J, Ramón Giménez JR. Chronic fatigue syndrome: aetiology, diagnosis and treatment. *BMC Psychiatry* 2009; **9** Suppl 1: S1 [PMID: 19857242 DOI: 10.1186/1471-244x-9-S1-S1]
162 **Weinstock LB**, Bosworth BP, Scherl EJ, Li E, Iroku U, Munsell MA, Mullen GE, Walters AS. Crohn's disease is associated with restless legs syndrome. *Inflamm Bowel Dis* 2010; **16**: 275-279 [PMID: 19575360 DOI: 10.1002/ibd.20992]
163 **Franco RA**, Ashwathnarayan R, Deshpandee A, Knox J, Daniel J, Eastwood D, Franco J, Saeian K. The high prevalence of restless legs syndrome symptoms in liver disease in an academic-based hepatology practice. *J Clin Sleep Med* 2008; **4**: 45-49 [PMID: 18350962]
164 **Patrick LR**. Restless legs syndrome: pathophysiology and the role of iron and folate. *Altern Med Rev* 2007; **12**: 101-112 [PMID: 17604457]
165 **Allen R**. Dopamine and iron in the pathophysiology of restless legs syndrome (RLS). *Sleep Med* 2004; **5**: 385-391 [PMID: 15222997]
166 **Rama AN**, Kushida CA. Restless legs syndrome and periodic limb movement disorder. *Med Clin North Am* 2004; **88**: 653-657, viii [PMID: 15087209]
167 **Baumgart DC**, Carding SR. Inflammatory bowel disease: cause and immunobiology. *Lancet* 2007; **369**: 1627-1640 [PMID: 17499605]
168 **Nemeth E**, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, Ganz T. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest* 2004; **113**: 1271-1276 [PMID: 15124018]
169 **Auger C**, Montplaisir J, Duquette P. Increased frequency of restless legs syndrome in a French-Canadian population with multiple sclerosis. *Neurology* 2005; **65**: 1652-1653 [PMID: 16301499]
170 **Hahn JS**, Berquist W, Alcorn DM, Chamberlain L, Bass D. Wernicke encephalopathy and beriberi during total parenteral nutrition attributable to multivitamin infusion shortage. *Pediatrics* 1998; **101**: E10 [PMID: 9417174]
171 **Larnaout A**, El-Euch G, Kchir N, Filali A, Hamida MB, Hentati F. Wernicke's encephalopathy in a patient with Crohn's disease: a pathological study. *J Neurol* 2001; **248**: 57-60 [PMID: 11266021]
172 **Kumar N**. Neurologic presentations of nutritional deficiencies. *Neurol Clin* 2010; **28**: 107-170 [PMID: 19932379 DOI: 10.1016/j.ncl.2009.09.006]

P- Reviewers: Cheifetz AS, Campos FG, Kato S, Papamichael KS
S- Editor: Zhai HH **L- Editor**: Kerr C **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8783

World J Gastroenterol 2014 July 21; 20(27): 8783-8789
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Primary sclerosing cholangitis as an independent risk factor for colorectal cancer in the context of inflammatory bowel disease: A review of the literature

Rosy Wang, Rupert W Leong

Rosy Wang, Rupert W Leong, Gastroenterology and Liver Services, Concord Hospital, Sydney, Concord NSW 2139, Australia
Author contributions: Wang R contributed to this work; Leong RW designed the research; Wang R performed the literature review and analysed the data; Wang R and Leong RW wrote the paper.
Supported by Career Development Fellowship of the National Health and Medical Research Council of Australia, to Leong RW
Correspondence to: Rupert W Leong, Associate Professor, Gastroenterology and Liver Services, Concord Hospital, Level 1 West, Hospital Rd, Sydney, Concord NSW 2139, Australia. rupertleong@outlook.com
Telephone: +61-2-9767 6111 Fax: +61-2-9767 6767
Received: October 13, 2013 Revised: January 21, 2014
Accepted: April 5, 2014
Published online: July 21, 2014

Abstract

To examine and evaluate recent evidence regarding the epidemiology, pathogenesis and management of colorectal cancer (CRC) development in inflammatory bowel disease (IBD)-primary sclerosing cholangitis (PSC) patients. Using the PubMed database, a literature search was conducted for relevant articles in English from the past 10 years. Relevant studies investigating PSC as a risk factor for CRC in IBD in the context of incidence and prevalence, pathogenesis, prevention and prognosis were included in this review. Recent evidence increasingly points to PSC as a significant risk factor in the development of CRC in patients with concomitant IBD. PSC may be an important risk factor for CRC in different populations worldwide. The mechanism for this increase in risk is still unclear. The efficacy of UDCA as a chemopreventive agent remains controversial. Liver transplantation does not halt the development of CRC, although there is not enough evidence to suggest that it is associated with increased incidence of CRC. While routine colonoscopic surveillance should be performed in patients with concurrent PSC and IBD, more high-level evidence is required to support the benefits of the procedure. While many new developments have taken place in the last decade, the pathogenesis and optimal management of CRC development in IBD-PSC patients remain unclear.

Key words: Primary sclerosing cholangitis; Ulcerative colitis; Crohn's; Inflammatory bowel disease; Colorectal cancer; Liver transplantation; Ursodeoxycholic acid

Core tip: The widely accepted risk factors for malignant transformation in inflammatory bowel disease (IBD) are disease duration and extent of inflammation. Since first proposed in 1992, one increasingly recognised independent risk factor for colorectal cancer development in IBD patients is concomitant primary sclerosing cholangitis.

Wang R, Leong RW. Primary sclerosing cholangitis as an independent risk factor for colorectal cancer in the context of inflammatory bowel disease: A review of the literature. *World J Gastroenterol* 2014; 20(27): 8783-8789 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8783.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8783

INTRODUCTION

Inflammatory bowel disease (IBD) is a widely accepted risk factor for colorectal cancer (CRC). The development of CRC complicating IBD only occurs in 1%-2% of CRC cases and has been reported to account for up to a third of mortality in ulcerative colitis (UC) patients[1].

Table 1 Summary of studies investigating colorectal cancer as a risk factor in patients with concurrent primary sclerosing cholangitis and inflammatory bowel disease

Ref.	Location	IBD-PSC patients (*n*)	Colorectal neoplasms (*n*)	Matched controls?	Study type	Is IBD-PSC a risk for CRC?
[24]	The Netherlands	IBD (126)	CRC (16)	No	Retrospective	Yes
[9]	Belgium	IBD (107)	CRC (10)	No	Retrospective	Yes
[12]	Sweden	IBD (152)	CRC/Dys (3)	No	Retrospective	No
[31]	Germany	IBD (120)	CRC (7)	No	Prospective	Only with dominant stenosis
[28]	Argentina	UC (39)	CRC (7)	Yes	Prospective	Yes
[30]	United States	UC (50)	N/S	Yes	Retrospective	No
[32]	Sweden	CD (28)	CRC/Dys (9)	Yes	Retrospective	Yes
[33]	United Kingdom	CD (35)	Dys (1)	No	Retrospective	No

UC: Ulcerative colitis; CD: Crohn's disease; Dys: Dysplasia; N/S: Not specified; CRC: Colorectal cancer; PSC: Primary sclerosing cholangitis; IBD: Inflammatory bowel disease.

Established risk factors for malignant transformation in IBD include disease duration and extent[2-4], family history of CRC[5,6], and concomitant primary sclerosing cholangitis (PSC).

PSC is a chronic syndrome of unknown aetiology. PSC is characterised by destruction and stenoses of intrahepatic and extrahepatic biliary ducts by inflammation and fibrosis, leading to cholestasis. As the disease progresses, portal tract fibrosis and biliary cirrhosis may develop, which may ultimately lead to death from hepatic cirrhosis and failure[7]. Besides CRC, PSC has been associated with other malignant conditions, including cholangiocarcinoma[8,9], pancreatic carcinoma[10], gallbladder cancer[11], hepatobilliary cancer[12], and hepatocellular carcinoma[10,13].

The incidence of PSC may be increasing, possibly due to earlier recognition and increasing index of suspicion[14]. The mean age of PSC diagnosis is 40 years, and the median survival time from diagnosis to death or liver transplantation is approximately 12 years[15]. PSC has a slightly male predominance[16]. Currently, the only definitive long-term treatment of PSC is liver transplantation[7].

The association of PSC with UC is stronger than with Crohn's disease (CD). The prevalence of concurrent PSC in UC patients is up to 8%, compared to only 1% to 3% for CD[17,18]. Evidence suggests that this figure varies according to the extensiveness of disease; the prevalence of PSC is approximately 5.5% in patients with pancolitis, but only 1% in those with distal colitis[18,19]. Overall the prevalence of PSC is approximately 10% in CD and 80% in UC[19,20]. Broomé *et al*[21,22] first proposed the association of PSC and CRC in UC patients in 1992 with a cumulative risk of 50% at 25 years of developing CRC in UC = PSC patients.

The suggestion that PSC is an independent risk factor for CRC in IBD patients is one that has widely debated. Currently, no explanation of how PSC increases the risk of CRC in IBD patients has been agreed upon. Numerous literature reviews, including a meta-analysis conducted in 2002[23], have evaluated earlier research concerning this topic. This review aims to evaluate research within the last decade and examine recent evidence concerning epidemiology, pathogenic mechanisms and management strategies of CRC development in IBD-PSC patients.

RESEARCH

A literature search was conducted using the PubMed database for relevant articles from January 2002 until January 2014. The keywords used were: primary sclerosing cholangitis, colorectal, cancer, neoplasia, carcinoma, inflammatory bowel disease, Crohn's disease and ulcerative colitis. Relevant studies investigating PSC as a risk factor for CRC in IBD in the context of incidence and prevalence, pathogenesis, prevention and management, and prognosis were included in this review. Articles not written in English, review articles and published abstracts were not included.

EVALUATION OF EVIDENCE

Since the initial proposal in 1992 by Broomé *et al*[21], recent research continue to support concurrent PSC as a key risk factor in the development of CRC in IBD. The studies evaluated in this section are summarised in Table 1. Many studies now recognise PSC as just an important risk factor for CRC development as previously established risk factors such as duration and extent of IBD.

Early studies of PSC patients were characterised by small cohorts, small statistical power and disparate measurable outcomes. The increasing awareness of the link between PSC and CRC has allowed larger and better designed studies to be conducted. A retrospective Dutch study in 2009 investigated 211 PSC patients. Of that cohort, 60% had concurrent IBD. The risk of CRC development was 14% at 10 years and 31% at 20 years in PSC patients with concurrent IBD, compared with a steady risk of 2.3% in patients without concurrent IBD ($P < 0.01$)[24]. The study also found that the majority of CRCs were located in the right colon, proximal to the splenic flexure, a result that has been confirmed by numerous other studies[25,26]. The same group went on to confirm this finding in a subsequent study, where the majority (67%) of IBD-PSC patients that developed CRC had tumours in the right-sided colon ($P < 0.01$) in contrast to patients with IBD alone. Based on this finding, a differ-

Table 2 Summary of studies investigating the efficacy of ursodeoxycholic acid a chemopreventive agent in primary sclerosing cholangitis patients with concurrent inflammatory bowel disease

Ref.	Location	UDCA (*n*)	CRN incidence UDCA (*n*)	No UDCA (*n*)	CRN incidence no UDCA (*n*)	Study type	Is UDCA chemopreventive?
[36]	United States	29	3	23	8	RCT	Yes
[37]	United States	28	3	92	13	Retrospective	No
[38]	Sweden	37	13	40	15	RCT	No
[39]	United States	25	9	31	3	RCT	No-high dose UDCA
[40]	Germany	120	7	N/A	N/A	Prospective	No-short term; yes-long term

CRN: Colorectal neoplasm (dysplasia and cancer); RCT: Randomised controlled trial; N/A: Not applicable; UDCA: Ursodeoxycholic acid; PSC: Primary sclerosing cholangitis; IBD: Inflammatory bowel disease.

ence in pathogenesis of CRC may occur in patients with PSC and concurrent IBD, compared to patients with IBD alone[27].

Other studies have also evaluated the cumulative risk of CRC development in the long-term. Fevery *et al*[9] studied 200 PSC patients in a long-term, single-centre study in Belgium, where 60% of the cohort had concomitant IBD. The cumulative incidence for the diagnosis of CRC after IBD diagnosis was found to be 2% in 5 years, 7% in 10 years and 15% in 20 years. Additionally, the median age of CRC diagnosis was 49.5 years, leading the authors to conclude malignancy to be the major cause of early mortality in patients with PSC.

Terg *et al*[28] prospectively recruited a Latin American PSC cohort from 1333 patients with UC. The prevalence of PSC was 2.9% and the cumulative risk of CRC after 10 and 20 years in these patients was 11% and 18% respectively, compared to 2% and 7% in UC without PSC ($P < 0.01$). Hence, it was confirmed that patients with UC and PSC indeed have a higher risk of CRC. An Asia-Pacific Consensus Group consisting of representatives from a host of countries, including India, China, Philippines and Australia published a paper in 2010 outlining various findings in UC patients in these countries. There was consensus on the statement that PSC associated with UC is less prevalent in the Asia-Pacific region compared to Western nations, though the level of evidence on this finding was classified as fairly weak. There was also some consensus on the statement that PSC in the setting of UC significantly increased the risk of development of CRC[29].

Some studies have not confirmed PSC as an independent risk factor of CRC in IBD. A 2006 case-control study investigating predictive and protective risk factors associated with CRC in UC patients did not find PSC to be significant[30]. A Swedish population-based cohort of 199 PSC patients revealed that while the disease was associated with a four-fold increase in mortality compared with the general population (SMR = 4.20, 95%CI: 3.01-5.69), the researchers unexpectedly could not confirm that PSC or PSC with concurrent IBD was associated with a higher incidence of CRC and colorectal dysplasia compared with the general population[12]. This cohort of patients was diagnosed from 1992 to 2005, which is relatively recent compared with other large-scale studies of PSC patients with cases recruited from the 1980s[24]. This led the researchers to postulate that the lowered incidence of CRC in this cohort was a result of better management of IBD in recent years[12].

Studies have also disease subtypes in the prediction of CRC. A prospective study of 171 PSC patients being treated with ursodeoxycholic acid (UDCA) found that IBD coexisting with dominant ISC bile duct stenosis had an increased CRC incidence, whereas IBD without dominant stenosis had no effect on the incidence of carcinoma ($P < 0.05$). The authors did not speculate whether this observation was a result of the interaction between dominant stenosis and IBD, or whether it was to do with UDCA treatment[31].

While the risk of CRC is established for UC-PSC patients, studies have also evaluated their association with CD. The overall risk of CRC development in CD-PSC is not as strong as UC-PSC. Lindström *et al*[32] studied the development of CRC in 28 patients with both PSC and CD, compared to controls with CD only. They found PSC to be a risk factor for development of CRC and dysplasia in CD (OR = 6.78, 95%CI: 1.65-27.9), but the study was limited by its small cohort and retrospective design. Another retrospective review of 166 PSC-IBD patients did not find an increased risk of CRC or dysplasia in CD[33].

PREVENTION OF CRC

The cause of the increased risk of CRC in PSC is largely unknown. Studies have evaluated whether the risk of CRC can be reduced. Strategies such as colonoscopic surveillance, UDCA and liver transplantation have been investigated as potential methods of preventing the development of CRC.

UDCA

The chemopreventative effects of UDCA against CRC in IBD-PSC patients remain controversial. UDCA is a synthetic, hydrophilic bile acid that purportedly prevents the carcinogenic effects of secondary bile acids in the colon[34,35]. A summary of recent data concerning the efficacy of UDCA is presented in Table 2.

A randomised, placebo-controlled trial evaluated the effect of UDCA on CRC and colorectal dysplasia in patients with concurrent UC and PSC[36]. Colorectal neoplasia developed in 10% of the patients assigned to the

UDCA group compared to 35% of the patients assigned to the placebo group (RR = 0.26, 95%CI: 0.06-0.92). Wolf *et al*[37], however, reported that the incidence of CRC and colorectal dysplasia was not significantly different between patients treated with UDCA and patients that were not, but the UDCA patients did report a lower mortality rate ($P < 0.05$).

A long-term, randomised placebo-controlled trial of IBD-PSC patients prescribed UDCA *vs* placebo followed-up patients for more than 10 years yielded no difference in the CRC rate between the UDCA (13%) and placebo (16%) groups. There also was no significant difference in cancer-free survival between the two groups[38]. Another long-term, randomised placebo-controlled trial assessed the effects of high dose UDCA (28 to 30 mg/kg per day) on the development of colorectal dysplasia and CRC in UC-PSC patients. The study found that UDCA had an adverse effect on neoplasia development where high dose UDCA significantly increased development of colorectal dysplasia and CRC compared to control (HR = 4.44, 95%CI: 1.30-20.1)[39].

Some studies have attempted to reconcile these conflicting findings. In a prospective cohort study conducted by Rudolph *et al*[40], the trend of colorectal carcinoma development in patients treated with UDCA was observed to increase up to 6 years after the start of treatment, plateaued between 6 to 9 years, and after treatment for more than 9 years (up to at least 12 years) no further colorectal carcinomas developed. This finding, together with others, suggests that the effects of UDCA in UC-PSC patients may not be straightforwardly beneficial or non-beneficial, and that longer term, placebo-controlled trials are needed to provide evidence for or against UDCA as a chemopreventative agent. Lower doses of UDCA have been used to avoid possible adverse events.

A recent meta-analysis reporting 177 cases of CRC in 763 patients with PSC-IBD failed to demonstrate significant protective association between UDCA use and CRC with OR = 0.81, 95%CI: 0.41-1.61. However, a significant chemopreventive effect was found on the risk of advanced neoplasia defined as CRC and/ or high-grade dysplasia (OR = 0.35, 95%CI: 0.17-0.73). Low-dose UDCA (8-15 mg/kg per day) did significantly reduce CRC (OR = 0.19, 95%CI: 0.08-0.49)[41]. Another meta-analysis of a similar dataset showed similar results[42].

Liver transplantation

Currently, liver transplantation remains the only effective treatment of PSC with end-stage liver disease. It follows that liver transplantation in PSC may offer prevention against CRC development by improving PSC status, however, the evidence is largely contrary.

A study from the United Kingdom identified 152 patients with PSC following liver transplantation. Of these patients, 5.3% developed CRC, of which all of them had concurrent IBD with an intact colon. The cumulative risk of CRC development in IBD-PSC patients was calculated to be 14% at 5 years and 17% at 10 years. The risk of developing CRC in PSC patients without IBD was 0% at 10 years[43]. Another study from the Cleveland Clinic found that coexistant IBD and PSC had a colorectal neoplasia incidence rate of 34% following liver transplantation, very similar to the incidence in matched IBD-PSC controls without liver transplantation (30%). However, the rate of colorectal neoplasia in IBD-PSC patients following liver transplantation was higher than if liver transplantation was performed for non-PSC indications (34% and 0%, respectively; $P < 0.05$)[44].

In a large-scale study, Dvorchik *et al*[45] identified 192 patients with both PSC and IBD, and found no increase in the risk of CRC in these patients ($P < 0.001$). van de Vrie *et al*[46] conducted a retrospective study of patients having had liver transplantation for PSC, and concluded that transplantation was not adversely affected by IBD, nor was the course of IBD different after liver transplantation. High incidence rates of CRC remain following liver transplantation for PSC according to a recently published meta-analysis. The incidence rates of CRC were 5.8 per 1000 person-years but increased to 13.5 per 1000 person-years in those with an intact colon at the time of transplantation. A long duration of IBD and extensive colitis were confirmed as risk factors for CRC but specific transplant-related factors that may increase CRC risk were not identified[47]. Overall, the evidence suggests that liver transplantation does not offer protection against CRC in PSC patients with concomitant IBD, and that post-liver transplantation patients are just as likely to develop CRC as non-transplanted patients. However, there is a lack of evidence to suggest that liver transplantation is an added risk factor for CRC development in IBD-PSC patients.

Surveillance

With increasing evidence conferring the increased risk of CRC in IBD-PSC patients, the importance of colonoscopic surveillance after the diagnosis of PSC in IBD patients has been stressed. The general consensus is that routine surveillance colonoscopy and random biopsies should be performed one to two years post PSC diagnosis in IBD patients[48-51].

Despite these recommended guidelines, research show that scheduled colonoscopy is rarely performed in UC-PSC patients. A Canadian study followed up IBD-PSC patients for five years, and found that only 36% of the expected annual surveillance colonoscopies were conducted. 33% of patients did not undergo a single colonoscopy, and 11% of patients developed colorectal dysplasia or CRC during the follow-up period[52]. Another study of 771 patients with an $\geqslant$ 8 years history of UC found the prevalence of annual surveillance amongst UC-PSC patients to be 38.5%, higher than that of the total study population (24.6%)[53]. A recent study from the Mayo Clinic showed that the rate of colorectal neoplasm (dysplasia and carcinoma) discovery within two years of diagnosis of coexisting IBD and PSC (21.5 per 100 patients) was similar to rate of discovery within eight to ten years (20.4

per 100 patients)[54]. This finding supports current guidelines for annual colonoscopic surveillance for IBD-PSC patients, starting from when concurrent PSC and IBD are diagnosed.

Rationale is lacking for the benefit of annual surveillance in IBD-PSC patients in the form of grade A supporting evidence, and current guidelines have been mainly based on expert opinion and retrospective studies. Little research has been done in the form of controlled trials to compare the rate of CRC diagnosis and prognosis between patients that undergo routine colonoscopy and those that do not. Data are unavailable that surveillance offers any prevention against CRC development or reduction in CRC mortality.

MECHANISMS OF PATHOGENESIS

Currently, the pathogenic mechanisms for the increased risk of CRC in UC-PSC patients remain unknown. One hypothesis suggests bile acids as the key culprit. PSC and other cholestatic conditions typically exhibit impaired hepatic excretion of bile acids, which may result in colonic build-up of secondary bile acids[55]. Bile acids have long been suspected as a carcinogen in human gastrointestinal cancers. Studies in animal models have shown secondary bile acids to cause DNA damage and promote cell mutation[56]. The observed increase in prevalence of CRC in the right proximal colon, where secondary bile acid concentrations are the highest suggests the role of bile acid in carcinogenesis[25-27]. The strongest evidence for this hypothesis comes from the beneficial role of UDCA. Ursodeoxycholic acid modifies the bile acid pool to reduce levels of the secondary bile acid deoxycholic acid, thereby purportedly reducing the carcinogenic potential of bile acid[57]. However, the preventative role of UDCA remains controversial, and similarly the role of bile acids in the development of CRC is also still up for debate.

Long-standing inflammation is a recognised risk factor in CRC development in IBD patients[4]. Studies have shown that coexisting IBD in PSC patients often exhibit milder clinical courses. Patients often require less use of steroids, immunomodulators and surgery, and have reduced disease activity or even asymptomatic disease[22,58]. Primary sclerosing cholangitis may be associated with a milder subclinical IBD for many years before diagnosis[4,19] and hence have had longer disease duration than apparent, increasing their risk of CRC development and requiring surveillance to commence immediately upon diagnosis of PSC[4].

CONCLUSION

Recent evidence increasingly points to PSC as a significant risk factor in the development of CRC in patients with concomitant IBD. Data suggest that the risk of CRC development can reach up to 30% at 20 years after diagnosis of concurrent IBD and PSC. PSC may be an important risk factor for CRC in different populations worldwide. The mechanism for this increase in risk is still unclear. Various methods to prevent CRC development have been extensively investigated. The efficacy of UDCA remains controversial, and more longer term randomised placebo-controlled trials are needed. Liver transplantation does not halt the development of CRC, although there is not enough evidence to suggest that it is associated with increased incidence of CRC. Patients with concurrent PSC and IBD should be educated about the risk of CRC, and while routine colonoscopic surveillance should be performed, more high-level evidence is required to support the benefits of the procedure.

While many new developments have taken place in the last decade, the pathogenesis and optimal management of CRC development in IBD-PSC patients remain unclear. Further research in these directions will lead to better insight into the relationship between IBD, PSC and CRC.

REFERENCES

1 **Weitz J**, Koch M, Debus J, Höhler T, Galle PR, Büchler MW. Colorectal cancer. *Lancet* 2005; **365**: 153-165 [PMID: 15639298 DOI: 10.1016/S0140-6736(05)17706-X]

2 **Ekbom A**, Helmick C, Zack M, Adami HO. Ulcerative colitis and colorectal cancer. A population-based study. *N Engl J Med* 1990; **323**: 1228-1233 [PMID: 2215606 DOI: 10.1056/NEJM199011013231802]

3 **Eaden J**. Review article: colorectal carcinoma and inflammatory bowel disease. *Aliment Pharmacol Ther* 2004; **20** Suppl 4: 24-30 [PMID: 15352890 DOI: 10.1111/j.1365-2036.2004.02046.x]

4 **Rutter M**, Saunders B, Wilkinson K, Rumbles S, Schofield G, Kamm M, Williams C, Price A, Talbot I, Forbes A. Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis. *Gastroenterology* 2004; **126**: 451-459 [PMID: 14762782 DOI: 10.1053/j.gastro.2003.11.010]

5 **Askling J**, Dickman PW, Karlén P, Broström O, Lapidus A, Löfberg R, Ekbom A. Family history as a risk factor for colorectal cancer in inflammatory bowel disease. *Gastroenterology* 2001; **120**: 1356-1362 [PMID: 11313305 DOI: 10.1053/gast.2001.24052]

6 **Itzkowitz SH**, Harpaz N. Diagnosis and management of dysplasia in patients with inflammatory bowel diseases. *Gastroenterology* 2004; **126**: 1634-1648 [PMID: 15168373 DOI: 10.1053/j.gastro.2004.03.025]

7 **Mendes F**, Lindor KD. Primary sclerosing cholangitis: overview and update. *Nat Rev Gastroenterol Hepatol* 2010; **7**: 611-619 [PMID: 20938459]

8 **Broomé U**, Olsson R, Lööf L, Bodemar G, Hultcrantz R, Danielsson A, Prytz H, Sandberg-Gertzén H, Wallerstedt S, Lindberg G. Natural history and prognostic factors in 305 Swedish patients with primary sclerosing cholangitis. *Gut* 1996; **38**: 610-615 [PMID: 8707097 DOI: 10.1136/gut.38.4.610]

9 **Fevery J**, Henckaerts L, Van Oirbeek R, Vermeire S, Rutgeerts P, Nevens F, Van Steenbergen W. Malignancies and mortality in 200 patients with primary sclerosering cholangitis: a long-term single-centre study. *Liver Int* 2012; **32**: 214-222 [PMID: 21745316 DOI: 10.1111/j.1478-3231.2011.02575.x]

10 **Bergquist A**, Ekbom A, Olsson R, Kornfeldt D, Lööf L, Danielsson A, Hultcrantz R, Lindgren S, Prytz H, Sandberg-Gertzén H, Almer S, Granath F, Broomé U. Hepatic and extrahepatic malignancies in primary sclerosing cholangitis. *J Hepatol* 2002; **36**: 321-327 [PMID: 11867174 DOI: 10.1016/S0168-8278(01)00288-4]

11 **Said K**, Glaumann H, Bergquist A. Gallbladder disease in patients with primary sclerosing cholangitis. *J Hepatol* 2008;

48: 598-605 [PMID: 18222013 DOI: 10.1016/j.jhep.2007.11.019]
12 **de Valle MB**, Björnsson E, Lindkvist B. Mortality and cancer risk related to primary sclerosing cholangitis in a Swedish population-based cohort. *Liver Int* 2012; **32**: 441-448 [PMID: 22098097]
13 **Harnois DM**, Gores GJ, Ludwig J, Steers JL, LaRusso NF, Wiesner RH. Are patients with cirrhotic stage primary sclerosing cholangitis at risk for the development of hepatocellular cancer? *J Hepatol* 1997; **27**: 512-516 [PMID: 9314129 DOI: 10.1016/S0168-8278(97)80356-X]
14 **Lindkvist B**, Benito de Valle M, Gullberg B, Björnsson E. Incidence and prevalence of primary sclerosing cholangitis in a defined adult population in Sweden. *Hepatology* 2010; **52**: 571-577 [PMID: 20683956 DOI: 10.1002/hep.23678]
15 **Boonstra K**, Beuers U, Ponsioen CY. Epidemiology of primary sclerosing cholangitis and primary biliary cirrhosis: a systematic review. *J Hepatol* 2012; **56**: 1181-1188 [PMID: 22245904 DOI: 10.1016/j.jhep.2011.10.025]
16 **Bambha K**, Kim WR, Talwalkar J, Torgerson H, Benson JT, Therneau TM, Loftus EV, Yawn BP, Dickson ER, Melton LJ. Incidence, clinical spectrum, and outcomes of primary sclerosing cholangitis in a United States community. *Gastroenterology* 2003; **125**: 1364-1369 [PMID: 14598252 DOI: 10.1016/j.gastro.2003.07.011]
17 **Rasmussen HH**, Fallingborg JF, Mortensen PB, Vyberg M, Tage-Jensen U, Rasmussen SN. Hepatobiliary dysfunction and primary sclerosing cholangitis in patients with Crohn's disease. *Scand J Gastroenterol* 1997; **32**: 604-610 [PMID: 9200295 DOI: 10.3109/00365529709025107]
18 **Olsson R**, Danielsson A, Järnerot G, Lindström E, Lööf L, Rolny P, Rydén BO, Tysk C, Wallerstedt S. Prevalence of primary sclerosing cholangitis in patients with ulcerative colitis. *Gastroenterology* 1991; **100**: 1319-1323 [PMID: 2013375]
19 **Loftus EV**, Harewood GC, Loftus CG, Tremaine WJ, Harmsen WS, Zinsmeister AR, Jewell DA, Sandborn WJ. PSC-IBD: a unique form of inflammatory bowel disease associated with primary sclerosing cholangitis. *Gut* 2005; **54**: 91-96 [PMID: 15591511 DOI: 10.1136/gut.2004.046615]
20 **Molodecky NA**, Kareemi H, Parab R, Barkema HW, Quan H, Myers RP, Kaplan GG. Incidence of primary sclerosing cholangitis: a systematic review and meta-analysis. *Hepatology* 2011; **53**: 1590-1599 [PMID: 21351115 DOI: 10.1002/hep.24247]
21 **Broomé U**, Lindberg G, Löfberg R. Primary sclerosing cholangitis in ulcerative colitis--a risk factor for the development of dysplasia and DNA aneuploidy? *Gastroenterology* 1992; **102**: 1877-1880 [PMID: 1587406]
22 **Broomé U**, Löfberg R, Veress B, Eriksson LS. Primary sclerosing cholangitis and ulcerative colitis: evidence for increased neoplastic potential. *Hepatology* 1995; **22**: 1404-1408 [PMID: 7590655]
23 **Soetikno RM**, Lin OS, Heidenreich PA, Young HS, Blackstone MO. Increased risk of colorectal neoplasia in patients with primary sclerosing cholangitis and ulcerative colitis: a meta-analysis. *Gastrointest Endosc* 2002; **56**: 48-54 [PMID: 12085034 DOI: 10.1067/mge.2002.125367]
24 **Claessen MM**, Vleggaar FP, Tytgat KM, Siersema PD, van Buuren HR. High lifetime risk of cancer in primary sclerosing cholangitis. *J Hepatol* 2009; **50**: 158-164 [PMID: 19012991 DOI: 10.1016/j.jhep.2008.08.013]
25 **Shetty K**, Rybicki L, Brzezinski A, Carey WD, Lashner BA. The risk for cancer or dysplasia in ulcerative colitis patients with primary sclerosing cholangitis. *Am J Gastroenterol* 1999; **94**: 1643-1649 [PMID: 10364038 DOI: 10.1111/j.1572-0241.1999.01156.x]
26 **Marchesa P**, Lashner BA, Lavery IC, Milsom J, Hull TL, Strong SA, Church JM, Navarro G, Fazio VW. The risk of cancer and dysplasia among ulcerative colitis patients with primary sclerosing cholangitis. *Am J Gastroenterol* 1997; **92**: 1285-1288 [PMID: 9260790]
27 **Claessen MM**, Lutgens MW, van Buuren HR, Oldenburg B, Stokkers PC, van der Woude CJ, Hommes DW, de Jong DJ, Dijkstra G, van Bodegraven AA, Siersema PD, Vleggaar FP. More right-sided IBD-associated colorectal cancer in patients with primary sclerosing cholangitis. *Inflamm Bowel Dis* 2009; **15**: 1331-1336 [PMID: 19229982 DOI: 10.1002/ibd.20886]
28 **Terg R**, Sambuelli A, Coronel E, Mazzuco J, Cartier M, Negreira S, Muñoz A, Gil A, Miguez C, Huernos S, Romero G, Goncalvez S, Levi D, Abecasis R. Prevalence of primary sclerosing cholangitis in patients with ulcerative colitis and the risk of developing malignancies. A large prospective study. *Acta Gastroenterol Latinoam* 2008; **38**: 26-33 [PMID: 18533354]
29 **Ooi CJ**, Fock KM, Makharia GK, Goh KL, Ling KL, Hilmi I, Lim WC, Kelvin T, Gibson PR, Gearry RB, Ouyang Q, Sollano J, Manatsathit S, Rerknimitr R, Wei SC, Leung WK, de Silva HJ, Leong RW. The Asia-Pacific consensus on ulcerative colitis. *J Gastroenterol Hepatol* 2010; **25**: 453-468 [PMID: 20370724 DOI: 10.1111/j.1440-1746.2010.06241.x]
30 **Velayos FS**, Loftus EV, Jess T, Harmsen WS, Bida J, Zinsmeister AR, Tremaine WJ, Sandborn WJ. Predictive and protective factors associated with colorectal cancer in ulcerative colitis: A case-control study. *Gastroenterology* 2006; **130**: 1941-1949 [PMID: 16762617 DOI: 10.1053/j.gastro.2006.03.028]
31 **Rudolph G**, Gotthardt D, Kloeters-Plachky P, Rost D, Kulaksiz H, Stiehl A. In PSC with dominant bile duct stenosis, IBD is associated with an increase of carcinomas and reduced survival. *J Hepatol* 2010; **53**: 313-317 [PMID: 20472317 DOI: 10.1016/j.jhep.2010.02.030]
32 **Lindström L**, Lapidus A, Ost A, Bergquist A. Increased risk of colorectal cancer and dysplasia in patients with Crohn's colitis and primary sclerosing cholangitis. *Dis Colon Rectum* 2011; **54**: 1392-1397 [PMID: 21979184 DOI: 10.1097/DCR.0b013e31822bbcc1]
33 **Braden B**, Halliday J, Aryasingha S, Sharifi Y, Checchin D, Warren BF, Kitiyakara T, Travis SP, Chapman RW. Risk for colorectal neoplasia in patients with colonic Crohn's disease and concomitant primary sclerosing cholangitis. *Clin Gastroenterol Hepatol* 2012; **10**: 303-308 [PMID: 22037429 DOI: 10.1016/j.cgh.2011.10.020]
34 **Earnest DL**, Holubec H, Wali RK, Jolley CS, Bissonette M, Bhattacharyya AK, Roy H, Khare S, Brasitus TA. Chemoprevention of azoxymethane-induced colonic carcinogenesis by supplemental dietary ursodeoxycholic acid. *Cancer Res* 1994; **54**: 5071-5074 [PMID: 7923119]
35 **Martinez JD**, Stratagoules ED, LaRue JM, Powell AA, Gause PR, Craven MT, Payne CM, Powell MB, Gerner EW, Earnest DL. Different bile acids exhibit distinct biological effects: the tumor promoter deoxycholic acid induces apoptosis and the chemopreventive agent ursodeoxycholic acid inhibits cell proliferation. *Nutr Cancer* 1998; **31**: 111-118 [PMID: 9770722 DOI: 10.1080/01635589809514689]
36 **Pardi DS**, Loftus EV, Kremers WK, Keach J, Lindor KD. Ursodeoxycholic acid as a chemopreventive agent in patients with ulcerative colitis and primary sclerosing cholangitis. *Gastroenterology* 2003; **124**: 889-893 [PMID: 12671884 DOI: 10.1053/gast.2003.50156]
37 **Wolf JM**, Rybicki LA, Lashner BA. The impact of ursodeoxycholic acid on cancer, dysplasia and mortality in ulcerative colitis patients with primary sclerosing cholangitis. *Aliment Pharmacol Ther* 2005; **22**: 783-788 [PMID: 16225486 DOI: 10.1111/j.1365-2036.2005.02650.x]
38 **Lindström L**, Boberg KM, Wikman O, Friis-Liby I, Hultcrantz R, Prytz H, Sandberg-Gertzén H, Sangfelt P, Rydning A, Folvik G, Gangsøy-Kristiansen M, Danielsson A, Bergquist A. High dose ursodeoxycholic acid in primary sclerosing cholangitis does not prevent colorectal neoplasia. *Aliment Pharmacol Ther* 2012; **35**: 451-457 [PMID: 22221173 DOI: 10.1111/j.1365-2036.2011.04966.x]
39 **Eaton JE**, Silveira MG, Pardi DS, Sinakos E, Kowdley KV,

Luketic VA, Harrison ME, McCashland T, Befeler AS, Harnois D, Jorgensen R, Petz J, Lindor KD. High-dose ursodeoxycholic acid is associated with the development of colorectal neoplasia in patients with ulcerative colitis and primary sclerosing cholangitis. *Am J Gastroenterol* 2011; **106**: 1638-1645 [PMID: 21556038 DOI: 10.1038/ajg.2011.156]

40 **Rudolph G**, Gotthardt DN, Kloeters-Plachky P, Kulaksiz H, Schirmacher P, Stiehl A. In PSC with colitis treated with UDCA, most colonic carcinomas develop in the first years after the start of treatment. *Dig Dis Sci* 2011; **56**: 3624-3630 [PMID: 21656181 DOI: 10.1007/s10620-011-1763-2]

41 **Singh S**, Khanna S, Pardi DS, Loftus EV, Talwalkar JA. Effect of ursodeoxycholic acid use on the risk of colorectal neoplasia in patients with primary sclerosing cholangitis and inflammatory bowel disease: a systematic review and meta-analysis. *Inflamm Bowel Dis* 2013; **19**: 1631-1638 [PMID: 23665966 DOI: 10.1097/MIB.0b013e318286fa61]

42 **Hansen JD**, Kumar S, Lo WK, Poulsen DM, Halai UA, Tater KC. Ursodiol and colorectal cancer or dysplasia risk in primary sclerosing cholangitis and inflammatory bowel disease: a meta-analysis. *Dig Dis Sci* 2013; **58**: 3079-3087 [PMID: 23896754 DOI: 10.1007/s10620-013-2772-0]

43 **Vera A**, Gunson BK, Ussatoff V, Nightingale P, Candinas D, Radley S, Mayer A, Buckels JA, McMaster P, Neuberger J, Mirza DF. Colorectal cancer in patients with inflammatory bowel disease after liver transplantation for primary sclerosing cholangitis. *Transplantation* 2003; **75**: 1983-1988 [PMID: 12829898 DOI: 10.1097/01.TP.0000058744.34965.38]

44 **Hanouneh IA**, Macaron C, Lopez R, Zein NN, Lashner BA. Risk of colonic neoplasia after liver transplantation for primary sclerosing cholangitis. *Inflamm Bowel Dis* 2012; **18**: 269-274 [PMID: 21425212 DOI: 10.1002/ibd.21692]

45 **Dvorchik I**, Subotin M, Demetris AJ, Fung JJ, Starzl TE, Wieand S, Abu-Elmagd KM. Effect of liver transplantation on inflammatory bowel disease in patients with primary sclerosing cholangitis. *Hepatology* 2002; **35**: 380-384 [PMID: 11826412 DOI: 10.1053/jhep.2002.30695]

46 **van de Vrie W**, de Man RA, van Buuren HR, Schouten WR, Tilanus HW, Metselaar HJ. Inflammatory bowel disease and liver transplantation for primary sclerosing cholangitis. *Eur J Gastroenterol Hepatol* 2003; **15**: 657-663 [PMID: 12840678 DOI: 10.1097/00042737-200306000-00013]

47 **Singh S**, Varayil JE, Loftus EV, Talwalkar JA. Incidence of colorectal cancer after liver transplantation for primary sclerosing cholangitis: a systematic review and meta-analysis. *Liver Transpl* 2013; **19**: 1361-1369 [PMID: 24019127 DOI: 10.1002/lt.23741]

48 **Chapman R**, Fevery J, Kalloo A, Nagorney DM, Boberg KM, Shneider B, Gores GJ. Diagnosis and management of primary sclerosing cholangitis. *Hepatology* 2010; **51**: 660-678 [PMID: 20101749 DOI: 10.1002/hep.23294]

49 **Eaden JA**, Mayberry JF. Guidelines for screening and surveillance of asymptomatic colorectal cancer in patients with inflammatory bowel disease. *Gut* 2002; **51** Suppl 5: V10-V12 [PMID: 12221032 DOI: 10.1136/gut.51.suppl_5.v10]

50 **Itzkowitz SH**, Present DH. Consensus conference: Colorectal cancer screening and surveillance in inflammatory bowel disease. *Inflamm Bowel Dis* 2005; **11**: 314-321 [PMID: 15735438 DOI: 10.1097/01.MIB.0000160811.76729.d5]

51 **Cairns SR**, Scholefield JH, Steele RJ, Dunlop MG, Thomas HJ, Evans GD, Eaden JA, Rutter MD, Atkin WP, Saunders BP, Lucassen A, Jenkins P, Fairclough PD, Woodhouse CR. Guidelines for colorectal cancer screening and surveillance in moderate and high risk groups (update from 2002). *Gut* 2010; **59**: 666-689 [PMID: 20427401 DOI: 10.1136/gut.2009.179804]

52 **Kaplan GG**, Heitman SJ, Hilsden RJ, Urbanski S, Myers RP, Lee SS, Burak KW, Swain M, Panaccione R. Population-based analysis of practices and costs of surveillance for colonic dysplasia in patients with primary sclerosing cholangitis and colitis. *Inflamm Bowel Dis* 2007; **13**: 1401-1407 [PMID: 17600816 DOI: 10.1002/ibd.20204]

53 **Velayos FS**, Liu L, Lewis JD, Allison JE, Flowers N, Hutfless S, Abramson O, Perry GS, Herrinton LJ. Prevalence of colorectal cancer surveillance for ulcerative colitis in an integrated health care delivery system. *Gastroenterology* 2010; **139**: 1511-1518 [PMID: 20659470 DOI: 10.1053/j.gastro.2010.07.039]

54 **Thackeray EW**, Charatcharoenwitthaya P, Elfaki D, Sinakos E, Lindor KD. Colon neoplasms develop early in the course of inflammatory bowel disease and primary sclerosing cholangitis. *Clin Gastroenterol Hepatol* 2011; **9**: 52-56 [PMID: 20920596 DOI: 10.1016/j.cgh.2010.09.020]

55 **Torres J**, Pineton de Chambrun G, Itzkowitz S, Sachar DB, Colombel JF. Review article: colorectal neoplasia in patients with primary sclerosing cholangitis and inflammatory bowel disease. *Aliment Pharmacol Ther* 2011; **34**: 497-508 [PMID: 21692821 DOI: 10.1111/j.1365-2036.2011.04753.x]

56 **Bernstein H**, Bernstein C, Payne CM, Dvorakova K, Garewal H. Bile acids as carcinogens in human gastrointestinal cancers. *Mutat Res* 2005; **589**: 47-65 [PMID: 15652226 DOI: 10.1016/j.mrrev.2004.08.001]

57 **Batta AK**, Salen G, Holubec H, Brasitus TA, Alberts D, Earnest DL. Enrichment of the more hydrophilic bile acid ursodeoxycholic acid in the fecal water-soluble fraction after feeding to rats with colon polyps. *Cancer Res* 1998; **58**: 1684-1687 [PMID: 9563483]

58 **Marelli L**, Xirouchakis E, Kalambokis G, Cholongitas E, Hamilton MI, Burroughs AK. Does the severity of primary sclerosing cholangitis influence the clinical course of associated ulcerative colitis? *Gut* 2011; **60**: 1224-1228 [PMID: 21402617 DOI: 10.1136/gut.2010.235408]

P- Reviewers: Farup PG, Vasilieva LE
S- Editor: Wen LL **L- Editor**: A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8790

World J Gastroenterol 2014 July 21; 20(27): 8790-8795
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Treatment of Crohn's disease in pregnant women: Drug and multidisciplinary approaches

Didia Bismara Cury, Alan C Moss

Didia Bismara Cury, Center of Inflammatory Bowel Disease, Federal University of São Paulo, Sao Paulo, SP 040023-062, Brazil
Didia Bismara Cury, Center of Inflammatory Bowel Disease, Clinica Scope, Campo Grande, MS 79002212, Brazil
Didia Bismara Cury, Beth Israel Medical Center of Harvard Medical School, Sao Paulo, CEP 04023-062, Brazil
Alan C Moss, Beth Israel Deaconess Medical Center, Division of Gastroenterology, Boston, MA 02215, United States
Alan C Moss, Harvard Medical School, Boston, MA 02215, United States
Author contributions: Cury DB and Moss AC contributed to the manuscript.
Correspondence to: Didia Bismara Cury, Director, Center of Inflammatory Bowel Disease, Clinica Scope, Campo Grande, MS 79002212, Brazil. didia_cury@uol.com.br
Telephone: +55-673-3256040 Fax: +55-673-3256040
Received: November 19, 2013 Revised: February 11, 2014
Accepted: April 5, 2014
Published online: July 21, 2014

Abstract

Inflammatory bowel disease affects a substantial number of women in their reproductive years. Pregnancy presents a number of challenges for clinicians and patients; the health of the baby needs to be balanced with the need to maintain remission in the mother. Historically, treatments for Crohn's disease (CD) were often discontinued during the pregnancy, or nursing period, due to concerns about teratogenicity. Fortunately, observational data has reported the relative safety of many agents used to treat CD, including 5-aminosalicylic acid, thiopurines, and tumor necrosis factor. Data on the long-term development outcomes of children exposed to these therapies *in utero* are still limited. It is most important that physicians educate the patient regarding the optimal time to conceive, discuss the possible risks, and together decide on the best management strategy.

Key words: Pregnancy; Drugs; Inflammatory bowel disease; Crohn's disease; Breastfeeding

Core tip: Patients should be encouraged to postpone conception until their Crohn's disease (CD) is in remission. Monitoring of nutritional status remains important in patients with small bowel CD; folic acid, vitamin D and vitamin B12 may all need to be supplemented. Most drug treatments are safe in pregnancy, based on observational data, including 5-aminosalicylic acid, thiopurines, anti-tumor necrosis factor, and anti-integrins. Methotrexate should be avoided due to its teratogenicity. Cesarean section is only indicated from a CD perspective in women with active perianal disease at the time of delivery; all others can have a normal vaginal delivery.

Cury DB, Moss AC. Treatment of Crohn's disease in pregnant women: Drug and multidisciplinary approaches. *World J Gastroenterol* 2014; 20(27): 8790-8795 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8790.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8790

INTRODUCTION

In recent years, great advances have been made in the management of inflammatory bowel diseases (IBD)[1]. Nevertheless, many questions arise for physicians and patients when women with IBD consider pregnancy. In this situation, an understanding of the nutritional, pharmacological and diagnostic considerations is important for treating physicians to minimize harm to the fetus, while ensuring the mother's disease remains in remission[2,3].

FERTILITY IN CROHN'S DISEASE

Fertility issues, risk of the disease in off-spring, and the impact of both the disease and medication on mother

Table 1 Food and Drug Administration drug administration categories for the use of medications in pregnancy

Category	Observations
A	Controlled studies both in humans and in animals have shown that there is no risk during the first trimester and the possibility of fetal harm is remote
B	Studies in animals have shown no risk to the fetus. However, no controlled studies have been carried out in pregnant women. In addition, studies in animals have revealed adverse effects which were not confirmed in pregnant women in the first trimester
C	There is no record of controlled studies in humans. Studies in animals have shown adverse effects. Moreover, studies in humans and animals showing that the benefit may outweigh the risk have not been validated
D	Evidence of risk for the fetus
X	Studies in animals and humans have shown fetal abnormalities, so these drugs are contraindicated

and child can all create fear in many patients wanting to have a child[2]. Some factors may influence the fertility rate in patients with Crohn's disease (CD); these include active disease and/or malnutrition, and drug-induced oligospermia in male partners[4]. Active CD in the colon or the terminal ileum or even surgery such as proctocolectomy with ileoanal anastomosis (colitis) have been associated with lower fertility rates[4-6]. The psychological burden of disease may also play a role; in perianal CD, low fertility has been attributed to dyspareunia, decreased libido and depression in some women[7].

Sulfasalazine is associated with infertility in men; in 80% of cases sperm motility is reduced and morphology is changed. Such effects are not reversible with the administration of folic acid, but they may reverse two months after the end of the sulfasalazine treatment[8,9]. In contrast to these data, mezalazine, which is also a 5-ASA, and immunosuppressants such as azathioprine, do not appear to affect spermiogenesis in patients[10].

EFFECTS OF INFLAMMATORY DISEASE ON PREGNANCY AND PREGNANCY EFFECTS ON INFLAMMATORY DISEASE

Even though most pregnant women with IBD may be classified as having high-risk pregnancies, the course of the disease in this group usually does not present major complications. The rate of premature births in this specific group is frequently double that in women without inflammatory disease[3]. The risk of congenital malformation in the general population ranges from 1% to 4.8% and there is no current evidence of an increased risk for CD patients[11]. Conversely, miscarriage is more frequent in women with IBD (above 35%), especially in those with active disease. The natural risk of fetal loss after 16 wk is approximately 1%, which is similar to the risk of healthy women[12]. Some studies have shown that women with active CD are more likely to bear children with low birth weight (less than 2500 g)[13].

The influence of pregnancy on the course of the disease is closely related to the disease status at the time of delivery *i.e.*, active or inactive. This status will determine the behavior of the disease itself, the clinical course, and the response to drug therapy[7]. Some studies show that 70% of women with active disease have worsening or persisting symptoms during pregnancy, whereas the risk of relapse in women who do not have active disease at conception is similar to women who are not pregnant[14-25]. A recent meta-analysis of these studies concluded that the risk of active disease during pregnancy is higher in women who conceive when their disease is active[26].

THERAPEUTIC DRUGS AND THEIR SAFETY DURING PREGNANCY AND BREASTFEEDING

Studies conducted in the last decades with women who underwent treatment during their pregnancy provides reassuring data for the specialist as well as for the patient both during the pregnancy and while breastfeeding. The Food and Drug Administration (FDA) classification for drugs according to their known or potential teratogenicity is reviewed in Table 1. Table 2 shows the safety of medications commonly prescribed for IBD during pregnancy.

Sulfasalazine and 5-aminosalicylic acid (category B)

5-aminosalicylic acid (5-ASA) is considered safe up to doses of 3 mg/d. Above this dose, the risk is considered uncertain[8-10]. Studies show that sulfasalazine and 5-ASA in doses below 3 g/d do not increase the risk of congenital malformation, premature birth and miscarriage in patients with CD or ulcerative colitis (UC). A post-marketing study showed that of 55 pregnant women who used mezalazine in doses of 1.6 to 4 g/d, three had fetal malformations, however, these data were not different from those found in the general population, which suggests that there is no greater risk of malformations with mezalazine use[9].

Azathioprine and 6-mercaptopurine (category D)

Thiopurines (azathioprine and mercaptopurine) both cross the placental barrier and can be identified in the umbilical cord blood, however, serum level in the baby is not significant. Animal studies showed the occurrence of cleft palate and skeletal and urogenital abnormalities in rats, and historical retrospective studies associated thiopurines with teratogenic effects in 5% of cases and the risk of preterm birth in 3%, in addition to the effects of low fetal weight and myelotoxicity[4].

More recent observational studies did not observe a

Table 2 Safety of medications prescribed for inflammatory bowel disease during pregnancy

Safe to use when indicated	Limited data but used when clinically indicated	Contraindicated
Mesalamine	Olsalazine	Methotrexate
Sulfasalazine	AZA/6 MP	Thalidomide
Balsalazide	Ciprofloxacin	
Corticosteroids	Metronidazole	
TPN	Biologics	
Loperamide	Cyclosporine	

TPN: Total parenteral nutrition; AZA: Azathioprine MP: Mercaptopurine.

higher risk of these events in women with IBD. A recent prospective study of 30 children, performed by de Meij *et al*[15], evaluated the effect of azathioprine on the uterus in relation to quality of life, psychosocial development and an increased risk of infection, and showed that this drug did not directly influence these factors when compared to children who had not undergone this therapy. The American Academy of Pediatrics recommends that breastfeeding mothers should not take immunosuppressants, as these drugs induce immunosuppression in children[16,22]. Most studies report that the most common adverse effect in pregnant women is related to low weight and miscarriage[4].

Antibiotics

Metronidazole and ciprofloxacin are often administered in the treatment of patients with IBD, especially perianal CD. Metronidazole is classified as Category B, and short-term use (7-10 d) is considered safe in pregnancy. In contrast, extended use in the third month of pregnancy has been associated with fetal cleft palate and cleft lip, and therefore prolonged use during pregnancy is contraindicated[4]. Ciprofloxacin is Category C, as quinolones act on the cartilage and in humans they can cause arthropathy and skeletal abnormalities of the fetus[27]. For this reason, they are not recommended for children under 18 or for pregnant or breastfeeding women.

Corticosteroids (category C)

Corticosteroids (prednisolone) cross the placental barrier, however, they represent a very small risk when used in the first trimester of pregnancy. Studies carried out in animals have shown that these drugs may increase the risk of cleft palate and cleft lip when administered in the first trimester[25]. Glucocorticoids should be administered with care and both blood pressure and blood glucose should be monitored due to their ability to induce gestational hypertension, diabetes, membrane rupture and preterm delivery.

Cyclosporine/tacrolimus (category C)

Cyclosporine and tacrolimus are both calcineurin inhibitors occasionally used in the management of CD. Both are category C, and can be employed in the treatment of fulminant colitis; their teratogenic action has not yet been proven. Doses above 25 mg/kg per day can induce renal damage in the fetus in animals and their use in humans requires serum monitoring of renal function and blood pressure because both drugs cross the human placenta, however, there are conflicting reports on this point[26].

Thalidomide (category X)

Thalidomide is rated as category X (FDA) for pregnant women due to its potential teratogenic effects. It is contraindicated in this population.

Methotrexate (category X)

This drug has also been classified as category X. It is clearly teratogenic and should not be considered for use in pregnant women and in women who want to conceive. Patients who are taking this medication should be instructed to delay conception for three to six months after its cessation. It can cause growth retardation and even mental retardation, among other effects.

Infliximab (category B)

Infliximab is a chimeric used in the treatment of CD and UC. It is known to cross the placental barrier after the second trimester, similar to all IgGs. Maternal and embryonic toxicity and increased teratogenicity have not been observed. Infliximab can be detected in high concentrations in the newborn up to 6 mo after delivery, but the clinical significance of this finding is unknown[19]. Caution with any type of live vaccine in this group of infants during the first 6 mo is necessary, particularly if the infant received anti-tumor necrosis factors (TNFs) during gestation. There were no lethal cases of TB in three-month-old children who received BCG[19].

Adalimumab (category B)

Considered by the FDA to be a category B drug, adalimumab has been approved for CD in induction, remission and maintenance phases. It exhibits similar behavior to infliximab, also crossing the placental barrier in the third month of pregnancy. There are few data on its use by pregnant women, and related birth defects have been reported, however, further studies are needed. Waage *et al*[20] conducted a review study of 126 women who had been subjected to treatment with adalimumab and no increased risk of congenital malformation was observed. Recent studies have indicated dose adjustments during pregnancy to reduce maternal exposure. It has been specifically recommended that the last dose should be given between 34 and 36 wk of gestation[16].

Certolizumab pegol (category B)

Certolizumab is a Fab fragment of a monoclonal antibody linked to a polyethylene glycol chain. It is used during CD in remission and maintenance and it is known to cross the placental barrier throughout the pregnancy at a low level. In a recent study (PIANO), no increased risks in pregnant women administered certolizumab were ob-

Table 3 Classification of the drugs concerning the fetal risk according to Food and Drug Administration

Drugs	Recommendation
Adalimumab	Pregnancy (low risk)
Category B	Breastfeeding (probably compatible)
Azathioprine/6-mercaptopurina	Pregnancy (low risk) when used in low doses and as mono-therapy
Category D	Breastfeeding (it is recommended to breastfeed 4 h after taking the drug)
Balsalazide	Pregnancy (low risk)
Category B	Breastfeeding (probably compatible)
Certolizumab	Pregnancy (low risk)
Category B	Breastfeeding (probably compatible)
Ciprofloxacin	Pregnancy (not recommended due to skeletal muscular dysfunction)
Category C	Breastfeeding (compatible)
Corticosteroids	Pregnancy (risk of adrenal insufficiency, premature rupture of membrane, in the first trimester although there is little risk of cleft palate)
Category C	Breastfeeding (probably compatible)
Cyclosporine	Pregnancy (no congenital abnormalities have been noticed)
Category C	Breastfeeding (contraindicated)
Infliximab	Gestation (low risk when administered as mono-therapy) (increased risk of infection when used in combination with azathioprine)
Category B	Breastfeeding (probably compatible)
Mezalazine	Pregnancy (asacol showed low risk of teratogenicity in animal models)
Category B	Breastfeeding (both probably compatible)
Asacol (category C)	
Methotrexate	Contraindicated in both conditions
Category X	
Metronidazole	Pregnancy (used in the first trimester increases the risk of cleft palate)
Category B	Breastfeeding (toxic)
Olsalazine category (C)	Pregnancy (limited risk)
	Breastfeeding (probably compatible)
Rifaximin	Pregnancy (animal studies show teratogenicity)
Category C	Lactation (its safety is unknown)
Sulfasalazine	Pregnancy (low risk if administered in conjunction with folic acid)
Category B	Breastfeeding (probably compatible)
Tacrolimus	Pregnancy (no increased risk described)
Category C	Breastfeeding (contraindicated)
Thalidomide	Contraindicated in both conditions
Category X	

served[21]. In addition, when breast milk was analyzed, it was noted that from 3 to 6 d post-birth, serum levels of the drug were not detected. Thus, it seems that this drug is safe in this phase[21].

Golimumab

Golimumab is a completely human monoclonal antibody which aims to block anti-TNF. It is administered subcutaneously and was approved in May, 2013 by the FDA for the treatment of severe ulcerative colitis. To date, there are no reports on the use of this drug in pregnant women[24].

Natalizumab (category C)

Natalizumab was recently approved for induction and maintenance treatment of CD in patients who do not respond to therapy with anti-TNF-α. Individual studies are necessary to prove that its use can be recommended for pregnant women. In a recent study with natalizumab, no increase in abnormalities were noted in pregnant women who had received the drug. A similar result was found in the PIANO study[22]. However, most studies did not consider the drug to be safe enough to be used during pregnancy; thus, it is contraindicated[23].

DRUGS AND THEIR SAFETY DURING PREGNANCY AND BREASTFEEDING

It is important to bear in mind which drugs can be used during pregnancy and lactation. These drugs are shown in Table 3. In general, 5-ASA derivatives are considered safe (EL 3b, RGB) as well as corticosteroids (L4, RGC). A low concentration of these steroids has been noted in breast milk. To minimize the effects of these drugs it has been suggested that mothers can breastfeed 4 h after their ingestion. The thiopurines are excreted in small amounts in milk, and are also considered safe. However, more studies are needed to fully confirm the safety of these drugs (EL 4, RGC). All anti-TNFs are also excreted in small amounts in the milk and there are few studies regarding the effects on children who are or were breastfed and consequently received these medications (EL5, RGC). Metronidazole and ciprofloxacin are also excreted in breast milk and are not considered appropriate during breastfeeding as their safety is unknown and these agents should, if possible, be avoided. Studies on tacrolimus

are limited and its safety is unconfirmed. Drugs such as thalidomide, methotrexate and cyclosporine are contraindicated as they have been found in breast milk and are consequently considered unsafe.

ENDOSCOPIC METHODS DURING PREGNANCY

Endoscopy, colonoscopy, retosigmoidoscopy and cholangiography are considered safe during pregnancy according to ECCO Statement 7G (EL4, RGC). However, caution is required in relation to these procedures and there must be a strong indication for these procedures to be carried out in the second trimester (EL5, RGD). Techniques for hemostasis are safe, but should be performed with caution (EL3, RGC).

RISK OF THROMBOEMBOLISM IN HOSPITALIZED PREGNANT WOMEN

Many studies have considered the increased risk of venous thromboembolism in pregnant women. This increased risk was noted in the first six weeks of the postnatal period, and is even higher in pregnant women with inflammatory bowel disease. The use of low-molecular-weight heparin is considered important to prevent this event and should be considered especially in women who have been or will be hospitalized (EL3 RGB).

CONCLUSION

Due to the current knowledge on inflammatory bowel disease, it is thought that the majority of drugs administered during pregnancy are safe for both the mother and the fetus. However, guidance in this group of patients (mothers-to-be) and control of disease activity before conception are essential for the prevention of miscarriage or premature birth. Most drugs are also safe for breastfeeding.

REFERENCES

1 **Katz S**. My treatment approach to the management of ulcerative colitis. *Mayo Clin Proc* 2013; **88**: 841-853 [PMID: 23910410 DOI: 10.1016/j.mayocp.2013.05.001]
2 **Friedman S**, McElrath TF, Wolf JL. Management of fertility and pregnancy in women with inflammatory bowel disease: a practical guide. *Inflamm Bowel Dis* 2013; **19**: 2937-2948 [PMID: 23945187 DOI: 10.1097/MIB.0b013e3182a0ea6f]
3 **Correia LM**, Bonilha DQ, Ramos JD, Ambrogini O, Miszputen SJ. Treatment of inflammatory bowel disease and pregnancy: a review of the literature. *Arq Gastroenterol* 2010; **47**: 197-201 [PMID: 20721468]
4 **Kierkuś J**, Szymańska E, Szymańska S, Kamińska E. [Influence of inflammatory bowel disease on pregnancy and fertility - optimal treatment and management]. *Med Wieku Rozwoj* 2013; **17**: 77-84 [PMID: 23749699]
5 **Sica GS**, Biancone L. Surgery for inflammatory bowel disease in the era of laparoscopy. *World J Gastroenterol* 2013; **19**: 2445-2448 [PMID: 23674844 DOI: 10.3748/wjg.v19.i16.2445]
6 **Ørding Olsen K**, Juul S, Berndtsson I, Oresland T, Laurberg S. Ulcerative colitis: female fecundity before diagnosis, during disease, and after surgery compared with a population sample. *Gastroenterology* 2002; **122**: 15-19 [PMID: 11781275]
7 **Pedersen N**, Bortoli A, Duricova D, D Inca R, Panelli MR, Gisbert JP, Zoli G, López-Sanromán A, Castiglione F, Riegler G, Annese V, Gionchetti P, Prada A, Pont ED, Timmer A, Felley C, Shuhaibar M, Tsianos EV, Dejaco C, Baert FJ, Jess T, Lebech M, Hommes DW, Munkholm P. The course of inflammatory bowel disease during pregnancy and postpartum: a prospective European ECCO-EpiCom Study of 209 pregnant women. *Aliment Pharmacol Ther* 2013; **38**: 501-512 [PMID: 23855425 DOI: 10.1111/apt.12412]
8 **Saha S**, Esposti SD. Reproductive issues in inflammatory bowel disease. *Med Health R I* 2009; **92**: 148-151 [PMID: 19452759]
9 **Feagins LA**, Kane SV. Sexual and reproductive issues for men with inflammatory bowel disease. *Am J Gastroenterol* 2009; **104**: 768-773 [PMID: 19223893 DOI: 10.1038/ajg.2008.90]
10 **Angelberger S**, Reinisch W, Messerschmidt A, Miehsler W, Novacek G, Vogelsang H, Dejaco C. Long-term follow-up of babies exposed to azathioprine in utero and via breastfeeding. *J Crohns Colitis* 2011; **5**: 95-100 [PMID: 21453877 DOI: 10.1016/j.crohns.2010.10.005]
11 **Dominitz JA**, Young JC, Boyko EJ. Outcomes of infants born to mothers with inflammatory bowel disease: a population-based cohort study. *Am J Gastroenterol* 2002; **97**: 641-648 [PMID: 11926208]
12 **Couve S**, Seksik P, Elefant E, Jian R, Marteau P. [Inflammatory bowel disease and pregnancy]. *Gastroenterol Clin Biol* 2003; **27**: 618-626 [PMID: 12910228]
13 **Moser MA**, Okun NB, Mayes DC, Bailey RJ. Crohn's disease, pregnancy, and birth weight. *Am J Gastroenterol* 2000; **95**: 1021-1026 [PMID: 10763954]
14 **Nørgård B**, Hundborg HH, Jacobsen BA, Nielsen GL, Fonager K. Disease activity in pregnant women with Crohn's disease and birth outcomes: a regional Danish cohort study. *Am J Gastroenterol* 2007; **102**: 1947-1954 [PMID: 17573787]
15 **de Meij TG**, Jharap B, Kneepkens CM, van Bodegraven AA, de Boer NK. Long-term follow-up of children exposed intrauterine to maternal thiopurine therapy during pregnancy in females with inflammatory bowel disease. *Aliment Pharmacol Ther* 2013; **38**: 38-43 [PMID: 23675854 DOI: 10.1111/apt.12334]
16 **Burri E**, Beglinger C. Faecal calprotectin -- a useful tool in the management of inflammatory bowel disease. *Swiss Med Wkly* 2012; **142**: w13557 [PMID: 22481443 DOI: 10.4414/smw.2012.13557]
17 **Kane S**, Ford J, Cohen R, Wagner C. Absence of infliximab in infants and breast milk from nursing mothers receiving therapy for Crohn's disease before and after delivery. *J Clin Gastroenterol* 2009; **43**: 613-616 [PMID: 19142167 DOI: 10.1097/MCG.0b013e31817f9367]
18 **Moffatt DC**, Bernstein CN. Drug therapy for inflammatory bowel disease in pregnancy and the puerperium. *Best Pract Res Clin Gastroenterol* 2007; **21**: 835-847 [PMID: 17889811]
19 **Ben-Hur H**, Pecht M, Netzer L, Borenstein R, Blickstein I, Burstein Y, Trainin N. Immune modulation exerted by thymic humoral factor (THF-gamma 2), on T-cell subsets and IL-2 production of umbilical cord blood lymphocytes. *Immunopharmacol Immunotoxicol* 1990; **12**: 123-133 [PMID: 2112568]
20 **Waage A**, Seidel C. [Thalidomide--a dreaded drug with new indications]. *Tidsskr Nor Laegeforen* 2001; **121**: 2954-2957 [PMID: 11715779]
21 **Ng SW**, Mahadevan U. Management of inflammatory bowel disease in pregnancy. *Expert Rev Clin Immunol* 2013; **9**: 161-173; quiz 174 [PMID: 23390947 DOI: 10.1586/eci.12.103]
22 **Zarrintan MH**, Teng CD, Groves MJ. The effect of compactional pressure on a wheat germ lipase preparation. *Pharm Res* 1990; **7**: 247-250 [PMID: 2339097]
23 **Saha S**, Wald A. Safety and efficacy of immunomodulators and biologics during pregnancy and lactation for the treat-

ment of inflammatory bowel disease. *Expert Opin Drug Saf* 2012; **11**: 947-957 [PMID: 22954378 DOI: 10.1517/14740338.2012.720970]
24 **Mazumdar S**, Greenwald D. Golimumab. *MAbs* 2009; **1**: 422-431 [PMID: 20065639]
25 **Van Assche G**, Dignass A, Bokemeyer B, Danese S, Gionchetti P, Moser G, Beaugerie L, Gomollón F, Häuser W, Herrlinger K, Oldenburg B, Panes J, Portela F, Rogler G, Stein J, Tilg H, Travis S, Lindsay JO. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 3: special situations. *J Crohns Colitis* 2013; **7**: 1-33 [PMID: 23040453 DOI: 10.1016/j.crohns.2012.09.005]
26 **Abhyankar A**, Ham M, Moss AC. Commentary: impact of disease activity at conception on disease activity during pregnancy in patients with inflammatory bowel disease--authors' reply. *Aliment Pharmacol Ther* 2013; **38**: 843 [PMID: 24001099 DOI: 10.1111/apt.12457]
27 **van der Woude CJ**, Kolacek S, Dotan I, Oresland T, Vermeire S, Munkholm P, Mahadevan U, Mackillop L, Dignass A. European evidenced-based consensus on reproduction in inflammatory bowel disease. *J Crohns Colitis* 2010; **4**: 493-510 [PMID: 21122553 DOI: 10.1016/j.crohns.2010.07.004]

P- Reviewers: Arroyo A, Owczarek D, Paydas S, Ranieri G
S- Editor: Qi Y **L- Editor**: Webster JR **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8796

World J Gastroenterol 2014 July 21; 20(27): 8796-8806
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

Irritable bowel syndrome: A concise review of current treatment concepts

Geoffrey C Wall, Ginelle A Bryant, Michelle M Bottenberg, Erik D Maki, Andrew R Miesner

Geoffrey C Wall, Ginelle A Bryant, Michelle M Bottenberg, Erik D Maki, Andrew R Miesner, Department of Pharmacy Practice, Drake University College of Pharmacy, Des Moines, IA 50311, United States

Author contributions: All authors participated in literature review, retrieval, synthesis as well as writing this paper

Correspondence to: Geoffrey C Wall, Pharm.D., FCCP, BCPS, CGP, Department of Pharmacy Practice, Drake University College of Pharmacy, 2507 University Ave., Des Moines, IA 50311, United States. geoff.wall@drake.edu

Telephone: +1-515-2414297 Fax: +1-515-2415085

Received: September 27, 2013 Revised: March 4, 2014
Accepted: June 14, 2014
Published online: July 21, 2014

Abstract

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal disorders causing patients to seek medical treatment. It is relatively resource intensive and the source of significant morbidity. Recent insights into the pathophysiology and treatment of IBS has given clinicians more options than ever to contend with this disorder. The purpose of our paper is to review older, "classic" treatments for IBS as well as newer agents and "alternative" therapies. We discuss the evidence base of these drugs and provide context to help develop appropriate treatment plans for IBS patients.

Key words: Irritable bowel syndrome; Probiotics; Rifaximin; Lubiprostone; Linaclotide; Peppermint oil

Core tip: Gastroenterology practitioners have more agents than ever before to treat the symptoms associated with irritable bowel syndrome. Unfortunately, despite advances in our understanding of the pathophysiology of this disorder, targeted treatments do not yet exist. This review summarizes the recent evidence-based treatment of this disorder, including, older and newer agents.

Wall GC, Bryant GA, Bottenberg MM, Maki ED, Miesner AR. Irritable bowel syndrome: A concise review of current treatment concepts. *World J Gastroenterol* 2014; 20(27): 8796-8806 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8796.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8796

INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal disorders causing patients to seek medical treatment. It exerts significant economic burden and is responsible for considerable morbidity in Western countries[1]. Despite these costs and numerous investigations into the pathophysiology and treatment of this disorder, our understanding of IBS is still incomplete. Over the last ten years, increasing insight into the enteric nervous system and how its dysfunction may play a role in IBS pathology has emerged[2]. Additionally our increasing understanding of the gut microbiome and how its potential disruption may lead to IBS symptoms has also been highlighted[3]. However, with few exceptions, these insights have yet to lead to targeted treatment strategies for IBS. Currently, many clinicians use a treatment approach based on the predominant symptoms of the patient: constipation (IBS-C), diarrhea (IBS-D), or mixed symptoms (IBS-M) (Table 1)[4]. Several new drugs have recently been examined for IBS using this symptom-based approach. Two agents for IBS-C, lubiprostone and linaclotide have been approved by the United States Food and Drug Administration (FDA) for that specific indication[5]. To improve the evidence by which drugs for IBS are approved, the FDA has recently proposed standardized outcomes for approval studies as is discussed later in this paper. The purpose of this paper is to provide the clinician with a concise review of pharmacotherapy strategies for IBS.

Table 1 Irritable bowel syndrome subtypes

Subtype	Definition (symptoms classified using Bristol stool form scale)
IBS with constipation (IBS-C)	> 25% of stools are hard or lumpy and < 25% of stools are loose/mushy or watery
IBS with diarrhea (IBS-D)	> 25% of stools are loose/mushy or watery stools and < 25% are hard or lumpy
Mixed IBS (IBS-M)	> 25% of stools are loose/mushy or watery stools and > 25% and hard or lumpy
Unsubtyped IBS	insufficient abnormality of stool consistency to meet criteria for IBS-C, D, or M (in the absence of antidiarrheals or laxatives)

IBS: Irritable bowel syndrome.

Consequently, it is divided into three sections: "classic" treatment options, "newer drugs," such as lubiprostone and linaclotide, and "alternative" treatments such as probiotics and peppermint oil. In the last section we will also discuss emerging information on the so-called "pre-cebo" effect in IBS.

CLASSIC TREATMENTS FOR IBS

Antidiarrheals

Loperamide is a synthetic opioid, which acts on intestinal muscles to prolong transit time and inhibit peristalsis. While loperamide has been studied in different subtypes of IBS, it may be particularly effective in IBS-D because of its ability to decrease fecal volume and transit time. A meta-analysis in 2000 found loperamide to be an effective agent in decreasing stool frequency and improving stool consistency, as well as demonstrating a modest improvement in global well being[6]. However, it does not appear that loperamide is effective in reducing abdominal pain in comparison to placebo. In fact, some studies show an increase in abdominal pain particularly when loperamide is used in IBS-C[7]. Other common antidiarrheal agents, such as diphenoxylate with atropine, have not been well studied in IBS and are likely to be less tolerated due to anticholinergic effects, such as sedation, dry mouth, constipation, and urinary retention.

Antidepressants

Antidepressants, such as the tricyclic antidepressants (TCA), selective serotonin reuptake inhibitors (SSRI), and serotonin norepinephrine reuptake inhibitors, have been utilized in the treatment of various functional gastrointestinal disorders. Current treatment guidelines endorse the use of either TCAs or SSRIs for patients with IBS, although duloxetine has also been studied in small trials for this population[8,9]. These agents are believed to act *via* centrally-mediated antinociceptive pathways decreasing abdominal pain associated with IBS. In addition, these agents may affect the gastrointestinal tract by peripheral means particularly in gut transit times[8]. A recent Cochrane review pooled 15 TCA and SSRI trials[10]. The antidepressant class as a whole significantly decreased pain, IBS symptom scores, and overall global assessment. A subgroup analysis revealed only TCAs remained statistically significant for abdominal pain and improvement in symptom scores. However, this may be due to a smaller number of patients and trials studying the use of SSRIs to treat IBS. In addition, an earlier meta-analysis demonstrated a reduction in pain, bloating, and other symptoms, although it contained mostly TCA trials[11].

Despite the large differences in the amount of supporting data, many clinicians are reticent to prescribe TCAs instead of SSRIs given the poor tolerability of these agents. In fact, one trial utilizing desipramine found nearly one in five subjects of that treatment arm dropped out due to adverse reactions[12]. Secondary amine tricyclic antidepressants (*e.g.*, nortriptyline) are typically better tolerated than tertiary amines (*e.g.*, amitriptyline) because of decreased anticholinergic adverse effects. In addition, lower doses of TCAs as compared to doses used to treat depression seem adequate to provide IBS symptom relief. Despite a more favorable side effect profile, SSRI use is more controversial in IBS patients as the supporting evidence is not nearly as robust. Clinical guidelines do suggest hypothetically that SSRIs may be of more utility in IBS-C and TCAs may be of more benefit in IBS-D due to their respective effects on whole gut transit times[8]. Clinicians await head-to-head trials with these agents.

Antispasmodics

Medications that relax smooth muscle *via* anticholinergic mechanisms or calcium channel antagonism have been commonly utilized for the treatment of IBS. Among these are alverine, dicyclomine (with or without cimetropium), hyoscyamine, otilonium, pinaverium, scopolamine, and trimebutine. The availability of many of these medications varies from country to country. Generally, antispasmodics have been utilized for their effects on gastrointestinal motility in attempts to reduce abdominal pain associated with IBS. They have also been evaluated in combination with agents such as acetaminophen, simethacone, and benzodiazepines in attempts to improve gastrointestinal discomfort[13-15].

Unfortunately many of the studies evaluating antispasmodics are small, suffer from methodological issues, and often fail to evaluate individual symptoms or effect on IBS subtypes. Only a small number of trials include active comparators. A recent Cochrane review of 29 antispasmodic trials for IBS suggested that some, but not all antispasmodics may decrease abdominal pain[10]. Similarly some, but not all, antispasmodics improved IBS symptom scores and global assessment. A subgroup analysis showed benefit of the use of trimebutine, pinaverium, and combined dicyclomine/cimetropium in the treatment of IBS. Anticholinergic side effects of these agents often include dose-related vision disturbances, dry mouth, and dizziness. Moreover, antispasmodics can also cause constipation, thus they should be used cautiously in patients with IBS-C. Prescribers should consider the limitations of these medications when using them for IBS.

Table 2 Response rates for lubiprostone 12 wk phase Ⅲ irritable bowel syndrome with constipation studies

	Lubiprostone	Placebo	*P* value
Overall responder	17.90%	10.10%	0.001
Month 1	10.80%	7.50%	0.078
Month 2	18.20%	11.40%	0.003
Month 3	22.00%	14.50%	0.003

Bulking agents

Several bulking agents have been examined in the treatment of IBS. These include psyllium, calcium polycarbophil, bran, and ispaghula husk. These synthetic and naturally occurring fiber supplements are often used for their ability to increase stool frequency, quality, and transit time. Consequently, they are often attractive options in all subtypes of IBS, particularly IBS-C. Most of the trials involving these agents have been small and as a result, multiple meta-analyses have been undertaken. An early systematic review found that there may be a significant improvement in global IBS symptoms with soluble fibers (psyllium, calcium polycarbophil, ispaghula), but worsening symptoms with insoluble fiber (bran)[16]. However, this review suffered from significant heterogeneity. Furthermore, a recent Cochrane review of 12 randomized control trials showed that fiber supplements do not improve abdominal pain, IBS symptom scores, or global assessment. Other meta-analyses have had similar results[17].

Osmotic laxatives

Osmotic laxatives are often used in the treatment of IBS-C due their efficacy in chronic idiopathic constipation. These agents, including polyethylene glycol (PEG) 3350 and lactulose, work by increasing water in the intestinal lumen to decrease intestinal transit time. PEG 3350 (with or without electrolytes) has been utilized in only a few randomized control trials for the treatment of IBS[18,19]. It has been shown to be effective for relieving constipation associated with IBS, but no more effective than placebo for reducing abdominal pain, bloating, or other symptoms associated with IBS[18]. Lactulose has not been rigorously studied in IBS. In addition, lactulose may cause bloating resulting from fermentation in the intestinal lumen. Thus it should not be recommended for patients with IBS.

NEWER TREATMENTS FOR IBS

Lubiprostone

Lubiprostone is a gastrointestinal chloride-channel activator (specifically at the chloride channel 2 receptor) that enhances intestinal fluid secretion which leads to increased intestinal motility and facilitation of stool passage[20]. It was FDA approved in 2006 for the treatment of chronic idiopathic constipation (CIC) at a dose of 24 μg taken twice per day. Subsequently in 2008, its use was approved for IBS-C in women older than 18 years of age at a dose of 8 μg taken twice per day. This approval was based on the results of two 12 wk randomized phase Ⅲ trials that were published in one manuscript in 2009[21]. The primary endpoint in this study was monthly responder status at three months. The definition of responder was developed between the study investigators and the FDA and thought to be more rigorous than previous trials of IBS treatments. In this study, a "monthly responder" was defined as subjects who reported moderate relief of IBS symptoms for four of four weeks or significant relief for more than two of 4 wk (Table 2). To be considered an "overall responder" (the primary efficacy endpoint), patients had to be a monthly responder for two of three months of the trial. Symptoms were recorded in a weekly electronic diary in which patients were asked "How would you rate your relief of IBS symptoms over the past week compared to how you felt before you entered the study?" Subjects' responses were recorded on a seven-point scale that ranged from "significantly worse" to "significantly relieved". Results of this study showed a statistically significant improvement in the primary efficacy endpoint (17.9% lubiprostone *vs* 10.1% placebo, $P = 0.001$; NNT = 13), as well as monthly response at months two and three (Table 2). Adverse events were frequent but similar between the lubiprostone and placebo groups, with gastrointestinal events occurring most frequently. There was no difference in serious adverse drug reactions (ADRs) or patients who discontinued treatment due to an adverse event (Table 3). ADRs to lubiprostone were reported at a lower rate in the IBS-C trials when compared to trials examining its other indications of opioid-induced constipation and CIC. This is likely due to lower systemic exposure (16 μg/d *vs* 48 μg/d) and the differences between disease states since placebo rates were higher in those trials.

Table 3 Adverse events for lubiprostone phase Ⅲ irritable bowel syndrome with constipation studies

	Lubiprostone 12 wk	Placebo 12 wk	Lubiprostone 36 wk
Serious	1%	1%	1.90%
Treatment related	22%	21%	25.40%
Nausea	8%	4%	11%
Diarrhea	6%	4%	11%
Abdominal distension	2%	2%	3.70%
Discontinuation due to ADR	5%	7%	4%

ADR: Adverse drug reaction.

Patients who completed the 12 wk study were eligible for an additional open-label 36 wk extension study if they had been at least 70% compliant with the study medication[22]. The primary objective of this study was to assess long-term safety and tolerability. Treatment related ADRs were more frequent but similar to the 12 wk study with nausea and diarrhea reported most commonly (Table 3). The drug was tolerated well with only 4% of patients withdrawing due to adverse events. This rate was lower than the 12 wk study, however this likely reflects some selection bias since patients were not treatment naive (except

those previously in the placebo arm).

Too few men with IBS-C were enrolled in the clinical trials with lubiprostone to draw any conclusions about its effectiveness in this population. Because the drug is associated with teratogenic effects in animals, the manufacturer recommends that women who could become pregnant have a negative pregnancy test before beginning therapy, as well as be able to comply with effective contraceptive measures during therapy. The drug is significantly more expensive than traditional laxatives, and should generally be reserved for patients who have failed other therapy for IBS-C.

Linaclotide

An agonist of guanylate cyclase, linaclotide is a unique agent which was recently approved by both American and European regulatory agencies for the treatment of IBS-C[23,24]. Stimulation of guanylate cyclase receptors leads to increased secretion of both guanylin and uroguanylin into the intestinal lumen where they act as a second messenger for both fluid and electrolyte release into the large bowel[25]. Linaclotide is minimally absorbed and has a strong affinity for the guanylate cyclase receptor. Preliminary clinical studies were conducted in the mid-2000s and found the drug to have significant effects on ascending colonic transit time and clinical symptoms related to stooling[26,27]. This led to phase Ⅲ studies that were submitted for regulatory approval. One such study was performed by Rao and colleagues in a randomized, double-blinded fashion on 800 patients with IBS-C[28]. These patients were randomized in this 12-wk trial to linaclotide 266 mcg (n = 405) *vs* placebo (n = 395). As with most IBS studies, the majority of patients were white females who had met Rome Ⅱ criteria for IBS-C. Exclusion criteria included cathartic colon, laxative or enema abuse, ischemic colitis, pelvic floor dysfunction, recent abdominal or pelvic surgery, or other conditions that would explain symptoms, such as inflammatory bowel disease. Of interest, this study was one of the first to use the United States FDA recommendations for trial design and outcomes in IBS studies[29]. Thus, one of the four primary outcomes in the trial was the combination of (1) an improvement of ≥ 30% from baseline in the average of the daily worst abdominal pain scores on standardized scales; and (2) an increase of ≥ 1 spontaneous bowel movements from baseline. Numerous secondary endpoints including patient assessed symptoms, such as abdominal discomfort, abdominal bloating, stool frequency and stool consistency were evaluated. In this study, the primary FDA endpoint was reached by 33.6% receiving linaclotide compared with 21.0% receiving placebo (OR = 1.9, 95%CI: 1.4-2.7, P < 0.0001; NNT = 8). All other primary and secondary efficacy endpoints were similar. Of interest was the group of patients who had improvement in abdominal pain of ≥ 30% (34.3% of linaclotide *vs* 27.1% placebo, OR = 1.4, 95%CI: 1.0-1.9, P = 0.0262; NNT = 14). This suggests that in addition to acting as a laxative, linaclotide has gut anti-nociceptive properties. The safety profile of linaclotide was favorable with diarrhea being the most common adverse effect reported (5.7% *vs* 0.3% in placebo-treated patients). Additionally, no serious or life-threatening adverse effects were reported in this study.

A similarly designed study was performed by Chey *et al*[30] to examine the long-term safety and efficacy of linaclotide in IBS-C. Subjects included 804 patients classified as having IBS-C by Rome Ⅱ criteria and were randomized to either linaclotide 290 mcg or placebo once daily for 26 wk. Exclusion criteria and outcomes were virtually identical to the study discussed above. In this 26 wk study, linaclotide achieved the FDA outcome more frequently than placebo (33.7% *vs* 13.9% respectively, P < 0.0001; NNT = 6). As with the 12 wk study, all other primary and secondary efficacy endpoints showed similar benefits with linaclotide. Diarrhea was again the most common adverse effect reported, with 5.7% of patients dropping out of the study due to this effect. This study not only helped confirm linaclotide's role in treating IBS-C, but it also showed durability of response, a notorious problem when addressing the evidence base of older treatments for this disorder. As mentioned above, these two studies were among the first to utilize the FDA recommended outcomes for IBS trials. It should be noted that other investigators have examined these outcomes and have suggested they may be conservative. Consequently, the true effect size of linaclotide in IBS-C may be greater than these studies suggest[31].

Most recently, a meta-analysis assessed all current randomized controlled trials of linaclotide for both chronic constipation as well as IBS-C[32]. For IBS-C, the investigators utilized the two studies listed above as well as a third trial for which the FDA primary outcome was compiled. When analyzing the data from these studies together, linaclotide was associated with a significant improvement in the FDA outcome [RR = 1.95 (95%CI: 1.3-2.9); NNT = 7 (95%CI: 5-11)]. The authors concluded that linaclotide was effective and had a robust effect size in treating IBS-C. Despite the growing evidence, the role of linaclotide for treating IBS-C in the United States is still uncertain. Given the published data, some experts have called for its placement as a first-line option for this disorder[33]. However, given its cost in the United States (roughly United States $900 monthly), and the reluctance of many third-party payers to cover it, its use will likely be reserved for those patients with IBS-C who have failed other treatments.

Rifaximin

As previously mentioned, a number of avenues concerning the pathogenesis of IBS have received considerable investigation in recent years. Among these lines of research is the relationship between host-gut microbiome. Disruption of this complex relationship, perhaps caused by small intestinal bacterial overgrowth (SIBO), may lead to symptoms attributed to IBS: constipation, abdominal pain and bloating, and change in bowel habit[34]. This

may explain the subset of IBS patients who develop symptoms after a gut infection (so-called "post-infectious IBS"). After disruption of the normal gut microbiome and overgrowth of the small bowel by bacteria, the resulting inflammation may lead to chronic IBS-like symptoms[35]. For the practicing clinician, this does raise interesting questions, such as is SIBO a cause of IBS, particularly the diarrhea-predominant version of the disorder[36]? Or conversely, are some patients labeled as having IBS in reality suffering from SIBO? In either event, a therapeutic strategy aimed at treating SIBO in select patients with IBS-D may be rational.

Since traditional bacterial culture of the entire small bowel is impractical, experts have recommended using breath tests, such as the hydrogen or lactulose test to assess the possibility of SIBO[37]. Selective utilization of these tests, combined with assessment of patient symptoms may help to delineate IBS patients with a SIBO component to their disorder. A recent review of this subject provides an excellent overview for the clinician[37]. Once the determination that SIBO may be playing a factor in a patient's IBS symptoms, should antimicrobials be used for treatment? And, if so, which agent would be preferred? The ideal agent would have little to no systemic absorption, would be active against common gut flora, and would have few adverse effects. Older agents traditionally used for bowel decontamination such as neomycin or metronidazole largely do not meet these criteria. Rifaximin is a drug chemically related to rifampin that has little to no systemic absorption and is well tolerated[38]. This agent has been used in patients with SIBO and has been examined in patients with IBS who do not have constipation. Currently, rifaximin is not FDA approved in the United States for IBS, however, several trials support its use for this indication.

An initial small randomized, controlled trial by Pimentel and co-workers in 87 patients with IBS suggested that a 10-d course of rifaximin 400 mg three times daily improved patient global scores of symptoms compared to placebo[39]. This improvement seemed to persist for the duration of the trial (10 wk) and led these investigators to confirm rifaximin's utility in two larger studies named TARGET-1 and TARGET-2. The results of these trials were combined and published in 2011[40]. Both studies were identically designed and enrolled patients with IBS as assessed by the Rome Ⅱ criteria. Key exclusion criteria included patients with a recent exposure to antibiotics, inflammatory bowel disease, diabetes, or use of other medications exclusively for IBS symptoms. Patients were randomized to rifaximin 550 mg twice daily for two wk or placebo and were followed for up to 10 wk after medication completion. The primary outcome was patients who reported qualitative relief of their global IBS symptoms. A key secondary endpoint was patient assessment of relief from abdominal bloating. A total of 1260 patients were enrolled in the two trials, making these studies among the largest in the IBS literature. In looking at the combined primary endpoint, 40.7% of rifaximin patients reported global improvement in symptoms compared to 31.7% of placebo patients ($P < 0.001$; NNT = 12) in the two studies combined. Numerous secondary endpoints, including abdominal bloating, were statistically better in the active treatment arm compared to placebo. This benefit was largely maintained throughout the study period, up to 10 wk after treatment ended. No significant adverse effects were reported in the rifaximin arm, and no cases of *Clostridium difficile*-associated diarrhea or ischemic colitis were seen. The authors concluded that a two-week course of rifaximin may provide lasting improvement of symptoms in patients with IBS without constipation.

One concern with the aforementioned study was the need to know durability of response to see if or when patients would need retreatment. The TARGET lead investigators performed a retrospective review of patients in their health-system who had received rifaximin for IBS[41]. Of the 71 patients evaluated, the majority did require retreatment for relapsing symptoms. However, patients who responded to one treatment generally also responded to subsequent ones. This is in accordance with a study in only SIBO patients that found a recurrence of symptoms in approximately half of patients nine months after rifaximin treatment[42]. Such patients may be required to receive multiple doses of an expensive antibiotic (roughly United States $700 per treatment course), raising the possibility of developing resistance[43].

Most recently, a meta-analysis was published examining the treatment effect of rifaximin in IBS patients[44]. The authors performed a systematic review that culminated in five articles subject to meta-analysis. The results of this analysis are consistent with individual trial data. Rifaximin was found to improve global IBS symptoms compared to placebo (OR = 1.57, 95%CI: 1.22-2.01; NNT = 11). Bloating symptoms also improved compared to placebo (OR = 1.55, 95%CI: 1.23-1.96; NNT = 11).

Given the price of rifaximin in the US, many patients or payers will be unwilling to assume the cost of the drug. Yet another cost consideration is whether all patients should undergo hydrogen or lactulose breath testing before rifaximin therapy. A recent study from Switzerland suggests that a high percentage of patients diagnosed with IBS will have positive breath testing, and when treated with rifaximin, will have a sustained response[45]. This suggests that, if available to the clinician, such testing should be performed to help guide therapy with rifaximin.

Other treatments, including prucalopride (a selective serotonin receptor agonist with prokinetic activity) may become viable options for IBS, but data to date are limited[46].

ALTERNATIVE TREATMENTS

Peppermint oil

Peppermint oil is an antispasmodic available over the counter in the United States that blocks calcium channels resulting in gastrointestinal smooth muscle relaxation. [8]According to the American College of Gastroen-

Table 4 Probiotic strains

Clinical condition	Effectiveness	Specific strain
IBS	B	*Bifidobacterium infantis* B5624
IBS	B	VSL33 (composite containing multiple strains): 3 strains of *Bifidobacterium*: *Bifidobacterium longum* *Bifidobacterium finfantis* *Bifidobacterium breve* 4 strains of *Lactobacillus*: *Lactobacillus acidophilus* *Lactobacillus casei* *Lactobacillus bulgaricus* *Lactobacillus plantarum* 1 strain of *Streptococcus salivarius*, subspecies *Thermophilus*
IBS	C	*Bifidobacterium animalis*
IBS	C	*Lactobacillus plantarum* 299V

IBS: Irritable bowel syndrome.

terology, peppermint oil may provide short-term relief of discomfort and abdominal pain in IBS and appears to be superior to placebo[17]. However, this conclusion is based on a small number of studies (Grade 2B), and there are no long-term studies to support chronic use. Additionally, there is large variation in the doses of oral peppermint oil (450-900 mg/d in 2-3 divided doses) and duration of therapy used in clinical trials (1-3 mo)[47-51]. The most common adverse effect reported with oral peppermint oil is gastroesophageal reflux. This is thought to be due in part to relaxation of the lower esophageal sphincter, and has led to the popularity of enteric-coated preparations that can bypass the upper gastrointestinal tract[52].

A 2008 meta-analysis including 4 trials (n = 392) provides support for the use of peppermint oil in IBS[17]. In this study, peppermint oil (n = 197) resulted in fewer patients reporting persistent symptoms compared to treatment with placebo (n = 195) for a duration of one to three months (26% *vs* 65% respectively, RR = 0.43, 95%CI: 0.32-0.59; NNT = 2.5). However, statistically significant heterogeneity was detected between studies (I^2 = 31.1%, P = 0.23). Only one of the trials (n = 57) reported the type of IBS according to stool pattern, as two of the four trials included predate the use of these subgroups which were developed with the publication of the Rome Ⅱ criteria in 1999. In this study, 25% of patients had predominant IBS-C and 75% had IBS-D[49]. Additionally, the treatment effect of peppermint oil was found to last for 4 wk after stopping therapy in over 50% of patients in this trial. Although other alternative therapies have been advocated to treat IBS, data on many of these treatments are limited. Oral capsaicin was examined in one small trial found a small improved in abdominal pain and bloating scores, but discontinuations due to initial intolerance was high[53].

Probiotics

Probiotics are dietary supplements that contain live or attenuated bacteria, or bacterial products, which when ingested, may have beneficial effects to a patient's health by altering the gastrointestinal flora[54]. The precise mechanism of action of probiotics is not known. It is hypothesized that inflammation or disproportion of the gastrointestinal bacterial flora may play a part in the pathogenesis of IBS. The probiotic theory suggests that supplementation of the gastrointestinal flora with the right types and numbers of live microorganisms can improve the gut flora and promote health[55]. Additionally, there is evidence to suggest that certain strains of probiotics may stimulate an anti-inflammatory response or improve visceral hypersensitivity, which could theoretically lead to an improvement in symptoms of IBS[56]. Probiotics may comprise a formulation containing a single or mixed-culture of live microbes and are obtainable in diverse preparations, including fermented milk drinks, food products (snacks, chocolates, *etc*.), capsules, pills, and powders[57]. Side effects are generally minimal, although there are risks for patients who are immune compromised[58].

Several strains of probiotics have been studied, but the most commonly used organisms are the lactobacillae and bifidobacteria (Table 4). Several clinical trials have evaluated the effectiveness of a variety of probiotics in patients with IBS, and in general, probiotics can be used for patients with all types of IBS (IBS-D, IBS-C, and IBS-M). Nonetheless, the supportive evidence for treating IBS with probiotics is weak due to the heterogeneity of the studies and the varying probiotics evaluated[59]. Relating and summarizing these trials is difficult due to differences in study design, patient populations, dosing regimens, probiotic species utilized, and reported clinical end points. Regardless of these limitations, some recent systematic reviews and meta-analyses concluded that probiotics seem to be effective in patients with IBS[60-63]. A systematic review of data pooled from 10 randomized controlled trials (RCTs) involving 918 patients with IBS showed a significant benefit for probiotics *vs* placebo in reducing IBS symptoms and decreasing pain and flatulence [RR = 0.71, 95%CI: 0.57-0.88, I^2 = 68%; NNT = 4 (95%CI: 3-12.5)][61]. An additional systematic review of 14 RCTs showed a moderate improvement in overall symptoms, abdominal pain, and flatulence in patients taking probiotics *vs* placebo (OR = 1.6; 95%CI: 1.2-2.2 for dichotomous data from seven trials and standardized mean difference = 0.23; 95%CI: 0.07-0.38 for continuous data from six trials)[60]. Several of the studies found improvement in primary end points compared with baseline, but only some were able to show significant improvement over placebo.

Two types of probiotics were granted the highest rating for efficacy in the treatment of IBS (level "B": based on positive, controlled studies and in spite of the presence of some negative studies) in the Recommendations for Probiotic Use from a Yale University Workshop[64]. The recommendation for *Bifidobacterium infantis* 35624 was concluded from two well-designed clinical trials[65,66] and has been labeled with the "B" rating since the 2008 update[67]. One particular mixture of probiotics, VSL#3

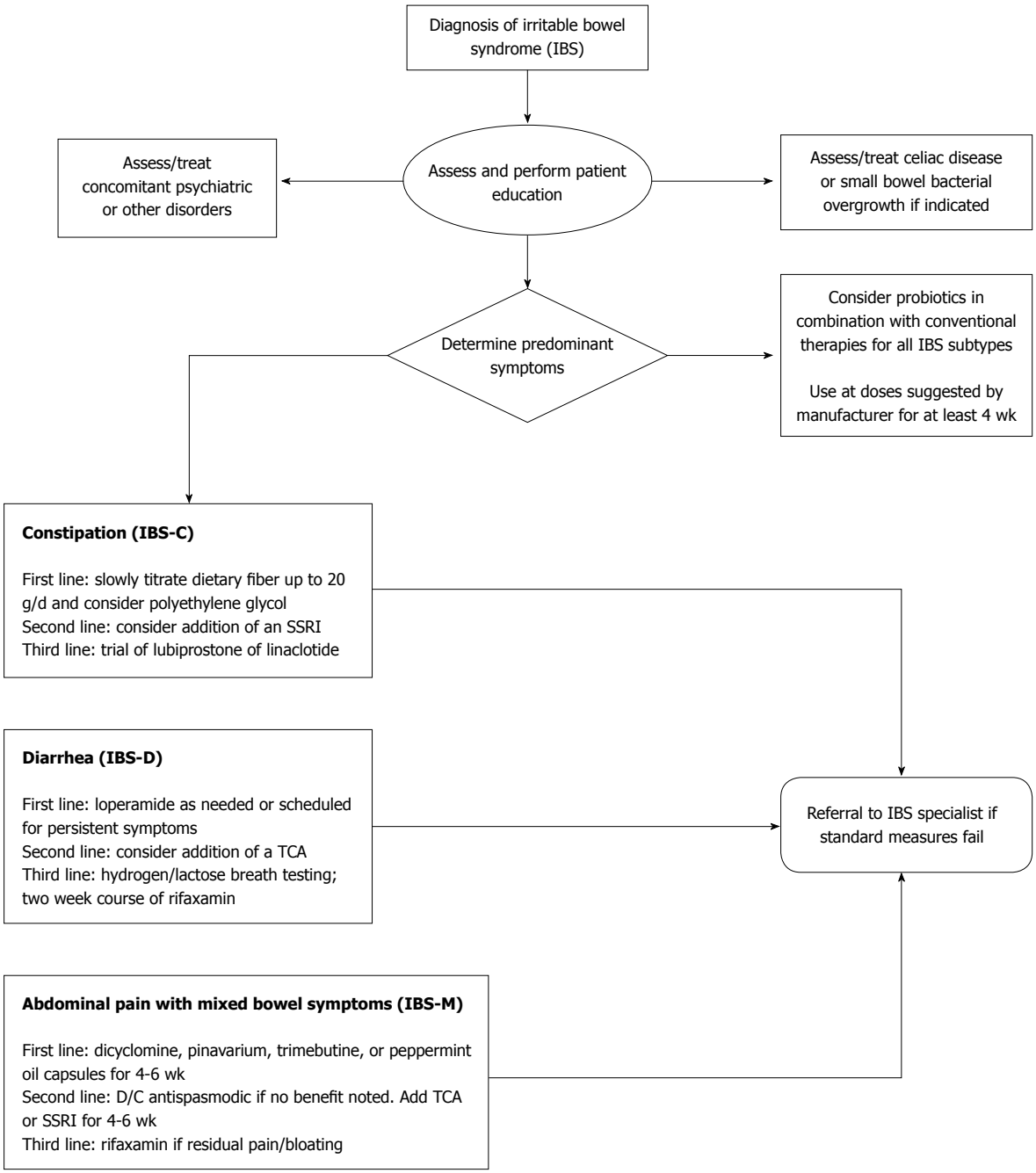


Figure 1 Treatment algorithm for irritable bowel syndrome. IBS: Irritable bowel syndrome; TCA: Tricyclic antidepressant; SSRI: Selective serotonin reuptake inhibitor; D/C: Discontinue.

moved up from a "C" rating to a "B" rating based on the results from two trials[68,69]. A more recent study evaluated patients randomized to receive either placebo or *Bifidobacterium bifidum* MIMBb75[70]. This particular probiotic type reduced the global assessment of IBS symptoms (on a 7-point Likert scale) by 0.88 points *vs* 0.16 points ($P < 0.0001$) and had adequate symptom relief in 47% *vs* 11% ($P < 0.0001$, NNT = 3).

It is important to note that in the United States, regulatory authorities consider probiotics as dietary supplements that are not intended to diagnose, treat, cure, or mitigate the effects of diseases. It is advised that consumers should consult with a health care professional before consuming these products. Many of the available products have not been sufficiently tested for their effectiveness in IBS in satisfactorily designed clinical trials. Another critical factor is the issue of the type products being sold to the public and if their content have enough viable amounts of organisms to make a clinical difference[71]. Furthermore, a study by Mercer *et al*[72] evaluated how patients with IBS viewed probiotics. In this study, patients conveyed frustration that their more traditional IBS medications had worked at first, but became less effective over time. Patients in this study considered probiotics as an appealing potential therapeutic approach for those running out of pharmaceutical options.

Further research is needed to help identify the most effective probiotic species and strains, and the ideal regimen. However, with limited available treatments for IBS, the overall safety of probiotics lowers the bar for trying probiotic products in patients with IBS. Clinicians should not recommend probiotics as monotherapy in symptomatic patients with IBS, but rather in combination with current conventional treatments[57]. Based on the limited evidence for the use of probiotics in patients with IBS, the following organizations have developed guidelines to aid clinicians in their recommendations of products to patients. The National Institute for Health and Clinical Excellence in the United Kingdom has the following recommendation about the use of probiotics in IBS: "Probiotics do not appear to be harmful (unless they come from an unreliable source) and they might benefit people with IBS; they should be advised to take the product at the dose recommended by the manufacturer for at least four wk while monitoring the effect[73]." Additionally, recommendations from the American College of Gastroenterology Task Force on IBS resolved that Bifidobacteria and certain combinations of probiotics demonstrate some efficacy, and that in single-organism studies, lactobacilli do not appear effective for patients with IBS[8].

"Pre-cebo" effect

The placebo effect in clinical trials has long been known, and because of the vague nature of IBS symptoms and the use of primary outcomes that are often subjective in nature, high placebo response rates have been noted in IBS trials. However, Kim *et al*[74] have also described the potential for a "pre-cebo" effect in IBS, which impacts the treatment outcome even before the study begins. The pre-cebo effect describes the impact of consent language used in clinical trials on expectations of benefit from the study medication. This was studied in 59 patients with IBS-D who were randomized to one of 3 medication questionnaires (desipramine, alosetron, or rifaximin). Subjects were asked to rate the percent (0%-100%) improvement in symptoms that would be sufficient for the subject to feel adequate relief. Patients anticipating therapy for any of the three drugs had very high expectations of benefit (> 70%), and patients anticipating rifaxamin treatment had the highest expectation of improvement needed for satisfactory symptom relief (87.3%) compared to desipramine (73.4%, $P < 0.001$) and alosetron (76.8%, $P = 0.049$). This was thought to be due both to the wording used in the consent process, as well as any preconceived ideas about the study medication. The authors note that the high overall expectations may be a challenge to positive outcomes of therapy in any study, and this is particularly noteworthy in IBS because many trials depend on subjective measures of improvement that are patient driven.

CONCLUSION

Gastroenterology practitioners have more agents than ever before to treat the symptoms associated with IBS. Unfortunately, despite advances in our understanding of the pathophysiology of this disorder, targeted treatments do not yet exist. Based on the literature reviewed in this paper, the authors have constructed an algorithm to guide practicing clinicians who encounter this disorder (Figure 1). This algorithm is stratified to symptoms, economic costs, and level of evidence. Using this, or another systematic approach will enable practitioners who treat IBS to do so more efficiently, yet provide relief to a significant number of their patients with this disorder.

REFERENCES

1 **Mitra D**, Davis KL, Baran RW. All-cause health care charges among managed care patients with constipation and comorbid irritable bowel syndrome. *Postgrad Med* 2011; **123**: 122-132 [PMID: 21566422 DOI: 10.3810/pgm.2011.05.2290]

2 **Stasi C**, Rosselli M, Bellini M, Laffi G, Milani S. Altered neuro-endocrine-immune pathways in the irritable bowel syndrome: the top-down and the bottom-up model. *J Gastroenterol* 2012; **47**: 1177-1185 [PMID: 22766747 DOI: 10.1007/s00535-012-0627-7]

3 **Simrén M**, Barbara G, Flint HJ, Spiegel BM, Spiller RC, Vanner S, Verdu EF, Whorwell PJ, Zoetendal EG. Intestinal microbiota in functional bowel disorders: a Rome foundation report. *Gut* 2013; **62**: 159-176 [PMID: 22730468 DOI: 10.1136/gutjnl-2012-302167]

4 **Tack J**, Fried M, Houghton LA, Spicak J, Fisher G. Systematic review: the efficacy of treatments for irritable bowel syndrome--a European perspective. *Aliment Pharmacol Ther* 2006; **24**: 183-205 [PMID: 16842448]

5 **Pohl D**, Tutuian R, Fried M. Pharmacologic treatment of constipation: what is new? *Curr Opin Pharmacol* 2008; **8**: 724-728 [PMID: 18687411 DOI: 10.1016/j.coph.2008.07.008]

6 **Jailwala J**, Imperiale TF, Kroenke K. Pharmacologic treatment of the irritable bowel syndrome: a systematic review of randomized, controlled trials. *Ann Intern Med* 2000; **133**: 136-147 [PMID: 10896640]

7 **Hovdenak N**. Loperamide treatment of the irritable bowel syndrome. *Scand J Gastroenterol Suppl* 1987; **130**: 81-84 [PMID: 3306904]

8 **Brandt LJ**, Chey WD, Foxx-Orenstein AE, Schiller LR, Schoenfeld PS, Spiegel BM, Talley NJ, Quigley EM. An evidence-based position statement on the management of irritable bowel syndrome. *Am J Gastroenterol* 2009; **104** Suppl 1: S1-S35 [PMID: 19521341 DOI: 10.1038/ajg.2008.122]

9 **Brennan BP**, Fogarty KV, Roberts JL, Reynolds KA, Pope HG, Hudson JI. Duloxetine in the treatment of irritable bowel syndrome: an open-label pilot study. *Hum Psychopharmacol* 2009; **24**: 423-428 [PMID: 19548294 DOI: 10.1002/hup.1038]

10 **Ruepert L**, Quartero AO, de Wit NJ, van der Heijden GJ, Rubin G, Muris JW. Bulking agents, antispasmodics and antidepressants for the treatment of irritable bowel syndrome. *Cochrane Database Syst Rev* 2011; (**8**): CD003460 [PMID: 21833945 DOI: 10.1002/14651858.CD003460.pub3]

11 **Jackson JL**, O'Malley PG, Tomkins G, Balden E, Santoro J, Kroenke K. Treatment of functional gastrointestinal disorders with antidepressant medications: a meta-analysis. *Am J Med* 2000; **108**: 65-72 [PMID: 11059442]

12 **Drossman DA**, Toner BB, Whitehead WE, Diamant NE, Dalton CB, Duncan S, Emmott S, Proffitt V, Akman D, Frusciante K, Le T, Meyer K, Bradshaw B, Mikula K, Morris CB, Blackman CJ, Hu Y, Jia H, Li JZ, Koch GG, Bangdiwala SI. Cognitive-behavioral therapy versus education and desipramine versus placebo for moderate to severe functional

bowel disorders. *Gastroenterology* 2003; **125**: 19-31 [PMID: 12851867]
13 **Mueller-Lissner S**, Tytgat GN, Paulo LG, Quigley EM, Bubeck J, Peil H, Schaefer E. Placebo- and paracetamol-controlled study on the efficacy and tolerability of hyoscine butylbromide in the treatment of patients with recurrent crampy abdominal pain. *Aliment Pharmacol Ther* 2006; **23**: 1741-1748 [PMID: 16817918]
14 **Wittmann T**, Paradowski L, Ducrotté P, Bueno L, Andro Delestrain MC. Clinical trial: the efficacy of alverine citrate/simeticone combination on abdominal pain/discomfort in irritable bowel syndrome--a randomized, double-blind, placebo-controlled study. *Aliment Pharmacol Ther* 2010; **31**: 615-624 [PMID: 20003095 DOI: 10.1111/j.1365-2036.2009.04216.x]
15 **Ritchie JA**, Truelove SC. Treatment of irritable bowel syndrome with lorazepam, hyoscine butylbromide, and ispaghula husk. *Br Med J* 1979; **1**: 376-378 [PMID: 32949]
16 **Bijkerk CJ**, Muris JW, Knottnerus JA, Hoes AW, de Wit NJ. Systematic review: the role of different types of fibre in the treatment of irritable bowel syndrome. *Aliment Pharmacol Ther* 2004; **19**: 245-251 [PMID: 14984370]
17 **Ford AC**, Talley NJ, Spiegel BM, Foxx-Orenstein AE, Schiller L, Quigley EM, Moayyedi P. Effect of fibre, antispasmodics, and peppermint oil in the treatment of irritable bowel syndrome: systematic review and meta-analysis. *BMJ* 2008; **337**: a2313 [PMID: 19008265 DOI: 10.1136/bmj.a2313]
18 **Chapman RW**, Stanghellini V, Geraint M, Halphen M. Randomized clinical trial: macrogol/PEG 3350 plus electrolytes for treatment of patients with constipation associated with irritable bowel syndrome. *Am J Gastroenterol* 2013; **108**: 1508-1515 [PMID: 23835436 DOI: 10.1038/ajg.2013.197]
19 **Awad RA**, Camacho S. A randomized, double-blind, placebo-controlled trial of polyethylene glycol effects on fasting and postprandial rectal sensitivity and symptoms in hypersensitive constipation-predominant irritable bowel syndrome. *Colorectal Dis* 2010; **12**: 1131-1138 [PMID: 19575740 DOI: 10.1111/j.1463-1318.2009.01990.x]
20 Amitiza Product Information 2013. Bethesda, MD: Sucampo Pharmaceuticals, Inc., 2013. Accessed 9/1/13. Available from: URL: http://general.takedapharm.com/content/file/PI.pdf?applicationCode=69CADEA4-354A-44BF-B5E7-017-BDF1333E&fileTypeCode=AMITIZAPI
21 **Drossman DA**, Chey WD, Johanson JF, Fass R, Scott C, Panas R, Ueno R. Clinical trial: lubiprostone in patients with constipation-associated irritable bowel syndrome--results of two randomized, placebo-controlled studies. *Aliment Pharmacol Ther* 2009; **29**: 329-341 [PMID: 19006537 DOI: 10.1111/j.1365-2036.2008.03881.x]
22 **Chey WD**, Drossman DA, Johanson JF, Scott C, Panas RM, Ueno R. Safety and patient outcomes with lubiprostone for up to 52 weeks in patients with irritable bowel syndrome with constipation. *Aliment Pharmacol Ther* 2012; **35**: 587-599 [PMID: 22251419 DOI: 10.1111/j.1365-2036.2011.4983.x]
23 Linzess Product Information 2013. Cambridge, MA: Ironwood Pharmaceuticals, Inc., 2013. Accessed 7/8/13. Available from: URL: http://www.frx.com/pi/linzess_pi.pdf
24 **European Medicines Agency**. Constella Information Page 2013. Accessed 7/8/13. Available from: URL: http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/002490/human_med_001597.jsp&mid=WC0b01ac058001d124
25 **Bryant AP**, Busby RW, Bartolini WP, Cordero EA, Hannig G, Kessler MM, Pierce CM, Solinga RM, Tobin JV, Mahajan-Miklos S, Cohen MB, Kurtz CB, Currie MG. Linaclotide is a potent and selective guanylate cyclase C agonist that elicits pharmacological effects locally in the gastrointestinal tract. *Life Sci* 2010; **86**: 760-765 [PMID: 20307554 DOI: 10.1016/j.lfs.2010.03.015]
26 **Andresen V**, Camilleri M, Busciglio IA, Grudell A, Burton D, McKinzie S, Foxx-Orenstein A, Kurtz CB, Sharma V, Johnston JM, Currie MG, Zinsmeister AR. Effect of 5 days linaclotide on transit and bowel function in females with constipation-predominant irritable bowel syndrome. *Gastroenterology* 2007; **133**: 761-768 [PMID: 17854590]
27 **Johnston JM**, Kurtz CB, Macdougall JE, Lavins BJ, Currie MG, Fitch DA, O'Dea C, Baird M, Lembo AJ. Linaclotide improves abdominal pain and bowel habits in a phase IIb study of patients with irritable bowel syndrome with constipation. *Gastroenterology* 2010; **139**: 1877-1886.e2 [PMID: 20801122 DOI: 10.1053/j.gastro.2010.08.041]
28 **Rao S**, Lembo AJ, Shiff SJ, Lavins BJ, Currie MG, Jia XD, Shi K, MacDougall JE, Shao JZ, Eng P, Fox SM, Schneier HA, Kurtz CB, Johnston JM. A 12-week, randomized, controlled trial with a 4-week randomized withdrawal period to evaluate the efficacy and safety of linaclotide in irritable bowel syndrome with constipation. *Am J Gastroenterol* 2012; **107**: 1714-1724; quiz p.1725 [PMID: 22986440]
29 Guidance for industry: irritable bowel syndrome - clinical evaluation of drugs for treatment Food and Drug Administration 2012. Accessed 7/8/13. Available from: URL: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM205269.pdf
30 **Chey WD**, Lembo AJ, Lavins BJ, Shiff SJ, Kurtz CB, Currie MG, MacDougall JE, Jia XD, Shao JZ, Fitch DA, Baird MJ, Schneier HA, Johnston JM. Linaclotide for irritable bowel syndrome with constipation: a 26-week, randomized, double-blind, placebo-controlled trial to evaluate efficacy and safety. *Am J Gastroenterol* 2012; **107**: 1702-1712 [PMID: 22986437]
31 **Macdougall JE**, Johnston JM, Lavins BJ, Nelson LM, Williams VS, Carson RT, Shiff SJ, Shi K, Kurtz CB, Baird MJ, Currie MG, Lembo AJ. An evaluation of the FDA responder endpoint for IBS-C clinical trials: analysis of data from linaclotide Phase 3 clinical trials. *Neurogastroenterol Motil* 2013; **25**: 481-486 [PMID: 23384406 DOI: 10.1111/nmo.12089]
32 **Videlock EJ**, Cheng V, Cremonini F. Effects of linaclotide in patients with irritable bowel syndrome with constipation or chronic constipation: a meta-analysis. *Clin Gastroenterol Hepatol* 2013; **11**: 1084-1092.e3; quiz e68 [PMID: 23644388 DOI: 10.1016/j.cgh.2013.04.032]
33 **Brenner DM**. Linaclotide for the treatment of irritable bowel syndrome with constipation: is it time to reshuffle the deck? *Gastroenterology* 2013; **145**: 476-478 [PMID: 23791794 DOI: 10.1053/j.gastro.2013.06.017]
34 **Azpiroz F**, Bouin M, Camilleri M, Mayer EA, Poitras P, Serra J, Spiller RC. Mechanisms of hypersensitivity in IBS and functional disorders. *Neurogastroenterol Motil* 2007; **19**: 62-88 [PMID: 17280586]
35 **Pimentel M**, Lezcano S. Irritable Bowel Syndrome: Bacterial Overgrowth--What's Known and What to Do. *Curr Treat Options Gastroenterol* 2007; **10**: 328-337 [PMID: 17761126]
36 **Carroll IM**, Ringel-Kulka T, Siddle JP, Ringel Y. Alterations in composition and diversity of the intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome. *Neurogastroenterol Motil* 2012; **24**: 521-530, e248 [PMID: 22339879 DOI: 10.1111/j.1365-2982.2012.01891.x]
37 **Ghoshal UC**. How to interpret hydrogen breath tests. *J Neurogastroenterol Motil* 2011; **17**: 312-317 [PMID: 21860825 DOI: 10.5056/jnm.2011.17.3.312]
38 **Basseri RJ**, Weitsman S, Barlow GM, Pimentel M. Antibiotics for the treatment of irritable bowel syndrome. *Gastroenterol Hepatol* (N Y) 2011; **7**: 455-493 [PMID: 22298980]
39 **Pimentel M**, Park S, Mirocha J, Kane SV, Kong Y. The effect of a nonabsorbed oral antibiotic (rifaximin) on the symptoms of the irritable bowel syndrome: a randomized trial. *Ann Intern Med* 2006; **145**: 557-563 [PMID: 17043337]
40 **Pimentel M**, Lembo A, Chey WD, Zakko S, Ringel Y, Yu J, Mareya SM, Shaw AL, Bortey E, Forbes WP. Rifaximin therapy for patients with irritable bowel syndrome without constipation. *N Engl J Med* 2011; **364**: 22-32 [PMID: 21208106

DOI: 10.1056/NEJMoa1004409]
41 **Pimentel M**, Morales W, Chua K, Barlow G, Weitsman S, Kim G, Amichai MM, Pokkunuri V, Rook E, Mathur R, Marsh Z. Effects of rifaximin treatment and retreatment in nonconstipated IBS subjects. *Dig Dis Sci* 2011; **56**: 2067-2072 [PMID: 21559740 DOI: 10.1007/s10620-011-1728-5]
42 **Lauritano EC**, Gabrielli M, Scarpellini E, Lupascu A, Novi M, Sottili S, Vitale G, Cesario V, Serricchio M, Cammarota G, Gasbarrini G, Gasbarrini A. Small intestinal bacterial overgrowth recurrence after antibiotic therapy. *Am J Gastroenterol* 2008; **103**: 2031-2035 [PMID: 18802998]
43 **Farrell DJ**. Rifaximin in the treatment of irritable bowel syndrome: is there a high risk for development of antimicrobial resistance? *J Clin Gastroenterol* 2013; **47**: 205-211 [PMID: 23340064]
44 **Menees SB**, Maneerattannaporn M, Kim HM, Chey WD. The efficacy and safety of rifaximin for the irritable bowel syndrome: a systematic review and meta-analysis. *Am J Gastroenterol* 2012; **107**: 28-35; quiz 36 [PMID: 22045120 DOI: 10.1038/ajg.2011.355]
45 **Meyrat P**, Safroneeva E, Schoepfer AM. Rifaximin treatment for the irritable bowel syndrome with a positive lactulose hydrogen breath test improves symptoms for at least 3 months. *Aliment Pharmacol Ther* 2012; **36**: 1084-1093 [PMID: 23066911 DOI: 10.1111/apt.12087]
46 **Manabe N**, Rao AS, Wong BS, Camilleri M. Emerging pharmacologic therapies for irritable bowel syndrome. *Curr Gastroenterol Rep* 2010; **12**: 408-416 [PMID: 20694841]
47 **Lech Y**, Olesen KM, Hey H, Rask-Pedersen E, Vilien M, Ostergaard O. [Treatment of irritable bowel syndrome with peppermint oil. A double-blind study with a placebo]. *Ugeskr Laeger* 1988; **150**: 2388-2389 [PMID: 3061106]
48 **Liu JH**, Chen GH, Yeh HZ, Huang CK, Poon SK. Enteric-coated peppermint-oil capsules in the treatment of irritable bowel syndrome: a prospective, randomized trial. *J Gastroenterol* 1997; **32**: 765-768 [PMID: 9430014]
49 **Cappello G**, Spezzaferro M, Grossi L, Manzoli L, Marzio L. Peppermint oil (Mintoil) in the treatment of irritable bowel syndrome: a prospective double blind placebo-controlled randomized trial. *Dig Liver Dis* 2007; **39**: 530-536 [PMID: 17420159]
50 **Capanni M**, Surrenti E, Biagini M, Milani S, Surrenti C, Galli A. Efficacy of peppermint oil in the treatment of irritable bowel syndrome: a randomized, controlled trial. *Gazz Med Ital* 2005; **164**: 119-126
51 **Merat S**, Khalili S, Mostajabi P, Ghorbani A, Ansari R, Malekzadeh R. The effect of enteric-coated, delayed-release peppermint oil on irritable bowel syndrome. *Dig Dis Sci* 2010; **55**: 1385-1390 [PMID: 19507027 DOI: 10.1007/s10620-009-0854-9]
52 **Kligler B**, Chaudhary S. Peppermint oil. *Am Fam Physician* 2007; **75**: 1027-1030 [PMID: 17427617]
53 **Bortolotti M**, Porta S. Effect of red pepper on symptoms of irritable bowel syndrome: preliminary study. *Dig Dis Sci* 2011; **56**: 3288-3295 [PMID: 21573941]
54 **Ford AC**, Talley NJ. Irritable bowel syndrome. *BMJ* 2012; **345**: e5836 [PMID: 22951548 DOI: 10.1136/bmj.e5836]
55 **Reid G**, Younes JA, Van der Mei HC, Gloor GB, Knight R, Busscher HJ. Microbiota restoration: natural and supplemented recovery of human microbial communities. *Nat Rev Microbiol* 2011; **9**: 27-38 [PMID: 21113182 DOI: 10.1038/nrmicro2473]
56 **McCarthy J**, O'Mahony L, O'Callaghan L, Sheil B, Vaughan EE, Fitzsimons N, Fitzgibbon J, O'Sullivan GC, Kiely B, Collins JK, Shanahan F. Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance. *Gut* 2003; **52**: 975-980 [PMID: 12801954]
57 **Ringel Y**, Ringel-Kulka T. The rationale and clinical effectiveness of probiotics in irritable bowel syndrome. *J Clin Gastroenterol* 2011; **45** Suppl: S145-S148 [PMID: 21992954 DOI: 10.1097/MCG.0b013e31822d32d3]
58 **Boyle RJ**, Robins-Browne RM, Tang ML. Probiotic use in clinical practice: what are the risks? *Am J Clin Nutr* 2006; **83**: 1256-1264; quiz 1446-1447 [PMID: 16762934]
59 **Wilkins T**, Pepitone C, Alex B, Schade RR. Diagnosis and management of IBS in adults. *Am Fam Physician* 2012; **86**: 419-426 [PMID: 22963061]
60 **Hoveyda N**, Heneghan C, Mahtani KR, Perera R, Roberts N, Glasziou P. A systematic review and meta-analysis: probiotics in the treatment of irritable bowel syndrome. *BMC Gastroenterol* 2009; **9**: 15 [PMID: 19220890 DOI: 10.1186/1471-230X-9-15]
61 **Moayyedi P**, Ford AC, Talley NJ, Cremonini F, Foxx-Orenstein AE, Brandt LJ, Quigley EM. The efficacy of probiotics in the treatment of irritable bowel syndrome: a systematic review. *Gut* 2010; **59**: 325-332 [PMID: 19091823 DOI: 10.1136/gut.2008.167270]
62 **Brenner DM**, Moeller MJ, Chey WD, Schoenfeld PS. The utility of probiotics in the treatment of irritable bowel syndrome: a systematic review. *Am J Gastroenterol* 2009; **104**: 1033-1049; quiz 1050 [PMID: 19277023 DOI: 10.1038/ajg.2009.25]
63 **McFarland LV**, Dublin S. Meta-analysis of probiotics for the treatment of irritable bowel syndrome. *World J Gastroenterol* 2008; **14**: 2650-2661 [PMID: 18461650]
64 **Floch MH**, Walker WA, Madsen K, Sanders ME, Macfarlane GT, Flint HJ, Dieleman LA, Ringel Y, Guandalini S, Kelly CP, Brandt LJ. Recommendations for probiotic use-2011 update. *J Clin Gastroenterol* 2011; **45** Suppl: S168-S171 [PMID: 21992958 DOI: 10.1097/MCG.0b013e318230928b]
65 **O'Mahony L**, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, O'Sullivan GC, Kiely B, Collins JK, Shanahan F, Quigley EM. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. *Gastroenterology* 2005; **128**: 541-551 [PMID: 15765388]
66 **Whorwell PJ**, Altringer L, Morel J, Bond Y, Charbonneau D, O'Mahony L, Kiely B, Shanahan F, Quigley EM. Efficacy of an encapsulated probiotic Bifidobacterium infantis 35624 in women with irritable bowel syndrome. *Am J Gastroenterol* 2006; **101**: 1581-1590 [PMID: 16863564]
67 **Floch MH**, Walker WA, Guandalini S, Hibberd P, Gorbach S, Surawicz C, Sanders ME, Garcia-Tsao G, Quigley EM, Isolauri E, Fedorak RN, Dieleman LA. Recommendations for probiotic use--2008. *J Clin Gastroenterol* 2008; **42** Suppl 2: S104-S108 [PMID: 18542033 DOI: 10.1097/MCG.0b013e31816b903f]
68 **Kim HJ**, Vazquez Roque MI, Camilleri M, Stephens D, Burton DD, Baxter K, Thomforde G, Zinsmeister AR. A randomized controlled trial of a probiotic combination VSL# 3 and placebo in irritable bowel syndrome with bloating. *Neurogastroenterol Motil* 2005; **17**: 687-696 [PMID: 16185307]
69 **Guandalini S**, Magazzù G, Chiaro A, La Balestra V, Di Nardo G, Gopalan S, Sibal A, Romano C, Canani RB, Lionetti P, Setty M. VSL#3 improves symptoms in children with irritable bowel syndrome: a multicenter, randomized, placebo-controlled, double-blind, crossover study. *J Pediatr Gastroenterol Nutr* 2010; **51**: 24-30 [PMID: 20453678 DOI: 10.1097/MPG.0b013e3181ca4d95]
70 **Guglielmetti S**, Mora D, Gschwender M, Popp K. Randomised clinical trial: Bifidobacterium bifidum MIMBb75 significantly alleviates irritable bowel syndrome and improves quality of life--a double-blind, placebo-controlled study. *Aliment Pharmacol Ther* 2011; **33**: 1123-1132 [PMID: 21418261 DOI: 10.1111/j.1365-2036.2011.04633.x]
71 **Whorwell PJ**. Do probiotics improve symptoms in patients with irritable bowel syndrome? *Ther Adv Gastroenterol* 2009; **2** (Suppl 1): S37-S44 [DOI: 10.1177/1756283X09335637]
72 **Mercer M**, Brinich MA, Geller G, Harrison K, Highland J, James K, Marshall P, McCormick JB, Tilburt J, Achkar JP, Farrell RM,

Sharp RR. How patients view probiotics: findings from a multicenter study of patients with inflammatory bowel disease and irritable bowel syndrome. *J Clin Gastroenterol* 2012; **46**: 138-144 [PMID: 21716123 DOI: 10.1097/MCG.0b013e318225f545]

73 NICE (2008) Irritable Bowel Syndrome. Accessed 9/16/13. Available from: URL: http://www.nice.org.uk/Guidance/CG61#summary

74 **Kim SE**, Kubomoto S, Chua K, Amichai MM, Pimentel M. "Pre-cebo": an unrecognized issue in the interpretation of adequate relief during irritable bowel syndrome drug trials. *J Clin Gastroenterol* 2012; **46**: 686-690 [PMID: 22739219 DOI: 10.1097/MCG.0b013e31825828a7]

P- Reviewers: Abraham P, Butterworth J, Bortolotti M, Stanghellini V, Wasim F
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8807

World J Gastroenterol 2014 July 21; 20(27): 8807-8820
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

Irritable bowel syndrome: A disease still searching for pathogenesis, diagnosis and therapy

Massimo Bellini, Dario Gambaccini, Cristina Stasi, Maria Teresa Urbano, Santino Marchi, Paolo Usai-Satta

Massimo Bellini, Dario Gambaccini, Maria Teresa Urbano, Santino Marchi, Gastrointestinal Unit, Department of Gastroenterology, University of Pisa, 56124 Pisa, Italy
Cristina Stasi, Department of Experimental and Clinical Medicine University of Florence, 50134 Florence, Italy
Cristina Stasi, Epidemiological Observatory, Health Agency of Tuscany, 50141 Florence, Italy
Paolo Usai-Satta, Gastrointestinal Unit, P. Brotzu Hospital, 09124 Cagliari, Italy
Author contributions: Bellini M contributed to the conception and design of the study and wrote the manuscript; Gambaccini D and Urbano MT contributed to the conception of the study and wrote the manuscript; Stasi C contributed to the conception of the study and revised the manuscript; Marchi S revised the manuscript; Usai-Satta P contributed to the study design and wrote the manuscript.
Correspondence to: Massimo Bellini, MD, PhD, Gastrointestinal Unit, Department of Gastroenterology, University of Pisa, via Paradisa n 2, 56124 Pisa, Italy. mbellini@med.unipi.it
Telephone: +39-50-997448 Fax: +39-50-997368
Received: October 22, 2013 Revised: January 21, 2014
Accepted: May 28, 2014
Published online: July 21, 2014

Abstract

Irritable bowel syndrome (IBS) is the most frequently diagnosed functional gastrointestinal disorder in primary and secondary care. It is characterised by abdominal discomfort, pain and changes in bowel habits that can have a serious impact on the patient's quality of life. The pathophysiology of IBS is not yet completely clear. Genetic, immune, environmental, inflammatory, neurological and psychological factors, in addition to visceral hypersensitivity, can all play an important role, one that most likely involves the complex interactions between the gut and the brain (gut-brain axis). The diagnosis of IBS can only be made on the basis of the symptoms of the Rome Ⅲ criteria. Because the probability of organic disease in patients fulfilling the IBS criteria is very low, a careful medical history is critical and should pay particular attention to the possible comorbidities. Nevertheless, the severity of the patient's symptoms or concerns sometimes compels the physician to perform useless and/or expensive diagnostic tests, transforming IBS into a diagnosis of exclusion. The presence of alarming symptoms (fever, weight loss, rectal bleeding, significant changes in blood chemistry), the presence of palpable abdominal masses, any recent onset of symptoms in patient aged over 50 years, the presence of symptoms at night, and a familial history of celiac disease, colorectal cancer and/or inflammatory bowel diseases all warrant investigation. Treatment strategies are based on the nature and severity of the symptoms, the degree of functional impairment of the bowel habits, and the presence of psychosocial disorders. This review examines and discusses the pathophysiological aspects and the diagnostic and therapeutic approaches available for patients with symptoms possibly related to IBS, pointing out controversial issues and the strengths and weaknesses of the current knowledge.

Key words: Irritable bowel syndrome; Pathogenesis; Diagnosis; Therapy

Core tip: The pathophysiology of irritable bowel syndrome (IBS) is not definitely known and many fundamental questions remain unanswered about its pathophysiology, diagnosis and therapy. Conflicting results reflect the largely overlapping data of healthy controls and the wide heterogeneity of the IBS patients. This review summarises the main pathophysiological aspects, practical diagnostic approaches and therapeutic management strategies for patients with symptoms possibly related to IBS, in addition to pointing out some controversial issues and pointing out the strengths and the weaknesses of our current knowledge.

Bellini M, Gambaccini D, Stasi C, Urbano MT, Marchi S, Usai-Satta P. Irritable bowel syndrome: A disease still searching for

pathogenesis, diagnosis and therapy. *World J Gastroenterol* 2014; 20(27): 8807-8820 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8807.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8807

INTRODUCTION

Irritable bowel syndrome (IBS) is quite prevalent in the general population (from 5% to 20%) and represents the functional gastrointestinal (GI) disorder most frequently encountered in primary and secondary care[1,2]. IBS is characterised by abdominal discomfort, pain and changes in bowel habits (constipation and/or diarrhoea)[3] that wax and wane over time. Moreover, it is often associated with other functional digestive and non-digestive disorders[4-8].

The pathophysiology of IBS is not definitely known but most likely involves central and peripheral mechanisms. A disruption of the so called "brain-gut axis" that determines changes in digestive motility and secretion, causes visceral hypersensitivity and leads to cellular and molecular abnormalities in the enteroendocrine and immune systems has been suggested. In addition, genetic factors, infections and alterations of the intestinal microbiota, inflammation and food intolerance and/or hypersensitivity could play a role by altering the integrity of the intestinal barrier and increasing intestinal permeability[9,10]. Up to now, unfortunately, conflicting results have been achieved, most likely reflecting the largely overlapping data of healthy controls and the wide heterogeneity of the IBS population.

The direct and indirect costs of the syndrome are significant, as IBS can have a serious impact on patient quality of life. Because there are not yet any available biological markers or resolving therapies, the patient may undergo expensive tests and treatments[11-13].

The therapeutic approach depends on the intensity of symptoms and the degree of psychosocial comorbidities. Initial treatment is directed towards education, reassurance and lifestyle modification. In a second phase, an appropriate pharmacotherapy can be proposed on the basis of individual or global intestinal symptoms and/or psychological disturbances.

Many different drugs have been suggested for IBS treatment, but their real benefits are very debatable. Based on the multifaceted pathophysiology of the disease, it is unlikely that drugs acting on a single receptor and/or a unique pathophysiologic mechanism would be able to provide any substantial therapeutic gain over a placebo in this disease, for which the placebo response rate is approximately 40%[14].

Essentially, we are still far from having discovered the magic bullet capable of treating all IBS symptoms. Although many papers have been published on this syndrome in recent years, up to now, many fundamental questions remain unanswered about its pathophysiology, diagnosis and therapy.

This review summarises the main pathophysiological aspects, practical diagnostic approaches and therapeutic management strategies for patients with symptoms possibly related to IBS, in addition to pointing out some controversial issues and pointing out the strengths and the weaknesses of our current knowledge.

A search of the literature was carried out using the online databases of PubMed, Medline and Cochrane to identify articles published in English concerning pathophysiology, diagnosis and treatment of IBS.

PATHOPHYSIOLOGICAL ASPECTS

The pathophysiology of IBS, as in all functional digestive disorders, is complicated because there is no clearly identified pathophysiological basis for the disease. In fact, IBS is identified by a combination of chronic or recurrent GI symptoms in the absence of structural abnormalities (radiological/endoscopic) or biomarkers capable of positively identifying this condition. Aside from these drawbacks, the clinical manifestations of IBS are themselves extremely heterogeneous, a sort of "semantic umbrella" under which different clinical situations related to phenotypic aspect (traditionally subtyped as diarrhoea predominant, constipation predominant and mixed type) and the modality of clinical onset (post-infectious, food-related, stress-linked, *etc.*) fall[15].

The aetiology of IBS is multifactorial. Many pathogenetic factors, in various combinations and not all necessarily present in each patient, can play an important role (Table 1). Genetics, immune factors, environmental influences, inflammatory and infective agents, neurological and psychological factors, hypersensitivity to food and to bile salts and altered intestinal microbiota and permeability can all influence the brain-gut axis, leading to abnormal GI function and motility. It is unclear which among these factors is the trigger or how these conditions converge to initiate the IBS; previous studies aiming to identify a factor as more of a trigger over the others all failed to distinguish any one trigger.

The genetic factors have been extensively studied. Up to 33% of IBS patients have a family history of IBS, compared to 2% of controls[16]. There is a higher prevalence of the disease in families of patients with IBS compared to the families of the spouses without IBS[17]. Moreover, some studies have reported a higher prevalence in monozygotic twins compared with heterozygotes, indicating a hypothetical genetic component[18]. However, other studies[19] demonstrated that having a parent with IBS was a better predictive factor than having a twin affected with IBS, suggesting that the environmental factor is more important.

The genetic factors involved in the pathogenesis of IBS has also been evaluated by a number of studies investigating the possible role of gene polymorphisms coding for serotonin (SERT), cholecystokinin (CCK) receptors 1, anti-inflammatory and pro-inflammatory interleukins and alpha 2 adrenergic receptors[20-22]. As sero-

Table 1 Factors potentially involved in the pathogenesis of irritable bowel syndrome

Altered intestinal motility
Food intolerance/allergy
Enteric infection/inflammation
Altered intestinal immunity
Altered gut microbiota
Genetics
Psychological distress and disorders; sexual abuse

tonin was involved in the regulation of digestive motility, secretion and visceral sensitivity, particular investigative emphasis has been placed on polymorphisms of the gene regulating the reuptake of serotonin (SERT), which can induce a variation of its synaptic concentration[23]. SERT polymorphisms are not related to the development or onset of IBS, but rather to a different clinical expression, a greater perception of abdominal pain and an increased dissatisfaction regarding bowel habits[24].

Recently, a "biopsychosocial" model[25,26] has been introduced, in an attempt to integrate and harmonise the different factors (genetic, environmental and psychological) acting in a synergistic way to produce these symptoms.

These deficiencies in understanding the pathophysiological mechanisms of IBS have a heavy negative effect on clinical practice and may explain the disappointing results of previous therapeutic attempts, as well as the high costs of management. Currently, there is no single drug that is able to treat all of the symptoms related to IBS; rather, a "drug cocktail" is administered, having different effects on different symptoms.

Previous studies[27] have considered this syndrome a result of alterations in the normal digestive motility pattern, the so-called "spastic colon". Subsequently, much interest was directed toward visceral hypersensitivity, under the hypothesis that IBS patients experienced visceral stimuli more strongly than healthy subjects. Later, IBS came to be considered a two-way interaction between the gut and the brain, with much interest directed not only toward the activation/deactivation of afferent and efferent nervous stimuli but also toward the effects of neuromodulators.

The possibility that IBS could be initiated after an enteric infection and the evidence that, in inflammatory bowel disease limited to the mucosa, patients suffer from enhanced sensory perception and motor dysfunction have driven researchers to study these as further potential causes of IBS.

Some previous studies[28,29] attempted to assess whether an abnormal motility pattern is typical in cases of IBS; however, despite identifying cluster contractions in phase Ⅱ of the migrating motor complex in the jejunum, propagated ileal contractions related to pain and an increased postprandial motor activity of the colon, up to now, all attempts made have failed to reach a single typical pattern.

An altered colonic transit rate [accelerated in IBS and diarrhoea (IBS-D) and slowed in IBS with constipation (IBS-C)] was described in some studies[30,31] but these results have not been confirmed by more recent studies[32,33]. Salvioli *et al*[34] reported a decreased capacity of the motor activity in the small intestine to eliminate intestinal gas, resulting in abdominal distension and typical symptoms of IBS. IBS patients likely experience psychological stress, foods, neurotransmitters and/or rectal or bowel distension, which can lead to an altered motor response that leads to the same motor events being perceived more strongly and painfully[35].

Visceral hypersensitivity in IBS patients is supported by several studies[36-38]. Verne *et al*[39] used functional nuclear magnetic resonance (RMN) to show that a mechanical stimulus (rectal distension) active different regions of the brain in healthy volunteers, compared to patients with IBS. Unfortunately, this technique is expensive and not widely available. Moreover, comorbidities, such as fibromyalgia and psychological disturbances, can significantly affect its outcome.

Psychological disorders, including sexual and physical abuse, result in a high percentage of patients with functional disorders. Even if the disorders are not directly responsible for the onset or progression of the IBS symptoms, they certainly determine a different perception of the symptoms and result in more frequent requests for medical aid. In fact, these disorders are more common in IBS patients who seek medical care than in patients who do not ask for medical help or healthy volunteers[15,40].

Psychological distress and disorders can affect the brain-gut axis, promoting the release of corticotropin-releasing hormone, which is able to influence mood, digestive motility, permeability, visceral sensitivity and inflammatory pathways *via* neuroendocrine and autonomic outflows[41-44]. Dinan *et al*[44] showed that physical and mental stress in IBS patients increased the levels of pro-inflammatory interleukins, activating both the hypothalamic-autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axes and consequently increasing the serological adreno-cortico-tropic-hormone and cortisol levels. Recent studies[44,45] introduced the hypothesis that IBS could be an inflammatory disorder that is supported by a dysregulation of the HPA.

On the other hand, it has been shown that physical and psychological stress activates different regions of the brain among patients with IBS than among healthy volunteers. In particular, IBS patients have a greater activation of the mid-anterior cingulate cortex, an area linked to anxiety, fear and hypervigilance[46]. This area is the target of many antidepressant drugs and psychotherapy. In healthy controls, stress instead activates the perigenual area, from which originate the descending inhibitory pathways that control visceral afferents to the posterior horn of the spinal cord[47].

A continuous and mutual interaction between the gut and the brain is made possible through the autonomic nervous system and the enteric nervous system *via* neuroendocrine mediators (VIP, 5HT, Ach, NO, NO, CCK, *etc.*); this system comprises the so-called "gut - brain axis".

Signals received from the GI tract affect the brain that, in turn, can affect the motility, secretion and immune functions of the digestive tract. Thus, alterations to this system may cause many digestive disorders, and particularly IBS, compared to normal, unaltered subjects[41,48,49].

The neuroendocrine system is potentially involved in the pathogenesis of IBS. This system is very complex and consists essentially of two components.

The endocrine cells (at least 14 endocrine or paracrine cell populations), which are distributed between the epithelial cells of the digestive mucosa and directly in contact with the intestinal lumen and its contents; and the nerve fibres (peptidergic, serotonergic, nitrergic, *etc.*) of the enteric nervous system[15].

Motility, secretion, absorption and intestinal microcirculation are all influenced by this system by the means of several mediators that have endocrine (released directly into the blood stream), autocrine/paracrine (local effects) or neuroendocrine (released from synapses into the bloodstream) functions[41].

An alteration to this system has been hypothesised, in which a decreased density of cells producing gastric inhibitory polypeptide (GIP) and somatostatin (in D-IBS and C-IBS) and in those producing secretin and CCK (in D-IBS) was reported in the small intestine, whereas a lower expression of cells producing 5-hydroxytryptamine and PYY was detected in the colons of patients with D-IBS and C-IBS[15,41]. An abnormal inflammatory response to different events (stress, infections, food, *etc.*) could be responsible for the abnormal cellularity in the colonic mucosa and the increased concentration of pro-inflammatory interleukins detected in the colons of some IBS patients[50]. These studies suggest that the activation of mast cells, macrophages or leukocytes producing inflammatory mediators is able to affect the motility, secretion, sensitive nerve endings and ultimate perception of pain.

Biopsies from the colons of IBS patients showed an increased activation of lymphocytes and mast cells in close proximity to the enteric neurons, with increased production of cytokines and other proinflammatory and vasoactive peptides[51,52]. Degranulation of these cells (especially mast cells) has been associated with the onset of the typical abdominal pain endured by IBS patients[53]. Moreover, the density of immunocompetent cells gradually increases on a spectrum from controls to patients with IBS, then to patients with microscopic colitis and, finally, to those with ulcerative colitis[54].

Inflammation can also result from a previous enteric infection. The onset of IBS follows an infection in approximately 10% of patients. In these patients, there are increases in the levels of CD3 serum lymphocytes, CD8 intraepithelial lymphocytes, and macrophage calprotectin-positive cells. Moreover, cells producing serotonin and CCK were found to be increased in the small bowel, while those producing serotonin and PYY were decreased in the colon. These alterations were usually transient but tended to persist in patients who developed IBS[55].

In post-infectious IBS and D-IBS, intestinal permeability has also been studied. The findings included a decreased expression and remodelling of the structural proteins constituting the epithelial "tight junctions" in the cells of the small intestine and colon. These changes increased the intestinal permeability, resulting in an easier passage of antigenic material through the epithelium and a stimulation of the intestinal immune system (especially mast cells) with the production of the proteases, histamine and prostanoids able to maintain the permeability and to produce abnormal neuronal responses, inducing the motor and sensory results typical in IBS[42].

Based on these results, it is evident that preserving, maintaining or restoring the normal composition of the intestinal microbiota is essential for good bowel function[42]. The intestinal microbiota is a major target of many therapeutic options for relieving IBS symptoms. The colon of each individual contains from 300 to 500 different species of bacteria. Thus, each of our microbiota is individual and unique. The microbiota is influenced by the environment, diet, previous infections, genetics, age, and antibiotic therapy. In normal conditions, the lactobacilli and bifidobacteria bind to epithelial cells, inhibiting the binding of pathogens and reinforcing the defences of the mucosal barrier. In addition, lactobacilli and bifidobacteria do not produce gas by fermenting carbohydrates and inhibiting the growth of the Clostridia species, which do produce this effect. Lactobacilli and bifidobacteria were found to be decreased in IBS patients, and their activities were found to be heavily compromised[56]. Moreover, some evidence indicates that probiotics affect intestinal fermentation and stabilise the intestinal microbiota, normalising the relationship between pro-inflammatory and anti-inflammatory cytokines with beneficial effects on intestinal inflammation, permeability and visceral sensitivity[57,58].

Unfortunately, at present, there are intrinsic difficulties in clearly establishing the role of the gut microbiota in the pathophysiology of IBS, both due to the great heterogeneity in the clinical presentation of IBS and to the limitations of the available studies (study design, length of observation, small sample, *etc.*).

Finally, the role of food in IBS merits specific mention. Patients with IBS tend to declare that their symptoms are often exacerbated by meals or by certain foods (sweeteners, fats, *etc.*). The classical IgE-mediated food allergy does not seem to play an important role in IBS. In the recent past, high levels of the specific IgG4 for wheat, beef, pork and lamb were found in IBS patients, compared to healthy subjects, and based on this, an exclusion diet was proposed[59]. On the other hand, this subgroup of Ig seems to be only an epiphenomenon of mucosal production, according to recent evidence[60].

In any case, up to 60% of patients with IBS reported a worsening of symptoms after food intake, in particular after specific foods like milk and dairy products, wheat, onions, beans, spices, cabbage, red meat, fried, smoked products, and caffeine. These foods represent the so-

Table 2 Most frequently reported comorbidities in irritable bowel syndrome patients

Functional dyspepsia and functional heartburn
Fibromyalgia
Chronic fatigue syndrome
Back pain
Multiple chemical sensitivity syndrome
Post-traumatic stress disorder
Psychological/psychiatric disorders
Sleep disturbances
Migraine and tension headaches

called fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs). However, studies supporting this are limited and demonstrate only a partial improvement in patients after the restriction of these foods. More frequently, IBS patients seem to have an exaggerated gastric-colic reflex after eating any item of food.

In recent years, it has been observed that the ingestion of gluten causes abdominal discomfort and IBS-like symptoms in subjects without a diagnosis of celiac disease (the so-called gluten sensitivity).

At the moment, the mechanisms responsible for these symptoms are not clear. Most likely, the gluten, as other well-known factors, alters the intestinal permeability, activating the enteric and autonomous nervous systems and producing the typical symptoms of IBS. Recently, authors have disagreed on the topic of gluten sensitivity, instead attempting to explain the problem with a simpler hypothesis: gluten-rich foods may cause symptoms with the same mechanisms of the FODMAPs[61,62]. The positive effect of the gluten-free diet on abdominal disorders could be due to the drastic reduction of FODMAPs that is inevitable in a diet of this type.

Up to now, the available results in the literature conflict; thus, further studies are needed to clarify this intriguing matter.

DIAGNOSTIC APPROACHES

A careful medical history is critical for the evaluation of a patient with a possible diagnosis of IBS. Particular attention has to be devoted to many different issues, such as dietary habits, therapies (especially the intake of drugs capable of altering the bowel frequency and/or causing abdominal pain), the degree of physical activity, comorbidities, previous surgical interventions, presence of symptoms suggesting anxiety or depression, and recent trips to exotic locations[3,63].

In the absence of accepted and shared biological markers, symptoms remain the cornerstone for the diagnosis of IBS.

Regarding the symptom "pain", it is useful to assess its type (cramping, tensive, stabbing, burning), localisation, frequency, duration, mode of occurrence and possible changes in relation to defecation, to food intake (or to intake of particular foods), to stressful events and to the menstrual cycle[63,64].

As for abdominal distension or tension, it is mandatory to ask the patient if it is visible from others or if it is otherwise measurable (changes in size, inability to tie the skirt or pants, *etc.*). Additionally, patients should be asked whether their pain gets worse at certain times or improves with evacuation or emission of the flatus.

It is also necessary to investigate the characteristics of the defecation: difficult or prolonged, painful or simply incomplete, the presence of a sensation of anorectal blocking, the need for manual help, the presence of ineffective attempts or, on the contrary, of an urgency at defecation and real episodes of faecal incontinence[64].

Moreover, it is important to check for the presence of blood, mucus or pus in the faeces and to assess the usual shape of the stool using the Bristol Scale that, by relating the rate of intestinal transit with faecal consistency, provides a visual aid to help the patient better classify a topic otherwise difficult to objectify[65].

Additionally, it is mandatory to look for the possible co-morbidities that can occur in a patient with IBS, because they can increase the perception of the disease severity[8,13,66,67].

In Table 2, the most frequent co-morbidities are represented. These share common characteristics, such as the following: (1) a higher prevalence in females; (2) pathophysiology linked to low-grade inflammation, stress, somatisation, hypersensitivity, changes in the central processing of peripheral afferents and/or alterations of substances acting as neuromodulators; (3) a diagnosis mainly based on symptoms; (4) possible responsiveness to antidepressant medications and cognitive-behavioural therapies; (5) frequent multidisciplinary management; and (6) a considerable reduction of the quality of life and high, direct and indirect, costs.

The presence of alarm symptoms, the so-called "red flags" like fever, weight loss, rectal bleeding, and significant changes in blood chemistry, should be investigated, as well as the presence of palpable abdominal masses, any recent onset of symptoms in patients aged over 50 years, the presence of symptoms at night, and a familiar history positive for celiac disease, colorectal cancer and/or inflammatory bowel disease[64,68].

Still, some authors[69] believe that the accuracy of the "alarm symptoms" is disappointing. In particular, rectal bleeding and nocturnal pain would be of little value in discriminating patients with IBS from patients with organic disease, while anaemia and weight loss would have low sensitivity, but high specificity, to identify an organic disease.

A physical examination would not be very rich in information, as it could only detect abdominal tenderness (localised or diffuse) and abdominal hypertympanism or bowel sounds at auscultation, but this practice reassures the patient and can provide a first, coarse exclusion of organic diseases (abdominal masses, *etc.*). The examination should include the inspection of the anorectal region and a digital rectal examination, preferably in the left-lateral decubitus, which would provide useful information

Table 3 Diseases and conditions considered in the differential diagnosis

Celiac disease and malabsorption
Lactose intolerance, fructose intolerance
Inflammatory bowel disease
Lymphocytic and collagenous colitis
Whipple's disease
Colonic cancer
Enteric infections
Metabolism disorders (*e.g.*, thyroid, diabetes, *etc.*)
Food allergy and intolerance
Endometriosis
SIBO
Neuroendocrine tumors
Drugs

SIBO: Small intestinal bacterial overgrowth.

about the dynamics of the pelvic floor, especially if any functional alteration is suspected. Thus, the presence of comorbidities and organic diseases can be detected[63,70-72].

The use of specifically dedicated scores to measure the impairment of the quality of life and symptom severity has been debated in clinical practice, both at the initial stages and later, in order to verify the effectiveness of the therapy administered[73]. Indeed, any such scoring systems are not widely used outside of clinical trials, even if they do not seem time-consuming or difficult to use[74-77].

Can a diagnosis of IBS be made only using only symptom-based criteria? The evidence from the literature seems reassuring in this respect, because the probability of organic disease arising in patients fulfilling the IBS criteria is very low[78]. Nevertheless, the nature and severity of the symptoms themselves, or of the patient's concerns and fears, sometimes compel the physician to perform unnecessary, useless, and/or expensive diagnostic tests, transforming IBS into a diagnosis of exclusion.

Indeed, in the differential diagnosis, the conditions reported in Table 3 will have to be considered with greater or lesser probability[68].

Unfortunately, there are no available biological markers that clearly identify IBS patients.

Some recent studies have examined faecal lactoferrin and calprotectin, which seem quite suitable to differentiate between infectious bursal disease and IBS but are not able to provide a certain diagnosis of IBS[79,80].

Recent studies have investigated some biomarkers involved in the pathophysiology of IBS[45,81]. A recent systematic review and meta-analysis examined the placebo response rate in treatment trials for IBS and demonstrated a high placebo response[82].

In the case of a patient with IBS-like chronic recurring abdominal symptoms, the presence of alarming symptoms should first be assessed[68,69,83,84]. In the presence of alarming symptoms, further investigation should be undertaken. On the contrary, in the case of Rome Ⅲ criteria positivity and in the absence of alarm symptoms, possible comorbidities (which are part of the IBS management) should be considered. Serological screening for celiac disease and a few basal blood tests have to be performed; if a negative result is returned, it is usually sufficient to reassure the patient and to offer advice on drug therapies, lifestyle habits and diet. A check-up after 8-12 wk should be offered, and in cases with sustained improvement, the patient will enter into a follow-up program (Figure 1).

In the case of a patient with symptoms in any way compatible with irritable bowel syndrome but that did not satisfy the Rome criteria, or in the case of a patient with a poor response to the therapy, depending on the prevailing symptoms (constipation, diarrhoea, abdominal pain/bloating), different options should be considered (Figure 1).

In the case of constipation, dietary habits and behaviours, as well as the use of laxatives, should be checked. In the case of the ineffectiveness of these measures, if not already performed, an assessment of the thyroid function, routine blood tests and screening for celiac disease are recommended. In the case of diarrhoea and abdominal pain/distention, lactose breath test (LBT) (or simply lactose withdrawal), a faecal blood test, faecal Calprotectin or Lactoferrin, stool culture, test for ova and parasites, a chemico-physical examination to test for Clostridium difficile toxins and an abdominal ultrasound aimed at studying the enteric loops should be considered.

If signs of a specific disease emerge from the investigation or from specific treatments, further investigation should be initiated. In the case of a negative outcome, it will become mandatory to proceed to the next steps, as follows (Figure 1): (1) in the case of constipation, the possibility arises of performing a colonoscopy, anorectal manometry, defecography, intestinal transit time and, in carefully selected cases, colonic and gastrojejunal manometry; (2) in the case of diarrhoea and abdominal pain, it will become appropriate to check and eventually change the patients' drugs; (3) in the case of a failed colonoscopy, biopsies may be useful; and (4) in the case of a negative outcome of a colonoscopy, the further investigations reported in Figure 1 should be considered.

Still, it is mandatory to emphasise that none of these investigations, even those that are costly and unusual, should be performed to achieve the diagnosis of IBS, which is essentially based on the Rome Ⅲ criteria, as reported above. On the contrary, these tools are to be taken into account only in a patient with abdominal symptoms that are IBS-like but Rome Ⅲ criteria-negative or -equivocal. They may also be used in IBS patients with very severe symptoms that require a careful reassessment of the clinical situation.

In IBS, the follow up should be tailored to the patient, because the disease is characterised by variable remissions and relapses, with symptoms waxing and waning over time, often oddly and sometimes in coincidence with stressful events, anxiety, the intake of certain foods, *etc.* IBS patients usually tend to avoid fixed controls, although, at least at the beginning, a clinical visit 2-3 mo after the diagnosis is advised to assess the patient's adher-

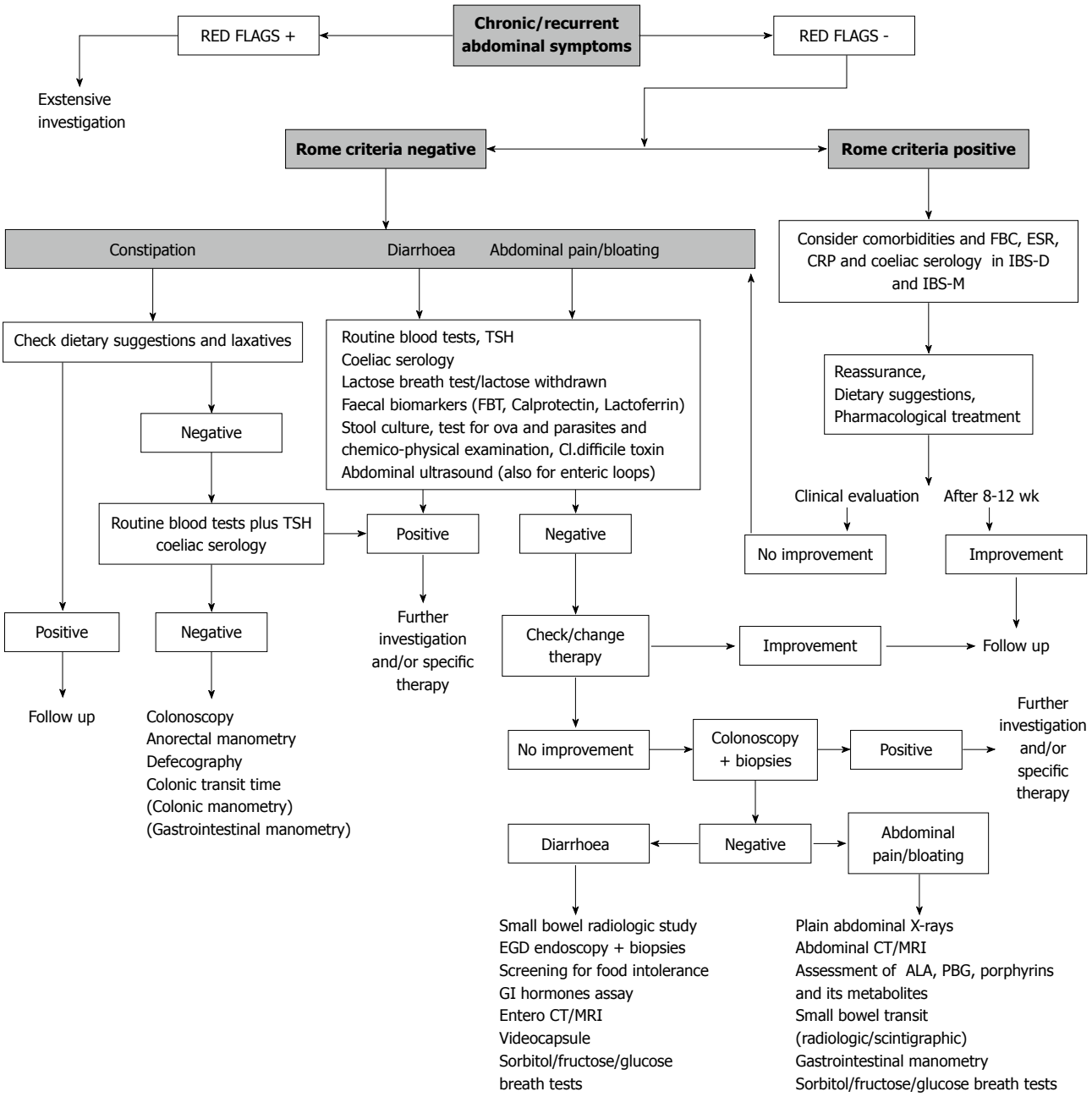


Figure 1 Diagnostic-therapeutic algorithm in a patient with abdominal symptoms possibly related to irritable bowel syndrome. FBT: Faecal blood test; FBC: Full blood count; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; PBG: Porphobilinogen; IBS: Irritable bowel syndrome; CT: Computed tomography; MRI: Magnetic resonance imaging.

ence to therapy and the dietary and behavioural recommendations.

The aim will be to help IBS patients perceive their symptoms as part of a chronic, intermittent disorder, learning to live with them. Thus, these patients can rejoin that "silent majority" of IBS patients who perceive her/his symptoms as no more than a nuisance and do not seek further special care, doctor visits, or additional diagnostic tests.

THERAPEUTIC PERSPECTIVES

Treatment strategies for IBS are based on the nature and severity of the symptoms, the degree of functional impairment of the bowel habits, and the presence of psychosocial comorbidity. In general, milder symptoms relate primarily to visceral hypersensitivity and are commonly treated symptomatically, with pharmacological agents directed at the gut. However, more severe symptoms are associated with greater levels of psychosocial problems and often require psychological and antidepressant medications.

There is limited evidence for the efficacy, safety and tolerability of the therapies currently available for the treatment of IBS. Overall, there is a limited availability of pharmacological agents licensed specifically for the treat-

Table 4 Indication of pharmacological agents in individual irritable bowel syndrome symptoms

Constipation	Diarrhoea	Pain
Soluble fibre	Opioid agents	Antispasmodics
Osmotic Laxative	5-HT$_3$ antagonists	Peppermint oil
5-HT$_4$ agonists	Probiotics	Serotoninergic drugs
Secretagogues	Antibiotics	Antidepressants
Probiotics	Mesalazine	Herbal therapy
SSRI	Colestyramine	Acupuncture
	Tricyclic antidepressants	

SSRI: Selective serotonin reuptake inhibitors.

ment of IBS subtypes, and new agents are eagerly awaited. In any case, it is difficult to achieve a significant therapeutic improvement in global IBS symptoms[64,69,71,85,86].

There is some evidence for improvements in individual IBS symptoms with the use of antidiarrhoeals, antispasmodics, bulking agents, laxatives, tricyclic antidepressants and behavioural therapy. Despite evidence that some pharmaceutical agents benefit the treatment of IBS in the short term, there is no medical intervention that has been proven to alter the long-term natural history of this condition. Further, there is no agreement on a gold-standard for the treatment of IBS. Finally, in functional GI disorders, in which the trial endpoints are likely to be less tangible than organic conditions, the placebo response rate may be very high (over 40%)[82]. Table 4 summarises the various drug categories and their relationships with individual IBS symptoms.

Education and reassurance

A strong physician-patient relationship should be the foundation for effective treatment and realistic expectations. Responding to all patient concerns and questions and spending time in the clinical visits validate their condition. A reassurance-based approach permits the patient to understand and accept his or her affliction and to participate in a care strategy. Using this approach, a decrease in the number of health care visits, a reduction in symptoms, and improved patient satisfaction can be easier obtained.

Diet

Patients with IBS commonly believe that specific dietary products contribute to their symptoms of abdominal discomfort, bloating, or alterations of bowel habits. The truth is that no specific food is likely implicated, as true food allergies and intolerances are rare. In many cases, IBS patients have an exaggerated gastric-colic reflex after eating certain foods.

Patients can associate with their complaints the ingestion of certain foods, such as fatty foods, caffeine, alcoholic beverages, carbonated foods, or gas-producing foods. Specifically, symptoms can be related to FODMAPs, such as fructans, galactans, lactose, fructose, sorbitol, xylitol, and mannitol[87]. Studies supporting this are limited and demonstrate a partial improvement in patients after the restriction of these foods. Otherwise, a lactose-restricted diet does not seem to produce a clear clinical benefit in IBS. Beyond this, recent evidence has shown that lactose intolerance was equally prevalent among IBS patients and the general population[64]. Finally, a recent study showed that patients with IBS but without celiac disease may reach satisfactory symptom control with a gluten-free diet but may suffer a symptom relapse after a gluten rechallenge[61]. Only a double-blind gluten challenge can discriminate between IBS and gluten-sensitivity patients. In any case, some care should be taken to avoid an unnecessarily restrictive diet with potentially serious nutritional consequences.

Fibre and bulking agents

Most physicians recommend the use of dietary fibre and bulking agents to regularise bowel function and to reduce meteorism and pain in patients with IBS. The quality of the evidence supporting this recommendation, however, is poor. Some randomised placebo controlled trials have compared the effectiveness of increasing the dietary content of soluble fibre (psyllium and ispaghula) or insoluble fibre (bran) in patients with IBS and constipation. There is some evidence that patients taking psyllium have significant symptom relief, whereas bran shows no clinical benefit and actually may worsen symptoms in many cases[64,69,71,73,85,86,88].

Antispasmodic agents

The rationale for using antispasmodic agents is to attenuate the postprandial abdominal pain seen in patients with IBS. The mechanisms of action of different antispasmodics can be divided broadly into those that directly affect the intestinal smooth muscle and those with anticholinergic/antimuscarinic effects[64,69,71,85,86]. The evidence for the effectiveness of these agents is not compelling.

One meta-analysis demonstrated an advantage of antispasmodics over placebo in terms of abdominal pain and distention[88]. Of all of the drugs studied, the most data were available for otilonium, trimebutine, cimetropium, hyoscine, and pinaverium. Trimebutine seemed to have no benefit over placebo in treating IBS, whereas the other four drugs all significantly reduced the risk of persistent symptoms after treatment. The anticholinergic side effects, including constipation, dry mouth, visual disturbances, and urinary retention, can lead to the discontinuation of these medications. Finally, there is evidence for the efficacy of some peppermint oil preparations (which may also act as antispasmodics) in IBS, but few data are available about the long-term results and adverse effects[88].

Anti-constipation agents

The presence or absence of abdominal pain should be more useful than other associated features for characterising IBS-C in comparison with chronic constipation. However, a clear clinical distinction is not always possible in clinical practice.

Traditional laxatives: Consistent with recent reviews, a therapeutic trial of traditional laxatives (*i.e.*, osmotic laxatives, stimulant laxatives), which are effective, safe, and generally inexpensive, should be considered for managing chronic constipation before newer agents (secretagogues, serotonin 5-HT$_4$ receptor agonists) are used[70,88]. In particular, polyethylene glycol (PEG) is more effective than lactulose in increasing stool frequency and improving stool consistency; thus, it is considered the first choice of treatment for chronic constipation[70].

However, no placebo-controlled, randomised study of laxatives in IBS has been published. Laxatives do not show a significant effect in reducing abdominal pain in IBS. A single small sequential study with PEG in adolescents with IBS-C showed an improvement in stool frequency[89].

Serotonin HT$_4$ agonists: 5-HT$_4$ receptor agonists induce fast excitatory postsynaptic potentials in intrinsic neurons, release acetylcholine, and induce mucosal secretion by activating submucosal neurons.

Tegaserod has been approved by the Food and Drug Administration (FDA) for the treatment of IBS-C in women. Tegaserod is also the only 5-HT$_4$ agonist that has been evaluated in an IBS-mixed population and showed an improvement of global symptoms. However, this drug was removed from the market in 2007 because cardiovascular events were found to be more frequent in tegaserod-treated patients than in placebo-treated patients[89,90].

Among the 5-HT$_4$ agonists for chronic constipation, the most evidence in humans is available for prucalopride[70,90]. The European Agency of Medicinal Products approved this medication for chronic constipation in women for whom laxatives fail to provide an adequate relief of their bowel habits. Prucalopride accelerates GI and colonic transit in constipation, but no placebo-controlled studies have been published, and no conclusive clinical evidence is available for IBS patients[90].

Intestinal secretagogues: By stimulating the efflux of ions and water into the intestinal lumen, secretagogues accelerate transit and facilitate defecation. Both lubiprostone and linaclotide increase intestinal chloride secretion by activating channels on the luminal enterocyte surface[90]. Lubiprostone works by activating apical CIC-2 chloride channels and does not affect colonic motor activity in healthy subjects. It is approved by the FDA for the treatment of women with IBS-C[91,92]. Linaclotide is a guanylyl cyclase C agonist that accelerates colonic transit in patients with IBS-C and chronic constipation[93]. In a recent randomised double-blind trial, linaclotide was shown to improve abdominal pain and discomfort in IBS-C, compared with placebo, over 12 and 26 wk[94]. In the same trial, diarrhoea was the most common adverse effect (19%), although few patients (5.7%) discontinued the drug as a result of this symptom. As of 2012, linaclotide is approved both by the FDA and also by the European Agency for the treatment of IBS-C.

Antidiarrhoeal agents

Opioid analogues: The opioid analogues loperamide and diphenoxylate stimulate inhibitory presynaptic receptors in the enteric nervous system, resulting in the inhibition of peristalsis and secretion. Loperamide has been shown to be effective in decreasing stool frequency and improving stool consistency across all studies[64,69,71,85,95], although it provided no significant improvement in global IBS symptoms (in particular, abdominal pain and distension) compared with placebo.

The simultaneous μ opioid agonist and δ opioid antagonist eluxadoline could reduce abdominal pain and diarrhoea in patients with IBS-D, compared with placebo, in a phase 2 study awaiting publication[96].

Serotonin HT3 antagonists: The 5-HT$_3$ receptor antagonists have been studied in IBS-D because they slow GI transit and decrease discomfort during the distension of the colon[64,69,71,85,86]. Ondansetron is the only 5-HT$_3$ receptor antagonist available in Europe and is licensed as an antiemetic, although it is not approved for use as a treatment for IBS[86]. The selective 5-HT$_3$ receptor antagonist alosetron was currently indicated for the treatment of women with severe IBS-D who had chronic symptoms of IBS[64,69,86,97].

Although it was originally approved by the FDA in 2000, alosetron was withdrawn from the market following reports of serious complications, including constipation, ischemic colitis, and bowel perforation, being associated with its use. Some evidence is available regarding other 5-HT$_3$ antagonists, such as cilansetron and ramosetron. In a recent double-blind randomised trial of 539 IBS-D patients, a positive response to ramosetron treatment was reported compared to patients receiving a placebo[98].

Bile acid binder: Some studies have indicated that a significant number of IBS-D patients can have mild to severe bile acid malabsorption. Several studies have shown a dose-response relationship between the severity of malabsorption and treatment with colestyramine, a bile acid binder[99].

Mesalazine: Mesalazine has intestinal anti-inflammatory properties, including cyclooxygenase and prostaglandin inhibition. A recent study showed that Mesalazine can reduce key symptoms of postinfectious IBS and noninfective IBS-D[100]. The results of an ongoing randomised trial of mesalazine in a group of IBS-D patients will be soon available[101].

Antibiotics and probiotics

Treatments aimed at altering or modifying the gut microbiota, including antibiotics and probiotics, have been the focus of a large number of recent studies on IBS patients[5,97,102,103].

Rifaximin is a semi-synthetic derivative of rifamycin with an additional benzimidazole ring that prevents its systemic absorption. A number of recent clinical trials

have evaluated the efficacy and safety of rifaximin in IBS patients (generally IBS-D). A recent systematic review and a meta-analysis[102,103] found rifaximin to be more efficacious than placebo for global IBS symptom improvement. The most common adverse events with rifaximin were headache, upper respiratory infection, diarrhoea, and abdominal pain. Serious side effects, however, were rare, and their prevalences were similar between rifaximin and placebo. Few data are available regarding other antibiotics. A subanalysis of a double-blind, randomised, placebo-controlled trial demonstrated that treatment with neomycin improved global symptoms in individuals with IBS-C compared with placebo[103].

Probiotics have demonstrated benefits for some symptoms, notably bloating and flatulence, and involve a variety of probiotic agents, including lactobacilli, bifidobacteria and streptococcus. Lactobacilli alone had no impact on symptoms, whereas probiotic combinations improved symptoms in IBS patients. Furthermore, there was a positive trend indicating that bifidobacteria improves IBS symptoms[71,85,86,96]. In a recent systematic review[104], probiotics appeared to be efficacious for IBS, but the magnitude of their benefit and the most effective species have not yet been completely established. Finally, probiotics have no serious side effects, and there is no significant difference in the observed adverse events between probiotics and placebo.

Psychological therapies

Among patients with IBS, the majority have anxiety, depression, or features of somatisation. Good patient compliance is necessary to achieve a successful clinical result after a psychotherapeutic approach or after the administration of antidepressants.

Psychotherapy: Among various psychological therapies, there is evidence for a benefit from cognitive behavioural therapy, dynamic psychotherapy, and hypnotherapy, but not from relaxation therapy[105-107]. The abnormal processing and enhanced perception of visceral stimuli in IBS can be normalised by psychological interventions. Psychotherapy is particularly successful in patients who reported a history of sexual abuse. Psychological therapies are not documented to have any serious adverse effects.

Tricyclic antidepressants: Tricyclic antidepressants (TCAs) are drugs with anticholinergic and non-selective serotonin reuptake inhibitor effects. Antidepressants could theoretically provide a benefit in IBS by both central and peripheral mechanisms[64,71,85,86,97]. Five tricyclic agents have been studied formally (amitriptyline, trimipramine, desipramine, clomipramine, and doxepin), and the effects of these agents are primarily related to pain. It has been suggested that patients with IBS-D obtain the greatest benefit from this approach[67]. The side effects of constipation, dry mouth, drowsiness, and fatigue occur in over one-third of IBS patients treated with TCAs, which often precludes good patient compliance.

Selective serotonin reuptake inhibitors, antidepressants: Physicians often prefer selective serotonin reuptake inhibitors (SSRIs) over TCAs because of their lower side-effect profiles. SSRIs, such as paroxetine and fluoxetine, can accelerate whole gut transit and are considered potentially effective in the treatment of IBS-C. A large trial[71] showed that a standard dose of an SSRI antidepressant led to a significant improvement in the health-related quality of life in patients with IBS, but no significant effects were observed in bowel habits or pain. However, in a double-blind randomised trial, fluoxetine was effective in decreasing global symptoms in the short-term therapy of a group of IBS-C patients[104].

Alternative approaches

Chinese herbal preparations have also been the subject of several trials[108]. By combining the effects of Iberis amara on smooth muscle tone with the spasmolytic effects of other plants, Iberogast, a popular combination of nine herbal plants, exerts a dual action on smooth muscle, stimulating or spasmolytic, depending on functional baseline conditions. These plant preparations have been shown to improve overall IBS scores and abdominal pain, but it is unclear which component is the active ingredient. A longer study of 16 wk with Chinese herbal preparations reported significant symptom improvement[109]. No conclusive data are available regarding any toxicity, especially regarding liver failure, of any Chinese herbal mixture.

Another popular alternative treatment concerns the use of acupuncture in IBS. A Cochrane review of six trials with a median sample size of 54 found insufficient evidence to determine whether acupuncture is an effective treatment for IBS[110]. In a recent open randomised trial, acupuncture for IBS provided an additional benefit over the usual care alone in a primary care experience[111].

Further studies are needed before any final recommendations on acupuncture or herbal therapy can be made.

CONCLUSION

Even though there is some evidence that changes in the digestive motility and secretion, visceral hypersensitivity, abnormalities of enteroendocrine and immune systems, genetic factors, infections, alterations of the intestinal microbiota and inflammation could play a role in IBS, its pathogenesis remains only partially understood. Thus, in clinical practice, its management is quite difficult. Because no biological markers are available, diagnoses can be made only on the basis of the symptoms described by the Rome Ⅲ criteria, for example. Unfortunately, many physicians do not use these criteria in their clinical practice and instead, driven by their own concerns or the concern of their patients, often prescribe many unnecessary diagnostic tests.

Furthermore, IBS therapy is far from satisfactory. The cornerstone for any effective treatment strategy should

be a solid patient-physician relationship; indeed, this relationship should be individualised for each patient. To achieve this goal, the use of combination drug therapies may be suggested. The data reviewed here indicate that there is limited evidence to support the individual efficacy of any of the agents currently available.

In conclusion, the pathogenesis, diagnosis and treatment of IBS remain subjects of much ongoing research. Further well-structured studies are needed to improve our knowledge about IBS and its management.

REFERENCES

1 **Bellini M**, Tosetti C, Costa F, Biagi S, Stasi C, Del Punta A, Monicelli P, Mumolo MG, Ricchiuti A, Bruzzi P, Marchi S. The general practitioner's approach to irritable bowel syndrome: from intention to practice. *Dig Liver Dis* 2005; **37**: 934-939 [PMID: 16243592 DOI: 10.1016/j.dld.2005.06.011]

2 **Videlock EJ**, Chang L. Irritable bowel syndrome: current approach to symptoms, evaluation, and treatment. *Gastroenterol Clin North Am* 2007; **36**: 665-685, x [PMID: 17950443 DOI: 10.1016/j.gtc.2007.07.002]

3 **Longstreth GF**, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology* 2006; **130**: 1480-1491 [PMID: 16678561 DOI: 10.1053/j.gastro.2005.11.061]

4 **Burbige EJ**. Irritable bowel syndrome: diagnostic approaches in clinical practice. *Clin Exp Gastroenterol* 2010; **3**: 127-137 [PMID: 21694856 DOI: 10.2147/CEG.S12596]

5 **Frissora CL**, Koch KL. Symptom overlap and comorbidity of irritable bowel syndrome with other conditions. *Curr Gastroenterol Rep* 2005; **7**: 264-271 [PMID: 16042909 DOI: 10.1007/s11894-005-0018-9]

6 **Whitehead WE**, Palsson O, Jones KR. Systematic review of the comorbidity of irritable bowel syndrome with other disorders: what are the causes and implications? *Gastroenterology* 2002; **122**: 1140-1156 [PMID: 11910364 DOI: 10.1053/gast.2002.32392]

7 **Aaron LA**, Burke MM, Buchwald D. Overlapping conditions among patients with chronic fatigue syndrome, fibromyalgia, and temporomandibular disorder. *Arch Intern Med* 2000; **160**: 221-227 [PMID: 10647761 DOI: 10.1001/archinte.160.2.221]

8 **Nellesen D**, Chawla A, Oh DL, Weissman T, Lavins BJ, Murray CW. Comorbidities in patients with irritable bowel syndrome with constipation or chronic idiopathic constipation: a review of the literature from the past decade. *Postgrad Med* 2013; **125**: 40-50 [PMID: 23816770 DOI: 10.3810/pgm.2013.03.2640]

9 **Spiller R**. Clinical update: irritable bowel syndrome. *Lancet* 2007; **369**: 1586-1588 [PMID: 17499587 DOI: 10.1016/S0140-6736(07)60726-0]

10 **Barbara G**, Cremon C, Carini G, Bellacosa L, Zecchi L, De Giorgio R, Corinaldesi R, Stanghellini V. The immune system in irritable bowel syndrome. *J Neurogastroenterol Motil* 2011; **17**: 349-359 [PMID: 22148103 DOI: 10.5056/jnm.2011.17.4.349]

11 **Maxion-Bergemann S**, Thielecke F, Abel F, Bergemann R. Costs of irritable bowel syndrome in the UK and US. *Pharmacoeconomics* 2006; **24**: 21-37 [PMID: 16445300 DOI: 10.2165/00019053-200624010-00002]

12 **Nyrop KA**, Palsson OS, Levy RL, Von Korff M, Feld AD, Turner MJ, Whitehead WE. Costs of health care for irritable bowel syndrome, chronic constipation, functional diarrhoea and functional abdominal pain. *Aliment Pharmacol Ther* 2007; **26**: 237-248 [PMID: 17593069 DOI: 10.1111/j.1365-2036.2007.03370.x]

13 **Whitehead WE**, Palsson OS, Levy RR, Feld AD, Turner M, Von Korff M. Comorbidity in irritable bowel syndrome. *Am J Gastroenterol* 2007; **102**: 2767-2776 [PMID: 17900326 DOI: 10.1111/j.1572-0241.2007.01540.x]

14 **De Ponti F**. Drug development for the irritable bowel syndrome: current challenges and future perspectives. *Front Pharmacol* 2013; **4**: 7 [PMID: 23378837 DOI: 10.3389/fphar.2013.00007]

15 **El-Salhy M**. Irritable bowel syndrome: diagnosis and pathogenesis. *World J Gastroenterol* 2012; **18**: 5151-5163 [PMID: 23066308 DOI: 10.3748/wjg.v18.i37.5151]

16 **Whorwell PJ**, McCallum M, Creed FH, Roberts CT. Non-colonic features of irritable bowel syndrome. *Gut* 1986; **27**: 37-40 [PMID: 3949235 DOI: 10.1136/gut.27.1.37]

17 **Kalantar JS**, Locke GR, Zinsmeister AR, Beighley CM, Talley NJ. Familial aggregation of irritable bowel syndrome: a prospective study. *Gut* 2003; **52**: 1703-1707 [PMID: 14633946 DOI: 10.1136/gut.52.12.1703]

18 **Bengtson MB**, Rønning T, Vatn MH, Harris JR. Irritable bowel syndrome in twins: genes and environment. *Gut* 2006; **55**: 1754-1759 [PMID: 17008364 DOI: 10.1136/gut.2006.097287]

19 **Mohammed I**, Cherkas LF, Riley SA, Spector TD, Trudgill NJ. Genetic influences in irritable bowel syndrome: a twin study. *Am J Gastroenterol* 2005; **100**: 1340-1344 [PMID: 15929767 DOI: 10.1111/j.1572-0241.2005.41700.x]

20 **Park MI**, Camilleri M. Genetics and genotypes in irritable bowel syndrome: implications for diagnosis and treatment. *Gastroenterol Clin North Am* 2005; **34**: 305-317 [PMID: 15862937 DOI: 10.1016/j.gtc.2005.02.009]

21 **Barkhordari E**, Rezaei N, Ansaripour B, Larki P, Alighardashi M, Ahmadi-Ashtiani HR, Mahmoudi M, Keramati MR, Habibollahi P, Bashashati M, Ebrahimi-Daryani N, Amirzargar AA. Proinflammatory cytokine gene polymorphisms in irritable bowel syndrome. *J Clin Immunol* 2010; **30**: 74-79 [PMID: 19844779 DOI: 10.1007/s10875-009-9342-4]

22 **Gonsalkorale WM**, Perrey C, Pravica V, Whorwell PJ, Hutchinson IV. Interleukin 10 genotypes in irritable bowel syndrome: evidence for an inflammatory component? *Gut* 2003; **52**: 91-93 [PMID: 12477767 DOI: 10.1136/gut.52.1.91]

23 **Van Kerkhoven LA**, Laheij RJ, Jansen JB. Meta-analysis: a functional polymorphism in the gene encoding for activity of the serotonin transporter protein is not associated with the irritable bowel syndrome. *Aliment Pharmacol Ther* 2007; **26**: 979-986 [PMID: 17877505 DOI: 10.1111/j.1365-2036.2007.03453.x]

24 **Colucci R**, Gambaccini D, Ghisu N, Rossi G, Costa F, Tuccori M, De Bortoli N, Fornai M, Antonioli L, Ricchiuti A, Mumolo MG, Marchi S, Blandizzi C, Bellini M. Influence of the serotonin transporter 5HTTLPR polymorphism on symptom severity in irritable bowel syndrome. *PLoS One* 2013; **8**: e54831 [PMID: 23393559 DOI: 10.1371/journal.pone.0054831]

25 **Palsson OS**, Drossman DA. Psychiatric and psychological dysfunction in irritable bowel syndrome and the role of psychological treatments. *Gastroenterol Clin North Am* 2005; **34**: 281-303 [PMID: 15862936 DOI: 10.1016/j.gtc.2005.02.004]

26 **Halpert A**, Drossman D. Biopsychosocial issues in irritable bowel syndrome. *J Clin Gastroenterol* 2005; **39**: 665-669 [PMID: 16082273 DOI: 10.1097/01.mcg.0000174024.81096.44]

27 **Drossman DA**, Lowman BC. Irritable bowel syndrome: epidemiology, diagnosis and treatment. *Clin Gastroenterol* 1985; **14**: 559-573 [PMID: 3877580]

28 **Evans PR**, Bennett EJ, Bak YT, Tennant CC, Kellow JE. Jejunal sensorimotor dysfunction in irritable bowel syndrome: clinical and psychosocial features. *Gastroenterology* 1996; **110**: 393-404 [PMID: 8566585 DOI: 10.1053/gast.1996.v110.pm8566585]

29 **Kellow JE**. Pathophysiology and management of irritable bowel syndrome. *Korean J Intern Med* 2001; **16**: 137-146 [PMID: 11769571]

30 **Vassallo MJ**, Camilleri M, Phillips SF, Steadman CJ, Talley NJ, Hanson RB, Haddad AC. Colonic tone and motility in patients with irritable bowel syndrome. *Mayo Clin Proc* 1992; **67**: 725-731 [PMID: 1434910 DOI: 10.1016/S0025-6196(12)60796-4]

31 **Stivland T**, Camilleri M, Vassallo M, Proano M, Rath D, Brown M, Thomforde G, Pemberton J, Phillips S. Scintigraphic measurement of regional gut transit in idiopathic constipation. *Gastroenterology* 1991; **101**: 107-115 [PMID: 2044899]

32 **Manabe N**, Wong BS, Camilleri M, Burton D, McKinzie S, Zinsmeister AR. Lower functional gastrointestinal disorders: evidence of abnormal colonic transit in a 287 patient cohort. *Neurogastroenterol Motil* 2010; **22**: 293-e82 [PMID: 20025692 DOI: 10.1111/j.1365-2982.2009.01442.x]

33 **Törnblom H**, Van Oudenhove L, Sadik R, Abrahamsson H, Tack J, Simrén M. Colonic transit time and IBS symptoms: what's the link? *Am J Gastroenterol* 2012; **107**: 754-760 [PMID: 22334251 DOI: 10.1038/ajg.2012.5]

34 **Salvioli B**, Serra J, Azpiroz F, Lorenzo C, Aguade S, Castell J, Malagelada JR. Origin of gas retention and symptoms in patients with bloating. *Gastroenterology* 2005; **128**: 574-579 [PMID: 15765392 DOI: 10.1053/j.gastro.2004.12.047]

35 **Di Stefano M**, Miceli E, Missanelli A, Mazzocchi S, Corazza GR. Meal induced rectosigmoid tone modification: a low caloric meal accurately separates functional and organic gastrointestinal disease patients. *Gut* 2006; **55**: 1409-1414 [PMID: 16434428 DOI: 10.1136/gut.2005.076323]

36 **Ritchie J**. Pain from distension of the pelvic colon by inflating a balloon in the irritable colon syndrome. *Gut* 1973; **14**: 125-132 [PMID: 4696535 DOI: 10.1136/gut.14.2.125]

37 **Camilleri M**, Lasch K, Zhou W. Irritable bowel syndrome: methods, mechanisms, and pathophysiology. The confluence of increased permeability, inflammation, and pain in irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* 2012; **303**: G775-G785 [PMID: 22837345 DOI: 10.1152/ajpgi.00155.2012]

38 **Mayer EA**. The neurobiology of stress and gastrointestinal disease. *Gut* 2000; **47**: 861-869 [PMID: 11076888 DOI: 10.1136/gut.47.6.861]

39 **Verne GN**, Himes NC, Robinson ME, Gopinath KS, Briggs RW, Crosson B, Price DD. Central representation of visceral and cutaneous hypersensitivity in the irritable bowel syndrome. *Pain* 2003; **103**: 99-110 [PMID: 12749964 DOI: 10.1016/S0304-3959(02)00416-5]

40 **Delvaux M**, Denis P, Allemand H. Sexual abuse is more frequently reported by IBS patients than by patients with organic digestive diseases or controls. Results of a multicentre inquiry. French Club of Digestive Motility. *Eur J Gastroenterol Hepatol* 1997; **9**: 345-352 [PMID: 9160196 DOI: 10.1097/00042737-199704000-00006]

41 **Stasi C**, Rosselli M, Bellini M, Laffi G, Milani S. Altered neuro-endocrine-immune pathways in the irritable bowel syndrome: the top-down and the bottom-up model. *J Gastroenterol* 2012; **47**: 1177-1185 [PMID: 22766747 DOI: 10.1007/s00535-012-0627-7]

42 **Matricon J**, Meleine M, Gelot A, Piche T, Dapoigny M, Muller E, Ardid D. Review article: Associations between immune activation, intestinal permeability and the irritable bowel syndrome. *Aliment Pharmacol Ther* 2012; **36**: 1009-1031 [PMID: 23066886 DOI: 10.1111/apt.12080]

43 **Fukudo S**, Nomura T, Hongo M. Impact of corticotropin-releasing hormone on gastrointestinal motility and adrenocorticotropic hormone in normal controls and patients with irritable bowel syndrome. *Gut* 1998; **42**: 845-849 [PMID: 9691924 DOI: 10.1136/gut.42.6.845]

44 **Dinan TG**, Quigley EM, Ahmed SM, Scully P, O'Brien S, O'Mahony L, O'Mahony S, Shanahan F, Keeling PW. Hypothalamic-pituitary-gut axis dysregulation in irritable bowel syndrome: plasma cytokines as a potential biomarker? *Gastroenterology* 2006; **130**: 304-311 [PMID: 16472586 DOI: 10.1053/j.gastro.2005.11.033]

45 **Stasi C**, Bellini M, Costa F, Mumolo MG, Ricchiuti A, Grosso M, Duranti E, Metelli MR, Gambaccini D, Bianchi L, Di Tanna GL, Laffi G, Taddei S, Marchi S. Neuroendocrine markers and psychological features in patients with irritable bowel syndrome. *Int J Colorectal Dis* 2013; **28**: 1203-1208 [PMID: 23377858 DOI: 10.1007/s00384-013-1646-4]

46 **Mertz H**. Role of the brain and sensory pathways in gastrointestinal sensory disorders in humans. *Gut* 2002; **51** Suppl 1: i29-i33 [PMID: 12077061 DOI: 10.1136/gut.51.suppl_1.i29]

47 **Mertz H**. Review article: visceral hypersensitivity. *Aliment Pharmacol Ther* 2003; **17**: 623-633 [PMID: 12641510 DOI: 10.1046/j.1365-2036.2003.01447.x]

48 **Naliboff BD**, Berman S, Suyenobu B, Labus JS, Chang L, Stains J, Mandelkern MA, Mayer EA. Longitudinal change in perceptual and brain activation response to visceral stimuli in irritable bowel syndrome patients. *Gastroenterology* 2006; **131**: 352-365 [PMID: 16890589 DOI: 10.1053/j.gastro.2006.05.014]

49 **Bellini M**, Alduini P, Bassotti G, Bove A, Bocchini R, Sormani MP, Bruzzi P, Pucciani F; Italian Constipation Study Group. Self-perceived normality in defecation habits. *Dig Liver Dis* 2006; **38**: 103-108 [PMID: 16263343]

50 **Collins SM**. Is the irritable gut an inflamed gut? *Scand J Gastroenterol Suppl* 1992; **192**: 102-105 [PMID: 1439559 DOI: 10.3109/00365529209095988]

51 **Barbara G**, De Giorgio R, Stanghellini V, Cremon C, Salvioli B, Corinaldesi R. New pathophysiological mechanisms in irritable bowel syndrome. *Aliment Pharmacol Ther* 2004; **20** Suppl 2: 1-9 [PMID: 15335408 DOI: 10.1111/j.1365-2036.2004.02058.x]

52 **Chadwick VS**, Chen W, Shu D, Paulus B, Bethwaite P, Tie A, Wilson I. Activation of the mucosal immune system in irritable bowel syndrome. *Gastroenterology* 2002; **122**: 1778-1783 [PMID: 12055584 DOI: 10.1053/gast.2002.33579]

53 **Barbara G**, Stanghellini V, De Giorgio R, Cremon C, Cottrell GS, Santini D, Pasquinelli G, Morselli-Labate AM, Grady EF, Bunnett NW, Collins SM, Corinaldesi R. Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. *Gastroenterology* 2004; **126**: 693-702 [PMID: 14988823 DOI: 10.1053/j.gastro.2003.11.055]

54 **Cremon C**, Gargano L, Morselli-Labate AM, Santini D, Cogliandro RF, De Giorgio R, Stanghellini V, Corinaldesi R, Barbara G. Mucosal immune activation in irritable bowel syndrome: gender-dependence and association with digestive symptoms. *Am J Gastroenterol* 2009; **104**: 392-400 [PMID: 19174797 DOI: 10.1038/ajg.2008.94]

55 **Barbara G**, Cremon C, Pallotti F, De Giorgio R, Stanghellini V, Corinaldesi R. Postinfectious irritable bowel syndrome. *J Pediatr Gastroenterol Nutr* 2009; **48** Suppl 2: S95-S97 [PMID: 19300138 DOI: 10.1097/MPG.0b013e3181a15e2e]

56 **Begtrup LM**, de Muckadell OB, Kjeldsen J, Christensen RD, Jarbøl DE. Long-term treatment with probiotics in primary care patients with irritable bowel syndrome--a randomised, double-blind, placebo controlled trial. *Scand J Gastroenterol* 2013; **48**: 1127-1135 [PMID: 23957590 DOI: 10.3109/00365521.2013.825314]

57 **Ohman L**, Simrén M. Intestinal microbiota and its role in irritable bowel syndrome (IBS). *Curr Gastroenterol Rep* 2013; **15**: 323 [PMID: 23580243 DOI: 10.1007/s11894-013-0323-7]

58 **Simrén M**, Barbara G, Flint HJ, Spiegel BM, Spiller RC, Vanner S, Verdu EF, Whorwell PJ, Zoetendal EG; Rome Foundation Committee. Intestinal microbiota in functional bowel disorders: a Rome foundation report. *Gut* 2013; **62**: 159-176 [PMID: 22730468 DOI: 10.1136/gutjnl-2012-302167]

59 **Atkinson W**, Sheldon TA, Shaath N, Whorwell PJ. Food elimination based on IgG antibodies in irritable bowel syndrome: a randomised controlled trial. *Gut* 2004; **53**: 1459-1464 [PMID: 15361495 DOI: 10.1136/gut.2003.037697]

60 **Zar S**, Benson MJ, Kumar D. Food-specific serum IgG4 and IgE titers to common food antigens in irritable bowel syndrome. *Am J Gastroenterol* 2005; **100**: 1550-1557 [PMID: 15984980 DOI: 10.1111/j.1572-0241.2005.41348.x]

61 **Biesiekierski JR**, Peters SL, Newnham ED, Rosella O, Muir JG, Gibson PR. No effects of gluten in patients with self-reported non-celiac gluten sensitivity after dietary reduc-

tion of fermentable, poorly absorbed, short-chain carbohydrates. *Gastroenterology* 2013; **145**: 320-8.e1-320-8.e3 [PMID: 23648697 DOI: 10.1053/j.gastro.2013.04.051]

62 **Barrett JS**, Gibson PR. Fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) and nonallergic food intolerance: FODMAPs or food chemicals? *Therap Adv Gastroenterol* 2012; **5**: 261-268 [PMID: 22778791 DOI: 10.1177/1756283X11436241]

63 **Quigley EM**, Abdel-Hamid H, Barbara G, Bhatia SJ, Boeckxstaens G, De Giorgio R, Delvaux M, Drossman DA, Foxx-Orenstein AE, Guarner F, Gwee KA, Harris LA, Hungin AP, Hunt RH, Kellow JE, Khalif IL, Kruis W, Lindberg G, Olano C, Moraes-Filho JP, Schiller LR, Schmulson M, Simrén M, Tzeuton C. A global perspective on irritable bowel syndrome: a consensus statement of the World Gastroenterology Organisation Summit Task Force on irritable bowel syndrome. *J Clin Gastroenterol* 2012; **46**: 356-366 [PMID: 22499071 DOI: 10.1097/MCG.0b013e318247157c]

64 **Spiller R**, Aziz Q, Creed F, Emmanuel A, Houghton L, Hungin P, Jones R, Kumar D, Rubin G, Trudgill N, Whorwell P; Clinical Services Committee of The British Society of Gastroenterology. Guidelines on the irritable bowel syndrome: mechanisms and practical management. *Gut* 2007; **56**: 1770-1798 [PMID: 17488783 DOI: 10.1136/gut.2007.119446]

65 **Lewis SJ**, Heaton KW. Stool form scale as a useful guide to intestinal transit time. *Scand J Gastroenterol* 1997; **32**: 920-924 [PMID: 9299672 DOI: 10.3109/00365529709011203]

66 **Sperber AD**, Drossman DA. Irritable bowel syndrome: a multidimensional disorder cannot be understood or treated from a unidimensional perspective. *Therap Adv Gastroenterol* 2012; **5**: 387-393 [PMID: 23152732 DOI: 10.1177/1756283X12460420]

67 **Bellini M**, Gemignani A, Gambaccini D, Toti S, Menicucci D, Stasi C, Costa F, Mumolo MG, Ricchiuti A, Bedini R, de Bortoli N, Marchi S. Evaluation of latent links between irritable bowel syndrome and sleep quality. *World J Gastroenterol* 2011; **17**: 5089-5096 [PMID: 22171143 DOI: 10.3748/wjg.v17.i46.5089]

68 **World Gastroenterology Organisation**. Global guideline. Irritable bowel syndrome: A global perspective, 2009. Available from: URL: http://www.worldgastroenterology.org/irritable-bowel-syndrome.html

69 **American College of Gastroenterology Task Force on Irritable Bowel Syndrome**; Brandt LJ, Chey WD, Foxx-Orenstein AE, Schiller LR, Schoenfeld PS, Spiegel BM, Talley NJ, Quigley EM. An evidence-based position statement on the management of irritable bowel syndrome. *Am J Gastroenterol* 2009; **104** Suppl 1: S1-35 [PMID: 19521341 DOI: 10.1038/ajg.2008.122]

70 **Bove A**, Pucciani F, Bellini M, Battaglia E, Bocchini R, Altomare DF, Dodi G, Sciaudone G, Falletto E, Piloni V, Gambaccini D, Bove V. Consensus statement AIGO/SICCR: diagnosis and treatment of chronic constipation and obstructed defecation (part I: diagnosis). *World J Gastroenterol* 2012; **18**: 1555-1564 [PMID: 22529683 DOI: 10.3748/wjg.v18.i14.1555]

71 **Drossman DA**, Camilleri M, Mayer EA, Whitehead WE. AGA technical review on irritable bowel syndrome. *Gastroenterology* 2002; **123**: 2108-2131 [PMID: 12454866 DOI: 10.1053/gast.2002.37095]

72 **Wong RK**, Drossman DA, Bharucha AE, Rao SS, Wald A, Morris CB, Oxentenko AS, Ravi K, Van Handel DM, Edwards H, Hu Y, Bangdiwala S. The digital rectal examination: a multicenter survey of physicians' and students' perceptions and practice patterns. *Am J Gastroenterol* 2012; **107**: 1157-1163 [PMID: 22858996 DOI: 10.1038/ajg.2012.23]

73 **Bijkerk CJ**, de Wit NJ, Muris JW, Jones RH, Knottnerus JA, Hoes AW. Outcome measures in irritable bowel syndrome: comparison of psychometric and methodological characteristics. *Am J Gastroenterol* 2003; **98**: 122-127 [PMID: 12526947 DOI: 10.1111/j.1572-0241.2003.07158.x]

74 **Mangel AW**, Hahn BA, Heath AT, Northcutt AR, Kong S, Dukes GE, McSorley D. Adequate relief as an endpoint in clinical trials in irritable bowel syndrome. *J Int Med Res* 1998; **26**: 76-81 [PMID: 9602985]

75 **Francis CY**, Morris J, Whorwell PJ. The irritable bowel severity scoring system: a simple method of monitoring irritable bowel syndrome and its progress. *Aliment Pharmacol Ther* 1997; **11**: 395-402 [PMID: 9146781 DOI: 10.1046/j.1365-2036.1997.142318000.x]

76 **Patrick DL**, Drossman DA, Frederick IO, DiCesare J, Puder KL. Quality of life in persons with irritable bowel syndrome: development and validation of a new measure. *Dig Dis Sci* 1998; **43**: 400-411 [PMID: 9512138 DOI: 10.1023/A:1018831127942]

77 **Wong E**, Guyatt GH, Cook DJ, Griffith LE, Irvine EJ. Development of a questionnaire to measure quality of life in patients with irritable bowel syndrome. *Eur J Surg Suppl* 1998; (**583**): 50-56 [PMID: 10027673]

78 **Cash BD**, Rubenstein JH, Young PE, Gentry A, Nojkov B, Lee D, Andrews AH, Dobhan R, Chey WD. The prevalence of celiac disease among patients with nonconstipated irritable bowel syndrome is similar to controls. *Gastroenterology* 2011; **141**: 1187-1193 [PMID: 21762658 DOI: 10.1053/j.gastro.2011.06.084]

79 **Pavlidis P**, Chedgy FJ, Tibble JA. Diagnostic accuracy and clinical application of faecal calprotectin in adult patients presenting with gastrointestinal symptoms in primary care. *Scand J Gastroenterol* 2013; **48**: 1048-1054 [PMID: 23883068 DOI: 10.3109/00365521.2013.816771]

80 **Costa F**, Mumolo MG, Bellini M, Romano MR, Ceccarelli L, Arpe P, Sterpi C, Marchi S, Maltinti G. Role of faecal calprotectin as non-invasive marker of intestinal inflammation. *Dig Liver Dis* 2003; **35**: 642-647 [PMID: 14563186 DOI: 10.1016/S1590-8658(03)00381-5]

81 **Chang L**, Adeyemo M, Karagiannides I, Videlock EJ, Bowe C, Shih W, Presson AP, Yuan PQ, Cortina G, Gong H, Singh S, Licudine A, Mayer M, Tache Y, Pothoulakis C, Mayer EA. Serum and colonic mucosal immune markers in irritable bowel syndrome. *Am J Gastroenterol* 2012; **107**: 262-272 [PMID: 22158028 DOI: 10.1038/ajg.2011.423]

82 **Ford AC**, Moayyedi P. Meta-analysis: factors affecting placebo response rate in the irritable bowel syndrome. *Aliment Pharmacol Ther* 2010; **32**: 144-158 [PMID: 20412064 DOI: 10.1111/j.1365-2036.2010.04328]

83 **Costa F**, Mumolo MG, Marchi S, Bellini M. Differential diagnosis between functional and organic intestinal disorders: is there a role for non-invasive tests? *World J Gastroenterol* 2007; **13**: 219-223 [PMID: 17226899]

84 **Spiller RC**, Thompson WG. Bowel disorders. *Am J Gastroenterol* 2010; **105**: 775-785 [PMID: 20372130 DOI: 10.1038/ajg.2010.69]

85 **Lesbros-Pantoflickova D**, Michetti P, Fried M, Beglinger C, Blum AL. Meta-analysis: The treatment of irritable bowel syndrome. *Aliment Pharmacol Ther* 2004; **20**: 1253-1269 [PMID: 15606387 DOI: 10.1111/j.1365-2036.2004.02267.x]

86 **Tack J**, Fried M, Houghton LA, Spicak J, Fisher G. Systematic review: the efficacy of treatments for irritable bowel syndrome--a European perspective. *Aliment Pharmacol Ther* 2006; **24**: 183-205 [PMID: 16842448 DOI: 10.1111/j.1365-2036.2006.02938.x]

87 **Staudacher HM**, Lomer MC, Anderson JL, Barrett JS, Muir JG, Irving PM, Whelan K. Fermentable carbohydrate restriction reduces luminal bifidobacteria and gastrointestinal symptoms in patients with irritable bowel syndrome. *J Nutr* 2012; **142**: 1510-1518 [PMID: 22739368 DOI: 10.3945/jn.112.159285]

88 **Ford AC**, Talley NJ, Spiegel BM, Foxx-Orenstein AE, Schiller L, Quigley EM, Moayyedi P. Effect of fibre, antispasmodics, and peppermint oil in the treatment of irritable bowel syndrome: systematic review and meta-analysis. *BMJ* 2008; **337**: a2313 [PMID: 19008265 DOI: 10.1136/bmj.a2313]

89 **Khoshoo V**, Armstead C, Landry L. Effect of a laxative with and without tegaserod in adolescents with constipation predominant irritable bowel syndrome. *Aliment Pharmacol Ther* 2006; **23**: 191-196 [PMID: 16393297 DOI: 10.1111/j.1365-2036.2006.02705.x]

90 **Bharucha AE**, Pemberton JH, Locke GR. American Gastroenterological Association technical review on constipation. *Gastroenterology* 2013; **144**: 218-238 [PMID: 23261065 DOI: 10.1053/j.gastro.2012.10.028]

91 **Drossman DA**, Chey WD, Johanson JF, Fass R, Scott C, Panas R, Ueno R. Clinical trial: lubiprostone in patients with constipation-associated irritable bowel syndrome--results of two randomized, placebo-controlled studies. *Aliment Pharmacol Ther* 2009; **29**: 329-341 [PMID: 19006537]

92 **Chey WD**, Drossman DA, Johanson JF, Scott C, Panas RM, Ueno R. Safety and patient outcomes with lubiprostone for up to 52 weeks in patients with irritable bowel syndrome with constipation. *Aliment Pharmacol Ther* 2012; **35**: 587-599 [PMID: 22251419 DOI: 10.1111/j.1365-2036.2011.04983.x]

93 **Rao S**, Lembo AJ, Shiff SJ, Lavins BJ, Currie MG, Jia XD, Shi K, MacDougall JE, Shao JZ, Eng P, Fox SM, Schneier HA, Kurtz CB, Johnston JM. A 12-week, randomized, controlled trial with a 4-week randomized withdrawal period to evaluate the efficacy and safety of linaclotide in irritable bowel syndrome with constipation. *Am J Gastroenterol* 2012; **107**: 1714-1724; quiz p.1725 [PMID: 22986440 DOI: 10.1038/ajg.2012.255]

94 **Quigley EM**, Tack J, Chey WD, Rao SS, Fortea J, Falques M, Diaz C, Shiff SJ, Currie MG, Johnston JM. Randomised clinical trials: linaclotide phase 3 studies in IBS-C - a prespecified further analysis based on European Medicines Agency-specified endpoints. *Aliment Pharmacol Ther* 2013; **37**: 49-61 [PMID: 23116208 DOI: 10.1111/apt.12123]

95 **Lavö B**, Stenstam M, Nielsen AL. Loperamide in treatment of irritable bowel syndrome--a double-blind placebo controlled study. *Scand J Gastroenterol Suppl* 1987; **130**: 77-80 [PMID: 3306903 DOI: 10.3109/00365528709091003]

96 **Dove LS**, Lembo A, Randall CW, Fogel R, Andrae D, Davenport JM, McIntyre G, Almenoff JS, Covington PS. Eluxadoline benefits patients with irritable bowel syndrome with diarrhea in a phase 2 study. *Gastroenterology* 2013; **145**: 329-338.e1 [PMID: 23583433 DOI: 10.1053/j.gastro.2013.04.006]

97 **Chang L**, Lacy BE, Spiegel BM. An Evidence-based Approach to Therapy in IBS-D: A Case Study Compendium. *Gastroenterol Hepatol* (N Y) 2010; **6**: 1-12 [PMID: 22570639]

98 **Chiba T**, Yamamoto K, Sato S, Suzuki K. Long-term efficacy and safety of ramosetron in the treatment of diarrhea-predominant irritable bowel syndrome. *Clin Exp Gastroenterol* 2013; **6**: 123-128 [PMID: 23922505 DOI: 10.2147/CEG.S32721]

99 **Wedlake L**, A'Hern R, Russell D, Thomas K, Walters JR, Andreyev HJ. Systematic review: the prevalence of idiopathic bile acid malabsorption as diagnosed by SeHCAT scanning in patients with diarrhoea-predominant irritable bowel syndrome. *Aliment Pharmacol Ther* 2009; **30**: 707-717 [PMID: 19570102 DOI: 10.1111/j.1365-2036.2009.04081.x]

100 **Bafutto M**, Almeida JR, Leite NV, Oliveira EC, Gabriel-Neto S, Rezende-Filho J. Treatment of postinfectious irritable bowel syndrome and noninfective irritable bowel syndrome with mesalazine. *Arq Gastroenterol* 2011; **48**: 36-40 [PMID: 21537540 DOI: 10.1590/S0004-28032011000100008]

101 **Leighton MP**, Lam C, Mehta S, Spiller RC. Efficacy and mode of action of mesalazine in the treatment of diarrhoea-predominant irritable bowel syndrome (IBS-D): study protocol for a randomised controlled trial. *Trials* 2013; **14**: 10 [PMID: 23302220 DOI: 10.1186/1745-6215-14-10]

102 **Pimentel M**, Lembo A, Chey WD, Zakko S, Ringel Y, Yu J, Mareya SM, Shaw AL, Bortey E, Forbes WP; TARGET Study Group. Rifaximin therapy for patients with irritable bowel syndrome without constipation. *N Engl J Med* 2011; **364**: 22-32 [PMID: 21208106 DOI: 10.1056/NEJMoa1004409]

103 **Menees SB**, Maneerattannaporn M, Kim HM, Chey WD. The efficacy and safety of rifaximin for the irritable bowel syndrome: a systematic review and meta-analysis. *Am J Gastroenterol* 2012; **107**: 28-35; quiz 36 [PMID: 22045120 DOI: 10.1038/ajg.2011.355]

104 **Moayyedi P**, Ford AC, Talley NJ, Cremonini F, Foxx-Orenstein AE, Brandt LJ, Quigley EM. The efficacy of probiotics in the treatment of irritable bowel syndrome: a systematic review. *Gut* 2010; **59**: 325-332 [PMID: 19091823 DOI: 10.1136/gut.2008.167270]

105 **Ford AC**, Talley NJ, Schoenfeld PS, Quigley EM, Moayyedi P. Efficacy of antidepressants and psychological therapies in irritable bowel syndrome: systematic review and meta-analysis. *Gut* 2009; **58**: 367-378 [PMID: 19001059 DOI: 10.1136/gut.2008.163162]

106 **Lindfors P**, Unge P, Arvidsson P, Nyhlin H, Björnsson E, Abrahamsson H, Simrén M. Effects of gut-directed hypnotherapy on IBS in different clinical settings-results from two randomized, controlled trials. *Am J Gastroenterol* 2012; **107**: 276-285 [PMID: 21971535 DOI: 10.1038/ajg.2011.340]

107 **Lowén MB**, Mayer EA, Sjöberg M, Tillisch K, Naliboff B, Labus J, Lundberg P, Ström M, Engström M, Walter SA. Effect of hypnotherapy and educational intervention on brain response to visceral stimulus in the irritable bowel syndrome. *Aliment Pharmacol Ther* 2013; **37**: 1184-1197 [PMID: 23617618 DOI: 10.1111/apt.12319]

108 **Madisch A**, Holtmann G, Plein K, Hotz J. Treatment of irritable bowel syndrome with herbal preparations: results of a double-blind, randomized, placebo-controlled, multicentre trial. *Aliment Pharmacol Ther* 2004; **19**: 271-279 [PMID: 14984373 DOI: 10.1111/j.1365-2036.2004.01859.x]

109 **Bensoussan A**, Talley NJ, Hing M, Menzies R, Guo A, Ngu M. Treatment of irritable bowel syndrome with Chinese herbal medicine: a randomized controlled trial. *JAMA* 1998; **280**: 1585-1589 [PMID: 9820260 DOI: 10.1001/jama.280.18.1585]

110 **Lim B**, Manheimer E, Lao L, Ziea E, Wisniewski J, Liu J, Berman B. Acupuncture for treatment of irritable bowel syndrome. *Cochrane Database Syst Rev* 2006; (**4**): CD005111 [PMID: 17054239]

111 **MacPherson H**, Tilbrook H, Bland JM, Bloor K, Brabyn S, Cox H, Kang'ombe AR, Man MS, Stuardi T, Torgerson D, Watt I, Whorwell P. Acupuncture for irritable bowel syndrome: primary care based pragmatic randomised controlled trial. *BMC Gastroenterol* 2012; **12**: 150 [PMID: 23095376 DOI: 10.1186/1471-230X-12-150]

P- Reviewers: Chen JX, Grundmann O, Guglielmetti F, Pasechnikov V, Sharara A, Soares RLS
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8821

World J Gastroenterol 2014 July 21; 20(27): 8821-8836
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

Methodological issues in the study of intestinal microbiota in irritable bowel syndrome

Valentina Taverniti, Simone Guglielmetti

Valentina Taverniti, Simone Guglielmetti, Division of Food Microbiology and Bioprocessing, Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, 20133 Milan, Italy
Author contributions: Taverniti V and Guglielmetti S performed the literature search and drafted the manuscript.
Correspondence to: Simone Guglielmetti, PhD, Division of Food Microbiology and Bioprocessing, Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, Via Celoria 2, 20133 Milan, Italy. simone.guglielmetti@unimi.it
Telephone: +39-2-50319136 Fax: +39-2-50319292
Received: October 31, 2013 Revised: April 10, 2014
Accepted: June 2, 2014
Published online: July 21, 2014

Abstract

Irritable bowel syndrome (IBS) is an intestinal functional disorder with the highest prevalence in the industrialized world. The intestinal microbiota (IM) plays a role in the pathogenesis of IBS and is not merely a consequence of this disorder. Previous research efforts have not revealed unequivocal microbiological signatures of IBS, and the experimental results are contradictory. The experimental methodologies adopted to investigate the complex intestinal ecosystem drastically impact the quality and significance of the results. Therefore, to consider the methodological aspects of the research on IM in IBS, we reviewed 29 relevant original research articles identified through a PubMed search using three combinations of keywords: "irritable bowel syndrome + microflora", "irritable bowel syndrome + microbiota" and "irritable bowel syndrome + microbiome". For each study, we reviewed the quality and significance of the scientific evidence obtained with respect to the experimental method adopted. The data obtained from each study were compared with all considered publications to identify potential inconsistencies and explain contradictory results. The analytical revision of the studies referenced in the present review has contributed to the identification of microbial groups whose relative abundance significantly alters IBS, suggesting that these microbial groups could be IM signatures for this syndrome. The identification of microbial biomarkers in the IM can be advantageous for the development of new diagnostic tools and novel therapeutic strategies for the treatment of different subtypes of IBS.

Key words: Intestinal dysfunction; Irritable bowel syndrome; Intestinal microbiota; Bifidobacteria; New generation DNA sequencing

Core tip: Irritable bowel syndrome (IBS) is the intestinal functional disorder with the highest prevalence in the industrialized world. The intestinal microbiota (IM) plays a role in its pathogenesis. Since the methodological aspects of the research on IM in IBS have never been considered in detail before, we carried out a revision of 29 original research articles. We reviewed the scientific microbiological message in light of the experimental method adopted. The analytical revision of the studies referenced in our review leaded to the identification of microbial groups whose relative abundance resulted significantly altered in IBS. Such microbial groups are potential IM signatures of IBS.

Taverniti V, Guglielmetti S. Methodological issues in the study of intestinal microbiota in irritable bowel syndrome. *World J Gastroenterol* 2014; 20(27): 8821-8836 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8821.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8821

INTRODUCTION

Irritable bowel syndrome (IBS) is a functional intestinal

disorder with the highest prevalence in the industrialized world[1,2]. Due to the absence of an evident pathogenesis, IBS is exclusively diagnosed based on the absence of mucosal, structural and biochemical diseases and the evaluation of specific symptoms according to Rome Ⅲ criteria[3,4]. The main symptoms that characterize IBS include abdominal pain and discomfort, accompanied by diarrhea (IBS-D), constipation (IBS-C), or a combination of the two (alternating IBS, IBS-A). The frequency and intensity of these symptoms largely varies, thereby affecting the quality of life of the patients[5].

The etiopathogenesis and pathophysiology of IBS are ambiguous and likely include many different factors, such as improper immune activation, visceral hypersensitivity, colon dysmotility, history of gastrointestinal infections, and psychological conditions[6-9]. In addition, many studies have also investigated a potential role for intestinal microbiota (IμB) in IBS.

Experimental observations showed that in IBS (1) toll-like receptor genes are upregulated[10]; (2) fecal levels of defensins are increased[11]; and (3) short chain fatty acid concentrations are frequently augmented[12-16]. Furthermore, it was shown that probiotics and antibiotics treatments could reduce IBS symptoms[17-19]. These data suggest that changes in the IμB are not only a consequence of IBS, but could also be a plausible causative factor. Nonetheless, current research efforts have not identified any definitive microbiological signatures of IBS and the experimental results are occasionally contradictory. The heterogeneity of the results on the role of IμB in IBS primarily reflects the high variability among various manifestations of IBS and marked differences in the IμB composition among subjects[20]. Moreover, the experimental methodologies employed and the specific protocols adopted to investigate complex ecosystems, such as the IμB, drastically impact the quality and significance of the results. To examine the methodological aspects of the research on the role of IμB in IBS, we reviewed 29 relevant original research articles obtained through a PubMed search using three combinations of keywords: "irritable bowel syndrome + microflora", "irritable bowel syndrome + microbiota" and "irritable bowel syndrome + microbiome". For each study, we reviewed the scientific evidence obtained with respect to the experimental technology adopted. The collected data from each study were compared among all considered studies to detect potential inconsistencies and explain contradictory results.

METHODOLOGIES EMPLOYED TO INVESTIGATE THE INTESTINAL MICROBIOTA IN IBS SUBJECTS

The 29 original research studies considered in the present review address the microbial community structure in the intestine of IBS subjects using several different experimental techniques. Only a few of these studies used classical (culture-based) strategies, which have extensively been replaced with molecular techniques (*i.e.*, strategies based on the analysis of nucleic acids).

The molecular methods employed in the selected studies primarily included (1) fluorescence *in situ* hybridization (FISH); (2) DNA microarrays; and (3) polymerase chain reaction (PCR)-based methods. The PCR-based methods can be further divided into three main groups: Real-time quantitative PCR (qPCR); Genetic fingerprinting [denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP)]; PCR fragment sequencing.

In the following paragraphs, the advantages and limitations of the technologies employed to correlate IμB to IBS are discussed. In addition, the experimental results obtained using each methodological strategy are presented and compared.

Culture-based methods

The classical strategies of microbial ecology, based on the cultivation of microorganisms, have been demonstrated as inappropriate for the analysis of complex microbial ecosystems, such as the intestinal environment, because the vast majority of the microorganisms (between 80% and 99%) in any environment are not cultivable using standard culturing techniques[21,22]. A few studies in the last 10 years, however, have adopted culture-dependent approaches to characterize the IμB of subjects with IBS (Table 1). For example, Mättö *et al*[23] found a moderate increase in the coliform bacteria concentration and aerobe/anaerobe ratio in fecal samples obtained from IBS patients (26 subjects: 12 IBS-D, 9 IBS-C and 5 IBS-A) compared with healthy controls (HCs, 25 subjects), whereas the bifidobacterial concentrations did not differ. More recently, Enck *et al*[24] applied culture-based analyses to examine fecal samples from more than 34000 subjects, including 7784 people with IBS. In contrast to Mättö[23], among the few bacterial groups considered, only bifidobacteria were significantly decreased in IBS samples. The differences in these results, however, are plausible, considering that Mattö used the Beerens medium[25], containing propionic acid, as a selective agent for bifidobacteria, whereas Enck *et al*[24] used DIC agar (Heipha GmbH, Germany), a commercial medium containing gentamycin and vancomycin as selective agents. Although bifidobacteria are considered resistant to these antibiotics, sensitivity has been reported for stressed cells belonging, for example, to the species *Bifidobacterium longum* (*B. longum*)[26]; therefore, the use of antibiotics as selective agents compromises the cultivation of viable bifidobacterial cells in a fecal sample. Furthermore, the bifidobacteria concentration was not significantly different in 10 IBS-D subjects compared with 10 healthy controls in another study[27] in which a medium similar to Beerens agar was used for the isolation. On the contrary, Chassard *et al*[28] detected reduced bifidobacteria concentrations in the fecal samples of 14 IBS-C women compared with 12 sex-matched HCs. However, in this study, bifidobacteria were isolated using de Man Rogosa Sharp (MRS) agar medium (pH 7.0),

Table 1 Outcomes of the selected original research studies (see text for details), which have been carried out to characterize the intestinal microbiota composition in irritable bowel syndrome

Participants	Rome criteria	Results	Sample	Technique	Ref.
27 IBS (20 F/7 M) 12 IBS-D 9 IBS-C 6 IBS-A Age: 20-65 22 HCs (15 F/7 M) Age: 25-64	II	↑ *Ruminococcus productus - Clostridium coccoides* ↓ *Lactobacillus* (IBS-D *vs* IBS-C) ↓ *Bifidobacterium* (IBS-D *vs* HC, IBD-C, IBS-A) ↓ *Desulfovibrio* (IBS-D *vs* HC, IBD-C, IBS-A) ↑ *Veillonella* (IBS-C *vs* HC)	Fecal	qPCR (SYBR Green)	[16]
26 IBS (19 F/7 M) 12 IBS-D 9 IBS-C 5 IBS-A Age: 20-65 25 HCs (18 F/7 M) Age: 23-63	II	More temporal instability in predominant bacterial population in IBS subjects Slight increase of coliforms in IBS and higher aerobe/anaerobe ratio in IBS ↑ *Clostridium* spp. ↓ *Eubacterium* spp.	Feces	DGGE Cloning and sequencing of 5 IBS and 4 HC samples (29 IBS and 16 HC clones); ABI PRISM BigDye terminator Cycle sequencing kit v. 3.0 Culture method	[23]
20 IBS (14 F/6 M) Mean age: 47.8 20 HCs (13 F/7 M) Mean age: 46.2	II	Mucosal bacteria concentration higher than 10^9 cells/mL in 65% of IBS subjects (35% in HC) Prevalence of *Eubacterium rectale-Clostridium coccoides* in IBS biofilm	Ileal and colonic biopsies	FISH	[33]
16 IBS (11 F/5 M) 7 IBS-D 6 IBS-C 3 IBS-A Age: 24-64 16 HCs (12 F/4 M) Age: 26-63	II	More temporal instability of predominant microbiota only in RNA-DGGE profiles in IBS *vs* HCs (not in DNA-DGGE) ↓ *C. coccoides - E. rectale* in IBS-C *vs* HC No differences in *Clostridium* population stability between IBS and HC	Feces	DGGE Multiplexed quantification of clostridial 16S rRNA through multiple transcript analysis with the aid of affinity capture (TRAC)	[49]
24 IBS 10 IBS-D 8 IBS-C 6 IBS-A Age: 21-65 23 HCs (16 F/7 M) Age: 26-64	II	Significant differences in microbiota composition in different IBS subcategories pooled in 3 groups on the basis of %GC (7-10-13 fractions) In fraction group 7: ↓ *Lactobacillus* in all IBS subgroups *vs* HC ↑ *Ruminococcus* in IBS-C and IBS-A ↑ *Streptococcus* in IBS-D In fraction group 13: ↓ *Collinsella* in IBS-C and IBS-D	Feces	16S rRNA gene cloning and sequencing of 3753 clones	[37]
20 IBS 8 IBS-D 8 IBS-C 4 IBS-A Age: 24-64 15 HCs Age: 25-64	II	↑ *Clostridium thermosuccinogenes* (IBS-A *vs* IBS-D) ↑ *Ruminococcus torques* 94% phylotype (IBS-D *vs* HCs and IBS-A) ↑ *Ruminococcus bromii*-like phylotype (IBS-C *vs* HCs) ↑ *Bacteroides intestinalis*-like and *C. cocleatum* (IBS-A and HCs *vs* IBS-D) ↓ *Clostridium aerofaciens*-like (IBS-D *vs* other groups)	Feces	qPCR (SYBR Green)	[46]
41 IBS (29 F/12 M) 14 IBS-D 11 IBS-C 16 IBS-A Mean age: 42 26 HCs (18 F/8 M) Mean age:32		↓ *Bifidobacterium* ↓ *B. catenulatum*	Feces Feces and duodenal brushes	FISH qPCR (Taqman)	[31]
10 IBS-D (6 F/4 M) Age average: 46.5 23 HCs Age average: 45 12 IBS-D (7 F/5 M) Age average: 46.5	II	Decreased diversity in the intestinal microbiota of IBS-D *vs* HCs ↑ Proteobacteria and Firmicutes ↑ Lachnospiraceae ↓ Actinobacteria and Bacteroidetes No significant differences in Enterobacteriaceae and *Eggerthella lenta*-type (*Atopobium*) phylotype between IBS-D and HCs	Feces	Genomic DNA fractioning on the basis of %GC (35%-40%/40%-45%/50%-55%/55%-60%/60-65/65%-70%/70%-75%); amplification of 16S rRNA gene; sequencing of 3267 clones for IBS subjects qPCR (SYBR Green)	[47]

22 HCs Age average: 45					
47 IBS (47 F) Age: 24-66	II	Significant difference in DGGE profile between IBS and HC, less microbial variation in IBS	Feces	DGGE of V1-V3 region of the 16S rRNA	[70]
33 HCs Age: 21-38		No significant intra and inter-differences in IBS subjects between luminal and mucosal microbiota. IBS impacts equally on both communities	Feces and colonic biopsies	DGGE of V6-V8 Region of the16S rRNA	
26 IBS (13 F/13 M) 8 IBS-D 11 IBS-C 7 IBS-A Age: 21.7 ± 2.0 26 HCs Age: 21.9 ± 2.9		↑ *Veillonella* ↑ *Lactobacillus* spp.	Feces	qPCR (SYBR Green) Culture method	[12]
10 IBS-D (8 F/2 M) Age: 23-50 10 HCs (6 F/4 M) Age: 21-54	III	↓ Aerobic counts in fecal samples of IBS-D No difference in mucosal samples between IBS-D and HC ↑ *Lactobacillus* spp. in fecal samples of IBS-D *vs* HC No difference in mucosal samples between IBS-D and HC	Feces samples and colonic biopsy	Culture method qPCR (SYBR Green)	[27]
11 IBS (7 F/4 M) Age: 25-64 22 HCs (17 F/5 M) Age: 21-61	II	Reduced biodiversity in IBS subjects Significant differences in profiles between IBS and HC subjects ↓ *B. vulgatus, B. ovatus, B. uniformis, Parabacteroides sp.* in IBS *vs* HC	Feces	DGGE on universal and specific primers for *Bacteroides* Sequencing of V3 region of the 16S rRNA genes	[69]
22 IBS (8 F/14 M) 1 IBS-D 13 IBS-C 8 IBS-A Age: 7-12 22 HCs (11 F/11 M)	Pediatric Rome III	No differences in total bacterial load between IBS and HCs Profile differences in IBS subtypes among each other, and between IBS and HCs In IBS: ↑ Proteobacteria ↑ γ-Proteobacteria ↑ *Haemophilus parainfluenzae* ↑ *Veillonella* ↑ *Dorea* ↓ *Eubacterium* ↓ *Anaerovorax* ↓ *Bacteroides vulgatus*	Feces	16S Metagenomics 454 Pyrosequencing (V1-V3 and V3-V5 regions of 16S rRNA) PhyloChip Microarray Hybridization on purified 27F and 1492R regions of 16S rRNA (on 28 IBS and 27 HC)	[35]
62 IBS (57 F/5 M) 25 IBS-D 19 IBS-C 19 IBS-A Age: 22-66 46 HCs (34 F/12 M) Age: 23-58	II	↑ Firmicutes/Bacteroidetes ratio ↑ *Bacillus* ↑ *Steptococcus* ↑ *Dorea* ↑ *Ruminococcus* ↑ *R. gnavus* ↑ *Blautia* ↑ *Clostridium* ↓ *Faecalibacterium* ↓ *Bacteroides* ↓ *B. vulgatus* ↓ *Prevotella* ↓ *Bifidobacterium* ↓ *B. gallicum* ↓ *B. pseudocatenulatum* ↓ *Methanobrevibacter* in IBS *vs* HC, particularly in IBS-C subgroup	Feces	HITChip phylogenetic microarray	[36]
11 IBS (5 F/6 M) 8 HCs (2 F/6 M) Age: 18-74	II	Greater biological variability of predominant bacteria among IBS subjects *vs* HC and higher microbial diversity (especially Bacteroides and lactobacilli) in IBS *vs* HC In IBS, Exclusive detection of *Eubacterium biforme* (absent in HC) ↑ *Bacteroidetes* ↑ *Lactobacillus* ↓ *Bifidobacterium* ↓ *C. coccoides*	Feces	qPCR (SYBR Green) DGGE on V3-V5 region of 16S rRNA gene qPCR (SYBR Green)	[63]
37 IBS (26 F/11 M) 13 IBS-D 13 IBS-C 13 IBS-A Age: 21.7 ± 2.0	II	No evident difference in predominant microbiota from profiles of both sample sites between IBS and HC ↑ *P. aeruginosa* in all subgroups if IBS and in both body niche samples	Duodenal brushes and feces	DGGE on V6-V8 region of 16S rRNA gene, generation of 51 clones and sequencing qPCR (Taqman)	[57]

20 HC (15 F/5 M) Age: 21.7 ± 2.0					
16 IBS-D (11 F/5 M) Age: 23-52	Ⅲ	Lower biodiversity in IBS-D *vs* HCs in fecal samples, no biodiversity differences in mucosal samples ↓ Clostridiales	Feces samples and colonic biopsy	T-RFLP	[71]
21 HCs (17 F/4 M) Age: 21-60		↓ Planctomycetaceae			[62]
81 IBS (69 F/27 M) 15 IBS-C Age: 20-73	Ⅰ and Ⅱ	*Staphylococcus aureus* detected only in IBS subjects, with higher prevalence in IBS-C Enterotoxin-encoding gene of *C. perfringens* detected only in IBS subjects	Feces	qPCR (SYBR Green)	
23 HCs (16 F/7 M) Age: 26-64		*Helicobacter pylori* detected in 3 IBS subjects, none in HCs		Sequencing of *S. aureus* amplicons	
23IBS-D (17 F/6 M) Age: 23-70 23 HCs (18/5 M) Age: 21-58	Ⅲ	Lower microbial richness in IBS-D Structural changes in IBS-D *vs* HC, from phylum to genus ↑ Proteobacteria ↑ γ-proteobacteria ↑ *Enterobacteriales* ↑ *Enterobacteriaceae* ↓ *Faecalibacterium* ↓ *F. prausnitzii*	Feces	454 Pyrosequencing of the V1-V3 and V6 regions of 16S rRNA gene qPCR (SYBR Green)	[54]
37 IBS (26 F/11 M) 15 IBS-D 10 IBS-C 12 IBS-A Age: 37 ± 12 20 HCs (13 F/7 M) Age: 39 ± 9	Ⅱ	Clustering by microbiota composition revealed subgroups of IBS patients: (1) a group (n = 15) with normal-like microbiota composition compared with HCs; and (2) a group (n = 22) with large microbiota-wide changes characterized by an increase of Firmicutes (mainly clostridia/Clostridiales) and a depletion of Bacteroidetes ↓ *Bacteroides* ↓ *Alistipes* ↑ *Lachnospiraceae incertae sedis* ↑ Butyrate-producing *Eubacterium halli* and *desmolans* ↑ *B. adolescentis*	Feces	Pyrosequencing of the V4 region of 16S rRNA gene	[77]
47 IBS 27 IBS-D 20 IBS-C Age average: 34.3 26 HCs Age average: 46.1	Ⅲ	Higher number of mucosa-associated bacteria in IBS ↑ *Bacteroides* ↑ *Eubacterium rectale-C. coccoides* ↓ *Bifidobacterium* in IBS-D than in IBS-C	Rectal biopsies	FISH	[32]
75 rural IBS Age: 4-18 20 Hurban HCs Age: 5-15	Ⅲ	↓ *Enterobacter* ↓ *Enterococcus* ↓ *Lactobacillus* ↓ *Bifidobacterium*	Feces	Culture-based analysis	[29]
22 IBS-D Age: 8-18 22 HCs Age: 11-18	Ⅱ	Higher variability among IBS subjects No difference between IBS-D and HCs at phylum level. No difference for *Clostridium* and *Faecalibacterium* ↑ *Veillonella* ↑ *Prevotella* ↑ *Enterobacter* ↑ *Lactobacillus* ↓ *Bifidobacterium* ↓ *Verrucomicrobium* Difference at species level in the genus *Bacteroides*: ↓ *B. fragilis* ↓ *B. thetaiotaomicron* ↑ *B. ovatus* ↑ *B. salyersiae* Positive abundance correlation between *Veillonella-Haemophilus* and *Streptococcus*; negative for *Ruminococcus* Confermation of data on Clostridia, Bacteroidetes, *Bifidobacterium* Confermation of data on *Bifidobacterium*, *Prevotella*, *Faecalibacterium*	Feces	Microbiota array Pyrosequencing (V1-V2-V3 region of 16S rRNA) FISH qPCR	[40]

14 IBS-C (14 F) 12 HCs (14 F) Age: 20-59	II	No differences in total strict and facultative anaerobes between IBS-C and HCs No difference in hydrolytic bacterial communities ↑ Lactate utilizing sulphate-reducing bacteria (SRB) ↓ Lactate non SRB (butyrate-producing) ↑ H_2-utilizing SRB ↓ H_2-utilizing non SRB (acetogenic, methanogens) ↑ *Enterobacteriaceae* ↓ *Bifidobacterium* ↓ *Lactobacillus* ↓ *Bifidobacterium* ↓ *Roseburia-E. rectale*	Feces	Culture-based analysis FISH	[28]
19 IBS 24 HCs Age: 33.6 ± 9.1	III	↑ Bifidobacteriaceae ↑ Lactobacillaceae ↑ *Clostridium* cluster IX ↑ *Eubacterium rectale* ↑ *Enterococcus faecium* ↑ *Clostridium difficile* ↑ *Bacillus cereus* and *B. clausii* ↑ *Campilobacter* spp. ↓ *Bacteroides/Prevotella* ↓ *Veillonella*	Feces	Microbiota Array	[42]
14 IBS-D(3 F/11 M) 18 HCs (7 F/11 M) Age: 18-65	III	↑ *E. coli* ↓ *Clostridium leptum* ↓ *Bifidobacterium*	Feces	qPCR (SYBR Green)	[58]
16 IBS 9 HCs		Reduced microbial diversity in IBS In mucosal samples: ↑ Bacteroidaceae In fecal samples: ↑ Rikenellaceae ↑ Porphyromonadaceae ↓ Ruminococcaceae IBS-D: ↑ *Acinetobacter, Leuconostoc, Butyricimonas, Odoribacter* (fecal) ↓ *Desulfovibrio, Oribacterium* (biopsies) IBS-C: ↑ *Alistipes, Butyricimonas* (feces) and *Bacteroides* (biopsies) ↓ *Fusobacterium, Eubacterium, Coprococcus, Eubacterium, Haemophilus, Neisseria, Streptococcus, Veillonella*	Colonic biopsies and feces	Pyrosequencing (V1-V2 regions of 16S rRNA)	[48]
2 IBS-D 1 HCs Several sampling over 6-8 wk	III	↑ *Alphaproteobacteria* ↑ Facultative anaerobe (Proteobacteria, *Streptococcus*) in days of acute diarrhea	Feces	Pyrosequencing (16S rRNA gene)	[89]

qPCR: Real time quantitative polymerase chain reaction; DGGE: Denaturing gradient gel electrophoresis; T-RFLP: Terminal restriction fragment length polymorphism; FISH: Fluorescence in situ hybridization; Ref.: Reference; IBS: Irritable bowel syndrome; IBS-D: Diarrhea-associated IBS; IBS-C: Constipation-associated IBS; IBS-A: Alternating symptoms IBS; HCs: Healthy controls. ↑: Increased presence in IBS; ↓: Reduced presence in IBS.

which is actually not a suitable selection medium for the isolation of these bacteria from feces.

In addition, Carroll *et al*[27] demonstrated a significant reduction in the concentration of aerobic bacteria in fecal samples from D-IBS patients compared with healthy controls. This result is not consistent with the results obtained by Mättö *et al*[23]. However, to determine the number of aerobes, Mättö *et al*[23] used nutrient agar, which is a particularly poor medium compared with the brain heart infusion agar, containing L-cysteine (0.05%) and hemin, adopted by Carroll *et al*[27]. Thus, the aerobic plate counts obtained from these two studies cannot be compared.

The inconsistencies in bacterial counts reflect the primary intrinsic flaw in culture-based methods: obtaining an appropriate selection medium for all members of a genus (or superior taxa).

The genus *Lactobacillus* is another microbial group often examined in microbiology. Tana *et al*[12] reported an increase in lactobacilli in fecal samples obtained from IBS patients (26 IBS subjects compared with 26 healthy controls). However, Chassard *et al*[28] reported that this same microbial group was reduced in IBS samples using MRS agar medium adjusted to pH 5.5 and incubation in aerobic

conditions, whereas Mättö *et al*[23] and Enck *et al*[24] reported that the lactobacilli concentrations were not significantly different using the same selective medium as Tana *et al*[12] (Rogosa agar) for the cultivation of these microbes. Therefore, the use of different culture media cannot explain the contradictory results concerning lactobacilli.

Moreover, culture-based analyses were used to characterize the fecal samples from 75 IBS children and adolescents living in rural areas in chernobyl compared with 20 healthy controls living in urban areas[29]. In this study, the researchers reported a lower abundance of all bacterial groups investigated, *i.e.*, the genera *Enterobacter*, *Enterococcus*, *Lactobacillus*, *Bifidobacterium*, in the IBS group. Thus, the choice of the selection medium profoundly affects the significance of the results obtained from analyses of the microbial ecology of a biological sample. The results of studies based on culture-dependent strategies suggest that changes in bifidobacteria, lactobacilli and the total aerobic count are typically associated with IBS. However, the intrinsic limitations of culture-based techniques, which do not examine a large majority of intestinal microorganisms, severely reduce the significance of these experimental data.

FISH

FISH in microbial ecology involves the detection of whole-microbial cells through the labeling of cellular rRNA using an oligonucleotide probe containing a fluorescent dye at the 5' end[30]. FISH probes, which commonly target 16S rRNA, are designed at various taxonomic levels, facilitating the *in situ* phylogenetic identification and enumeration of individual microbial cells. The FISH technique does not require PCR amplification; therefore, FISH does not have the potential problems associated with the nonspecific amplification of DNA during the PCR reaction.

Limitations: Similar to qPCR, FISH requires the preliminary selection of a target microbial taxonomic group (ribotype); therefore, only a limited number of previously known microbial groups can be analyzed. More importantly, FISH involves a labor-intensive protocol that includes intricate steps, such as the *in situ* acquisition of the target. Consequently, low signal intensity and background fluorescence are common problems.

In FISH experiments, reduced bifidobacteria concentrations have been detected in the fecal samples obtained from 41 IBS patients compared with 26 HCs[31], and in 14 IBS-C subjects compared with 12 HCs[28]. In addition, Parkes *et al*[32] showed reduced bifidobacteria concentrations in IBS-D patients compared with HCs and IBS-C patients.

FISH was also applied for the analysis of ileal and colonic biopsies, revealing a higher number of mucosa-associated bacteria in IBS patients (n = 20) compared with HCs (n = 20)[33]. This same study revealed the prevalence of *Eubacterium rectal* (*E. rectal*)-*Clostridium coccoides* (*C. coccoides*) in IBS. Similarly, higher mucosa-associated bacteria and increased numbers of *E. rectal*-*C. coccoides* were detected in rectal biopsies from IBS patients (n = 47) compared with those from HCs (n = 26)[32]. Furthermore, FISH analyses revealed increased *Bacteroides*[32] and reduced butyrate-producing bacteria, such as *Roseburia*-*E. rectal* (belonging to the family Lachnospiraceae), in IBS[28].

DNA microarrays (PhyloChip)

DNA microarray methods are based on the direct hybridization of PCR products amplified from total environmental DNA[34]. Therefore, the PCR amplicons are initially fluorescently labeled, and after hybridization, the signal intensity, which is directly proportional to the abundance of hybridization (*i.e.*, the amount of a specific sequence in the sample), is monitored through confocal laser scanning microscopy. The DNA microarrays used in microbial ecology are commonly based on the analysis of a pool of 16S rRNA gene fragments amplified through PCR from the total environmental DNA (PhyloChip). This technology facilitates the rapid high-throughput analysis of hundreds of microbial species in an environmental sample.

Limitations: Similarly to the binding of a primer to a nonspecific DNA target in PCR, cross hybridization is a major limitation of microarray technology. In addition, only those taxa included in the microarray can be analyzed; therefore, similar to qPCR and FISH, the ecological importance of a taxon that has not been previously selected could be erroneously omitted. Moreover, the results obtained solely through microarray are not considered sufficiently reliable, unless confirmation of these data is provided through other techniques, particularly qPCR.

Saulnier *et al*[35] did not detect a difference in the microbial richness between groups using high-resolution Phylochip Microarray on 28 IBS children and 27 HCs and the majority of taxa in IBS belonged to γ-Proteobacteria, particularly *Haemophylus parainfluenzae*. The results of the Phylochip Microarray analysis also showed the prevalence of the genera *Dorea* and *Veillonella* in IBS, similar to the results obtained for the same samples using 454 Pyrosequencing (see paragraph 2.4.3 for more details). Moreover, IBS children harbored lower levels of *Bacteroides*, including *B. vulgatus*. A previous study based on HITChip Phylogenetic Microarray showed reduced *Bacteroides* spp., including *B. vulgatus*, in IBS patients[36]. In this study, significant differences in the microbiota composition between 62 IBS patients and 46 HCs based on 129 phylotypes were revealed; specifically, IBS subjects presented a higher Firmicutes/Bacteroidetes ratio and increased numbers of *Bacillus*, *Streptococcus*, *Dorea*, *Blautia*, *Clostridium* and *Ruminococcus*. A significant abundance in the phylotype *Ruminococcus gnavus* (*R. gnavus*), including the species *Ruminococcus torques* (*R. torques*) (now reclassified as *Blautia torques*), was also detected. These findings suggested that *R. torques* and *R. gnavus* are potential IBS biomarkers. In addition, other phylotypes related to the genus *Ruminococcus* (*e.g.*, *R. productus*) were increased in IBS. However, IBS

patients presented reduced levels of *Faecalibacterium*, *Prevotella* and *Bifidobacterium*, with high significant differences in *B. gallicum* and *B. pseudocatenolatum*. Interestingly, the authors also showed a positive correlation with IBS symptoms, thus confirming the results of previous data[37-39].

To characterize the IμB of young IBS-D patients, Rigsbee *et al*[40] used the Microbiota Array Affymetrix, a platform containing sets of phylogenetic 16S rRNA gene probes, for the detection of 775 bacterial phylotypes in the human IμB[41]. In this study, IBS-D samples contained lower levels of the genus *Bifidobacterium* and higher levels of the genera *Veillonella*, *Prevotella* and *Lactobacillus*. Although there was no difference in the abundance of the complete *Bacteroides* genus between IBS patients and HCs, significant differences were observed for certain species, such as reduced *B. fragilis* and *B. thetaiotaomicron* and increased *B. ovatus* and *B. salyersiae*.

Similarly, Maccaferri *et al*[42] detected higher amounts of Lactobacillaceae in 19 IBS subjects compared with HCs using a fully validated high taxonomic fingerprint microbiota array. In the same study, a higher Bifidobacteriaceae concentration and a lower *Veillonella* concentration were detected in the IBS samples. Notably, the enrichment of several pathobiont bacterial species[43], such as *E. rectal*, *Enterococcus faecium*, *Campilobacter* spp. and *C. difficile*, was also reported in this study.

Culture-independent, PCR-based methods

Most culture-independent methods include PCR for the amplification of a specific DNA region from the total (metagenomic) DNA isolated from an environmental sample (*e.g.*, feces or intestinal biopsies). Although alternative genes are available, nearly all of the molecular methods used in these studies include an analysis of the gene encoding the ribosomal RNA subunit 16S (16S rRNA). The 16S rRNA gene is a conserved region of the bacterial chromosome that has been extensively used in microbial ecology research, as this gene is present in all bacterial genomes and contains both highly conserved and variable regions[22]. The highly conserved sequences, therefore, can be used as target regions for universal oligonucleotide probes (named universal primers) in the PCR amplification of the 16S rRNA gene from virtually all bacteria. Except for FISH, which is based on the direct *in situ* hybridization of an oligonucleotide probe onto rRNA targets, all the molecular methodologies reported here include the initial PCR amplification of the 16S rRNA gene using specific or universal primers. Consequently, all molecular biology protocols described herein inevitably require the extraction of nucleic acids from an environmental sample, which are subsequently used as templates for the characterization of microorganisms.

The protocol employed for DNA extraction affects the results of the downstream reactions. An efficient DNA extraction, producing high-quality genomic DNA, is essential to properly reflect the actual microbial diversity of a complex ecosystem and detect less represented microbial populations[44,45]. In the studies reviewed herein, different DNA extraction protocols have been adopted, including home-made methods[46,47] and commercial kits, such as the QIAamp DNA Stool Mini Kit (Qiagen)[48], the Fast DNAII spin kit (BIO 101)[23], the FastDNA Spin Kit (*QBIO* gene)[49], the ZR Fecal DNA Isolation kit (Zymo Research Corporation)[40], and the AccuPrep Genomic DNA Extraction Kit (Bioneer)[50]. Different kits generate diverse results in terms of DNA yield, purity and integrity, significantly affecting the microbial profiles[51] and differently impacting microbial diversity scores detected on the basis of the downstream techniques employed[52]. Understanding how an extraction protocol affects an analysis is difficult and outside of the scope of this review. However, other studies have addressed this technical issue[53,54].

qPCR: Specific oligonucleotides for the quantification of particular taxa *via* PCR (qPCR method) have been extensively used to overcome the problems of microbial cultivation.

The qPCR technique has clear advantages, such as the high sensitivity (*i.e.*, also limited concentrations of bacteria can be detected). Furthermore, qPCR facilitates the analysis of a large number of samples in a short time. Another important feature of qPCR is the design of primers that potentially target genes at any taxonomic level; thus, the identification of unique genetic signatures also facilitates quantification at the strain level, which is important when analyzing particular microbial behaviors, such as the fate of a probiotic strain in the gastrointestinal tract[50,55].

Limitations: However, the specificity of primers, particularly those targeting conserved ubiquitous genes, such as 16S rRNA, significantly varies depending on the experimental conditions of the assay. In other words, the protocol for a pair of primers targeting a specific group of microorganisms could lose specificity when using a different thermocycler[56] because even small changes in the reaction conditions could lead to the amplification of the genes from related taxa. Specificity problems can be drastically reduced using TaqMan fluorophore-quencher probes. However, with only two exceptions[31,57], the studies considered in this review exclusively used intercalating fluorescent dyes, such as SYBR Green, to measure the accumulation of amplicons in real time during each PCR cycle for the analysis of the IμB in IBS. The main limitation of qPCR is that this technique can only analyze one microbial group per reaction. Furthermore, the microbial groups are selected in advance, thereby limiting the potential identification of microbial groups that were not initially considered but might play an important role.

We selected 13 manuscripts published in the last 10 years that employed qPCR to characterize the IμB associated with IBS. Malinen *et al*[16] considered 20 different microbial groups ranging from the species and genus levels to supra-generic groups. This study showed several significant differences among IBS and HCs. Particularly, these authors showed a higher concentration of *Ruminococcus productus*/*C. coccoides* in IBS patients (n = 27) than in

the controls (n = 26). Several other differences were exclusively observed for diarrhea-predominant IBS patients (IBS-D, n = 12), including a reduced concentration of *Lactobacillus* spp., compared with IBS-C subjects (n = 9), and diminished *Bifidobacterium* spp. and *Desulfuvibrio* ssp., compared with controls and IBS-A subjects. Moreover, the Clostridiales genus *Veillonella* was more abundantly represented in IBS-C patients than in controls[16]. The qPCR analysis also showed a significant increase in the *Veillonella* spp. concentration in 26 young IBS patients (Age: 21.7 ± 2.0; 8 IBS-D, 11 IBS-C, 7 IBS-A) compared with age-matched HCs (n = 26)[12]. A significant decrease of bifidobacteria in diarrhea-predominant IBS patients was also observed in other studies using qPCR (22 IBS-D *vs* 22 HCs[40]; 14 IBS-D *vs* 18 HCs[58]). In another study, qPCR with Taqman technology was used to detect differences in the abundance of four different *Bifidobacterium* species in adult IBS patients (n = 19) and age-matched HCs (n = 19)[31]. These analyses revealed a significant reduction in the abundance of *B. catenulatum* in fecal specimens and duodenal mucosa brush samples obtained from IBS subjects. Although differences among the bifidobacterial species have been shown[59,60], the study of Kerckhoffs *et al*[57] is one of the very few that investigated bifidobacteria at intra-genus level in IBS (another example is[36]). Bifidobacteria are frequently analyzed in qPCR experiments, as these microbes are univocally recognized as health-promoting bacteria[61]. Thus, the available data obtained from bifidobacterial research, and reported herein, support the idea that a reduction of bifidobacteria is associated with IBS.

Interestingly, based on a previous study[37], Lyra *et al*[46] used qPCR to quantify 14 phylotypes in the fecal samples obtained from 20 IBS patients (8 IBS-D, 8 IBS-C, 4 IBS-A) and 15 healthy controls. Specifically, the abundance of several phylotypes, including the Clostridiales genera *Clostridium* and *Ruminococcus*, significantly differed among these subjects (Table 1). Moreover, in this study, the authors proposed *C. thermosuccinogenes* and *R. torques*-like phylotypes as potential biomarkers for IBS[38].

Rinttilä *et al*[62] used qPCR on samples obtained from IBS subjects (81 patients) to detect the presence of pathogens, such as *S. aureus* (with higher prevalence in IBS-C), *C. perfringens* and *H. pylori*, which were not identified in any of the control subjects (23 HCs).

Lactobacilli have often been included in qPCR analyses for the characterization of the IμB associated with IBS. In contrast to data concerning bifidobacteria, studies concerning lactobacilli have generated less convincing results, as previously shown for the culture-dependent studies described above. Malinen *et al*[16] reported reduced concentrations of *Lactobacillus* spp. in IBS-D patients (n = 12) compared with IBS-C patients (n = 9) but no differences were observed when compared with HCs (n = 22). In contrast, more recent studies have shown that lactobacilli were increased in the fecal samples of IBS-D patients (n = 10[27]) and IBS (n = 11[63]) compared with HCs (n = 10 and 8, respectively). Notably, in these studies, the same qPCR chemistry (SYBR Green) and primers[64] were used for the quantification of lactobacilli. Therefore, the observed differences might more accurately reflect actual differences in microbiota composition rather than methodological biases. The limited number of recruited subjects should also be considered to analyze these results.

Most studies have exclusively considered microbial groups belonging to the Bacteria superkingdom (also called "Eubacteria"). Experiments based on qPCR, however, have also revealed potential differences in the IμB associated with IBS in Archeabacteria. For instance, the reduced abundance of the genus *Methanobrevibacter* was reported in IBS subjects[36], particularly the IBS-C subgroup, consistent with the results of a previous study[65].

DGGE/T-RFLP: DGGE and T-RFLP are molecular techniques that produce an electrophoretic profile of microbial communities. Specifically, in DGGE, PCR products are obtained from environmental DNA using primers for a specific molecular marker (most commonly the 16S rRNA gene) and subsequently electrophoresed on a polyacrylamide gel under denaturing conditions using a chemical denaturant (*e.g.*, urea and formamide[66,67]).

In T-RFLP, the DNA fragments are obtained through PCR using a fluorescently labeled primer, followed by digestion with one or more restriction enzymes, and separated on an automated DNA sequencer[68] that only detects terminal fluorescently labeled restriction fragments, thereby simplifying the banding pattern and facilitating the analysis of complex microbial communities.

DGGE and TGGE are rapid and inexpensive techniques. These methods facilitate the simultaneous analysis and comparison of multiple samples. Different from qPCR, DGGE and TGGE facilitate the examination of different microbial groups in the same analysis.

Limitations: DGGE and T-RFLP are based on the PCR amplification of a specific genetic target; therefore, these methods have the same limitations concerning primer specificity as described for qPCR. Furthermore, DGGE does not provide direct taxonomic identification and involves the separation of DNA bands (excision from electrophoretic gel), cloning and sequencing. The separation of all DNA amplicons, however, is practically impossible because the PCR amplification of a target gene, such like the 16S rRNA gene from DNA isolated from an environmental sample, such as human feces, generates numerous DNA fragments. Consequently, only the most represented amplicons can be visualized in electrophoresis, and several DNA fragments might have similar melting points. Finally, the abundance of a specific microbial group can be exclusively estimated on the basis of the band intensity in electrophoresis. Thus, only those microbial groups represented with dominant bands in electrophoresis and showing markedly different abundance between the two conditions investigated can be identified as significant in DGGE. In T-RFLP, the separation of DNA amplicons through the amplification of the 16S rRNA gene is facilitated using an automated DNA sequencer; however, no more than approximately

100 fragments can be resolved per analysis, and more importantly, the taxonomic identification and quantification of the detected ribotypes can be deeply distorted by the fact that different bacterial species can share the same terminal restriction fragment length.

Concerning the characterization of the IμB associated with IBS using DGGE, an increase in *Clostridium* spp. and *Eubacterium* spp. and a decrease *Parabacteroides* spp. and several *Bacteroides* species in IBS samples was reported[69]. Furthermore, Kerckhoffs *et al*[57] showed the augmented presence of *Pseudomonas* spp. in duodenal mucosal brush and fecal samples from 37 IBS patients compared to 20 healthy subjects. Subsequent qPCR experiments confirmed the increased abundance of *Pseudomonas aeruginosa* in the same samples. In addition, DGGE technique displayed reduced biodiversity in IBS subjects, consistent with the results obtained by Noor *et al*[69]. In contrast, a Korean study showed that IBS subjects (n = 11) had a significantly higher diversity of total bacteria than HCs (n = 8)[64]. Maukonen *et al*[49] and Kerckhoffs *et al*[57] detected no significant differences in the microbiota variability between IBS patients and HCs. However, Codling *et al*[70] showed higher variability in HC subjects compared with IBS patients. The results of the DGGE analysis concerning microbial biodiversity in IBS are contradictory. In these studies, however, the general biodiversity was calculated according to the numbers and relative intensities of the bands detected among individual samples. Thus, this analysis has intrinsic technical limitations. Indeed, many taxa could be present at low levels and could be therefore only marginally amplified, generating bands that cannot be easily visualized on the electrophoretic gel. Therefore, DDGE profiles are not adequate for the determination of the biodiversity of a complex microbial ecosystem. Thus, the use of primers for the amplification of a specific group of bacteria (*e.g.*, genus-specific primers), generating a reduced number of taxa, could improve the significance of the evaluation of microbial diversity using DGGE. Indeed, Ponnusamy *et al*[63] used group-specific and detected the increased diversity of Bacteroidetes and lactobacilli and the decreased diversity of bifidobacteria and *C. coccoides* in IBS samples.

T-RFLP fingerprinting of the bacterial 16S rRNA gene was used to analyze the microbiota in fecal and mucosal samples from 16 IBS-D patients and 21 HCs, revealing lower biodiversity and the reduced abundance of Gram-positive Clostridiales and Gram-negative Planctomycetaceae in the IBS-D fecal samples[71]. These data are partially inconsistent with the results of the studies cited above, which showed an increase in certain taxa belonging to Clostridiales in IBS using qPCR. This inconsistency might reflect the fact that T-RFLP potentially included all taxa belonging to the Clostridiales, whereas qPCR analyses only quantified selected genera. Furthermore, the intrinsic limitations of T-RFLP fingerprinting distort the results.

16S rRNA gene library (clone library method): The preparation of a clone library containing microbial DNA fragments derived from an environmental sample is the "gold standard" for microbial community analyses. The most widely used methods include the PCR amplification of the 16S rRNA genes from an environmental sample, followed by cloning and sequencing of the individual DNA fragments[72]. The obtained sequences are subsequently compared with known sequences database, such as GenBank or the Ribosomal Database Project. For the data analysis, each clone sequence is assigned to a taxonomic lineage according to sequence similarity cut-off values (*e.g.*, cut-off values of 80%, 85%, 90%, 92%, 94%, and 97% for phylum, class, order, family, subfamily, and species, respectively)[72].

16S rRNA clone libraries facilitate the initial survey of the microbial diversity in an environmental sample, and differently from the methodologies described above, these libraries contribute to the identification of novel taxa.

Limitations: Environments characterized by complex microbial ecosystems, such as soil or feces, might require more than 40000 clones to document 50% of the richness[73]. However, until recently, 16S rRNA clone libraries rarely contained numbers of sequences of this magnitude. Therefore, these studies only revealed a small portion of the microbial biodiversity present in an environmental sample. This problem directly reflects the fact that the clone library method was, until recently, a time-consuming, labor-intensive and particularly expensive microbial ecology strategy.

Consistent with the limitations described above, the quality of the first studies employing clone libraries to characterize the IμB in IBS was drastically affected by the limited number of sequenced clones. Indeed, Mättö *et al*[23] sequenced the partial 16S rRNA gene from only 45 amplicons (29 amplicons from 5 IBS patients and 16 amplicons from 4 HCs), revealing the increased prevalence of *Clostridium* spp. and reduced prevalence of *Eubacterium* in IBS patients. Kerckhoffs *et al*[57] also evaluated a limited number of clones (n = 51) and did not detect significant differences between in the microbiota composition of both duodenal biopsies and fecal samples from IBS patients and HCs, except for an increase of *Pseudomonas aeruginosa* in IBS.

Kassinen *et al*[37] made an important contribution to the field of microbial ecology in IBS through 16S rRNA cloning and sequencing using a conventional sequencer (ABI PRISM® BigDye™ Terminator Cycle Sequencing, Applied Biosystems), generating 3753 sequences from the analysis of the fecal samples obtained from 24 IBS patients (10 IBS-D, 8 IBS-and 6 IBS-A patients) and 23 HCs. This study overcame the intrinsic problem inherent in most experimental approaches using PCR with universal primers, such as the 16S rRNA amplification, for the preparation of a clone library. Indeed, biases in favor targets with low guanine and cytosine (%GC) contents are observed in PCR amplification from a pool of 16 rRNA gene targets containing different se-

quences[74]. Therefore, the numbers of bacteria characterized by higher %GC in the 16S rRNA gene, such as bifidobacteria, might be underestimated. To overcome this problem, Kassinen *et al*[37] used cesium chloride gradient centrifugation to separate the genomic DNA from IBS and HC samples into three fractions based on %GC: fraction 7 (with a %GC between 25% and 30%), fraction 10 (%GC: 40%-45%), and fraction 13 (%GC: 55%-60%). Using this strategy, significant differences in the microbiota composition were detected among different IBS subcategories. In fraction 7, the members of the genus *Lactobacillus* were reduced in all IBS subgroups, whereas the *Ruminococcus* was higher in IBS-C and IBS-A patients, and *Streptococcus* was higher in IBS-D patients. Furthermore, in fraction 13, the high %GC bacterium *Collinsella*, phylum Actinobacteria (similar to bifidobacteria), was less abundant in IBS-C and IBS-D patients. This research group used a similar strategy to separate the genomic DNA obtained from 10 IBS-D subjects into 7 fractions based on %GC[47]. The sequences of 3267 clones were subsequently compared with an analogous HC library of 23 subjects, revealing an increase in Proteobacteria and *Firmicutes* (in particular, the family *Lachnospiraceae*) and a decrease in *Actinobacteria* and *Bacteroidetes* in IBS-D patients; decreased diversity in IBS-D was also observed.

Despite these efforts, studies based on the use of the clone library method have not completely overcome the problem of the limited bacterial diversity observed in intestinal samples, as only a limited number of clone sequences are observed. Thus, next-generation DNA sequencing technologies, such as the pyrosequencing, have made significant advancements.

Pyrosequencing: Pyrosequencing is a sequencing strategy based on the production of light from luciferase for the detection of individual nucleotides added to the nascent DNA; the resulting data are subsequently used to generate sequence read-outs. The rapid technological development of this strategy facilitates massive parallel high-throughput sequencing, which is applied to microbial ecology to sequence the hypervariable regions of 16S rRNA genes in large numbers. The use of pyrosequencing technology generates at least 100 times higher coverage of microbial diversity in a sample compared with typical Sanger sequencing. With this technology, the sequences of the hypervariable regions are generally short (100-350 bases) but provide sufficient phylogenetic information to determine the taxonomic level of genus.

In recent years, 454 Pyrosequencing has been used to study the microbial ecology of IBS. Carroll *et al*[75] used this technology to characterize the fecal DNA isolated from 23 IBS-D patients and 23 HCs. To this aim, the variable regions V1-V3 (an average of 8232 reads per sample) and V6 (an average of 6591 reads per sample) of the 16S rRNA gene were sequenced, revealing less microbial richness and a higher presence of the phylum Proteobacteria (particularly the class γ-Proteobacteria and the family Enterobacteriaceae) in the IBS-D population. Furthermore, the genus *Faecalibacterium* was less abundant in IBS-D samples, consistent with a significant reduction of the anti-inflammatory species *Faecalibacterium prausnitzii*[76], determined through qPCR. Saulnier *et al*[35] obtained analogous results concerning increased γ-Proteobacteria[35]. In this study, the 16S rRNA gene fragments from the fecal samples of 22 pediatric IBS patients and 22 HCs were sequenced through pyrosequencing, generating an average of 54287 reads per sample. The data analysis showed an abundance of γ-Proteobacteria and particularly, the species *Haemophilus parainfluenzae*. In addition, the Firmicutes genera *Dorea* and *Veillonella* were significantly represented in IBS patients. Similarly, Rigsbee *et al*[40] showed that the genus *Veillonella* was increased in pediatric IBS-D patients.

Moreover, Durbán *et al*[48] used pyrosequencing to study the microbiota population in feces and colon mucosa samples obtained from 16 IBS patients and 9 HCs. In this study, DNA was extracted from three types of samples per subject: biopsies of the ascending and the descending colon mucosa, and feces. Prior to pyrosequencing, the 16S rRNA genes were amplified from the extracted DNA, and equal amounts of the PCR products from different samples were pooled. The analysis of approximately 268000 reads showed reduced microbial diversity in the IBS samples and significant differences in the representation of several microbial taxa between IBS patients and HCs. Particularly, the families *Rikenellaceae* and *Porphyromonadaceae* were increased and *Ruminococcaceae* spp. were decreased in the fecal samples of IBS subjects. Furthermore, the family *Bacteroidaceae* was more abundant in mucosal samples. Several other taxa were diversely represented in IBS-D and IBS-C samples compared with HCs. This study, therefore, indicated several potential microbial signatures for IBS and IBS subtypes. However, these results were based on a limited number of sequence reads per subject (approximately 3500).

CONCLUSION

Intestinal microbiota plays a role in the pathogenesis of IBS and is not merely a consequence of the disorder[77]. A number of factors profoundly influence the identification of specific microbial modifications etiologically associated with IBS: The etiology of this disorder is heterogeneous and might profoundly vary among individuals. There is great variability among different subgroups of IBS (diarrhea, constipation-predominant and alternating IBS). The technologies adopted to characterize the IμB have intrinsic pitfalls associated with particular biases.

Despite these limitations, the analytical revision of the studies referenced in the present review resulted in the identification of microbial groups whose relative abundance, consistent with different studies using diverse methodological approaches, significantly altered IBS. These results suggest that the following microbial groups are potential IμB signatures of IBS, as briefly summarized below.

Bifidobacterium

Lower levels of members of the genus *Bifidobacterium*

have predominantly been identified in studies on IμB in IBS. Indeed, almost all of the studies analyzed in the present review (with only one exception[42]) suggest that bifidobacteria are underrepresented in IBS, particularly in the diarrhea-predominant type. Interestingly, most probiotic preparations shown as effective in managing IBS symptoms contain bifidobacteria (particularly, the species *B. animalis* subsp. *lactis*, *B. bifidum*, *B. breve* and *B. longum* subsp. *B. infantis*)[17,78], suggesting a preventing role for these microorganisms in IBS.

A mechanism underlying the beneficial role of bifidobacteria in IBS might depend on the presence of serine protease inhibitors (SERPINs) in these bacteria[79]. Indeed, supernatants obtained from IBS biopsy samples have high levels of these proteases (derived from the host or potentially produced by certain members of the phylum Firmicutes[80]). Such proteases have been implicated in the observed over-stimulation of sub-mucosal neurons in IBS subjects[81]. Therefore, the SERPINs from bifidobacteria might act on extra-cellular proteases to suppress the activity of these enzymes.

Veillonella

Different studies have shown an increase in the Firmicutes genus *Veillonella* in IBS patients[16,12,35,40] using different techniques (qPCR, Pyrosequencing and Microbiota Array Hybridization). Particularly, Tana *et al*[12] showed higher levels of *Veillonella* in IBS-C patients and demonstrated a correlation with severity of pain and increased levels of acetate and propionate in the feces of subjects. Interestingly, it has been demonstrated that *Veillonella* is abundant in jejunal samples of IBS patients and this bacteria might be involved in small-intestine bacterial overgrowth (SIBO)[82]. SIBO is defined as a malabsorption syndrome resulting from the presence of abnormal bacterial load in the small intestine (greater than 10^5 CFU per mL of intestinal aspirate and/or colonic-type species). Several studies have reported the prevalence of SIBO in IBS patients, although conflicting data have also been reported[83-85].

Furthermore, Rigsbee *et al*[40] showed a positive correlation among *Veillonella*, *Haemophilus* and *Streptococcus*, suggesting that *Veillonella* forms co-aggregation complexes with other bacteria present in the small intestine, such as *Streptococcus* and *Haemophilus*[12,86,87]. Higher proportions of *Haemophilus* and *Veillonella* have also been observed in microbiomes associated with esophagitis[88]. Thus, these data suggest that *Veillonella* might play a role in the onset of gastro-intestinal disorders, such as IBS.

γ-Proteobacteria

The studies described in the present review have presented non-controversial data concerning the increased prevalence of members the phylum Proteobacteria in IBS subjects[35,47,54,89]. Some studies have reported a significant increase in the abundance of the class γ-Proteobacteria in IBS[35]. Notably, *Haemophilus* was most represented among γ-Proteobacteria, and *Haemophilus parainfluenzae* was the predominant species.

The class γ-Proteobacteria comprises several families that include pathogenic bacteria (*e.g.*, Enterobacteriaceae, Legionellaceae, Aeromonadaceae, Vibrionaceae). Particularly, Enterobacteriaceae were increased in IBS[54]. Thus, it is likely that these bacteria are among those (potential) pathogens (also known as pathobionts) that contribute to the onset and maintenance of IBS.

Clostridiales/Blautia

Clostridiales is a wide and heterogenic Firmicutes order that includes several bacterial groups differently represented in IBS. Clostridiales also include the family Lachnospiraceae, a group of microorganisms that normally occur in the gut of humans and animals. This family comprises the genus *Blautia*, which comprises several misclassified species belonging to the *Clostridium* cluster XIVa, including *C. coccoides* and several *Ruminococcus* species related to *R. gnavus* (that also include *R. torques*)[90]. In several studies described herein, the increased presence of these bacteria has been demonstrated in IBS patients[16,32,33,36,46].

Clostridia abundantly colonize mucin[91], and it was proposed that an increase in these bacteria might reflect the increased production of rectal mucus in both IBS-C and IBS-D patients[92]. Particularly, *Clostridium* cluster XIVa has previously been associated with IBS[93]. More specifically, Jeffery *et al*[77] showed that the butyrate-producing clostridia of cluster XIVa are associated with IBS. Butyrate has been shown to cause visceral hypersensitivity[94]; thus, it is likely that an increase in butyrate-producing bacteria might promote sensory dysfunctions typical of IBS[77].

Faecalibacterium

Reduced levels of *Faecalibacterium* spp. has been shown in two studies reported in this review. Rajilić-Stojanović *et al*[36] showed that *Faecalibacterium* was the only microbial group within the phylum Firmicutes that was significantly underrepresented in both IBS-C and IBS-A subjects. Interestingly, *Faecalibacterium prausnitzii* possess anti-inflammatory properties[77], suggesting that the presence of this bacterium might modulate inflammatory conditions associated with IBS.

The available experimental data indicate modifications in the IBS IμB composition at the phylum level. Specifically, a general increase in *Firmicutes* and Proteobacteria with a concomitant reduction of *Bacteroidetes* and *Actinobacteria* has been associated with IBS.

Concluding remarks

The progress in DNA sequencing technologies offers promise to microbial ecology studies, facilitating the adequate detection and quantification of less represented microorganisms within the large microbial biodiversity in the intestinal ecosystem. Thus, sufficient research studies for the investigation of the IμB should include the following basic elements: New generation DNA sequencing technologies, such as 454 Pyrosequencing and Ion Tor-

rent[95], to obtain a high number of reads to satisfy the biodiversity requirements specified through rarefaction curves. Confirmation of the results using other methods, preferentially qPCR. An investigation of the microbiota components other than eubacteria, such as archaebacteria, fungi, yeasts and viruses.

The identification of microbial biomarkers in the IμB will contribute to the development of new diagnostic tools and novel therapeutic strategies for the treatment of different subtypes of IBS.

REFERENCES

1 **Paré P**, Gray J, Lam S, Balshaw R, Khorasheh S, Barbeau M, Kelly S, McBurney CR. Health-related quality of life, work productivity, and health care resource utilization of subjects with irritable bowel syndrome: baseline results from LOGIC (Longitudinal Outcomes Study of Gastrointestinal Symptoms in Canada), a naturalistic study. *Clin Ther* 2006; **28**: 1726-1735; discussion 1710-1711 [PMID: 17157129 DOI: 10.1016/j.clinthera.2006.10.010]

2 **Quigley EM**, Abdel-Hamid H, Barbara G, Bhatia SJ, Boeckxstaens G, De Giorgio R, Delvaux M, Drossman DA, Foxx-Orenstein AE, Guarner F, Gwee KA, Harris LA, Hungin AP, Hunt RH, Kellow JE, Khalif IL, Kruis W, Lindberg G, Olano C, Moraes-Filho JP, Schiller LR, Schmulson M, Simrén M, Tzeuton C. A global perspective on irritable bowel syndrome: a consensus statement of the World Gastroenterology Organisation Summit Task Force on irritable bowel syndrome. *J Clin Gastroenterol* 2012; **46**: 356-366 [PMID: 22499071 DOI: 10.1097/MCG.0b013e318247157c]

3 **Drossman DA**. The functional gastrointestinal disorders and the Rome III process. *Gastroenterology* 2006; **130**: 1377-1390 [PMID: 16678553 DOI: 10.1053/j.gastro.2006.03.008]

4 **Drossman DA**. Rome III: the new criteria. *Chin J Dig Dis* 2006; **7**: 181-185 [PMID: 17054578 DOI: 10.1111/j.1443-9573.2006.00265.x]

5 **El-Salhy M**. Irritable bowel syndrome: diagnosis and pathogenesis. *World J Gastroenterol* 2012; **18**: 5151-5163 [PMID: 23066308]

6 **Liebregts T**, Adam B, Bredack C, Röth A, Heinzel S, Lester S, Downie-Doyle S, Smith E, Drew P, Talley NJ, Holtmann G. Immune activation in patients with irritable bowel syndrome. *Gastroenterology* 2007; **132**: 913-920 [PMID: 17383420 DOI: 10.1053/j.gastro.2007.01.046]

7 **Gasbarrini A**, Lauritano EC, Garcovich M, Sparano L, Gasbarrini G. New insights into the pathophysiology of IBS: intestinal microflora, gas production and gut motility. *Eur Rev Med Pharmacol Sci* 2008; **12** Suppl 1: 111-117 [PMID: 18924450]

8 **Fukudo S**. Stress and visceral pain: focusing on irritable bowel syndrome. *Pain* 2013; **154** Suppl 1: S63-S70 [PMID: 24021863]

9 **Ringel Y**, Maharshak N. Intestinal microbiota and immune function in the pathogenesis of irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* 2013; **305**: G529-G541 [PMID: 23886861 DOI: 10.1152/ajpgi.00207.2012]

10 **Brint EK**, MacSharry J, Fanning A, Shanahan F, Quigley EM. Differential expression of toll-like receptors in patients with irritable bowel syndrome. *Am J Gastroenterol* 2011; **106**: 329-336 [PMID: 21102570 DOI: 10.1038/ajg.2010.438]

11 **Langhorst J**, Junge A, Rueffer A, Wehkamp J, Foell D, Michalsen A, Musial F, Dobos GJ. Elevated human beta-defensin-2 levels indicate an activation of the innate immune system in patients with irritable bowel syndrome. *Am J Gastroenterol* 2009; **104**: 404-410 [PMID: 19174795 DOI: 10.1038/ajg.2008.86]

12 **Tana C**, Umesaki Y, Imaoka A, Handa T, Kanazawa M, Fukudo S. Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome. *Neurogastroenterol Motil* 2010; **22**: 512-519, e114-e115 [PMID: 19903265]

13 **Ahmed I**, Greenwood R, Costello Bde L, Ratcliffe NM, Probert CS. An investigation of fecal volatile organic metabolites in irritable bowel syndrome. *PLoS One* 2013; **8**: e58204 [PMID: 23516449 DOI: 10.1371/journal.pone.0058204]

14 **Mortensen PB**, Andersen JR, Arffmann S, Krag E. Short-chain fatty acids and the irritable bowel syndrome: the effect of wheat bran. *Scand J Gastroenterol* 1987; **22**: 185-192 [PMID: 3033815 DOI: 10.3109/00365528708991878]

15 **Treem WR**, Ahsan N, Kastoff G, Hyams JS. Fecal short-chain fatty acids in patients with diarrhea-predominant irritable bowel syndrome: in vitro studies of carbohydrate fermentation. *J Pediatr Gastroenterol Nutr* 1996; **23**: 280-286 [PMID: 8890079 DOI: 10.1097/00005176-199610000-00013]

16 **Malinen E**, Rinttilä T, Kajander K, Mättö J, Kassinen A, Krogius L, Saarela M, Korpela R, Palva A. Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. *Am J Gastroenterol* 2005; **100**: 373-382 [PMID: 15667495 DOI: 10.1111/j.1572-0241.2005.40312.x]

17 **Guglielmetti S**, Mora D, Gschwender M, Popp K. Randomised clinical trial: Bifidobacterium bifidum MIMBb75 significantly alleviates irritable bowel syndrome and improves quality of life--a double-blind, placebo-controlled study. *Aliment Pharmacol Ther* 2011; **33**: 1123-1132 [PMID: 21418261 DOI: 10.1111/j.1365-2036.2011.04633.x]

18 **Pimentel M**, Lembo A, Chey WD, Zakko S, Ringel Y, Yu J, Mareya SM, Shaw AL, Bortey E, Forbes WP. Rifaximin therapy for patients with irritable bowel syndrome without constipation. *N Engl J Med* 2011; **364**: 22-32 [PMID: 21208106 DOI: 10.1056/NEJMoa1004409]

19 **Sachdev AH**, Pimentel M. Antibiotics for irritable bowel syndrome: rationale and current evidence. *Curr Gastroenterol Rep* 2012; **14**: 439-445 [PMID: 22945316 DOI: 10.1007/s11894-012-0284-2]

20 **Arumugam M**, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, Bertalan M, Borruel N, Casellas F, Fernandez L, Gautier L, Hansen T, Hattori M, Hayashi T, Kleerebezem M, Kurokawa K, Leclerc M, Levenez F, Manichanh C, Nielsen HB, Nielsen T, Pons N, Poulain J, Qin J, Sicheritz-Ponten T, Tims S, Torrents D, Ugarte E, Zoetendal EG, Wang J, Guarner F, Pedersen O, de Vos WM, Brunak S, Doré J, Antolín M, Artiguenave F, Blottiere HM, Almeida M, Brechot C, Cara C, Chervaux C, Cultrone A, Delorme C, Denariaz G, Dervyn R, Foerstner KU, Friss C, van de Guchte M, Guedon E, Haimet F, Huber W, van Hylckama-Vlieg J, Jamet A, Juste C, Kaci G, Knol J, Lakhdari O, Layec S, Le Roux K, Maguin E, Mérieux A, Melo Minardi R, M'rini C, Muller J, Oozeer R, Parkhill J, Renault P, Rescigno M, Sanchez N, Sunagawa S, Torrejon A, Turner K, Vandemeulebrouck G, Varela E, Winogradsky Y, Zeller G, Weissenbach J, Ehrlich SD, Bork P. Enterotypes of the human gut microbiome. *Nature* 2011; **473**: 174-180 [PMID: 21508958 DOI: 10.1038/nature09944]

21 **Eckburg PB**, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the human intestinal microbial flora. *Science* 2005; **308**: 1635-1638 [PMID: 15831718 DOI: 10.1126/science.1110591]

22 **Hugenholtz P**. Exploring prokaryotic diversity in the genomic era. *Genome Biol* 2002; **3**: REVIEWS0003 [PMID: 11864374]

23 **Mättö J**, Maunuksela L, Kajander K, Palva A, Korpela R, Kassinen A, Saarela M. Composition and temporal stability of gastrointestinal microbiota in irritable bowel syndrome--a longitudinal study in IBS and control subjects. *FEMS Immunol Med Microbiol* 2005; **43**: 213-222 [PMID: 15747442 DOI:

10.1016/j.femsim.2004.08.009]
24 **Enck P**, Zimmermann K, Rusch K, Schwiertz A, Klosterhalfen S, Frick JS. The effects of ageing on the colonic bacterial microflora in adults. *Z Gastroenterol* 2009; **47**: 653-658 [PMID: 19606407 DOI: 10.1055/s-0028-1109055]
25 **Beerens H**. An elective and selective isolation medium for Bifidobacterium spp. *Lett Appl Microbiol* 1990; **11**: 155-157 [DOI: 10.1111/j.1472-765X.1990.tb00148.x]
26 **Kheadr E**, Dabour N, Le Lay C, Lacroix C, Fliss I. Antibiotic susceptibility profile of bifidobacteria as affected by oxgall, acid, and hydrogen peroxide stress. *Antimicrob Agents Chemother* 2007; **51**: 169-174 [PMID: 17060530 DOI: 10.1128/AAC.00261-06]
27 **Carroll IM**, Chang YH, Park J, Sartor RB, Ringel Y. Luminal and mucosal-associated intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome. *Gut Pathog* 2010; **2**: 19 [PMID: 21143915 DOI: 10.1186/1757-4749-2-19]
28 **Chassard C**, Dapoigny M, Scott KP, Crouzet L, Del'homme C, Marquet P, Martin JC, Pickering G, Ardid D, Eschalier A, Dubray C, Flint HJ, Bernalier-Donadille A. Functional dysbiosis within the gut microbiota of patients with constipated-irritable bowel syndrome. *Aliment Pharmacol Ther* 2012; **35**: 828-838 [PMID: 22315951 DOI: 10.1111/j.1365-2036.2012.05007.x]
29 **Sheikh Sajjadieh MR**, Kuznetsova LV, Bojenko VB. Dysbiosis in ukrainian children with irritable bowel syndrome affected by natural radiation. *Iran J Pediatr* 2012; **22**: 364-368 [PMID: 23400266]
30 **Amann RI**, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* 1995; **59**: 143-169 [PMID: 7535888]
31 **Kerckhoffs AP**, Samsom M, van der Rest ME, de Vogel J, Knol J, Ben-Amor K, Akkermans LM. Lower Bifidobacteria counts in both duodenal mucosa-associated and fecal microbiota in irritable bowel syndrome patients. *World J Gastroenterol* 2009; **15**: 2887-2892 [PMID: 19533811 DOI: 10.3748/wjg.15.2887]
32 **Parkes GC**, Rayment NB, Hudspith BN, Petrovska L, Lomer MC, Brostoff J, Whelan K, Sanderson JD. Distinct microbial populations exist in the mucosa-associated microbiota of sub-groups of irritable bowel syndrome. *Neurogastroenterol Motil* 2012; **24**: 31-39 [PMID: 22070725 DOI: 10.1111/j.1365-2982.2011.01803.x]
33 **Swidsinski A**, Weber J, Loening-Baucke V, Hale LP, Lochs H. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *J Clin Microbiol* 2005; **43**: 3380-3389 [PMID: 16000463 DOI: 10.1128/JCM.43.7.3380-3389.2005]
34 **Gentry TJ**, Wickham GS, Schadt CW, He Z, Zhou J. Microarray applications in microbial ecology research. *Microb Ecol* 2006; **52**: 159-175 [PMID: 16897303 DOI: 10.1007/s00248-006-9072-6]
35 **Saulnier DM**, Riehle K, Mistretta TA, Diaz MA, Mandal D, Raza S, Weidler EM, Qin X, Coarfa C, Milosavljevic A, Petrosino JF, Highlander S, Gibbs R, Lynch SV, Shulman RJ, Versalovic J. Gastrointestinal microbiome signatures of pediatric patients with irritable bowel syndrome. *Gastroenterology* 2011; **141**: 1782-1791 [PMID: 21741921 DOI: 10.1053/j.gastro.2011.06.072]
36 **Rajilić-Stojanović M**, Biagi E, Heilig HG, Kajander K, Kekkonen RA, Tims S, de Vos WM. Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. *Gastroenterology* 2011; **141**: 1792-1801 [PMID: 21820992 DOI: 10.1053/j.gastro.2011.07.043]
37 **Kassinen A**, Krogius-Kurikka L, Mäkivuokko H, Rinttilä T, Paulin L, Corander J, Malinen E, Apajalahti J, Palva A. The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. *Gastroenterology* 2007; **133**: 24-33 [PMID: 17631127 DOI: 10.1053/j.gastro.2007.04.005]
38 **Lyra A**, Krogius-Kurikka L, Nikkilä J, Malinen E, Kajander K, Kurikka K, Korpela R, Palva A. Effect of a multispecies probiotic supplement on quantity of irritable bowel syndrome-related intestinal microbial phylotypes. *BMC Gastroenterol* 2010; **10**: 110 [PMID: 20849659 DOI: 10.1186/1471-230X-10-110]
39 **Salonen A**, de Vos WM, Palva A. Gastrointestinal microbiota in irritable bowel syndrome: present state and perspectives. *Microbiology* 2010; **156**: 3205-3215 [PMID: 20705664 DOI: 10.1099/mic.0.043257-0]
40 **Rigsbee L**, Agans R, Shankar V, Kenche H, Khamis HJ, Michail S, Paliy O. Quantitative profiling of gut microbiota of children with diarrhea-predominant irritable bowel syndrome. *Am J Gastroenterol* 2012; **107**: 1740-1751 [PMID: 22986438 DOI: 10.1038/ajg.2012.287]
41 **Paliy O**, Kenche H, Abernathy F, Michail S. High-throughput quantitative analysis of the human intestinal microbiota with a phylogenetic microarray. *Appl Environ Microbiol* 2009; **75**: 3572-3579 [PMID: 19363078 DOI: 10.1128/AEM.02764-08]
42 **Maccaferri S**, Candela M, Turroni S, Centanni M, Severgnini M, Consolandi C, Cavina P, Brigidi P. IBS-associated phylogenetic unbalances of the intestinal microbiota are not reverted by probiotic supplementation. *Gut Microbes* 2012; **3**: 406-413 [PMID: 22713265 DOI: 10.4161/gmic.21009]
43 **Cerf-Bensussan N**, Gaboriau-Routhiau V. The immune system and the gut microbiota: friends or foes? *Nat Rev Immunol* 2010; **10**: 735-744 [PMID: 20865020 DOI: 10.1038/nri2850]
44 **Ariefdjohan MW**, Savaiano DA, Nakatsu CH. Comparison of DNA extraction kits for PCR-DGGE analysis of human intestinal microbial communities from fecal specimens. *Nutr J* 2010; **9**: 23 [PMID: 20492702 DOI: 10.1186/1475-2891-9-23]
45 **Smith B**, Li N, Andersen AS, Slotved HC, Krogfelt KA. Optimising bacterial DNA extraction from faecal samples: comparison of three methods. *Open Microbiol J* 2011; **5**: 14-17 [PMID: 21643498 DOI: 10.2174/1874285801105010014]
46 **Lyra A**, Rinttilä T, Nikkilä J, Krogius-Kurikka L, Kajander K, Malinen E, Mättö J, Mäkelä L, Palva A. Diarrhoea-predominant irritable bowel syndrome distinguishable by 16S rRNA gene phylotype quantification. *World J Gastroenterol* 2009; **15**: 5936-5945 [PMID: 20014457 DOI: 10.3748/wjg.15.5936]
47 **Krogius-Kurikka L**, Lyra A, Malinen E, Aarnikunnas J, Tuimala J, Paulin L, Mäkivuokko H, Kajander K, Palva A. Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers. *BMC Gastroenterol* 2009; **9**: 95 [PMID: 20015409 DOI: 10.1186/1471-230X-9-95]
48 **Durbán A**, Abellán JJ, Jiménez-Hernández N, Salgado P, Ponce M, Ponce J, Garrigues V, Latorre A, Moya A. Structural alterations of faecal and mucosa-associated bacterial communities in irritable bowel syndrome. *Environ Microbiol Rep* 2012; **4**: 242-247 [PMID: 23757279 DOI: 10.1111/j.1758-2229.2012.00327.x]
49 **Maukonen J**, Satokari R, Mättö J, Söderlund H, Mattila-Sandholm T, Saarela M. Prevalence and temporal stability of selected clostridial groups in irritable bowel syndrome in relation to predominant faecal bacteria. *J Med Microbiol* 2006; **55**: 625-633 [PMID: 16585652 DOI: 10.1099/jmm.0.46134-0]
50 **Yoon JS**, Sohn W, Lee OY, Lee SP, Lee KN, Jun DW, Lee HL, Yoon BC, Choi HS, Chung WS, Seo JG. Effect of multispecies probiotics on irritable bowel syndrome: a randomized, double-blind, placebo-controlled trial. *J Gastroenterol Hepatol* 2014; **29**: 52-59 [PMID: 23829297]
51 **Maukonen J**, Simões C, Saarela M. The currently used commercial DNA-extraction methods give different results of clostridial and actinobacterial populations derived from human fecal samples. *FEMS Microbiol Ecol* 2012; **79**: 697-708 [PMID: 22098067 DOI: 10.1111/j.1574-6941.2011.01257.x]

52 **Claassen S**, du Toit E, Kaba M, Moodley C, Zar HJ, Nicol MP. A comparison of the efficiency of five different commercial DNA extraction kits for extraction of DNA from faecal samples. *J Microbiol Methods* 2013; **94**: 103-110 [PMID: 23684993 DOI: 10.1016/j.mimet.2013.05.008]

53 **Pan Y**, Bodrossy L, Frenzel P, Hestnes AG, Krause S, Lüke C, Meima-Franke M, Siljanen H, Svenning MM, Bodelier PL. Impacts of inter- and intralaboratory variations on the reproducibility of microbial community analyses. *Appl Environ Microbiol* 2010; **76**: 7451-7458 [PMID: 20870788 DOI: 10.1128/AEM.01595-10]

54 **Carroll IM**, Ringel-Kulka T, Siddle JP, Klaenhammer TR, Ringel Y. Characterization of the fecal microbiota using high-throughput sequencing reveals a stable microbial community during storage. *PLoS One* 2012; **7**: e46953 [PMID: 23071673 DOI: 10.1371/journal.pone.0046953]

55 **Kajander K**, Krogius-Kurikka L, Rinttilä T, Karjalainen H, Palva A, Korpela R. Effects of multispecies probiotic supplementation on intestinal microbiota in irritable bowel syndrome. *Aliment Pharmacol Ther* 2007; **26**: 463-473 [PMID: 17635381 DOI: 10.1111/j.1365-2036.2007.03391.x]

56 **Fraga D**, Meulia T, Fenster S. Real time PCR. In: Current Protocols Essential Laboratory Techiques. Online: Wiley online Library, 2014: 10:10.3:10.3.1-10:10.3:10.3.40 [DOI: 10.1002/9780470089941.et1003s08]

57 **Kerckhoffs AP**, Ben-Amor K, Samsom M, van der Rest ME, de Vogel J, Knol J, Akkermans LM. Molecular analysis of faecal and duodenal samples reveals significantly higher prevalence and numbers of Pseudomonas aeruginosa in irritable bowel syndrome. *J Med Microbiol* 2011; **60**: 236-245 [PMID: 20947663 DOI: 10.1099/jmm.0.022848-0]

58 **Duboc H**, Rainteau D, Rajca S, Humbert L, Farabos D, Maubert M, Grondin V, Jouet P, Bouhassira D, Seksik P, Sokol H, Coffin B, Sabaté JM. Increase in fecal primary bile acids and dysbiosis in patients with diarrhea-predominant irritable bowel syndrome. *Neurogastroenterol Motil* 2012; **24**: 513-520, e246-e247 [PMID: 22356587]

59 **Canzi E**, Guglielmetti S, Mora D, Tamagnini I, Parini C. Conditions affecting cell surface properties of human intestinal bifidobacteria. *Antonie Van Leeuwenhoek* 2005; **88**: 207-219 [PMID: 16284927 DOI: 10.1007/s10482-005-6501-3]

60 **Guglielmetti S**, Fracassetti D, Taverniti V, Del Bo' C, Vendrame S, Klimis-Zacas D, Arioli S, Riso P, Porrini M. Differential modulation of human intestinal bifidobacterium populations after consumption of a wild blueberry (Vaccinium angustifolium) drink. *J Agric Food Chem* 2013; **61**: 8134-8140 [PMID: 23883473 DOI: 10.1021/jf402495k]

61 **Turroni F**, Serafini F, Foroni E, Duranti S, O'Connell Motherway M, Taverniti V, Mangifesta M, Milani C, Viappiani A, Roversi T, Sánchez B, Santoni A, Gioiosa L, Ferrarini A, Delledonne M, Margolles A, Piazza L, Palanza P, Bolchi A, Guglielmetti S, van Sinderen D, Ventura M. Role of sortase-dependent pili of Bifidobacterium bifidum PRL2010 in modulating bacterium-host interactions. *Proc Natl Acad Sci USA* 2013; **110**: 11151-11156 [PMID: 23776216 DOI: 10.1073/pnas.1303897110]

62 **Rinttilä T**, Lyra A, Krogius-Kurikka L, Palva A. Real-time PCR analysis of enteric pathogens from fecal samples of irritable bowel syndrome subjects. *Gut Pathog* 2011; **3**: 6 [PMID: 21518462 DOI: 10.1186/1757-4749-3-6]

63 **Ponnusamy K**, Choi JN, Kim J, Lee SY, Lee CH. Microbial community and metabolomic comparison of irritable bowel syndrome faeces. *J Med Microbiol* 2011; **60**: 817-827 [PMID: 21330412 DOI: 10.1099/jmm.0.028126-0]

64 **Walter J**, Hertel C, Tannock GW, Lis CM, Munro K, Hammes WP. Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Appl Environ Microbiol* 2001; **67**: 2578-2585 [PMID: 11375166 DOI: 10.1128/AEM.67.6.2578-2585.2001]

65 **Rana SV**, Sharma S, Sinha SK, Kaur H, Sikander A, Singh K. Incidence of predominant methanogenic flora in irritable bowel syndrome patients and apparently healthy controls from North India. *Dig Dis Sci* 2009; **54**: 132-135 [PMID: 18493854 DOI: 10.1007/s10620-008-0315-x]

66 **Muyzer G**. Genetic fingerprinting of microbial communities - present status and future perspectives. Methods of microbial community analysis. Proceedings of the 8th international symposium on microbial Ecology. Halifax, Canada: Atlantic Canada Society for Microbial Ecology, 1999

67 **Muyzer G**, de Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 1993; **59**: 695-700 [PMID: 7683183]

68 **Thies JE**. Soil microbial community analysis using terminal restriction fragment length polymorphisms. *Soil Sci Soc Am J* 2007; **71**: 579-591 [DOI: 10.2136/sssaj2006.0318]

69 **Noor SO**, Ridgway K, Scovell L, Kemsley EK, Lund EK, Jamieson C, Johnson IT, Narbad A. Ulcerative colitis and irritable bowel patients exhibit distinct abnormalities of the gut microbiota. *BMC Gastroenterol* 2010; **10**: 134 [PMID: 21073731 DOI: 10.1186/1471-230X-10-134]

70 **Codling C**, O'Mahony L, Shanahan F, Quigley EM, Marchesi JR. A molecular analysis of fecal and mucosal bacterial communities in irritable bowel syndrome. *Dig Dis Sci* 2010; **55**: 392-397 [PMID: 19693670 DOI: 10.1007/s10620-009-0934-x]

71 **Carroll IM**, Ringel-Kulka T, Keku TO, Chang YH, Packey CD, Sartor RB, Ringel Y. Molecular analysis of the luminal- and mucosal-associated intestinal microbiota in diarrhea-predominant irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* 2011; **301**: G799-G807 [PMID: 21737778 DOI: 10.1152/ajpgi.00154.2011]

72 **DeSantis TZ**, Brodie EL, Moberg JP, Zubieta IX, Piceno YM, Andersen GL. High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. *Microb Ecol* 2007; **53**: 371-383 [PMID: 17334858 DOI: 10.1007/s00248-006-9134-9]

73 **Dunbar J**, Barns SM, Ticknor LO, Kuske CR. Empirical and theoretical bacterial diversity in four Arizona soils. *Appl Environ Microbiol* 2002; **68**: 3035-3045 [PMID: 12039765 DOI: 10.1128/AEM.68.6.3035-3045.2002]

74 **Reysenbach AL**, Giver LJ, Wickham GS, Pace NR. Differential amplification of rRNA genes by polymerase chain reaction. *Appl Environ Microbiol* 1992; **58**: 3417-3418 [PMID: 1280061]

75 **Carroll IM**, Ringel-Kulka T, Siddle JP, Ringel Y. Alterations in composition and diversity of the intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome. *Neurogastroenterol Motil* 2012; **24**: 521-530, e248 [PMID: 22339879]

76 **Sokol H**, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux JJ, Blugeon S, Bridonneau C, Furet JP, Corthier G, Grangette C, Vasquez N, Pochart P, Trugnan G, Thomas G, Blottière HM, Doré J, Marteau P, Seksik P, Langella P. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci USA* 2008; **105**: 16731-16736 [PMID: 18936492 DOI: 10.1073/pnas.0804812105]

77 **Jeffery IB**, O'Toole PW, Öhman L, Claesson MJ, Deane J, Quigley EM, Simrén M. An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. *Gut* 2012; **61**: 997-1006 [PMID: 22180058 DOI: 10.1136/gutjnl-2011-301501]

78 **Parkes GC**, Sanderson JD, Whelan K. Treating irritable bowel syndrome with probiotics: the evidence. *Proc Nutr Soc* 2010; **69**: 187-194 [PMID: 20236566 DOI: 10.1017/S002966511000011X]

79 **Ivanov D**, Emonet C, Foata F, Affolter M, Delley M, Fisseha M,

Blum-Sperisen S, Kochhar S, Arigoni F. A serpin from the gut bacterium Bifidobacterium longum inhibits eukaryotic elastase-like serine proteases. *J Biol Chem* 2006; **281**: 17246-17252 [PMID: 16627467 DOI: 10.1074/jbc.M601678200]

80 **Macfarlane GT**, Allison C, Gibson SA, Cummings JH. Contribution of the microflora to proteolysis in the human large intestine. *J Appl Bacteriol* 1988; **64**: 37-46 [PMID: 3127369 DOI: 10.1111/j.1365-2672.1988.tb02427.x]

81 **Buhner S**, Li Q, Vignali S, Barbara G, De Giorgio R, Stanghellini V, Cremon C, Zeller F, Langer R, Daniel H, Michel K, Schemann M. Activation of human enteric neurons by supernatants of colonic biopsy specimens from patients with irritable bowel syndrome. *Gastroenterology* 2009; **137**: 1425-1434 [PMID: 19596012 DOI: 10.1053/j.gastro.2009.07.005]

82 **Bouhnik Y**, Alain S, Attar A, Flourié B, Raskine L, Sanson-Le Pors MJ, Rambaud JC. Bacterial populations contaminating the upper gut in patients with small intestinal bacterial overgrowth syndrome. *Am J Gastroenterol* 1999; **94**: 1327-1331 [PMID: 10235214 DOI: 10.1111/j.1572-0241.1999.01016.x]

83 **Pimentel M**, Chow EJ, Lin HC. Eradication of small intestinal bacterial overgrowth reduces symptoms of irritable bowel syndrome. *Am J Gastroenterol* 2000; **95**: 3503-3506 [PMID: 11151884 DOI: 10.1111/j.1572-0241.2000.03368.x]

84 **Pimentel M**, Chow EJ, Lin HC. Normalization of lactulose breath testing correlates with symptom improvement in irritable bowel syndrome. a double-blind, randomized, placebo-controlled study. *Am J Gastroenterol* 2003; **98**: 412-419 [PMID: 12591062]

85 **Walters B**, Vanner SJ. Detection of bacterial overgrowth in IBS using the lactulose H2 breath test: comparison with 14C-D-xylose and healthy controls. *Am J Gastroenterol* 2005; **100**: 1566-1570 [PMID: 15984983 DOI: 10.1111/j.1572-0241.2005.40795.x]

86 **Tuyau JE**, Sims W, Williams RA. The acid end-products of glucose metabolism of oral and other haemophili. *J Gen Microbiol* 1984; **130**: 1787-1793 [PMID: 6332175]

87 **Chalmers NI**, Palmer RJ, Cisar JO, Kolenbrander PE. Characterization of a Streptococcus sp.-Veillonella sp. community micromanipulated from dental plaque. *J Bacteriol* 2008; **190**: 8145-8154 [PMID: 18805978 DOI: 10.1128/JB.00983-08]

88 **Yang L**, Lu X, Nossa CW, Francois F, Peek RM, Pei Z. Inflammation and intestinal metaplasia of the distal esophagus are associated with alterations in the microbiome. *Gastroenterology* 2009; **137**: 588-597 [PMID: 19394334 DOI: 10.1053/j.gastro.2009.04.046]

89 **Durbán A**, Abellán JJ, Jiménez-Hernández N, Artacho A, Garrigues V, Ortiz V, Ponce J, Latorre A, Moya A. Instability of the faecal microbiota in diarrhoea-predominant irritable bowel syndrome. *FEMS Microbiol Ecol* 2013; **86**: 581-589 [PMID: 23889283 DOI: 10.1111/1574-6941.12184]

90 **Liu C**, Finegold SM, Song Y, Lawson PA. Reclassification of Clostridium coccoides, Ruminococcus hansenii, Ruminococcus hydrogenotrophicus, Ruminococcus luti, Ruminococcus productus and Ruminococcus schinkii as Blautia coccoides gen. nov., comb. nov., Blautia hansenii comb. nov., Blautia hydrogenotrophica comb. nov., Blautia luti comb. nov., Blautia producta comb. nov., Blautia schinkii comb. nov. and description of Blautia wexlerae sp. nov., isolated from human faeces. *Int J Syst Evol Microbiol* 2008; **58**: 1896-1902 [PMID: 18676476 DOI: 10.1099/ijs.0.65208-0]

91 **Macfarlane S**, Woodmansey EJ, Macfarlane GT. Colonization of mucin by human intestinal bacteria and establishment of biofilm communities in a two-stage continuous culture system. *Appl Environ Microbiol* 2005; **71**: 7483-7492 [PMID: 16269790 DOI: 10.1128/AEM.71.11.7483-7492.2005]

92 **Thompson WG**, Longstreth GF, Drossman DA, Heaton KW, Irvine EJ, Müller-Lissner SA. Functional bowel disorders and functional abdominal pain. *Gut* 1999; **45** Suppl 2: II43-II47 [PMID: 10457044 DOI: 10.1136/gut.45.2008.ii43]

93 **Rajilić-Stojanović M**. Diversity of the Human Gastrointestinal Microbiota-Novel Perspectives from High Throughput Analyses.[PhD thesis]. Wageningen: Wageningen University, 2007

94 **Bourdu S**, Dapoigny M, Chapuy E, Artigue F, Vasson MP, Dechelotte P, Bommelaer G, Eschalier A, Ardid D. Rectal instillation of butyrate provides a novel clinically relevant model of noninflammatory colonic hypersensitivity in rats. *Gastroenterology* 2005; **128**: 1996-2008 [PMID: 15940632 DOI: 10.1053/j.gastro.2005.03.082]

95 **Milani C**, Hevia A, Foroni E, Duranti S, Turroni F, Lugli GA, Sanchez B, Martín R, Gueimonde M, van Sinderen D, Margolles A, Ventura M. Assessing the fecal microbiota: an optimized ion torrent 16S rRNA gene-based analysis protocol. *PLoS One* 2013; **8**: e68739 [PMID: 23869230 DOI: 10.1371/journal.pone.0068739]

P- Reviewers: Fouad YM, Rao VS, Saei AA **S- Editor**: Gou SX
L- Editor: A **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8837

World J Gastroenterol 2014 July 21; 20(27): 8837-8845
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

Irritable bowel syndrome and food interaction

Rosario Cuomo, Paolo Andreozzi, Francesco Paolo Zito, Valentina Passananti, Giovanni De Carlo, Giovanni Sarnelli

Rosario Cuomo, Paolo Andreozzi, Francesco Paolo Zito, Valentina Passananti, Giovanni De Carlo, Giovanni Sarnelli, Department Of Clinical Medicine and Surgery, Federico Ⅱ University Hospital, School of Medicine, 80131 Naples, Italy

Author contributions: Cuomo R and Sarnelli G ideated the project; Cuomo R wrote the first draft of the manuscript; Andreozzi P, Zito FP, De Carlo G and Passananti V critically reviewed the manuscript; all authors approved the final version of the manuscript.

Correspondence to: Rosario Cuomo, MD, Associate Professor of Gastroenterology, Department of Clinical Medicine and Surgery, Federico Ⅱ University Hospital, School of Medicine, Via S. Pansini 5, Building 6, 80131 Naples, Italy. rcuomo@unina.it

Telephone: +39-81-7463892 Fax: +39-339-7221830
Received: February 11, 2014 Revised: April 3, 2014
Accepted: June 2, 2014
Published online: July 21, 2014

Abstract

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal disorders in Western countries. Despite the high prevalence of this disorders, the therapeutic management of these patients is often unsatisfactory. A number of factors have been suggested to be involved in the pathogenesis of IBS, including impaired motility and sensitivity, increased permeability, changes in the gut microbiome and alterations in the brain-gut axis. Also food seems to play a critical role: the most of IBS patients report the onset or the exacerbation of their symptoms after the meals. Recently, an increasing attention has been paid to the role of food in IBS. In this review we summarize the most recent evidences about the role of diet on IBS symptoms. A diet restricted in fermentable, poorly absorbed carbohydrates and sugar alcohols has beneficial effects on IBS symptoms. More studies are needed to improve our knowledge about the relationship between food and IBS. However, in the foreseeable future, dietary strategies will represent one of the key tools in the therapeutic management of patients with IBS.

Key words: Irritable bowel syndrome; Fermentable, poorly absorbed carbohydrates and sugar alcohols; Gut microbiota; Food intolerance; Gluten

Core tip: The most of irritable bowel syndrome patients reported food as a trigger of gastrointestinal symptoms and self-referred intolerance to certain food items. However, it is difficult identify which items are involved in symptoms triggering because food is a complex milieu of several chemicals, almost all potentially able to induce symptoms *via* several ways. It has been proposed three pathogenic mechanisms by which food items might induce symptoms: *via* immune activation (food hypersensitivity), *via* direct action of bioactive molecules (food chemicals) and *via* luminal distension.

Cuomo R, Andreozzi P, Zito FP, Passananti V, De Carlo G, Sarnelli G. Irritable bowel syndrome and food interaction. *World J Gastroenterol* 2014; 20(27): 8837-8845 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8837.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8837

INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gastrointestinal (GI) disorder characterized by abdominal pain or discomfort associated with abnormal bowel habit. Since the absence of reliable biomarkers, Rome Ⅲ diagnostic criteria define IBS as recurrent abdominal pain or discomfort for at least 3 d per month in the past 3 mo, associated with 2 or more of the following: improvement with defecation, onset associated with a change in the frequency of stool or onset associated with a change in the form (appearance) of stool. Based on stool form, IBS is

classified in IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), mixed IBS (IBS-M) and unsubtyped IBS (IBS-U)[1]. Prevalence of IBS in the industrialized world is approximately 10%-15%, which makes IBS one of the most common GI disorders[2]. The pathogenesis of IBS is not completely understood, but several factors seem to play a role in the pathogenesis of IBS, including dysregulation of the brain-gut axis with impaired gut motility and sensibility, psyco-social factors, genetic factors, impaired gut barrier function and changes in the gut microbiome[3].

Food plays a key role in IBS: more than 60% of patients with IBS report the onset or worsening of symptoms after meals, within 15 min in 28% and within 3 h in 93% of these patients[4]. The most of IBS patients (84%) reported meal-related symptoms to at least one food item. In addition, self-reported food intolerance is associated with higher symptoms severity score and reduced quality of life[5-7]. In line with this, patients try to identify and remove the food items they do not tolerate: a cross-sectional study showed that 62% of IBS patients limited or excluded food items from the diet[8].

The role of food as trigger of GI symptoms in functional disorders is well-known, while it is much more difficult to pinpoint what food groups or items are involved in symptoms onset or worsening in IBS. For this reason, dietary recommendations for functional gastrointestinal disorders (FGIDs) are limited and largely based more upon empiricism or pathophysiology knowledge rather than randomized clinical trials or guideline consensus. The lack of a specific nutritional training and scientific evidences explains the skepticism of most primary care practitioners and gastroenterologists about dietary advices, that often are limited to change fiber intake or to reduce lipids consumption.

In the last years, the potential role of food in the management of IBS has been revisited[9]. Searching PubMed (MeDLINe) database using the terms "food" and "irritable bowel syndrome", we have found that the number of published papers increased from 7 in 1997 to 108 in 2011. This renewed interest has led to new advances in the pathophysiology and management of IBS, but also to new sources of confusion. For example, increasing attention has been paid to the role of wheat in GI symptoms. Recent studies have supported the existence of a subgroup of IBS patients with undiagnosed non-celiac gluten sensitivity, defined as a condition of morphological, immunological, or functional disorder that responds to gluten exclusion[10]. However, the existence of a objective clinical entity is controversial and recent evidences seem to reappraise the role of gluten in GI symptoms in these patients, focusing the attention on fermentable, poorly absorbed, short-chain carbohydrates present in wheat[11].

In this paper, we performed a literature review about the putative pathogenic mechanisms of food in IBS as well as the recent evidences supporting the role of food as a means of therapeutic strategies in the management of IBS. Since a great number of papers have been published in the last years, we focused mainly on high-quality works.

PUTATIVE MECHANISMS

It should be remembered that food is a complex milieu of nutrients. On the other side, the ingestion of food activates a complex response of GI tract that allows the transfer of nutrients from the intestinal lumen to the systemic circulation through the processes of digestion, absorption and expulsion of needless elements. The great complexity of food composition and GI physiology explain why it is difficult to identify single food items involved in IBS symptoms triggering or worsening.

Several mechanisms have been proposed to explain how food triggers GI symptoms in IBS. Gibson propose at least three pathogenic mechanisms by which food items might induce GI symptoms in functional bowel disorders: *via* immune activation (food hypersensitivity), *via* direct action of bioactive molecules (food chemicals), and *via* luminal distension[12].

A long-standing debate is whether or not immunological mechanisms are involved in the pathogenesis of IBS. In the last decades, it has been suggested that increased epithelial barrier permeability leads to immune activation and low-grade inflammation, that could play a crucial role in the pathogenesis of IBS[13]. Since the gut is the gatekeeper that controls nutrients access, it is not difficult to imagine that food antigens in definite conditions could trigger low-grade inflammation that would change the motor and sensory function of the gut in a group of susceptible individuals[14].

Adverse food reactions may play an important role in GI symptoms triggering, as many patients report an exacerbation of symptoms after food ingestion[15]. There is no international consensus for the terms of "food intolerance". This expression should be referred to non-immunological non-toxic adversion to food[16]. Chemicals with potential bioactivity such as salicylates, amines and glutamates are natural, pharmacologically active substances that are believed to cause adverse reactions, such as anaphylactoid reactions, urticaria, and asthma in susceptible individuals by a non-immune direct effect on mast cells to produce cysteinyl leukotrienes. However, bioactive chemicals would be able to trigger GI symptoms including nausea, vomiting, abdominal pain, bloating or diarrhea[17] and a line of evidences support the role of these molecules in IBS[15]. Although several mechanisms have been proposed to explain the pathogenesis of these symptoms, it has been hypothesized that chronic exposure to food chemicals may induce visceral hypersensitivity to luminal stimuli through the activation and overexpression of TRP channels on enteric nervous system neurons. In addition, some evidences in murine model suggest that salicylate intolerance may involve mast cells production of cysteynil leukotriens, which promote smooth muscle contraction and increase vascular permeability[18]. Salicylates, glutamates and amines have been the principal targets of elimination diet treating groups, with

Table 1 Estimated food allergy rates in North America[21]

Prevalence	Infant/child	Adult
Milk	2.5%	0.3%
Egg	1.5%	0.2%
Peanut	1.0%	0.6%
Tree nuts	0.5%	0.6%
Fish	0.1%	0.4%
Shellfish	0.1%	2.0%
Wheat, soy	0.4%	0.3%
Sesame	0.1%	0.1%
Overall	5.0%	3%-4%

Table 2 Pathophysiologic classification of allergic reactions to food[19]

Immunopathology	Disorder
IgE dependent	Urticaria and atopic eczema
	Rhinitis or asthma
	Oral allergy syndrome (angioedema of lips and tongue)
	Nausea, abdominal pain, diarrhea or constipation
Non IgE dependent	Atopic dermatitis
	Celiac disease
	Eosinophilic esophagitis, gastritis, gastroenteritis, enterocolitis and proctitis

contrasting results[15].

Luminal distension is another mechanism by which food induces GI symptoms. It is well-known the presence of visceral hypersensitivity in the majority of patients with IBS, resulting in a lower painful threshold of gut wall stretching. The presence of certain nutrients in food, in particular short chain carbohydrates, could induce or worsen GI symptoms in IBS patients *via* two main actions. First, these small molecules are osmotically active and increase luminal water volume in distal ileum and colon. Secondly, short chain carbohydrates are substrates for colonic bacterial fermentation, resulting in the production of gas. The increase of intra-luminal water and gas volume leads to luminal distension that induces GI symptoms in subjects with lower pain threshold or impaired motility pattern such as patients with functional GI disorders[19].

FOOD HYPERSENSITIVITY

The term food allergy is used to describe an adverse immune response to food. Food allergy can be classified on the basis of immunopathologic mechanisms in IgE-mediated (considered type I hypersensitivity) and non-IgE-mediated reactions (including type Ⅲ and Ⅳ hypersensitivity)[20]. In the Table 1 are reported estimated rates of food allergies in North America[21].

Classic IgE-mediated food allergies are classified as type- I immediate hypersensitivity reaction. These allergic reactions have an acute onset (from seconds to one hour) and may have extremely heterogeneous clinical manifestations[20]. Although it is relatively easy to recognize the allergic manifestations of skin, such as urticaria and atopic eczema, and respiratory tract, such as rhinitis or asthma, the GI tract can be affected by food allergies in several ways: oral allergy syndrome (angioedema of lips and tongue), nausea, abdominal pain, diarrhea or constipation. Rarely, food allergy manifested as signs and symptoms that can occur in IBS (diarrhea associated to abdominal pain)[22]. The spectrum of food allergies also includes delayed-onset diseases, that can be mediated by intestinal mucosal mechanisms involving not only IgE but also T cells, mast cells and eosinophils that produce proinflammatory mediators. Belong of this kind of disease: atopic dermatitis, celiac disease or eosinophilic GI diseases, such as esophagitis, gastritis, gastroenteritis, enterocolitis and proctitis (Table 2).

The increased prevalence of atopic conditions in patients with diarrhea-predominant IBS[23] and the positive response to oral sodium cromoglycate treatmentin these patients[24]. suggest that food hypersensitivity could play a role in pathogenesis of IBS.

An equivalent of prick test in the gut mucosa, the so-called colonoscopic allergen provocation test (COLAP), showed promising initial results. Food antigens selected according to the patients' history of food intolerance and the presence of specific IgE in serum were injected into the mucosa of the cecum during colonoscopy in seventy adult patients with chronic abdominal symptoms and suspected gastrointestinal food allergy and in five healthy volunteers. COLAP test was positive in response to at least one food antigen in 77% of patients, whereas no reaction was detected in the five healthy volunteers. Moreover, in the clinical follow up over a period of at least 6 months, a food eliminiation diet induced a significant improvement of symptoms in 29 of 35 patients (83%) with positive COLAP test. The researchers concluded that allergic reactions may play a part in a subgroup of patients with irritable bowel syndrome and COLAP test may improve the clinical management of these patients, supporting this "intestinal prick test" as a valuable diagnostic tool of GI food allergy[25].

Several researchers focused on the role of food-specific IgG and IgG4. Although dietary antigens physiologically induce the production of IgG4, the hypothesis that these immunoglobulin are involved in IBS stems from the observation that IBS patients had higher IgG4 titers to certain antigens, such as wheat, beef, pork and lamb, compared to controls[26]. Moreover, two studies revealed that a food elimination diet based on serum IgG/IgG4 antibodies is able to improve overall symptoms in IBS[26,27]. Despite the initial promising results of COLAP test and elimination diet based on serum IgG/IgG4 antibodies, there have been no further published reports of these tests[28].

In conclusion, the role of hypersensitivity in IBS remains uncertain. Clinical trials, *in vitro* and epidemiological studies have suggested a potential role of allergic mechanisms in the pathogenesis of IBS, but further studies are needed to elucidate this relationship. To date, food allergy and IBS should be considered as two distinct clini-

cal entities. Food allergy should be considered in case of uncontrolled symptoms in patients with IBS-like symptoms and when a clear allergic response to a specific food has been identified. Unfortunately, there is not a gold standard procedure for food allergy diagnosis. At present, skin prick tests and the radioallergosorbent test, the most used tests to investigate IgE-mediated allergy, suggest only individual sensitization but they are not sufficient *per se* to diagnose food allergy. For this reason, suspected food allergy needs to be confirmed by a double-blind, placebo-controlled food challenge[20].

Fat hypersensitivity

Lipids are a complex group of chemical substances including triglycerides, and its constituent fatty acid, as well as cholesterol, phospholipids and sterol. Fat is not a simply nutrient, in fact, lipids are able to modulate the responses of the gut to various stimuli. In patients suffering from FGIDs, such as irritable bowel syndrome, some of these modulatory mechanisms, being abnormal, may lead to the onset of gastrointestinal symptoms[29]. In fact, it has been hypothesized that in irritable bowel syndrome, as well as in other FGIDs, patients display intestinal hypersensitivity and exaggerated reflexes after normal stimuli, for example after fat ingestion[30]. These patients complain symptoms such as fullness, bloating and nausea after lipids intake much more frequently and at lower fat load than healthy subjects. It has been described that lipids through the inhibition of small bowel motility and the delaying of intestinal transit may cause gas retention and, then, abdominal bloating[31]. On the other hand, many evidences show that lipids stimulate colonic motor activity through a mechanism known as "gastrocolonic reflex". Such reflex seems to be upregulated in IBS patients and may lead to post-prandial diarrhea. Simrén *et al*[32] have, also, demonstrated that duodenal lipid load increased rectal sensitivity and perception of rectal distension in IBS patients, inducing different symptoms in the constipated and diarrheal subtypes of IBS with the same mechanism. In fact, if C-IBS patients experience rectal distension as pain, D-IBS subjects report primarily rectal urgency. However, although association between lipids intake and gastrointestinal symptoms has been observed, only few studies report lower dietary fat consumption in IBS patients if compared to healthy subjects.

FOOD CHEMICALS

Salicylates

Although, aspirin and other non steroidal anti-inflammatory drugs are the best studied compounds belonging to salicylates, salicylic acid and its derivates are present in many foods in different concentrations[33]. Salicylate intolerance is defined as a nonspecific antigen-induced pseudoallergic hypersensitivity reaction characterized by systemic and local manifestations[15,18-33].

Symptoms of acetylsalicylic acid intolerance are caused by overproduction of leukotriene metabolites (leukotrien B4, C4, D4, E4) and a reduction of prostaglandin, prostacyclin and thromboxan as consequence of cyclooxygenase inhibition. It has been hypothesized that in patients intolerant to salicylate the inhibition of these enzymes may be higher than in healthy subjects[34].

The typical triad of intolerance to salicylic acid comprises the occurrence of polyposis nasi, nonallergic asthma and angioedema as well as laryngeal edema following contact with substances containing acetylsalicylic acid. Further clinical manifestations of salicylate intolerance may include gastrointestinal symptoms such as abdominal pain, swelling, meteorism, colitis and diarrhea.

However, the presentation of such gastrointestinal symptoms, accompanied or not by typical systemic manifestations, may create diagnostic difficulties; in fact, the diagnosis of salicylate intolerance may be considered once other causes have been excluded.

The variety of symptoms of salicylate intolerance is linked to the different expression and concentration of cytokines in the different tissues. In fact, for example, leukotriene B4 is primarily involved in inflammation, leukotriene C4 is responsible of typical pseudo allergic mechanisms while leukotrienes C4, D4 and E4 cause bronchoconstriction, bronchial hyperreactivity, mucus production and vasodilatation[35]. These mechanisms have been used to explain intolerance to acetylsalicylic acid and, although it is possible to hypothesize that other salicylate derivates may induce symptoms sharing similar pathogenesis, further studies are needed.

At the moment, elimination diet represents the best way to diagnose and manage salicylate as well as other food chemicals intolerance, but, considered the widespread presence of these substances in foods (Table 3), too severe alimentary restrictions should be avoided for the risk of unpalatable diets and malnutrition. Moreover, all studies, which report that dietary manipulation may be a valid treatment choice in IBS patients, have important limitations in their trial designs, including inadequate patient selection, appropriateness and duration of exclusion diets, and methods of food challenge[15].

LUMINAL DISTENSION

Milk

The enzyme activity of lactase, a β-galactosidase present on the apical surface of enterocytes in the small intestinal brush border, physiologically starts to decline within the first few months of life in most of mammalian. In humans, approximately 70% of the adult population has a decreased lactase activity[36]. In people with lactase deficiency, lactose is not hydrolyzed and absorbed in the small bowel, but passes through the gastrointestinal tract into the colon into where bacterial fermentation produces gas and short-chain fatty acids and other products that can cause luminal distension and induce GI symptoms[37].

The typical symptoms of lactose intolerance are similar to those in IBS and include abdominal pain, bloating, flatus, diarrhoea, borborygmi. Conversely, patients with

Table 3 Food sources of salicylate reported in literature[15,18,33]

Food	State	Significant source of salicylate
Pepper (red chili)	Fresh	1.20
Sweet potato (white)	Fresh	0.50
Apricot	Fresh	2.58
Apricot	Canned	1.42
Apricot	Nectar	0.14
Orange	Fresh	2.39
Pineapple	Fresh	2.10
Almonds	Fresh	3.0
Raspberries	Fresh	3.14
Dates	Fresh	3.73

IBS more frequently report perceived intolerance to milk or dairy products compared to healthy individuals[8].

Despite the similarity between IBS and lactose intolerance, the prevalence of lactose intolerance in IBS patients is similar compared to controls[38] and testing patients for lactose intolerance or the use of lactase supplementation is not justified[39].

On the other side, subjective perception of intolerance for milk is not a useful criteria to identify people with lactose malabsorption. Vernia *et al*[40] tried to define the relationship between self-referred perception of milk intolerance and lactose intolerance. In this study, 475 consecutive IBS patients underwent a hydrogen breath test after an oral load of lactose. Data analysis of 201 age- and sex-matched pairs of IBS patients classified according to self-reported milk tolerance/intolerance showed that the prevalence of positive HBT was similar in milk "tolerant" (68.6%) and "intolerant" patients (75.6%), confirming that self-reported milk intolerance does not help in identifying lactose intolerance in IBS patients.

However, it is plausible to hypothesize that not lactose but milk-specific component may play a role in IBS symptoms and reducing milk and dairy products in the diet could represent an appropriate strategy in the management of IBS.

Fermentable oligosaccharides, disaccharides, monosaccharides and polyols

In the last couple of years, increasing evidences support the efficacy for the management of IBS of a diet with lower amounts of fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs)[41]. Scientific evidences showed that they are individually involved as a trigger for symptoms in patients with functional disorders[42-44]. At the base of the concept of enclosing these sugars into one group would be the common pathogenetic mechanism by which they contribute to symptoms burden in IBS: when FODMAPs are poorly absorbed through the small intestine, they pass in the bowel and increase intestinal luminal water content through their osmotic activity and induce gas production due to fermentation by gut bacteria. The increased content of water and gas causes luminal distension that induces GI symptoms in IBS patients. In addition, products of fermentation such as short-chain fatty acids could be involved in symptom generation[12].

The low FODMAP diet approach involves the reduction, not complete avoidance, of these sugars in the diet. Based on the knowledge of the FODMAP status of foods, foods are classified into high and low FODMAP content and the latter food consumption is encouraged (Table 4). In a first period of 6-8 wk, all known or suspected types of food with high content in FODMAP groups are strictly restricted from the diet, in order to determine the benefit of FODMAP restriction. Subsequently, individual FODMAPs are reintroduced to test their individual tolerance of each FODMAP *via* a series for food challenges[44]. As the authors rightfully acknowledge, restricting the intake of FODMAPs excludes a wide variety of foods from the diet with the potential risk to affect nutrient intake.

Several studies supported the potential benefits of restricting a spectrum of FODMAPs in the diet in IBS[45,46]. Recently, in a randomized double-blind controlled crossover study, Halmos *et al*[47] demonstrated that a diet low in FODMAPs for a 3 wk period effectively reduced overall gastrointestinal symptoms -abdominal pain, bloating and bowel habit dissatisfaction- in a group of 30 unselected IBS patients, compared to a standard Australian diet.

In a non-randomized study, the low FODMAP diet was more effective than United Kingdom national dietary guidelines for symptom control in a series of consecutive patients with IBS who attended a follow-up dietetic outpatient visit for dietary management of their symptoms[45].

Other studies are needed to assess the long-term efficacy and safety of FODMAP restriction as well as to identify patient profiles that predict dietary response. However, low FODMAP diet represents the one of the most promising emerging strategies in the management of IBS.

Wheat

Many individuals complaining GI symptoms benefit from gluten withdrawal, although they cannot be classified as either celiac diseases or wheat allergy[48,49]. The hypothesis that gluten is able to induce IBS-like symptoms in non-coeliac people is not new[50,51]. Gluten has been considered the culprit of the causal relationship between wheat ingestion and GI symptoms. Indeed, recent literature has supported the existence of a subgroup of IBS patients with undiagnosed non-celiac gluten sensitivity, defined as a condition of morphological, immunological, or functional disorder that responds to gluten exclusion[10]. The existence of this condition is suggested by clinical trials showing that gluten-free diet was able to relieve GI symptoms in a randomized, double-blind, placebo-controlled, rechallenge trials[52]. Biesiekierski *et al*[52] confirmed the existence of gluten sensitivity in patients with IBS-D in a randomized, double-blind, placebo-controlled, rechallenge trial. In this study, 34 IBS patients who reported symptomatic relief after a GFD for at least 6 wk were enrolled. Nineteen patients received 16 g of non fermentable gluten per day *via* bread and a muffin, whereas the

Table 4 Food sources of fermentable oligosaccharides, disaccharides, monosaccharides and polyols[44]

	High FODMAP food source	Low-FODMAP food source
Excess fructose	Fruits (apples, pears, nashi pears, clingstone peaches, mango, sugar snap peas, watermelon, tinned fruit in natural juice) Honey Sweeteners (fructose)	Fruit (banana, blueberry, carambola, durian, grapefruit, grape, honeydew melon, kiwifruit, lemon, lime, mandarin, orange, passionfruit, paw paw, raspberry, rockmelon, strawberry, tangelo) Honey substitutes (maple syrup, golden syrup) Sweeteners (any except polyols)
Lactose oligosaccharides	Milk (cow, goat and sheep) Ice cream Yoghurt Soft cheeses	Milk (lactose-free, rice milk) Cheese (hard cheeses, camembert) Yoghurt (lactose-free) Ice cream substitutes (gelati, sorbet) Butter
Polyols	Vegetables (artichokes, asparagus, beetroot, Brussels sprout, broccoli, cabbage, fennel, garlic, leeks, okra, onions, peas, shallots) Cereals (wheat and rye when eaten in large amounts) Legumes (chickpeas, lentils, red kidney beans, baked beans) Fruits (watermelon, custard apple, white peaches, rambutan, persimmon)	Vegetables (bamboo shoots, bokchoy, carrot, celery, capsicum, choko, choy sum, corn, eggplant, green beans, lettuce, chives, parsnip, pumpkin, silverbeet, spring onion, tomato) Onion/garlic substitutes (garlic-infused oil) Cereals (gluten-free and spelt bread/cereal products)
Fructans and/or galactans	Fruits (apples, apricots, cherries, longon, lychee, nashi pears, nectarine, pears, peaches, plums, prunes, watermelon) Vegetables (avocado, cauliflower, mushrooms, snow peas) Sweeteners (sorbitol, mannitol, xylitol, maltitol, isomalt	Fruits (banana, blueberry, carambola, durian, grapefruit, grape, honeydew melon, kiwifruit, lemon, lime, mandarin, orange, passionfruit, paw paw, raspberry, rockmelon) Sweeteners (sucrose, glucose)

other 15 patients received gluten-free bread and a muffin. In the gluten group, 68% reported that symptoms were not adequately controlled compared with 40% in gluten-free group ($P = 0.0001$). Moreover, patients in the gluten-free group reported significantly greater improvements in GI symptoms such as pain, bloating, stool consistency and tiredness compared to patients in gluten group. Researchers suggested that gluten sensitivity may be a distinct clinical entity in a subset of patients with IBS.

Following studies failed to find a specific marker or pathogenetic mechanisms supporting the idea that gluten sensitivity is an objective clinical entity. Despite the lack of evidences, the mass media have publicized the advantages of GFD leading many patients to exclude gluten from diet. Two years later, the same group of researchers conducted a placebo-controlled, crossover rechallenge study in 37 subjects with gluten sensitivity and IBS. After a two weeks run-in on a gluten-free and low FODMAP diet test, subjects were placed on high-gluten (16 g gluten/d), low-gluten (2 g gluten/d and 14 g whey protein/d), or control (16 g whey protein/d) diets for 1 wk, followed by a washout period of at least 2 wk. Twenty-two participants then crossed over to groups given gluten (16 g/d), whey (16 g/d), or control (no additional protein) diets for 3 d. In all participants, gastrointestinal symptoms improved during reduced FODMAP intake and similarly worsened when their diets included gluten or whey protein. Participants were then rechallenged gluten (16 g/d), whey (16 g/d), or control (no additional protein) diets for 3 d and during this rechallenge symptoms increased by similar levels among groups, again regardless of the protein source. The researchers concluded that gluten sensitivity might not be a discrete entity and that gluten might not be a specific trigger of functional gut symptoms once dietary FODMAPs are reduced[11].

In conclusion, no clear evidences support that gluten may induce GI symptoms in individuals without CD. The observed effects of GFD in GI symptoms relief may be due to the fact that many gluten-containing cereals are high in fermentable, poorly absorbed,short-chain carbohydrates that seem to have a critical role in triggering IBS symptoms[12].

DIET AND GUT MICROBIOTA

Gut microbiota is individual-specific and is influenced by the genetic and environmental factors. In particular is well-known the role of nutrition in changes of gut bacteria[53,54]. In a recent study, researchers found that gut microbiota is able to rapidly switch between herbivorous and carnivorous functional profiles after a short-term macronutrient changes in diet[55].

Recently, the intestinal microbiota has been proposed as an etiological factor in physiopathology and pathogenesis of IBS[56]. Supporting the role of gut bacteria in IBS are studies that document the onset of IBS symptoms after an acute gastroenteritis and the qualitative and quantitative changes of bacteria composition that occur in IBS subtypes[57]. In a recent study, researchers aimed to assess the microbiota composition by molecular analysis of fecal samples from 62 patients with IBS patients and 46 healthy individuals. They found that gut microbiota of IBS patients differed significantly from that of controls. In particular, the microbiota of IBS patients had a 2-fold increased ratio of the Firmicutes to Bacteroidetes[58]. However, the role of microbiota is still unclear due to

methodological problems, influence of confounding factors and large differences between studies.

In agreement with this observation, we can speculate that diet-induced changes to the gut microbiota may contribute to the onset or worsening of IBS symptoms, as well as beneficial effects of certain nutrients on IBS symptoms could be, at least partially, mediated by changes in gut bacteria.

CONCLUSION

Food is able to trigger IBS symptoms in a great part of patients. Food related mechanisms involved in to trigger symptoms seem generally referred to food hypersensitivity, action of bioactive molecules and luminal distension. Intestinal microbiota aberration has a crucial role in luminal distension and considering that microbiota is often modified by dietary habits so we have closed the circle. The food changes the microbiota which in turn induces the abnormal fermentation of food ingested. A great attention is now directed to food containing FODMAP that are able to determine IBS symptoms both *via* microbiota aberration and luminal distension. Finally, studies oriented to define relationship between IBS and food could be a comprehensive strategy to improve medical therapy of IBS.

ACKNOWLEDGMENTS

The authors would like to express gratitude to Emanuela De Santis for her help with the preparation of the manuscript.

REFERENCES

1 **Drossman DA**. The functional gastrointestinal disorders and the Rome III process. *Gastroenterology* 2006; **130**: 1377-1390 [PMID: 16678553 DOI: 10.1053/j.gastro.2006.03.008]
2 **Spiller R**, Aziz Q, Creed F, Emmanuel A, Houghton L, Hungin P, Jones R, Kumar D, Rubin G, Trudgill N, Whorwell P. Guidelines on the irritable bowel syndrome: mechanisms and practical management. *Gut* 2007; **56**: 1770-1798 [PMID: 17488783 DOI: 10.1136/gut.2007.119446corr1]
3 **Camilleri M**, Lasch K, Zhou W. Irritable bowel syndrome: methods, mechanisms, and pathophysiology. The confluence of increased permeability, inflammation, and pain in irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* 2012; **303**: G775-G785 [PMID: 22837345 DOI: 10.1152/ajpgi.00155.2012]
4 **Simrén M**, Månsson A, Langkilde AM, Svedlund J, Abrahamsson H, Bengtsson U, Björnsson ES. Food-related gastrointestinal symptoms in the irritable bowel syndrome. *Digestion* 2001; **63**: 108-115 [PMID: 11244249 DOI: 10.1159/000051878]
5 **Böhn L**, Störsrud S, Törnblom H, Bengtsson U, Simrén M. Self-reported food-related gastrointestinal symptoms in IBS are common and associated with more severe symptoms and reduced quality of life. *Am J Gastroenterol* 2013; **108**: 634-641 [PMID: 23644955 DOI: 10.1038/ajg.2013.105]
6 **El-Salhy M**, Ostgaard H, Gundersen D, Hatlebakk JG, Hausken T. The role of diet in the pathogenesis and management of irritable bowel syndrome (Review). *Int J Mol Med* 2012; **29**: 723-731 [PMID: 22366773]
7 **Zigich S**, Heuberger R. The relationship of food intolerance and irritable bowel syndrome in adults. *Gastroenterol Nurs* 2013; **36**: 275-282 [PMID: 23899486 DOI: 10.1097/SGA.0b013e31829ed911]
8 **Monsbakken KW**, Vandvik PO, Farup PG. Perceived food intolerance in subjects with irritable bowel syndrome-- etiology, prevalence and consequences. *Eur J Clin Nutr* 2006; **60**: 667-672 [PMID: 16391571 DOI: 10.1038/sj.ejcn.1602367]
9 **Gibson PR**, Shepherd SJ. Food choice as a key management strategy for functional gastrointestinal symptoms. *Am J Gastroenterol* 2012; **107**: 657-666; quiz 667 [PMID: 22488077 DOI: 10.1038/ajg.2012.49]
10 **Verdu EF**, Armstrong D, Murray JA. Between celiac disease and irritable bowel syndrome: the "no man's land" of gluten sensitivity. *Am J Gastroenterol* 2009; **104**: 1587-1594 [PMID: 19455131 DOI: 10.1038/ajg.2009.188]
11 **Biesiekierski JR**, Peters SL, Newnham ED, Rosella O, Muir JG, Gibson PR. No effects of gluten in patients with self-reported non-celiac gluten sensitivity after dietary reduction of fermentable, poorly absorbed, short-chain carbohydrates. *Gastroenterology* 2013; **145**: 320-8.e1-320-8.e3 [PMID: 23648697 DOI: 10.1053/j.gastro.2013.04.051]
12 **Gibson PR**. Food intolerance in functional bowel disorders. *J Gastroenterol Hepatol* 2011; **26** Suppl 3: 128-131 [PMID: 21443725 DOI: 10.1111/j.1440-1746.2011.06650.x]
13 **Dunlop SP**, Hebden J, Campbell E, Naesdal J, Olbe L, Perkins AC, Spiller RC. Abnormal intestinal permeability in subgroups of diarrhea-predominant irritable bowel syndromes. *Am J Gastroenterol* 2006; **101**: 1288-1294 [PMID: 16771951 DOI: 10.1111/j.1572-0241.2006.00672.x]
14 **Ohman L**, Simrén M. Pathogenesis of IBS: role of inflammation, immunity and neuroimmune interactions. *Nat Rev Gastroenterol Hepatol* 2010; **7**: 163-173 [PMID: 20101257 DOI: 10.1038/nrgastro.2010.4]
15 **Niec AM**, Frankum B, Talley NJ. Are adverse food reactions linked to irritable bowel syndrome? *Am J Gastroenterol* 1998; **93**: 2184-2190 [PMID: 9820394 DOI: 10.1111/j.1572-0241.1998.00531.x]
16 **Johansson SG**, Hourihane JO, Bousquet J, Bruijnzeel-Koomen C, Dreborg S, Haahtela T, Kowalski ML, Mygind N, Ring J, van Cauwenberge P, van Hage-Hamsten M, Wüthrich B. A revised nomenclature for allergy. An EAACI position statement from the EAACI nomenclature task force. *Allergy* 2001; **56**: 813-824 [PMID: 11551246 DOI: 10.1111/j.1398-9995.2001.00002.x-i1]
17 **Allen DH**, Van Nunen S, Loblay R, Clarke L, Swain A. Adverse reactions to foods. *Med J Aust* 1984; **141**: S37-S42 [PMID: 6482784]
18 **Raithel M**, Baenkler HW, Naegel A, Buchwald F, Schultis HW, Backhaus B, Kimpel S, Koch H, Mach K, Hahn EG, Konturek PC. Significance of salicylate intolerance in diseases of the lower gastrointestinal tract. *J Physiol Pharmacol* 2005; **56** Suppl 5: 89-102 [PMID: 16247191]
19 **Shepherd SJ**, Lomer MC, Gibson PR. Short-chain carbohydrates and functional gastrointestinal disorders. *Am J Gastroenterol* 2013; **108**: 707-717 [PMID: 23588241 DOI: 10.1038/ajg.2013.96]
20 **Brandtzaeg P**. Food allergy: separating the science from the mythology. *Nat Rev Gastroenterol Hepatol* 2010; **7**: 380-400 [PMID: 20606633 DOI: 10.1038/nrgastro.2010.80]
21 **Sicherer SH**, Sampson HA. Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. *J Allergy Clin Immunol* 2014; **133**: 291-307; quiz 308 [PMID: 24388012 DOI: 10.1016/j.jaci.2013.11.020]
22 **Bischoff S**, Crowe SE. Gastrointestinal food allergy: new insights into pathophysiology and clinical perspectives. *Gastroenterology* 2005; **128**: 1089-1113 [PMID: 15825090 DOI: 10.1053/j.gastro.2004.08.015]
23 **White AM**, Stevens WH, Upton AR, O'Byrne PM, Collins SM. Airway responsiveness to inhaled methacholine in patients with irritable bowel syndrome. *Gastroenterology* 1991;

100: 68-74 [PMID: 1983850]
24 **Stefanini GF**, Saggioro A, Alvisi V, Angelini G, Capurso L, di Lorenzo G, Dobrilla G, Dodero M, Galimberti M, Gasbarrini G. Oral cromolyn sodium in comparison with elimination diet in the irritable bowel syndrome, diarrheic type. Multicenter study of 428 patients. *Scand J Gastroenterol* 1995; **30**: 535-541 [PMID: 7569760 DOI: 10.3109/00365529509089786]
25 **Bischoff SC**, Mayer J, Wedemeyer J, Meier PN, Zeck-Kapp G, Wedi B, Kapp A, Cetin Y, Gebel M, Manns MP. Colonoscopic allergen provocation (COLAP): a new diagnostic approach for gastrointestinal food allergy. *Gut* 1997; **40**: 745-753 [PMID: 9245928 DOI: 10.1136/gut.40.6.745]
26 **Zar S**, Benson MJ, Kumar D. Food-specific serum IgG4 and IgE titers to common food antigens in irritable bowel syndrome. *Am J Gastroenterol* 2005; **100**: 1550-1557 [PMID: 15984980 DOI: 10.1111/j.1572-0241.2005.41348.x]
27 **Atkinson W**, Sheldon TA, Shaath N, Whorwell PJ. Food elimination based on IgG antibodies in irritable bowel syndrome: a randomised controlled trial. *Gut* 2004; **53**: 1459-1464 [PMID: 15361495 DOI: 10.1136/gut.2003.037697]
28 **Park MI**, Camilleri M. Is there a role of food allergy in irritable bowel syndrome and functional dyspepsia? A systematic review. *Neurogastroenterol Motil* 2006; **18**: 595-607 [PMID: 16918724 DOI: 10.1111/j.1365-2982.2005.00745.x]
29 **Feinle-Bisset C**, Azpiroz F. Dietary lipids and functional gastrointestinal disorders. *Am J Gastroenterol* 2013; **108**: 737-747 [PMID: 23567355 DOI: 10.1038/ajg.2013.76]
30 **Accarino AM**, Azpiroz F, Malagelada JR. Modification of small bowel mechanosensitivity by intestinal fat. *Gut* 2001; **48**: 690-695 [PMID: 11302970 DOI: 10.1136/gut.48.5.690]
31 **Serra J**, Salvioli B, Azpiroz F, Malagelada JR. Lipid-induced intestinal gas retention in irritable bowel syndrome. *Gastroenterology* 2002; **123**: 700-706 [PMID: 12198695 DOI: 10.1053/gast.2002.35394]
32 **Simrén M**, Simms L, D'Souza D, Abrahamsson H, Björnsson ES. Lipid-induced colonic hypersensitivity in irritable bowel syndrome: the role of 5-HT3 receptors. *Aliment Pharmacol Ther* 2003; **17**: 279-287 [PMID: 12534414 DOI: 10.1046/j.1365-2036.2003.01399.x]
33 **Swain AR**, Dutton SP, Truswell AS. Salicylates in foods. *J Am Diet Assoc* 1985; **85**: 950-960 [PMID: 4019987]
34 **Worm M**, Vieth W, Ehlers I, Sterry W, Zuberbier T. Increased leukotriene production by food additives in patients with atopic dermatitis and proven food intolerance. *Clin Exp Allergy* 2001; **31**: 265-273 [PMID: 11251628 DOI: 10.1046/j.1365-2222.2001.00979.x]
35 **Schäfer D**, Schmid M, Göde UC, Baenkler HW. Dynamics of eicosanoids in peripheral blood cells during bronchial provocation in aspirin-intolerant asthmatics. *Eur Respir J* 1999; **13**: 638-646 [PMID: 10232440 DOI: 10.1183/09031936.99.13363899]
36 **Savaiano DA**, Levitt MD. Milk intolerance and microbe-containing dairy foods. *J Dairy Sci* 1987; **70**: 397-406 [PMID: 3553256]
37 **Lomer MC**, Parkes GC, Sanderson JD. Review article: lactose intolerance in clinical practice--myths and realities. *Aliment Pharmacol Ther* 2008; **27**: 93-103 [PMID: 17956597 DOI: 10.1111/j.1365-2036.2007.03557.x]
38 **Farup PG**, Monsbakken KW, Vandvik PO. Lactose malabsorption in a population with irritable bowel syndrome: prevalence and symptoms. A case-control study. *Scand J Gastroenterol* 2004; **39**: 645-649 [PMID: 15370685 DOI: 10.1080/00365520410005405]
39 **Parker TJ**, Woolner JT, Prevost AT, Tuffnell Q, Shorthouse M, Hunter JO. Irritable bowel syndrome: is the search for lactose intolerance justified? *Eur J Gastroenterol Hepatol* 2001; **13**: 219-225 [PMID: 11293439]
40 **Vernia P**, Marinaro V, Argnani F, Di Camillo M, Caprilli R. Self-reported milk intolerance in irritable bowel syndrome: what should we believe? *Clin Nutr* 2004; **23**: 996-1000 [PMID: 15380888 DOI: 10.1016/j.clnu.2003.12.005]
41 **Simrén M**. Diet as a therapy for irritable bowel syndrome: progress at last. *Gastroenterology* 2014; **146**: 10-12 [PMID: 24275241 DOI: 10.1053/j.gastro.2013.11.027]
42 **Yao CK**, Tan HL, van Langenberg DR, Barrett JS, Rose R, Liels K, Gibson PR, Muir JG. Dietary sorbitol and mannitol: food content and distinct absorption patterns between healthy individuals and patients with irritable bowel syndrome. *J Hum Nutr Diet* 2014; **27** Suppl 2: 263-275 [PMID: 23909813 DOI: 10.1111/jhn.12144]
43 **Shepherd SJ**, Gibson PR. Fructose malabsorption and symptoms of irritable bowel syndrome: guidelines for effective dietary management. *J Am Diet Assoc* 2006; **106**: 1631-1639 [PMID: 17000196 DOI: 10.1016/j.jada.2006.07.010]
44 **Gibson PR**, Shepherd SJ. Evidence-based dietary management of functional gastrointestinal symptoms: The FODMAP approach. *J Gastroenterol Hepatol* 2010; **25**: 252-258 [PMID: 20136989 DOI: 10.1111/j.1440-1746.2009.06149.x]
45 **Staudacher HM**, Whelan K, Irving PM, Lomer MC. Comparison of symptom response following advice for a diet low in fermentable carbohydrates (FODMAPs) versus standard dietary advice in patients with irritable bowel syndrome. *J Hum Nutr Diet* 2011; **24**: 487-495 [PMID: 21615553 DOI: 10.1111/j.1365-277X.2011.01162.x]
46 **Staudacher HM**, Lomer MC, Anderson JL, Barrett JS, Muir JG, Irving PM, Whelan K. Fermentable carbohydrate restriction reduces luminal bifidobacteria and gastrointestinal symptoms in patients with irritable bowel syndrome. *J Nutr* 2012; **142**: 1510-1518 [PMID: 22739368 DOI: 10.3945/jn.112.159285]
47 **Halmos EP**, Power VA, Shepherd SJ, Gibson PR, Muir JG. A diet low in FODMAPs reduces symptoms of irritable bowel syndrome. *Gastroenterology* 2014; **146**: 67-75.e5 [PMID: 24076059 DOI: 10.1053/j.gastro.2013.09.046]
48 **Kaukinen K**, Turjanmaa K, Mäki M, Partanen J, Venäläinen R, Reunala T, Collin P. Intolerance to cereals is not specific for coeliac disease. *Scand J Gastroenterol* 2000; **35**: 942-946 [PMID: 11063153]
49 **Usai P**, Manca R, Cuomo R, Lai MA, Boi MF. Effect of gluten-free diet and co-morbidity of irritable bowel syndrome-type symptoms on health-related quality of life in adult coeliac patients. *Dig Liver Dis* 2007; **39**: 824-828 [PMID: 17644056 DOI: 10.1016/j.dld.2007.05.017]
50 **Ellis A**, Linaker BD. Non-coeliac gluten sensitivity? *Lancet* 1978; **1**: 1358-1359 [PMID: 78118 DOI: 10.1016/S0140-6736(78)92427-3]
51 **Cooper BT**, Holmes GK, Ferguson R, Thompson RA, Allan RN, Cooke WT. Gluten-sensitive diarrhea without evidence of celiac disease. *Gastroenterology* 1980; **79**: 801-806 [PMID: 7419003]
52 **Biesiekierski JR**, Newnham ED, Irving PM, Barrett JS, Haines M, Doecke JD, Shepherd SJ, Muir JG, Gibson PR. Gluten causes gastrointestinal symptoms in subjects without celiac disease: a double-blind randomized placebo-controlled trial. *Am J Gastroenterol* 2011; **106**: 508-514; quiz 515 [PMID: 21224837 DOI: 10.1038/ajg.2010.487]
53 **Wu GD**, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, Sinha R, Gilroy E, Gupta K, Baldassano R, Nessel L, Li H, Bushman FD, Lewis JD. Linking long-term dietary patterns with gut microbial enterotypes. *Science* 2011; **334**: 105-108 [PMID: 21885731 DOI: 10.1126/science.1208344]
54 **Duncan SH**, Belenguer A, Holtrop G, Johnstone AM, Flint HJ, Lobley GE. Reduced dietary intake of carbohydrates by obese subjects results in decreased concentrations of butyrate and butyrate-producing bacteria in feces. *Appl Environ Microbiol* 2007; **73**: 1073-1078 [PMID: 17189447 DOI: 10.1128/AEM.02340-06]
55 **David LA**, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ. Diet rapidly and reproducibly alters the human gut microbiome. *Na-*

ture 2014; **505**: 559-563 [PMID: 24336217 DOI: 10.1038/nature12820]
56 **Bonfrate L**, Tack J, Grattagliano I, Cuomo R, Portincasa P. Microbiota in health and irritable bowel syndrome: current knowledge, perspectives and therapeutic options. *Scand J Gastroenterol* 2013; **48**: 995-1009 [PMID: 23964766 DOI: 10.3109/00365521.2013.799220]
57 **Simrén M**, Barbara G, Flint HJ, Spiegel BM, Spiller RC, Vanner S, Verdu EF, Whorwell PJ, Zoetendal EG. Intestinal microbiota in functional bowel disorders: a Rome foundation report. *Gut* 2013; **62**: 159-176 [PMID: 22730468 DOI: 10.1136/gutjnl-2012-302167]
58 **Rajilić-Stojanović M**, Biagi E, Heilig HG, Kajander K, Kekkonen RA, Tims S, de Vos WM. Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. *Gastroenterology* 2011; **141**: 1792-1801 [PMID: 21820992 DOI: 10.1053/j.gastro.2011.07.043]

P- Reviewers: Frontela-Saseta C, Li YY, Rahimi R
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8846

World J Gastroenterol 2014 July 21; 20(27): 8846-8858
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

Convergence of neuro-endocrine-immune pathways in the pathophysiology of irritable bowel syndrome

Maria M Buckley, Siobhain M O'Mahony, Dervla O'Malley

Maria M Buckley, Department of Physiology, Western Gateway Building, University College Cork, Cork, Ireland
Siobhain M O'Mahony, Department of Anatomy and Neuroscience and Alimentary Pharmabiotic Centre, University College Cork, Cork, Ireland
Dervla O'Malley, Department of Physiology and Alimentary Pharmabiotic Centre, University College Cork, Cork, Ireland
Author contributions: Buckley MM, O'Mahony SM and O'Malley D contributed equally to this work, reviewing the literature and writing the paper; O'Malley D reviewed the contributions and integrated the sections.
Correspondence to: Dervla O'Malley, BSc, DIS, PhD, Department of Physiology, Western Gateway Building, University College Cork, Western Rd, Cork, Ireland. d.omalley@ucc.ie
Telephone: +353-21-4205483 Fax: +353-21-4205370
Received: October 25, 2013 Revised: February 13, 2014
Accepted: May 28, 2014
Published online: July 21, 2014

Abstract

Disordered signalling between the brain and the gut are generally accepted to underlie the functional bowel disorder, irritable bowel syndrome (IBS). However, partly due to the lack of disease-defining biomarkers, understanding the aetiology of this complex and multifactorial disease remains elusive. This common gastrointestinal disorder is characterised by alterations in bowel habit such as diarrhoea and/or constipation, bloating and abdominal pain, and symptom exacerbation has been linked with periods of stress, both psychosocial and infection-related. Indeed, a high level of comorbidity exists between IBS and stress-related mood disorders such as anxiety and depression. Moreover, studies have observed alterations in autonomic output and neuro-endocrine signalling in IBS patients. Accumulating evidence indicates that a maladaptive stress response, probably mediated by the stress hormone, corticotropin-releasing factor contributes to the initiation, persistence and severity of symptom flares. Other risk factors for developing IBS include a positive family history, childhood trauma, dietary factors and prior gastrointestinal infection. An emerging role has been attributed to the importance of immune factors in the pathophysiology of IBS with evidence of altered cytokine profiles and increased levels of mucosal immune cells. These factors have also been shown to have direct effects on neural signalling. This review discusses how pathological changes in neural, immune and endocrine pathways, and communication between these systems, contribute to symptom flares in IBS.

Key words: Stress; Corticotropin-releasing factor; Pro-inflammatory cytokines; Enteric nervous system; Vagus

Core tip: Irritable bowel syndrome (IBS) is a disorder characterised by symptoms such as diarrhoea and/or constipation, bloating and abdominal pain. However the underlying pathophysiology of this common disorder remains unclear. Nonetheless, a number of mechanisms have been proposed to contribute to the initiation, exacerbation and persistence of symptoms. Alterations in brain-gut communication, stress, previous infections, abnormal microbiota, altered cytokine profiles and increased intestinal permeability have all been proposed as contributors to IBS and indeed, we propose that complex interactions between neural, endocrine and immune factors underlie the heterogeneity of symptoms that is characteristic of IBS.

Buckley MM, O'Mahony SM, O'Malley D. Convergence of neuro-endocrine-immune pathways in the pathophysiology of irritable bowel syndrome. *World J Gastroenterol* 2014; 20(27): 8846-8858 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8846.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8846

INTRODUCTION

Irritable bowel syndrome (IBS) is the most common functional digestive condition with a worldwide prevalence rate of 10%-20% in the general population[1,2]. As with other functional disorders it is often difficult to identify an unequivocal organic cause, at least with the diagnostic tools available. This disorder accounts for approximately 3% all general practice consultation and up to 40% of gastrointestinal (GI) referrals[3] leading to a large economic burden. At the level of the individual, IBS significantly impinges on the quality of life of a patient causing recurrent abdominal pain or discomfort coupled with disturbed bowel habits[4]. IBS is subtyped according to bowel habit pattern, therefore patients are classified as diarrhoea-(IBS-D) or constipation-predominant IBS (IBS-C) or an alternating subtype (IBS-A), which fluxes between the two states[5]. Some reports suggest that IBS-D and IBS-A are more prevalent[6] while others show an equal distribution between all three subtypes[7]. Although little mortality is associated with IBS a satisfactory treatment still does not exist, primarily due to the fact that the aetiology and pathophysiology of IBS are incompletely understood. Nonetheless, dysfunctional brain-gut axis signalling is hypothesised to be at the heart of symptoms of IBS[8] and this incorporates three major systems, neural, endocrine and immune signalling. In this review we discuss the contribution of each system to IBS symptoms and how convergence and interplay between factors from each system may provide a better understanding of the heterogeneity of IBS.

NEURAL SIGNALLING IN IBS PATHOPHYSIOLOGY

Autonomic regulation of the gut

The two major persisting symptoms of IBS are visceral hypersensitivity and altered bowel habit[9] each of which are entwined within the nervous system. Functions of the GI tract are modulated by both intrinsic and extrinsic innervation[10]. Extrinsic innervation includes both branches of the autonomic nervous system, which are anatomically and functionally integrated within the brain-gut axis and are responsible for homeostatic regulation of GI function[11]. The parasympathetic nervous system stimulates smooth muscle and secretory actions while the sympathetic element inhibits motor and secretory activity of the GI tract. The parasympathetic afferent pathway runs primarily with the vagus and terminates in the nucleus solitary tact, which sends information regarding non-nociceptive information, including gastric accommodation and gastric-colic reflex, to corticolimbic structures[12]. The sympathetic afferent pathways mediates mainly nociceptive signals through spinal pathways primarily to the thalamus and then to the sensory cortex and pain matrix[13]. Information is also sent to specific brain regions such as the hippocampus, amygdala, prefrontal cortex[14] and the hypothalamus[15] for processing. These central regions which are capable of modulating gut function are also involved in emotional (*e.g.* mood, anxiety, pain) and cognitive behaviours (*e.g.* memory, decision making) and hence, in the development of coping strategies and general well-being[16]. A descending pain inhibitory pathway from the brainstem also exists in order to control the signals reaching the brain. The intrinsic or enteric nervous system works somewhat independently providing local reflexes, such as migrating motor complex and peristaltic reflexes, and y*et al*so receives input from the central nervous system (CNS) *via* the autonomic nervous system.

Autonomic dysfunction

A growing body of evidence suggests the existence of autonomic dysfunction in IBS[17-19] and some have shown correlations with symptoms[20]. Low vagal activity can lead to a reduction in bowel contractions, reduced motility, and constipation, while high vagal activity can result in increased contractions and diarrhoea[21]. The sympatho-vagal balance was found to be disturbed in IBS patients compared to healthy controls[22]. Furthermore, a study assessing female IBS patients with constipation and severe abdominal pain showed lower vagal activity than controls[23], which correlates with a study showing an increased parasympathetic tone in women with IBS-D compared to those with IBS-C[24]. Given the close association between the stress axis and the autonomic nervous system, increased sympathetic tone as seen with constipation may be due to the increase in corticotropin-releasing factor (CRF) expression[10], which is discussed in more detail in the next section. Indeed, the psychological disorders that often co-occur with IBS are also associated with altered autonomic balance[25].

Underlying neural causes of visceral hypersensitivity

Visceral hypersensitivity, as seen in a subset of IBS patients, is an exaggerated response to a stimulus such as colorectal distension, was first noted by Ritchie[26], 1973. Several neural theories have been proposed for this increased sensitivity, including sensitisation of primary afferent pathways, increased activity of endogenous pain facilitation and reduced engagement of endogenous pain inhibition[27]. IBS patients have significantly elevated levels of anxiety, interpersonal sensitivity, depression, hostility and somatization of effect, which can impact on pain perception[28]. Some studies indicate a difference in sensitivity to colon and rectal distension between diarrhoea and control subjects[29,30], while patients with constipation showed conflicting results[31,32]. However, a comparison between constipation and diarrhoea predominant IBS patients revealed no significant difference in pain threshold[33].

Peripheral mechanisms-sensitisation of primary afferents

Preclinical studies of acute gut inflammation have shown that sensitization of primary afferent pathways can result in visceral hyperalgesia[34-36]. A subset of IBS patients develop symptoms following an acute GI infection[37]. Usu-

ally peripheral sensitization is temporary and response properties of primary afferents return to normal state after resolution of the inflammation[27]. Evidence from human mucosal biopsies suggests neuroplastic remodelling in the epithelium[38]. Such plastic changes can affect the response properties of primary afferents which include spinal and vagal afferents[39]. Changes in afferent nerve terminals could affect responsiveness to visceral stimuli and interfere with the release of neuropeptides from these terminals resulting in neurogenic inflammation[27].

Central pain amplification

In turn then there are multiple mechanisms by which the CNS can modulate afferent signals from the viscera, including increased activation of endogenous pain facilitation and reduced engagement of endogenous pain inhibition[27]. Of course, these modulatory systems are also influenced by stress and mood[27]. Neuroimaging studies consistently support a role for altered neural processing of visceral stimuli[40]. Indeed, some sophisticated studies now incorporate the contribution of emotional factors and cognitive influences such as expectation, attention and learning, to their analyses of functional connectivity between brain regions and actual CNS structural changes[40].

It was noted that a thinning of the anterior mid-cingulate and insular cortices was evident in IBS patients[41], these areas being associated with perception of the internal state. Moreover, regional structural changes including decreased grey matter in the medial and ventrolateral prefrontal cortex, thalamus and periaqueductal grey are seen in IBS patients as compared to healthy controls[42]. These may point towards an impaired ability to activate the descending pain inhibition system. This hypothesis is supported by the observation that the reduction in grey matter in the ventrolateral prefrontal cortex was only found in the patients presenting with a high level of pain[42]. Central areas involved in the processing of the affective component of pain such as the pregenual anterior cingulate cortex and the orbital frontal cortex showed an increase in grey matter in IBS patients, which was abolished once data was corrected for anxiety and depression in these patients[40]. These findings further confirm the involvement of emotional systems in the processing of visceral pain. Consistent with this, Chen *et al*[43] showed that white matter aberrations are seen in the anterior cingulate cortex and the insula. However, as it is still not known whether these changes are present before symptoms emerge, or are actually acquired due to altered visceral signalling, these results should be interpreted with caution.

Enhanced CNS responses

A meta-analysis of functional magnetic resonance imaging studies in IBS patients reported differences in CNS response to colorectal distension[44]. The differences were seen in areas associated with visceral afferent signalling, attention and emotional arousal. The anterior cingulate cortex is one of the most commonly reported cortical areas that displays pain evoked activation during acute stimulation in patients[43]. Mertz *et al*[45] demonstrated that the anterior cingulate cortex, thalamus, the insula and the prefrontal cortex were more activated in IBS patients than controls and that the pattern of activation was dependent on previous experience. A greater activation of the thalamic, striatal and dorsolateral prefrontal cortex was seen in controls as compared to IBS patients during rectal distension indicating an abnormal descending modulation in IBS[46]. It has also been shown that female IBS patients have a greater engagement of the emotional arousal system during expectation of visceral pain than males[47]. These studies highlight the importance of the emotional status of patients in pain perception and that the female predominance may be in part due to the gender differences in the activation of circuits involved in stress and arousal[27]. Taken together these results indicate a role for both structural and functional abnormalities in the CNS in IBS pathophysiology.

ENDOCRINE PATHWAYS IN IBS PATHOPHYSIOLOGY

CRF

Stress is a pervasive condition that effects everyone and is defined as a "stereotyped body response to any demand"[48]. However, the high co-morbidity of stress-associated mood disorders such as anxiety and depression and altered bowel function in IBS patients[49], suggests that these individuals are more sensitive to the effects of stress. Indeed, the relationship between severe and chronic stress and symptom intensity in IBS patients[50] is linked to chronic stress, with the onset and duration of symptoms increased[51]. As noted above, this may mediated *via* altered autonomic signalling[52], however the key signalling factor initiated by stress is an endocrine hormone, CRF.

CRF is the vital hormone in the body's response to stress, activating the hypothalamic-pituitary-adrenal (HPA) axis in reaction to a variety of physical and psychological stressors. This results in enhanced levels of adrenocorticotropic hormone and cortisol in IBS patients as compared to healthy subjects[53,54]. CRF is secreted by the paraventricular nucleus (PVN) of the hypothalamus and its release is regulated by the amygdala, which is part of the limbic system[51].

CRF exerts its biological effects through activation of CRF1 and CRF2 receptors (CRFR1 and CRFR2), which are members of the seven transmembrane G-protein coupled receptor superfamily[55]. CRFR1 is prevalent in brain regions associated with affective, stress and nociceptive circuitries including the PVN, locus coeruleus and amygdala[56,57]. CRF neurons project from the PVN to the spinal cord, where they can alter the function of innervated organs[58].

CRF in the GI tract

Although much of the influence of CRF on GI function

is mediated centrally, the presence of CRF ligands and its receptors in the colon[55,59,60] suggests that organ-specific activation of these receptors may also be important for stress-induced changes in bowel function. CRFR1 is expressed on enteric neurons and in the mucosal layer[59] and is likely to be the focal mechanism by which stress induces changes in GI function including delayed gastric emptying[61], accelerated colonic transit[62] and motility[63]. The importance of CRF to these effects has been demonstrated using the non-selective CRF receptor antagonist, α-helical CRF[64]. Furthermore, the use of CRFR1 -/- mice has revealed the importance of the CRFR1 subtype in IBS like-symptoms, as these knock-out mice exhibit decreased visceral sensitivity[65], as well as decreased anxiety and an impaired stress response[66]. In addition CRF-evoked defecation in rats[67] is inhibited by blocking CRF1 receptors[68]. These results translate to IBS patients, where peripheral administration of CRF1 receptor antagonists reduces abdominal pain and anxiety[64]. In contrast to CRFR1-mediated increases in GI contractile activity, stimulation of CRFR2 is likely to result in inhibition of GI motility[69,70] and contribute to stress-induced colonic permeability dysfunction[71].

Effects of CRF on visceral hypersensitivity and colonic function

Some of the key symptoms of IBS, such as colonic motility, alterations in bowel habit and abdominal pain associated with gut hypersensitivity may be a consequence of CRFR1 signalling[72]. Consistent with this is increased thalamic expression of CRFR1 following colonic distension in the maternally separated rat model of IBS[73]. Moreover, central administration of CRF increases pain behaviours in response to colonic distension in rats[74] demonstrating the bidirectional signalling between the CNS and the gut. Activation of the CRFR1 signalling pathways causes increases in colonic motor activity and visceral pain[75,76], and conversely, activation of central and peripheral CRFR2 receptors delays gastric emptying[70]. Furthermore, activation of either CRFR1 or CRFR2 causes increased colonic permeability and inflammation[77]. The pathophysiology of stress-induced exacerbation of IBS symptoms may be due to central hypersecretion of CRF, as it has been shown that inhibition of CRFR1[78] as well as central inhibition with CRF antagonists decreases the response to water avoidance stress[79]. Also, stress increases intestinal permeability, visceral hypersensitivity, causes alterations in gastrointestinal motility and leads to profound activation of mast cells, resulting in the release of many pro-inflammatory mediators[80-82] which will be discussed in more detail later. Williams *et al*[67] illustrated that acute restraint stress increased large intestine transit rates and stimulated defecation and this was associated with mucosal mast cell activation[83,84], which was mediated by CRF[83]. These studies imply that the brain-gut axis of IBS patients has a magnified response to CRF. Thus, targeting CRF signalling molecules has been proposed as a potential treatment for IBS[70]. However, thus far, clinical trials using a CRFR1 antagonist have been disappointing[85].

Corticosteroids

Mineralocorticoids and glucocorticoids are steroid hormones which mediate the actions of the adrenal hormone, cortisol in the initiation and termination of the stress response, respectively[86]. Cortisol, which is the natural ligand for corticosteroid receptors, is elevated in IBS patients both at baseline and in response to stress[53,54]. In rodent studies, application of corticosterone to the amygdala induces colonic hypersensitivity and anxiety[87,88] and alters colonic transit[89], actions that are mediated through both mineralocorticoid and glucocorticoid receptors[90]. These studies demonstrate that central signalling by corticosteroids are potential targets for treating bowel dysfunction in IBS.

Glucagon-like peptide 1

A precipitating factor in symptom exacerbation is food ingestion, frequently in the form of abdominal pain and gas[91]. Although food intolerance has not been shown to cause IBS, ingestion of certain foods can result in abdominal pain, bloating, flatus and diarrhoea[92,93], especially carbohydrates, including gluten and lactose and fat-rich meals[92]. This appears to be more common in females and those who display increased anxiety levels again demonstrating the multi-factorial nature of IBS[93]. Prolonged and exaggerated colonic motor responses following a meal has been observed in IBS patients[94] and balloon distension in the jejunum demonstrated increased sensitivity in IBS patients following a meal[95]. Recent studies have reported the success of diets low in fermentable oligosaccharides, disaccharides, monosaccharides and polyols[96-98]. Reduction of poorly absorbed short-chain carbohydrates such as lactose, fructose and sorbitol, fructo-oligosaccharides, galacto-oligosaccharides and incompletely absorbed sugar polyols such as sorbitol and mannitol relieves symptoms of IBS, such as bloating, distension, abdominal pain, excessive flatus[98] and osmotic diarrhoea[99]. Although release of gas by fermentation is normal, the sensitivity of IBS bowels to distension results in visceral pain. The pathophysiological changes resulting in these symptoms are not yet clear. However, an important physiological response to the arrival of food in the GI tract is the secretion of incretin hormones such as glucagon-like peptide 1 (GLP-1) which is secreted by L-cells. The biological activities of GLP-1 include stimulation of glucose-dependent insulin secretion and insulin biosynthesis, inhibition of glucagon secretion and gastric emptying, and the inhibition of food intake. One report has related GLP-1 to IBS pathophysiology by demonstrating that a GLP-1 mimetic alleviated some of the pathophysiological symptoms of IBS with antispasmodic and pain-relieving properties[100]. Although, the molecular mechanisms by which GLP-1 achieves this outcome are not completely understood, it is thought to act in a neurocrine fashion. Indeed, GLP-1 has been found to increase firing rates in afferent vagal nerves[101]

and also decreasing neurally-evoked chloride secretion[102]. Interestingly, GLP-1 can also modulate GI secretion of cytokines and alter central CRF pathways that regulate stress-induced alterations in colonic transit[103]. GLP-1-expressing neurons are found in the enteric nervous system but also in brain regions such as the nucleus tractus solitarius and the ventrolateral medulla[104], revealing that the action of GLP-1 on gut function may be central or peripheral. GLP-1 activates the HPA axis through CRF neuronal stimulation, which may be important in the suppression of feeding behaviour[105]. Other GI hormones, such as motilin[106], which regulates the migrating motor complex in the fasting period and cholecystokinin[107] are elevated in IBS. In contrast, colonic expression of peptide YY[108] and circulating neuropeptide Y are lower in IBS patients[107]. Consideration must therefore be given to these and other GI factors in the pathogenesis of IBS.

ALTERATIONS IN IMMUNE FUNCTION IN IBS

Mounting evidence suggests that alterations in immune status such as elevations in mucosal mast cell numbers, pro-inflammatory cytokines and increased intestinal permeability are frequently noted in IBS patients[109]. Potential biomarkers of the disorder include alterations in cytokine profiles, mucosal and muscular infiltration of immune cells, changes in intestinal permeability and luminal microbiota which are discussed below.

Post-infectious IBS

Gross morphological evidence of inflammation is absent from IBS mucosal biopsies and other indicators of inflammation such as faecal levels of calprotectin and lactoferrin are not elevated[110,111]. Nonetheless, evidence is mounting on the important contribution of immune activation to the development of this syndrome. Indeed, one of clearest predictors of developing IBS is a prior history of bacterial or viral gastroenteritis[37,112], with one study showing a sevenfold increase in the risk of developing the functional bowel disorder following gastrointestinal infection[113]. Samples from individuals with post-infectious IBS show persistent increases in mucosal mononuclear immune cells[114], T-lymphocytes[115] and mast cells[116], which degranulate following stimulation releasing compounds such as histamine, tryptase and chymase. The extent of immune activation is an indicator of the severity of the infective gastroenteritis episode and the subsequent risk of developing IBS[114,117].

Immune cells and cytokines

Expression of lymphocytes and mast cells are elevated in IBS mucosal samples[118,119], although not all studies detected increased numbers of mucosal mast cells[118,120,121]. Nonetheless, soluble mediators released from degranulated mast cells were found to induce excitation of rat sensory neurons[121]. This has implications for GI sensory and motor function, with one study demonstrating that the colon is more susceptible to effects of stress on enteric nerve function following a prior bout of inflammation[122].

Evidence of immune activation in IBS includes elevated levels of pro-inflammatory cytokines such as interleukin (IL)-6 and IL-8[53,123-125], although not all studies detected such increases[120,126]. Furthermore, in peripheral blood mononuclear cells isolated from IBS patients, abnormal secretion of pro-inflammatory cytokines in response to immune challenges was observed[123,125,127]. Studies reporting changes in mucosal levels of pro-inflammatory cytokines in IBS biopsies varied with some studies describing an upregulation[116,128] but several others describing down-regulation of these cytokines[54,129]. That said, anti-inflammatory cytokines such as IL-10 and transforming growth factor β appear to be decreased in IBS colonic and rectal biopsies[54,128,129]. Expression of chemokines, including IL-8, CXCL-9 and monocyte chemoattractant protein-1, which are important in mucosal defence, were also decreased in IBS biopsies[129].

The source of these immune messengers are likely to be from mast cells, the numbers of which are elevated in IBS[128,130,131] and can secrete IL-6 and IL-1β[132] in addition to histamine, tryptase, chymase and proteases. Indeed, Buhner *et al*[131] described how excitation of non-IBS submucosal neurons with IBS biopsy secretions was dependent on serotonin, tryptase and histamine. Furthermore, the proximity of activated mast cells to colonic nerves was found to correlate with visceral pain severity[130].

Cytokines have been shown to have neuromodulatory effects with IL-6[133], IL-1β[134] and tumour necrosis factor (TNF) α[135] stimulating submucosal secretomotor neurons. This may result in changes in gut function including contractility[136], absorption and/or secretion[133]. IL-6 and IL-1β also influence mucosal ion transport and epithelial permeability and enhance cholinergically-mediated neurotransmission[133,137,138]. Furthermore, IL-6 has a potential role in neurogenic secretory diarrhoea[125] as this cytokine can suppress the inhibitory and anti-secretory effects of norepinephrine by blocking its release from sympathetic fibres[139]. Others have provided evidence that IL-6 attenuates the pre-synaptic inhibition of noradrenalin release, thereby releasing the sympathetic brake[134], which further contributes to a pro-secretory state. Aside from altered GI motility, the other main debilitating symptom of IBS is visceral pain sensitivity. Given the demonstrated effects of cytokines on enteric neuron excitability[133-135] and proven roles in nociception and sensory pain pathways[140], activation of enteric neurons and subsequent evocation of visceral pain make cytokines attractive candidates for mediating the visceral pain-related features of IBS.

Epithelial barrier integrity

The permeability of the epithelial layer which acts as a barrier between the external environment of the gut lumen and the body's internal milieu is an important consideration in immune activation in IBS. Indeed, some IBS patients have increased intestinal permeability[141], which

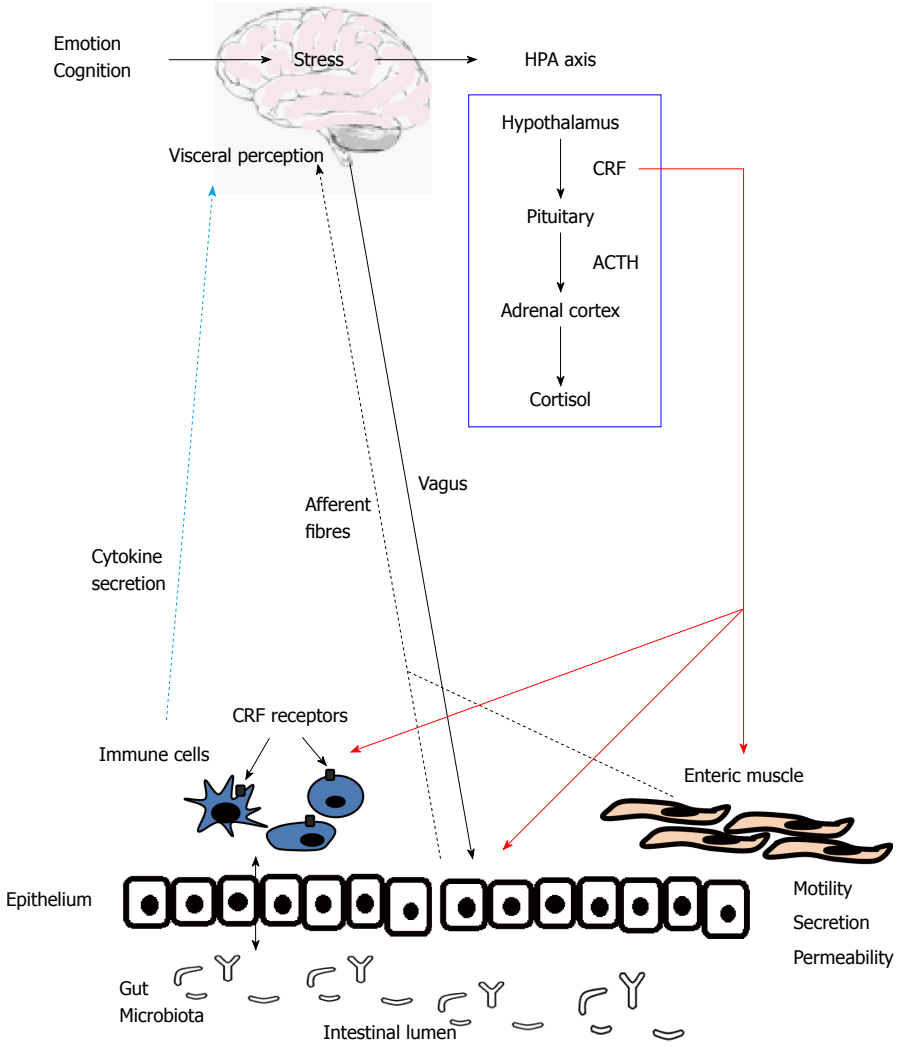


Figure 1 Convergence of neural endocrine and immune signalling pathways in bowel dysfunction. The figure illustrates the complex nature of functional bowel syndromes such as IBS. It illustrates interaction between three major bodily systems, neural pathways between the brain and gut, hormonal release, primarily from stress-induced activation of the HPA axis and secretion of immune factors such as cytokines. ACTH: Adrenocorticotropic hormone; CRF: Corticotropin-releasing factor; HPA: Hypothalamic-pituitary-adrenal; IBS: Irritable bowel syndrome.

may be due to proteasomal degradation of tight junction proteins[128,142]. Additionally, altered secretion of inflammatory cytokines may affect barrier function and permeability[129]. Breakdown of the mucosal barrier by IL-6 and other pro-inflammatory cytokines[137,143] may provide access for foreign proteins, thus initiating an immune response in the GI muscle layers resulting in changes in bowel function. In IBS, where circulating IL-6 levels are elevated and the HPA axis is hyper-activated[53], a coincident compromise of the mucosal barrier is observed. Thus, increased permeability of the mucosal barrier and the subsequent initiation of an immune response may contribute to the increase in sensitivity to visceral pain in IBS patients[144].

Microbiota

An additional factor contributing to brain-gut axis signalling in IBS currently gaining considerable attention is the importance of disrupting the luminal microbiota[145,146]. Indeed, microbiota dysbiosis, which may facilitate the adhesion of enteric pathogens in the human gut, has been reported in several IBS studies[147-150]. This virtual organ is integrated into the bi-directional communication in the brain-gut axis with studies demonstrating that microbiota dysbiosis exists in IBS patients and manipulation of the microbial environment with probiotics may lead to symptom improvement[151]. Probiotics have been shown to modulate the immune response in IBS, suppressing pro-inflammatory cytokines[152], maintaining intestinal barrier integrity[153], causing down-regulation of T cells and inhibition of nuclear factor kappa B[154]. Moreover, probiotics prevented adhesion of enteric pathogens to the wall of the GI tract[155]. However, more recent longer-term studies did not detect an improvement in symptoms[156,157]. Other members of the innate immune system that are altered in IBS include the pattern recognition receptors, toll-like receptors (TLRs), which recognise and respond to pathogens. Altered expression of TLR4, 5, 7 and 8 in mucosal biopsies from IBS patients further supports the importance of interactions between the luminal flora and

the host in this disorder[158].

CONVERGENCE OF PATHWAYS

The pathophysiology of altered bowel function in IBS patients remains unclear, however a number of mechanisms have been proposed to contribute to the initiation, exacerbation and persistence of symptoms. Alterations in brain-gut communication[159], stress[70], previous infections[57], abnormal microbiota[160], altered cytokine profiles[53] and increased intestinal permeability[142] have all been discussed. However, we believe that complex interactions between neural, endocrine and immune factors underlie the heterogeneity of symptoms that is characteristic of IBS as diagrammed in Figure 1.

For example, a perceived threat or stressor, which frequently precedes symptom flares, evokes responses from both the immune and stress systems. In healthy individuals this is a crucial response for the adaptation and ultimate survival of an organism. However, in the case of PI-IBS, co-morbidity with anxiety or depression and the occurrence of stressful life events around the time of exposure to the enteric pathogen have been independent predictors of risk for the development of IBS[114,161,162], although not all studies, including the Walkerton cohort[112], detected this association. IBS patients are more likely to be stress-sensitive, as measured by the Holmes and Rahe stress scale, and exhibit elevated numbers of colonic mucosal mast cells[130]. Moreover, acute stress causes increases in the numbers of white blood cells, natural killer cells and CD8+ T-lymphocytes, decreases B cell numbers and stimulates secretion of pro-inflammatory cytokines[163,164], whereas secretion of glucocorticoids and an associated decrease in secretion of pro-inflammatory cytokines is noted in cases of chronic stress[165]. Patients with IBS often exhibit concurrent increases in markers of a hyperactive stress response and immune upregulation such as CRF-stimulated HPA axis hyper-responsivity which is related to the elevation in IL-6 levels[53]. CRF also stimulates the recruitment and activation of granulocytes[166] and mast cells[167] to the gut mucosa.

Immune cells express receptors for several different stress-related peptides including CRF[168]. Indeed, we have detected both CRFR1 and IL-6 receptors on T-helper cells[169]. CRF peptides have potent immunomodulatory actions[170], including degranulation of mast cells[171] and secretion of cytokines[53,172], although it is not yet clear whether these effects are pro-[173] or anti-inflammatory[174,175].

In terms of crosstalk between the stress system and the neural response, many of the psychological disorders frequently found to be co-morbid with IBS also have the capacity to disrupt autonomic balance[52] and indeed, anxiety and depression are associated with depressed parasympathetic activity in IBS patients. Enteric neurons, which directly regulate absorpto-secretory function and gut motility have been shown to express both CRF receptors and IL-6 receptors[169]. Indeed, cytokines such as IL-6 can directly induce excitation of enteric neurons in animal models of IBS[133,176]. IL-6[133], IL-1β[134] and TNFα[135] can cause activation of submucosal secretomotor neurons thereby acting as neuromodulatory factors that can directly influence such gut functions as motility, absorption, secretion and blood flow. IL-6 and IL-1β also have effects on mucosal ion transport and epithelial permeability, in addition to enhancing cholinergically-mediated neurotransmission[133,137,138]. Indeed, soluble mediators released from mast cells in IBS biopsies were found to have excitatory effects on rat sensory neurons[121].

Although the pathophysiology of IBS still requires further elucidation, recent progress in the field has demonstrated the importance of molecular factors such as the stress hormone, CRF and cytokine release and their influence on neural communication between the brain and gut. Further research will hopefully reveal the aberrant signalling between endocrine, immune and neural systems in IBS patients and pave the way towards effective new therapies for this common bowel disorder.

REFERENCES

1 **Drossman DA**, Camilleri M, Mayer EA, Whitehead WE. AGA technical review on irritable bowel syndrome. *Gastroenterology* 2002; **123**: 2108-2131 [PMID: 12454866 DOI: 10.1053/gast.2002.37095]

2 **Longstreth GF**, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology* 2006; **130**: 1480-1491 [PMID: 16678561]

3 **Thompson WG**, Heaton KW, Smyth GT, Smyth C. Irritable bowel syndrome in general practice: prevalence, characteristics, and referral. *Gut* 2000; **46**: 78-82 [PMID: 10601059]

4 **El-Serag HB**, Olden K, Bjorkman D. Health-related quality of life among persons with irritable bowel syndrome: a systematic review. *Aliment Pharmacol Ther* 2002; **16**: 1171-1185 [PMID: 12030961]

5 **Su AM**, Shih W, Presson AP, Chang L. Characterization of symptoms in irritable bowel syndrome with mixed bowel habit pattern. *Neurogastroenterol Motil* 2014; **26**: 36-45 [PMID: 23991913 DOI: 10.1111/nmo.12220]

6 **Andrews EB**, Eaton SC, Hollis KA, Hopkins JS, Ameen V, Hamm LR, Cook SF, Tennis P, Mangel AW. Prevalence and demographics of irritable bowel syndrome: results from a large web-based survey. *Aliment Pharmacol Ther* 2005; **22**: 935-942 [PMID: 16268967]

7 **Saito YA**, Schoenfeld P, Locke GR. The epidemiology of irritable bowel syndrome in North America: a systematic review. *Am J Gastroenterol* 2002; **97**: 1910-1915 [PMID: 12190153 DOI: 10.1111/j.1572-0241.2002.05913.x]

8 **Collins SM**, Bercik P. The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease. *Gastroenterology* 2009; **136**: 2003-2014 [PMID: 19457424 DOI: 10.1053/j.gastro.2009.01.075]

9 **Drossman DA**, Chang L, Bellamy N, Gallo-Torres HE, Lembo A, Mearin F, Norton NJ, Whorwell P. Severity in irritable bowel syndrome: a Rome Foundation Working Team report. *Am J Gastroenterol* 2011; **106**: 1749-1759; quiz 1760 [PMID: 21747417 DOI: 10.1038/ajg.2011.201]

10 **Stasi C**, Rosselli M, Bellini M, Laffi G, Milani S. Altered neuro-endocrine-immune pathways in the irritable bowel syndrome: the top-down and the bottom-up model. *J Gastroenterol* 2012; **47**: 1177-1185 [PMID: 22766747 DOI: 10.1007/s00535-012-0627-7]

11 **Aggarwal A**, Cutts TF, Abell TL, Cardoso S, Familoni B,

Bremer J, Karas J. Predominant symptoms in irritable bowel syndrome correlate with specific autonomic nervous system abnormalities. *Gastroenterology* 1994; **106**: 945-950 [PMID: 8143999]
12 **Mayer EA**, Tillisch K, Bradesi S. Review article: modulation of the brain-gut axis as a therapeutic approach in gastrointestinal disease. *Aliment Pharmacol Ther* 2006; **24**: 919-933 [PMID: 16948804]
13 **Fukudo S**. Stress and visceral pain: focusing on irritable bowel syndrome. *Pain* 2013; **154** Suppl 1: S63-S70 [PMID: 24021863]
14 **Saper CB**. The central autonomic nervous system: conscious visceral perception and autonomic pattern generation. *Annu Rev Neurosci* 2002; **25**: 433-469 [PMID: 12052916 DOI: 10.1146/annurev.neuro.25.032502.111311]
15 **Fukudo S**, Kanazawa M. Gene, environment, and brain-gut interactions in irritable bowel syndrome. *J Gastroenterol Hepatol* 2011; **26** Suppl 3: 110-115 [PMID: 21443722 DOI: 10.1111/j.1440-1746.2011.06631.x]
16 **Gillanders S**, Wild M, Deighan C, Gillanders D. Emotion regulation, affect, psychosocial functioning, and well-being in hemodialysis patients. *Am J Kidney Dis* 2008; **51**: 651-662 [PMID: 18371541 DOI: 10.1053/j.ajkd.2007.12.023]
17 **Spaziani R**, Bayati A, Redmond K, Bajaj H, Mazzadi S, Bienenstock J, Collins SM, Kamath MV. Vagal dysfunction in irritable bowel syndrome assessed by rectal distension and baroreceptor sensitivity. *Neurogastroenterol Motil* 2008; **20**: 336-342 [PMID: 18179607 DOI: 10.1111/j.1365-2982.2007.01042.x]
18 **Spetalen S**, Sandvik L, Blomhoff S, Jacobsen MB. Autonomic function at rest and in response to emotional and rectal stimuli in women with irritable bowel syndrome. *Dig Dis Sci* 2008; **53**: 1652-1659 [PMID: 17990112 DOI: 10.1007/s10620-007-0066-0]
19 **van Orshoven NP**, Andriesse GI, van Schelven LJ, Smout AJ, Akkermans LM, Oey PL. Subtle involvement of the parasympathetic nervous system in patients with irritable bowel syndrome. *Clin Auton Res* 2006; **16**: 33-39 [PMID: 16477493 DOI: 10.1007/s10286-006-0307-x]
20 **Adeyemi EO**, Desai KD, Towsey M, Ghista D. Characterization of autonomic dysfunction in patients with irritable bowel syndrome by means of heart rate variability studies. *Am J Gastroenterol* 1999; **94**: 816-823 [PMID: 10086672 DOI: 10.1111/j.1572-0241.1999.00861.x]
21 **Lomax AE**, Sharkey KA, Furness JB. The participation of the sympathetic innervation of the gastrointestinal tract in disease states. *Neurogastroenterol Motil* 2010; **22**: 7-18 [PMID: 19686308 DOI: 10.1111/j.1365-2982.2009.01381.x]
22 **Pellissier S**, Dantzer C, Canini F, Mathieu N, Bonaz B. Psychological adjustment and autonomic disturbances in inflammatory bowel diseases and irritable bowel syndrome. *Psychoneuroendocrinology* 2010; **35**: 653-662 [PMID: 19910123 DOI: 10.1016/j.psyneuen.2009.10.004]
23 **Cain KC**, Jarrett ME, Burr RL, Hertig VL, Heitkemper MM. Heart rate variability is related to pain severity and predominant bowel pattern in women with irritable bowel syndrome. *Neurogastroenterol Motil* 2007; **19**: 110-118 [PMID: 17244165]
24 **Heitkemper M**, Jarrett M, Cain KC, Burr R, Levy RL, Feld A, Hertig V. Autonomic nervous system function in women with irritable bowel syndrome. *Dig Dis Sci* 2001; **46**: 1276-1284 [PMID: 11414305]
25 **Jarrett ME**, Burr RL, Cain KC, Hertig V, Weisman P, Heitkemper MM. Anxiety and depression are related to autonomic nervous system function in women with irritable bowel syndrome. *Dig Dis Sci* 2003; **48**: 386-394 [PMID: 12643620]
26 **Ritchie J**. Pain from distension of the pelvic colon by inflating a balloon in the irritable colon syndrome. *Gut* 1973; **14**: 125-132 [PMID: 4696535]
27 **Mayer EA**, Tillisch K. The brain-gut axis in abdominal pain syndromes. *Annu Rev Med* 2011; **62**: 381-396 [PMID: 21090962 DOI: 10.1146/annurev-med-012309-103958]
28 **Whitehead WE**, Engel BT, Schuster MM. Irritable bowel syndrome: physiological and psychological differences between diarrhea-predominant and constipation-predominant patients. *Dig Dis Sci* 1980; **25**: 404-413 [PMID: 7379673]
29 **Prior A**, Maxton DG, Whorwell PJ. Anorectal manometry in irritable bowel syndrome: differences between diarrhoea and constipation predominant subjects. *Gut* 1990; **31**: 458-462 [PMID: 2338274]
30 **Simrén M**, Abrahamsson H, Björnsson ES. An exaggerated sensory component of the gastrocolonic response in patients with irritable bowel syndrome. *Gut* 2001; **48**: 20-27 [PMID: 11115818]
31 **Harraf F**, Schmulson M, Saba L, Niazi N, Fass R, Munakata J, Diehl D, Mertz H, Naliboff B, Mayer EA. Subtypes of constipation predominant irritable bowel syndrome based on rectal perception. *Gut* 1998; **43**: 388-394 [PMID: 9863485]
32 **Slater BJ**, Plusa SM, Smith AN, Varma JS. Rectal hypersensitivity in the irritable bowel syndrome. *Int J Colorectal Dis* 1997; **12**: 29-32 [PMID: 9112147]
33 **Steens J**, Van Der Schaar PJ, Penning C, Brussee J, Masclee AA. Compliance, tone and sensitivity of the rectum in different subtypes of irritable bowel syndrome. *Neurogastroenterol Motil* 2002; **14**: 241-247 [PMID: 12061908]
34 **Chen J**, Winston JH, Sarna SK. Neurological and cellular regulation of visceral hypersensitivity induced by chronic stress and colonic inflammation in rats. *Neuroscience* 2013; **248C**: 469-478 [PMID: 23806714 DOI: 10.1016/j.neuroscience.2013.06.024]
35 **Christianson JA**, Bielefeldt K, Altier C, Cenac N, Davis BM, Gebhart GF, High KW, Kollarik M, Randich A, Undem B, Vergnolle N. Development, plasticity and modulation of visceral afferents. *Brain Res Rev* 2009; **60**: 171-186 [PMID: 19150371 DOI: 10.1016/j.brainresrev.2008.12.004]
36 **Christianson JA**, Bielefeldt K, Malin SA, Davis BM. Neonatal colon insult alters growth factor expression and TRPA1 responses in adult mice. *Pain* 2010; **151**: 540-549 [PMID: 20850221 DOI: 10.1016/j.pain.2010.08.029]
37 **Spiller R**, Garsed K. Postinfectious irritable bowel syndrome. *Gastroenterology* 2009; **136**: 1979-1988 [PMID: 19457422]
38 **Vergnolle N**. Postinflammatory visceral sensitivity and pain mechanisms. *Neurogastroenterol Motil* 2008; **20** Suppl 1: 73-80 [PMID: 18402644 DOI: 10.1111/j.1365-2982.2008.01110.x]
39 **Moore BA**, Stewart TM, Hill C, Vanner SJ. TNBS ileitis evokes hyperexcitability and changes in ionic membrane properties of nociceptive DRG neurons. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G1045-G1051 [PMID: 12016130 DOI: 10.1152/ajpgi.00406.2001]
40 **Elsenbruch S**. Abdominal pain in Irritable Bowel Syndrome: a review of putative psychological, neural and neuro-immune mechanisms. *Brain Behav Immun* 2011; **25**: 386-394 [PMID: 21094682]
41 **Blankstein U**, Chen J, Diamant NE, Davis KD. Altered brain structure in irritable bowel syndrome: potential contributions of pre-existing and disease-driven factors. *Gastroenterology* 2010; **138**: 1783-1789 [PMID: 20045701 DOI: 10.1053/j.gastro.2009.12.043]
42 **Seminowicz DA**, Labus JS, Bueller JA, Tillisch K, Naliboff BD, Bushnell MC, Mayer EA. Regional gray matter density changes in brains of patients with irritable bowel syndrome. *Gastroenterology* 2010; **139**: 48-57.e2 [PMID: 20347816 DOI: 10.1053/j.gastro.2010.03.049]
43 **Chen JY**, Blankstein U, Diamant NE, Davis KD. White matter abnormalities in irritable bowel syndrome and relation to individual factors. *Brain Res* 2011; **1392**: 121-131 [PMID: 21466788 DOI: 10.1016/j.brainres.2011.03.069]
44 **Tillisch K**, Mayer EA, Labus JS. Quantitative meta-analysis identifies brain regions activated during rectal distension in irritable bowel syndrome. *Gastroenterology* 2011; **140**: 91-100 [PMID: 20696168 DOI: 10.1053/j.gastro.2010.07.053]

45 **Mertz H**, Morgan V, Tanner G, Pickens D, Price R, Shyr Y, Kessler R. Regional cerebral activation in irritable bowel syndrome and control subjects with painful and nonpainful rectal distention. *Gastroenterology* 2000; **118**: 842-848 [PMID: 10784583]

46 **Hall GB**, Kamath MV, Collins S, Ganguli S, Spaziani R, Miranda KL, Bayati A, Bienenstock J. Heightened central affective response to visceral sensations of pain and discomfort in IBS. *Neurogastroenterol Motil* 2010; **22**: 276-e80 [PMID: 20003075 DOI: 10.1111/j.1365-2982.2009.01436.x]

47 **Labus JS**, Naliboff BN, Fallon J, Berman SM, Suyenobu B, Bueller JA, Mandelkern M, Mayer EA. Sex differences in brain activity during aversive visceral stimulation and its expectation in patients with chronic abdominal pain: a network analysis. *Neuroimage* 2008; **41**: 1032-1043 [PMID: 18450481 DOI: 10.1016/j.neuroimage.2008.03.009]

48 **Selye H**. A syndrome produced by diverse nocuous agents. 1936. *J Neuropsychiatry Clin Neurosci* 1998; **10**: 230-231 [PMID: 9722327]

49 **Folks DG**. The interface of psychiatry and irritable bowel syndrome. *Curr Psychiatry Rep* 2004; **6**: 210-215 [PMID: 15142474]

50 **Bennett EJ**, Tennant CC, Piesse C, Badcock CA, Kellow JE. Level of chronic life stress predicts clinical outcome in irritable bowel syndrome. *Gut* 1998; **43**: 256-261 [PMID: 10189854]

51 **Gué M**, Tekamp A, Tabis N, Junien JL, Buéno L. Cholecystokinin blockade of emotional stress- and CRF-induced colonic motor alterations in rats: role of the amygdala. *Brain Res* 1994; **658**: 232-238 [PMID: 7834346]

52 **Tougas G**. The autonomic nervous system in functional bowel disorders. *Gut* 2000; **47** Suppl 4: iv78-i80; discussion iv87 [PMID: 11076926]

53 **Dinan TG**, Quigley EM, Ahmed SM, Scully P, O'Brien S, O' Mahony L, O'Mahony S, Shanahan F, Keeling PW. Hypothalamic-pituitary-gut axis dysregulation in irritable bowel syndrome: plasma cytokines as a potential biomarker? *Gastroenterology* 2006; **130**: 304-311 [PMID: 16472586]

54 **Chang L**, Sundaresh S, Elliott J, Anton PA, Baldi P, Licudine A, Mayer M, Vuong T, Hirano M, Naliboff BD, Ameen VZ, Mayer EA. Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis in irritable bowel syndrome. *Neurogastroenterol Motil* 2009; **21**: 149-159 [PMID: 18684212 DOI: 10.1111/j.1365-2982.2008.01171.x]

55 **Kostich WA**, Chen A, Sperle K, Largent BL. Molecular identification and analysis of a novel human corticotropin-releasing factor (CRF) receptor: the CRF2gamma receptor. *Mol Endocrinol* 1998; **12**: 1077-1085 [PMID: 9717834]

56 **Sánchez MM**, Young LJ, Plotsky PM, Insel TR. Autoradiographic and in situ hybridization localization of corticotropin-releasing factor 1 and 2 receptors in nonhuman primate brain. *J Comp Neurol* 1999; **408**: 365-377 [PMID: 10340512]

57 **Reyes BA**, Valentino RJ, Van Bockstaele EJ. Stress-induced intracellular trafficking of corticotropin-releasing factor receptors in rat locus coeruleus neurons. *Endocrinology* 2008; **149**: 122-130 [PMID: 17947354]

58 **Swanson LW**, Sawchenko PE, Rivier J, Vale WW. Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study. *Neuroendocrinology* 1983; **36**: 165-186 [PMID: 6601247]

59 **Chatzaki E**, Crowe PD, Wang L, Million M, Taché Y, Grigoriadis DE. CRF receptor type 1 and 2 expression and anatomical distribution in the rat colon. *J Neurochem* 2004; **90**: 309-316 [PMID: 15228587]

60 **Muramatsu Y**, Fukushima K, Iino K, Totsune K, Takahashi K, Suzuki T, Hirasawa G, Takeyama J, Ito M, Nose M, Tashiro A, Hongo M, Oki Y, Nagura H, Sasano H. Urocortin and corticotropin-releasing factor receptor expression in the human colonic mucosa. *Peptides* 2000; **21**: 1799-1809 [PMID: 11150640]

61 **Barquist E**, Zinner M, Rivier J, Taché Y. Abdominal surgery-induced delayed gastric emptying in rats: role of CRF and sensory neurons. *Am J Physiol* 1992; **262**: G616-G620 [PMID: 1566844]

62 **Mönnikes H**, Schmidt BG, Taché Y. Psychological stress-induced accelerated colonic transit in rats involves hypothalamic corticotropin-releasing factor. *Gastroenterology* 1993; **104**: 716-723 [PMID: 8440432]

63 **Gue M**, Junien JL, Bueno L. Conditioned emotional response in rats enhances colonic motility through the central release of corticotropin-releasing factor. *Gastroenterology* 1991; **100**: 964-970 [PMID: 2001832]

64 **Sagami Y**, Shimada Y, Tayama J, Nomura T, Satake M, Endo Y, Shoji T, Karahashi K, Hongo M, Fukudo S. Effect of a corticotropin releasing hormone receptor antagonist on colonic sensory and motor function in patients with irritable bowel syndrome. *Gut* 2004; **53**: 958-964 [PMID: 15194643]

65 **Trimble N**, Johnson AC, Foster A, Greenwood-van Meerveld B. Corticotropin-releasing factor receptor 1-deficient mice show decreased anxiety and colonic sensitivity. *Neurogastroenterol Motil* 2007; **19**: 754-760 [PMID: 17539891]

66 **Smith GW**, Aubry JM, Dellu F, Contarino A, Bilezikjian LM, Gold LH, Chen R, Marchuk Y, Hauser C, Bentley CA, Sawchenko PE, Koob GF, Vale W, Lee KF. Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. *Neuron* 1998; **20**: 1093-1102 [PMID: 9655498]

67 **Williams CL**, Peterson JM, Villar RG, Burks TF. Corticotropin-releasing factor directly mediates colonic responses to stress. *Am J Physiol* 1987; **253**: G582-G586 [PMID: 2821826]

68 **Lu L**, Liu D, Ceng X, Ma L. Differential roles of corticotropin-releasing factor receptor subtypes 1 and 2 in opiate withdrawal and in relapse to opiate dependence. *Eur J Neurosci* 2000; **12**: 4398-4404 [PMID: 11122350]

69 **Martínez V**, Wang L, Rivier J, Grigoriadis D, Taché Y. Central CRF, urocortins and stress increase colonic transit via CRF1 receptors while activation of CRF2 receptors delays gastric transit in mice. *J Physiol* 2004; **556**: 221-234 [PMID: 14755002]

70 **Taché Y**, Bonaz B. Corticotropin-releasing factor receptors and stress-related alterations of gut motor function. *J Clin Invest* 2007; **117**: 33-40 [PMID: 17200704]

71 **Teitelbaum AA**, Gareau MG, Jury J, Yang PC, Perdue MH. Chronic peripheral administration of corticotropin-releasing factor causes colonic barrier dysfunction similar to psychological stress. *Am J Physiol Gastrointest Liver Physiol* 2008; **295**: G452-G459 [PMID: 18635602]

72 **Martinez V**, Taché Y. CRF1 receptors as a therapeutic target for irritable bowel syndrome. *Curr Pharm Des* 2006; **12**: 4071-4088 [PMID: 17100612]

73 **Tjong YW**, Ip SP, Lao L, Wu J, Fong HH, Sung JJ, Berman B, Che CT. Neonatal maternal separation elevates thalamic corticotrophin releasing factor type 1 receptor expression response to colonic distension in rat. *Neuro Endocrinol Lett* 2010; **31**: 215-220 [PMID: 20424584]

74 **Gué M**, Del Rio-Lacheze C, Eutamene H, Théodorou V, Fioramonti J, Buéno L. Stress-induced visceral hypersensitivity to rectal distension in rats: role of CRF and mast cells. *Neurogastroenterol Motil* 1997; **9**: 271-279 [PMID: 9430796]

75 **Holsboer F**, Ising M. Central CRH system in depression and anxiety--evidence from clinical studies with CRH1 receptor antagonists. *Eur J Pharmacol* 2008; **583**: 350-357 [PMID: 18272149 DOI: 10.1016/j.ejphar.2007.12.032]

76 **Taché Y**, Million M, Nelson AG, Lamy C, Wang L. Role of corticotropin-releasing factor pathways in stress-related alterations of colonic motor function and viscerosensibility in female rodents. *Gend Med* 2005; **2**: 146-154 [PMID: 16290887]

77 **Larauche M**, Kiank C, Tache Y. Corticotropin releasing factor signaling in colon and ileum: regulation by stress and pathophysiological implications. *J Physiol Pharmacol* 2009; **60** Suppl 7: 33-46 [PMID: 20388944]

78 **Million M**, Grigoriadis DE, Sullivan S, Crowe PD, McRoberts JA, Zhou H, Saunders PR, Maillot C, Mayer EA, Taché Y. A novel water-soluble selective CRF1 receptor antagonist, NBI 35965, blunts stress-induced visceral hyperalgesia and colonic motor function in rats. *Brain Res* 2003; **985**: 32-42 [PMID: 12957366]

79 **Taché Y**, Martinez V, Million M, Rivier J. Corticotropin-releasing factor and the brain-gut motor response to stress. *Can J Gastroenterol* 1999; **13** Suppl A: 18A-25A [PMID: 10202204]

80 **Söderholm JD**, Perdue MH. Stress and gastrointestinal tract. II. Stress and intestinal barrier function. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G7-G13 [PMID: 11123192]

81 **Nakade Y**, Fukuda H, Iwa M, Tsukamoto K, Yanagi H, Yamamura T, Mantyh C, Pappas TN, Takahashi T. Restraint stress stimulates colonic motility via central corticotropin-releasing factor and peripheral 5-HT3 receptors in conscious rats. *Am J Physiol Gastrointest Liver Physiol* 2007; **292**: G1037-G1044 [PMID: 17158256 DOI: 10.1152/ajpgi.00419.2006]

82 **Konturek SJ**, Brzozowski T, Konturek PC, Zwirska-Korczala K, Reiter RJ. Day/night differences in stress-induced gastric lesions in rats with an intact pineal gland or after pinealectomy. *J Pineal Res* 2008; **44**: 408-415 [PMID: 18086146 DOI: 10.1111/j.1600-079X.2007.00543.x]

83 **Castagliuolo I**, Lamont JT, Qiu B, Fleming SM, Bhaskar KR, Nikulasson ST, Kornetsky C, Pothoulakis C. Acute stress causes mucin release from rat colon: role of corticotropin releasing factor and mast cells. *Am J Physiol* 1996; **271**: G884-G892 [PMID: 8944704]

84 **Castagliuolo I**, Wershil BK, Karalis K, Pasha A, Nikulasson ST, Pothoulakis C. Colonic mucin release in response to immobilization stress is mast cell dependent. *Am J Physiol* 1998; **274**: G1094-G1100 [PMID: 9696710]

85 **Griebel G**, Holsboer F. Neuropeptide receptor ligands as drugs for psychiatric diseases: the end of the beginning? *Nat Rev Drug Discov* 2012; **11**: 462-478 [PMID: 22596253 DOI: 10.1038/nrd3702]

86 **de Kloet ER**, Joëls M, Holsboer F. Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* 2005; **6**: 463-475 [PMID: 15891777 DOI: 10.1038/nrn1683]

87 **Gunter WD**, Shepard JD, Foreman RD, Myers DA, Greenwood-Van Meerveld B. Evidence for visceral hypersensitivity in high-anxiety rats. *Physiol Behav* 2000; **69**: 379-382 [PMID: 10869605]

88 **Myers B**, Dittmeyer K, Greenwood-Van Meerveld B. Involvement of amygdaloid corticosterone in altered visceral and somatic sensation. *Behav Brain Res* 2007; **181**: 163-167 [PMID: 17481745 DOI: 10.1016/j.bbr.2007.03.031]

89 **Venkova K**, Foreman RD, Greenwood-Van Meerveld B. Mineralocorticoid and glucocorticoid receptors in the amygdala regulate distinct responses to colorectal distension. *Neuropharmacology* 2009; **56**: 514-521 [PMID: 18977372 DOI: 10.1016/j.neuropharm.2008.10.004]

90 **Myers B**, Greenwood-Van Meerveld B. Elevated corticosterone in the amygdala leads to persistent increases in anxiety-like behavior and pain sensitivity. *Behav Brain Res* 2010; **214**: 465-469 [PMID: 20573588 DOI: 10.1016/j.bbr.2010.05.049]

91 **Simrén M**, Abrahamsson H, Björnsson ES. Lipid-induced colonic hypersensitivity in the irritable bowel syndrome: the role of bowel habit, sex, and psychologic factors. *Clin Gastroenterol Hepatol* 2007; **5**: 201-208 [PMID: 17174611]

92 **Ragnarsson G**, Bodemar G. Pain is temporally related to eating but not to defaecation in the irritable bowel syndrome (IBS). Patients' description of diarrhea, constipation and symptom variation during a prospective 6-week study. *Eur J Gastroenterol Hepatol* 1998; **10**: 415-421 [PMID: 9619389]

93 **Simrén M**, Månsson A, Langkilde AM, Svedlund J, Abrahamsson H, Bengtsson U, Björnsson ES. Food-related gastrointestinal symptoms in the irritable bowel syndrome. *Digestion* 2001; **63**: 108-115 [PMID: 11244249]

94 **Rogers J**, Henry MM, Misiewicz JJ. Increased segmental activity and intraluminal pressures in the sigmoid colon of patients with the irritable bowel syndrome. *Gut* 1989; **30**: 634-641 [PMID: 2731756]

95 **Evans PR**, Kellow JE. Physiological modulation of jejunal sensitivity in health and in irritable bowel syndrome. *Am J Gastroenterol* 1998; **93**: 2191-2196 [PMID: 9820395 DOI: 10.1111/j.1572-0241.1998.00534.x]

96 **Barrett JS**, Gibson PR. Fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) and nonallergic food intolerance: FODMAPs or food chemicals? *Therap Adv Gastroenterol* 2012; **5**: 261-268 [PMID: 22778791 DOI: 10.1177/1756283X11436241]

97 **Gearry RB**, Irving PM, Barrett JS, Nathan DM, Shepherd SJ, Gibson PR. Reduction of dietary poorly absorbed short-chain carbohydrates (FODMAPs) improves abdominal symptoms in patients with inflammatory bowel disease-a pilot study. *J Crohns Colitis* 2009; **3**: 8-14 [PMID: 21172242 DOI: 10.1016/j.crohns.2008.09.004]

98 **Staudacher HM**, Whelan K, Irving PM, Lomer MC. Comparison of symptom response following advice for a diet low in fermentable carbohydrates (FODMAPs) versus standard dietary advice in patients with irritable bowel syndrome. *J Hum Nutr Diet* 2011; **24**: 487-495 [PMID: 21615553 DOI: 10.1111/j.1365-277X.2011.01162.x]

99 **Barrett JS**, Gearry RB, Muir JG, Irving PM, Rose R, Rosella O, Haines ML, Shepherd SJ, Gibson PR. Dietary poorly absorbed, short-chain carbohydrates increase delivery of water and fermentable substrates to the proximal colon. *Aliment Pharmacol Ther* 2010; **31**: 874-882 [PMID: 20102355 DOI: 10.1111/j.1365-2036.2010.04237.x]

100 **Hellström PM**, Näslund E, Edholm T, Schmidt PT, Kristensen J, Theodorsson E, Holst JJ, Efendic S. GLP-1 suppresses gastrointestinal motility and inhibits the migrating motor complex in healthy subjects and patients with irritable bowel syndrome. *Neurogastroenterol Motil* 2008; **20**: 649-659 [PMID: 18298441]

101 **McKee DP**, Quigley EM. Intestinal motility in irritable bowel syndrome: is IBS a motility disorder? Part 1. Definition of IBS and colonic motility. *Dig Dis Sci* 1993; **38**: 1761-1772 [PMID: 8404395]

102 **Quigley EM**. Antibiotics for irritable bowel syndrome: hitting the target, but what is it? *Gastroenterology* 2011; **141**: 391-393 [PMID: 21620844]

103 **Nakade Y**, Tsukamoto K, Iwa M, Pappas TN, Takahashi T. Glucagon like peptide-1 accelerates colonic transit via central CRF and peripheral vagal pathways in conscious rats. *Auton Neurosci* 2007; **131**: 50-56 [PMID: 16938493]

104 **Lim GE**, Huang GJ, Flora N, LeRoith D, Rhodes CJ, Brubaker PL. Insulin regulates glucagon-like peptide-1 secretion from the enteroendocrine L cell. *Endocrinology* 2009; **150**: 580-591 [PMID: 18818290 DOI: 10.1210/en.2008-0726]

105 **Larsen PJ**, Tang-Christensen M, Jessop DS. Central administration of glucagon-like peptide-1 activates hypothalamic neuroendocrine neurons in the rat. *Endocrinology* 1997; **138**: 4445-4455 [PMID: 9322962]

106 **Simrén M**, Björnsson ES, Abrahamsson H. High interdigestive and postprandial motilin levels in patients with the irritable bowel syndrome. *Neurogastroenterol Motil* 2005; **17**: 51-57 [PMID: 15670264 DOI: 10.1111/j.1365-2982.2004.00582.x]

107 **Zhang H**, Yan Y, Shi R, Lin Z, Wang M, Lin L. Correlation of gut hormones with irritable bowel syndrome. *Digestion* 2008; **78**: 72-76 [PMID: 18948690 DOI: 10.1159/000165352]

108 **Simrén M**, Stotzer PO, Sjövall H, Abrahamsson H, Björnsson ES. Abnormal levels of neuropeptide Y and peptide YY in the colon in irritable bowel syndrome. *Eur J Gastroenterol Hepatol* 2003; **15**: 55-62 [PMID: 12544695]

109 **Matricon J**, Meleine M, Gelot A, Piche T, Dapoigny M, Muller E, Ardid D. Review article: Associations between immune activation, intestinal permeability and the irritable

bowel syndrome. *Aliment Pharmacol Ther* 2012; **36**: 1009-1031 [PMID: 23066886 DOI: 10.1111/apt.12080]
110 **Schoepfer AM**, Trummler M, Seeholzer P, Seibold-Schmid B, Seibold F. Discriminating IBD from IBS: comparison of the test performance of fecal markers, blood leukocytes, CRP, and IBD antibodies. *Inflamm Bowel Dis* 2008; **14**: 32-39 [PMID: 17924558 DOI: 10.1002/ibd.20275]
111 **Sidhu R**, Drew K, McAlindon ME, Lobo AJ, Sanders DS. Elevated serum chromogranin A in irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD): a shared model for pathogenesis? *Inflamm Bowel Dis* 2010; **16**: 361 [PMID: 19575362 DOI: 10.1002/ibd.20982]
112 **Marshall JK**, Thabane M, Garg AX, Clark WF, Salvadori M, Collins SM. Incidence and epidemiology of irritable bowel syndrome after a large waterborne outbreak of bacterial dysentery. *Gastroenterology* 2006; **131**: 445-450; quiz 660 [PMID: 16890598]
113 **Halvorson HA**, Schlett CD, Riddle MS. Postinfectious irritable bowel syndrome--a meta-analysis. *Am J Gastroenterol* 2006; **101**: 1894-1899; quiz 1942 [PMID: 16928253]
114 **Gwee KA**, Leong YL, Graham C, McKendrick MW, Collins SM, Walters SJ, Underwood JE, Read NW. The role of psychological and biological factors in postinfective gut dysfunction. *Gut* 1999; **44**: 400-406 [PMID: 10026328]
115 **Kai Y**, Takahashi I, Ishikawa H, Hiroi T, Mizushima T, Matsuda C, Kishi D, Hamada H, Tamagawa H, Ito T, Yoshizaki K, Kishimoto T, Matsuda H, Kiyono H. Colitis in mice lacking the common cytokine receptor gamma chain is mediated by IL-6-producing CD4+ T cells. *Gastroenterology* 2005; **128**: 922-934 [PMID: 15825075]
116 **Wang LH**, Fang XC, Pan GZ. Bacillary dysentery as a causative factor of irritable bowel syndrome and its pathogenesis. *Gut* 2004; **53**: 1096-1101 [PMID: 15247174 DOI: 10.1136/gut.2003.021154]
117 **Neal KR**, Hebden J, Spiller R. Prevalence of gastrointestinal symptoms six months after bacterial gastroenteritis and risk factors for development of the irritable bowel syndrome: postal survey of patients. *BMJ* 1997; **314**: 779-782 [PMID: 9080994]
118 **Chadwick VS**, Chen W, Shu D, Paulus B, Bethwaite P, Tie A, Wilson I. Activation of the mucosal immune system in irritable bowel syndrome. *Gastroenterology* 2002; **122**: 1778-1783 [PMID: 12055584]
119 **Goral V**, Kucukoner M, Buyukbayram H. Mast cells count and serum cytokine levels in patients with irritable bowel syndrome. *Hepatogastroenterology* 2010; **57**: 751-754 [PMID: 21033222]
120 **Chang L**, Adeyemo M, Karagiannides I, Videlock EJ, Bowe C, Shih W, Presson AP, Yuan PQ, Cortina G, Gong H, Singh S, Licudine A, Mayer M, Tache Y, Pothoulakis C, Mayer EA. Serum and colonic mucosal immune markers in irritable bowel syndrome. *Am J Gastroenterol* 2012; **107**: 262-272 [PMID: 22158028 DOI: 10.1038/ajg.2011.423]
121 **Barbara G**, Wang B, Stanghellini V, de Giorgio R, Cremon C, Di Nardo G, Trevisani M, Campi B, Geppetti P, Tonini M, Bunnett NW, Grundy D, Corinaldesi R. Mast cell-dependent excitation of visceral-nociceptive sensory neurons in irritable bowel syndrome. *Gastroenterology* 2007; **132**: 26-37 [PMID: 17241857]
122 **Collins SM**. The immunomodulation of enteric neuromuscular function: implications for motility and inflammatory disorders. *Gastroenterology* 1996; **111**: 1683-1699 [PMID: 8942751]
123 **McKernan DP**, Gaszner G, Quigley EM, Cryan JF, Dinan TG. Altered peripheral toll-like receptor responses in the irritable bowel syndrome. *Aliment Pharmacol Ther* 2011; **33**: 1045-1052 [PMID: 21453321 DOI: 10.1111/j.1365-2036.2011.04624.x]
124 **Dinan TG**, Clarke G, Quigley EM, Scott LV, Shanahan F, Cryan J, Cooney J, Keeling PW. Enhanced cholinergic-mediated increase in the pro-inflammatory cytokine IL-6 in irritable bowel syndrome: role of muscarinic receptors. *Am J Gastroenterol* 2008; **103**: 2570-2576 [PMID: 18785949]
125 **Liebregts T**, Adam B, Bredack C, Röth A, Heinzel S, Lester S, Downie-Doyle S, Smith E, Drew P, Talley NJ, Holtmann G. Immune activation in patients with irritable bowel syndrome. *Gastroenterology* 2007; **132**: 913-920 [PMID: 17383420]
126 **Kindt S**, Van Oudenhove L, Broekaert D, Kasran A, Ceuppens JL, Bossuyt X, Fischler B, Tack J. Immune dysfunction in patients with functional gastrointestinal disorders. *Neurogastroenterol Motil* 2009; **21**: 389-398 [PMID: 19126184 DOI: 10.1111/j.1365-2982.2008.01220.x]
127 **Elsenbruch S**, Holtmann G, Oezcan D, Lysson A, Janssen O, Goebel MU, Schedlowski M. Are there alterations of neuroendocrine and cellular immune responses to nutrients in women with irritable bowel syndrome? *Am J Gastroenterol* 2004; **99**: 703-710 [PMID: 15089905 DOI: 10.1111/j.1572-0241.2004.04138.x]
128 **Coëffier M**, Gloro R, Boukhettala N, Aziz M, Lecleire S, Vandaele N, Antonietti M, Savoye G, Bôle-Feysot C, Déchelotte P, Reimund JM, Ducrotté P. Increased proteasome-mediated degradation of occludin in irritable bowel syndrome. *Am J Gastroenterol* 2010; **105**: 1181-1188 [PMID: 19997094]
129 **Macsharry J**, O'Mahony L, Fanning A, Bairead E, Sherlock G, Tiesman J, Fulmer A, Kiely B, Dinan TG, Shanahan F, Quigley EM. Mucosal cytokine imbalance in irritable bowel syndrome. *Scand J Gastroenterol* 2008; **43**: 1467-1476 [PMID: 18752146]
130 **Barbara G**, Stanghellini V, De Giorgio R, Cremon C, Cottrell GS, Santini D, Pasquinelli G, Morselli-Labate AM, Grady EF, Bunnett NW, Collins SM, Corinaldesi R. Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. *Gastroenterology* 2004; **126**: 693-702 [PMID: 14988823]
131 **Buhner S**, Li Q, Vignali S, Barbara G, De Giorgio R, Stanghellini V, Cremon C, Zeller F, Langer R, Daniel H, Michel K, Schemann M. Activation of human enteric neurons by supernatants of colonic biopsy specimens from patients with irritable bowel syndrome. *Gastroenterology* 2009; **137**: 1425-1434 [PMID: 19596012]
132 **Galli SJ**, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. *Nat Immunol* 2005; **6**: 135-142 [PMID: 15662442]
133 **O'Malley D**, Liston M, Hyland NP, Dinan TG, Cryan JF. Colonic soluble mediators from the maternal separation model of irritable bowel syndrome activate submucosal neurons via an interleukin-6-dependent mechanism. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**: G241-G252 [PMID: 21109592]
134 **Xia Y**, Hu HZ, Liu S, Ren J, Zafirov DH, Wood JD. IL-1beta and IL-6 excite neurons and suppress nicotinic and noradrenergic neurotransmission in guinea pig enteric nervous system. *J Clin Invest* 1999; **103**: 1309-1316 [PMID: 10225974]
135 **Rehn M**, Hübschle T, Diener M. TNF-alpha hyperpolarizes membrane potential and potentiates the response to nicotinic receptor stimulation in cultured rat myenteric neurones. *Acta Physiol Scand* 2004; **181**: 13-22 [PMID: 15086448]
136 **Zhang L**, Hu L, Chen M, Yu B. Exogenous interleukin-6 facilitated the contraction of the colon in a depression rat model. *Dig Dis Sci* 2013; **58**: 2187-2196 [PMID: 23589140 DOI: 10.1007/s10620-013-2656-3]
137 **Natale L**, Piepoli AL, De Salvia MA, De Salvatore G, Mitolo CI, Marzullo A, Portincasa P, Moschetta A, Palasciano G, Mitolo-Chieppa D. Interleukins 1 beta and 6 induce functional alteration of rat colonic motility: an in vitro study. *Eur J Clin Invest* 2003; **33**: 704-712 [PMID: 12864781]
138 **Kindt S**, Vanden Berghe P, Boesmans W, Roosen L, Tack J. Prolonged IL-1beta exposure alters neurotransmitter and electrically induced Ca(2+) responses in the myenteric plexus. *Neurogastroenterol Motil* 2010; **22**: 321-e85 [PMID: 19796332]

139 **Rühl A**, Hurst S, Collins SM. Synergism between interleukins 1 beta and 6 on noradrenergic nerves in rat myenteric plexus. *Gastroenterology* 1994; **107**: 993-1001 [PMID: 7926489]

140 **De Jongh RF**, Vissers KC, Meert TF, Booij LH, De Deyne CS, Heylen RJ. The role of interleukin-6 in nociception and pain. *Anesth Analg* 2003; **96**: 1096-1103, table of contents [PMID: 12651667]

141 **Dunlop SP**, Hebden J, Campbell E, Naesdal J, Olbe L, Perkins AC, Spiller RC. Abnormal intestinal permeability in subgroups of diarrhea-predominant irritable bowel syndromes. *Am J Gastroenterol* 2006; **101**: 1288-1294 [PMID: 16771951]

142 **Piche T**, Barbara G, Aubert P, Bruley des Varannes S, Dainese R, Nano JL, Cremon C, Stanghellini V, De Giorgio R, Galmiche JP, Neunlist M. Impaired intestinal barrier integrity in the colon of patients with irritable bowel syndrome: involvement of soluble mediators. *Gut* 2009; **58**: 196-201 [PMID: 18824556]

143 **Hiribarren A**, Heyman M, L'Helgouac'h A, Desjeux JF. Effect of cytokines on the epithelial function of the human colon carcinoma cell line HT29 cl 19A. *Gut* 1993; **34**: 616-620 [PMID: 8504961]

144 **Zhou Q**, Zhang B, Verne GN. Intestinal membrane permeability and hypersensitivity in the irritable bowel syndrome. *Pain* 2009; **146**: 41-46 [PMID: 19595511]

145 **Cremonini F**, Talley NJ. Treatments targeting putative mechanisms in irritable bowel syndrome. *Nat Clin Pract Gastroenterol Hepatol* 2005; **2**: 82-88 [PMID: 16265125]

146 **Bravo JA**, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, Bienenstock J, Cryan JF. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci USA* 2011; **108**: 16050-16055 [PMID: 21876150 DOI: 10.1073/pnas.1102999108]

147 **Mättö J**, Maunuksela L, Kajander K, Palva A, Korpela R, Kassinen A, Saarela M. Composition and temporal stability of gastrointestinal microbiota in irritable bowel syndrome--a longitudinal study in IBS and control subjects. *FEMS Immunol Med Microbiol* 2005; **43**: 213-222 [PMID: 15747442]

148 **Malinen E**, Rinttilä T, Kajander K, Mättö J, Kassinen A, Krogius L, Saarela M, Korpela R, Palva A. Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. *Am J Gastroenterol* 2005; **100**: 373-382 [PMID: 15667495 DOI: 10.1111/j.1572-0241.2005.40312.x]

149 **Kassinen A**, Krogius-Kurikka L, Mäkivuokko H, Rinttilä T, Paulin L, Corander J, Malinen E, Apajalahti J, Palva A. The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. *Gastroenterology* 2007; **133**: 24-33 [PMID: 17631127]

150 **Jeffery IB**, O'Toole PW, Öhman L, Claesson MJ, Deane J, Quigley EM, Simrén M. An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. *Gut* 2012; **61**: 997-1006 [PMID: 22180058 DOI: 10.1136/gutjnl-2011-301501]

151 **Yoon JS**, Sohn W, Lee OY, Lee SP, Lee KN, Jun DW, Lee HL, Yoon BC, Choi HS, Chung WS, Seo JG. Effect of multispecies probiotics on irritable bowel syndrome: a randomized, double-blind, placebo-controlled trial. *J Gastroenterol Hepatol* 2014; **29**: 52-59 [PMID: 23829297 DOI: 10.1111/jgh.12322]

152 **O'Mahony L**, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, O'Sullivan GC, Kiely B, Collins JK, Shanahan F, Quigley EM. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. *Gastroenterology* 2005; **128**: 541-551 [PMID: 15765388]

153 **Prakash S**, Rodes L, Coussa-Charley M, Tomaro-Duchesneau C. Gut microbiota: next frontier in understanding human health and development of biotherapeutics. *Biologics* 2011; **5**: 71-86 [PMID: 21847343 DOI: 10.2147/BTT.S19099]

154 **O'Mahony C**, Scully P, O'Mahony D, Murphy S, O'Brien F, Lyons A, Sherlock G, MacSharry J, Kiely B, Shanahan F, O'Mahony L. Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB activation. *PLoS Pathog* 2008; **4**: e1000112 [PMID: 18670628 DOI: 10.1371/journal.ppat.1000112]

155 **Lee BJ**, Bak YT. Irritable bowel syndrome, gut microbiota and probiotics. *J Neurogastroenterol Motil* 2011; **17**: 252-266 [PMID: 21860817 DOI: 10.5056/jnm.2011.17.3.252]

156 **Begtrup LM**, de Muckadell OB, Kjeldsen J, Christensen RD, Jarbøl DE. Long-term treatment with probiotics in primary care patients with irritable bowel syndrome--a randomised, double-blind, placebo controlled trial. *Scand J Gastroenterol* 2013; **48**: 1127-1135 [PMID: 23957590 DOI: 10.3109/00365521.2013.825314]

157 **Roberts LM**, McCahon D, Holder R, Wilson S, Hobbs FD. A randomised controlled trial of a probiotic 'functional food' in the management of irritable bowel syndrome. *BMC Gastroenterol* 2013; **13**: 45 [PMID: 23496803 DOI: 10.1186/1471-230X-13-45]

158 **Bloom JR**, Stewart SL, Chang S, Banks PJ. Then and now: quality of life of young breast cancer survivors. *Psychooncology* 2004; **13**: 147-160 [PMID: 15022150 DOI: 10.1002/pon.794]

159 **Mayer EA**. The neurobiology of stress and gastrointestinal disease. *Gut* 2000; **47**: 861-869 [PMID: 11076888]

160 **Barbara G**, Stanghellini V, Cremon C, De Giorgio R, Gargano L, Cogliandro R, Pallotti F, Corinaldesi R. Probiotics and irritable bowel syndrome: rationale and clinical evidence for their use. *J Clin Gastroenterol* 2008; **42** Suppl 3 Pt 2: S214-S217 [PMID: 18685517]

161 **Dunlop SP**, Jenkins D, Neal KR, Spiller RC. Relative importance of enterochromaffin cell hyperplasia, anxiety, and depression in postinfectious IBS. *Gastroenterology* 2003; **125**: 1651-1659 [PMID: 14724817]

162 **Gwee KA**, Graham JC, McKendrick MW, Collins SM, Marshall JS, Walters SJ, Read NW. Psychometric scores and persistence of irritable bowel after infectious diarrhoea. *Lancet* 1996; **347**: 150-153 [PMID: 8544549]

163 **Pawlak CR**, Jacobs R, Mikeska E, Ochsmann S, Lombardi MS, Kavelaars A, Heijnen CJ, Schmidt RE, Schedlowski M. Patients with systemic lupus erythematosus differ from healthy controls in their immunological response to acute psychological stress. *Brain Behav Immun* 1999; **13**: 287-302 [PMID: 10600217]

164 **Ackerman KD**, Martino M, Heyman R, Moyna NM, Rabin BS. Immunologic response to acute psychological stress in MS patients and controls. *J Neuroimmunol* 1996; **68**: 85-94 [PMID: 8784264]

165 **Leonard BE**. Changes in the immune system in depression and dementia: causal or co-incidental effects? *Int J Dev Neurosci* 2001; **19**: 305-312 [PMID: 11337199]

166 **Zheng PY**, Feng BS, Oluwole C, Struiksma S, Chen X, Li P, Tang SG, Yang PC. Psychological stress induces eosinophils to produce corticotrophin releasing hormone in the intestine. *Gut* 2009; **58**: 1473-1479 [PMID: 19651632 DOI: 10.1136/gut.2009.181701]

167 **Söderholm JD**, Yang PC, Ceponis P, Vohra A, Riddell R, Sherman PM, Perdue MH. Chronic stress induces mast cell-dependent bacterial adherence and initiates mucosal inflammation in rat intestine. *Gastroenterology* 2002; **123**: 1099-1108 [PMID: 12360472]

168 **Song C**, Earley B, Leonard BE. Behavioral, neurochemical, and immunological responses to CRF administration. Is CRF a mediator of stress? *Ann N Y Acad Sci* 1995; **771**: 55-72 [PMID: 8597429]

169 **O'Malley D**, Cryan JF, Dinan TG. Crosstalk between interleukin-6 and corticotropin-releasing factor modulate submucosal plexus activity and colonic secretion. *Brain Behav Immun* 2013; **30**: 115-124 [PMID: 23369733 DOI: 10.1016/j.bbi.2013.01.078]

170 **Fekete EM**, Zorrilla EP. Physiology, pharmacology, and therapeutic relevance of urocortins in mammals: ancient CRF paralogs. *Front Neuroendocrinol* 2007; **28**: 1-27 [PMID: 17083971]
171 **Wu Y**, Hu J, Zhang R, Zhou C, Xu Y, Guan X, Li S. Enhanced intracellular calcium induced by urocortin is involved in degranulation of rat lung mast cells. *Cell Physiol Biochem* 2008; **21**: 173-182 [PMID: 18209484]
172 **Ando T**, Rivier J, Yanaihara H, Arimura A. Peripheral corticotropin-releasing factor mediates the elevation of plasma IL-6 by immobilization stress in rats. *Am J Physiol* 1998; **275**: R1461-R1467 [PMID: 9791062]
173 **Saruta M**, Takahashi K, Suzuki T, Torii A, Kawakami M, Sasano H. Urocortin 1 in colonic mucosa in patients with ulcerative colitis. *J Clin Endocrinol Metab* 2004; **89**: 5352-5361 [PMID: 15531481]
174 **Tsatsanis C**, Androulidaki A, Dermitzaki E, Charalampopoulos I, Spiess J, Gravanis A, Margioris AN. Urocortin 1 and Urocortin 2 induce macrophage apoptosis via CRFR2. *FEBS Lett* 2005; **579**: 4259-4264 [PMID: 16054139]
175 **Tsatsanis C**, Androulidaki A, Dermitzaki E, Gravanis A, Margioris AN. Corticotropin releasing factor receptor 1 (CRF1) and CRF2 agonists exert an anti-inflammatory effect during the early phase of inflammation suppressing LPS-induced TNF-alpha release from macrophages via induction of COX-2 and PGE2. *J Cell Physiol* 2007; **210**: 774-783 [PMID: 17117478]
176 **O'Malley D**, Dinan TG, Cryan JF. Altered expression and secretion of colonic interleukin-6 in a stress-sensitive animal model of brain-gut axis dysfunction. *J Neuroimmunol* 2011; **235**: 48-55 [PMID: 21565410 DOI: 10.1016/j.jneuroim.2011.04.003]

P- Reviewers: Chen L, Hoensch HP, Gonzalez-Reimers E, Pozo MJ, Zhu YL
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8859

World J Gastroenterol 2014 July 21; 20(27): 8859-8866
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

Microbiota-host interactions in irritable bowel syndrome: Epithelial barrier, immune regulation and brain-gut interactions

Niall P Hyland, Eamonn MM Quigley, Elizabeth Brint

Niall P Hyland, Eamonn MM Quigley, Elizabeth Brint, Alimentary Pharmabiotic Centre, University College Cork, 30 Cork, Ireland
Niall P Hyland, Department of Pharmacology and Therapeutics, University College Cork, 30 Cork, Ireland
Eamonn MM Quigley, Houston Methodist Hospital, Houston, TX 77030, United States
Elizabeth Brint, Department of Pathology, University College Cork, 30 Cork, Ireland
Author contributions: Hyland NP and Quigley EMM contributed equally to this manuscript; all the authors approved the final version.
Correspondence to: Elizabeth Brint, PhD, Department of Pathology, University College Cork, Coláiste Na Hollscoile, Bóthar an Choláiste, Co. Corcaigh, 30 Cork, Ireland. e.brint@ucc.ie
Telephone: +353-21-4205045
Received: November 27, 2013 Revised: March 19, 2014
Accepted: May 12, 2014
Published online: July 21, 2014

Abstract

Irritable bowel syndrome (IBS) is a common, sometimes debilitating, gastrointestinal disorder worldwide. While altered gut motility and sensation, as well as aberrant brain perception of visceral events, are thought to contribute to the genesis of symptoms in IBS, a search for an underlying aetiology has, to date, proven unsuccessful. Recently, attention has been focused on the microbiota as a possible factor in the pathogenesis of IBS. Prompted by a number of clinical observations, such as the recognition of the *de novo* development of IBS following enteric infections, as well as descriptions of changes in colonic bacterial populations in IBS and supported by clinical responses to interventions, such as antibiotics and probiotics, that modify the microbiota, various approaches have been taken to investigating the microbiota-host response in IBS, as well as in animal models thereof. From such studies a considerable body of evidence has accumulated to indicate the activation or upregulation of both factors involved in bacterial engagement with the host as well host defence mechanisms against bacteria. Alterations in gut barrier function, occurring in response, or in parallel, to changes in the microbiota, have also been widely described and can be seen to play a pivotal role in generating and sustaining host immune responses both within and beyond the gut. In this manner a plausible hypothesis, based on an altered microbiota and/or an aberrant host response, for the pathogenesis, of at least some instances of IBS, can be generated.

Key words: Microbiota; Irritable bowel syndrome; Toll-like receptor; Epithelial barrier; Gut-brain axis

Core tip: Recent discoveries have kindled an interest in microbiota-host interactions in irritable bowel syndrome (IBS) and have led to new lines of research into this common and elusive disorder. It is clear that the microbiota is altered in IBS and that such alterations could well contribute to the pathogenesis of the disorder through, for example, increased permeability, an altered immune profile, effects on the central nervous system and modulation of gut neuromuscular function. This review will explore these host-microbe interactions and their relevance to the pathogenesis of IBS. This review will explore these interactions and their relevance to the pathogenesis of IBS.

Hyland NP, Quigley EMM, Brint E. Microbiota-host interactions in irritable bowel syndrome: Epithelial barrier, immune regulation and brain-gut interactions. *World J Gastroenterol* 2014; 20(27): 8859-8866 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8859.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8859

INTRODUCTION

The importance of the microbiota in the pathogenesis of irritable bowel syndrome (IBS) has only recently begun to be understood with alterations in the composition of the gut microbiota being increasingly investigated as a factor in the pathogenesis and pathophysiology of IBS. The human microbiota is a complex ecosystem which may contain as many as 1000 to 1150 bacterial species and between 10^{13} to 10^{14} microorganisms with the greatest density and diversity of bacteria being found in the distal small bowel and colon[1]. The number of bacteria within the gut is about 10 times that of all cells in the human body. While data remains limited, it is evident that IBS patients have an altered microbiota relative to healthy individuals. Bacterial diversity is reduced[2] and more detailed analyses have identified differences at species and strain level[3] among both children and adults with IBS. Not surprisingly, given the heterogeneity of the IBS phenotype, these results have not been consistent and the sizes of the study populations involved have not been large enough to encompass the entire symptom and demographic spectrum that is IBS. Other clinical evidence also supports a role for the microbiota in IBS, including the role of enteric infections as well as the well documented symptom responses to antibiotics, such as rifaximin, and certain probiotics[4].

IBS is one of the most common gastrointestinal ailments worldwide affecting anywhere from 5%-15% of adults in the general population[5]. Despite considerable effort, a biomarker(s) specific for IBS has not been identified[6] and its definition remains entirely clinical, based on the presence of abdominal pain/or discomfort associated with altered bowel habit, often accompanied by symptoms of bloating and distension[7]. The spectrum of symptom severity in IBS is broad with the majority of those affected never seeking medical advice but self-medicating or instituting dietary or life-style measures to control symptoms. At the other end of the spectrum are a smaller number of affected individuals whose symptoms are debilitating and impose a very significant impact on quality of life. IBS is commonly associated with other gastrointestinal ailments such as gastroesophageal reflux, functional dyspepsia and extra-intestinal disorders[8]. Over the years, altered motility, visceral hypersensitivity, immune alterations and, more recently, compromised epithelial barrier function have all been invoked to explain the genesis of symptoms in IBS. Whether considered individually or collectively, these factors undoubtedly play a role in the onset and exacerbation of symptoms in IBS, although none can satisfactorily claim to be a fundamental cause of IBS[9]. Indeed, one of the few true causes of IBS that has been identified is enteric infection; several large series attest to the de novo development of IBS following acute enteric bacterial, viral and parasitic infections[10]. This latter observation kindled an interest in microbiota-host interactions in IBS and has led to a new and surprising line of research into this common and elusive disorder. This review will explore these interactions and their relevance to the pathogenesis of IBS.

INTESTINAL EPITHELIAL BARRIER: AN INTERFACE FOR HOST-MICROBE INTERACTIONS IN IBS

Given the size of the intestine and the density of the commensal flora, the gut represents an enormous interface between the host and its' environment, and, thereby, functions as a barrier between the external environment and the internal milieu and is essential in maintaining health and preventing disease[11]. The intestinal epithelial barrier comprises a thick mucus layer and a single layer of intestinal epithelial cells (IECs) which separate commensal bacteria from the underlying submucosa and as such are a critical component of commensal-host interactions[12]. It is now well understood that IECs are not an inert component of this interaction but are both effected by, and themselves effect, the microbiota. The commensal flora has been shown to directly affect the epithelial barrier through its regulation of tight junction proteins. Examples of this include, increased expression and distribution of zonula ocludin-2[13] as well asupregulation of other gap junction proteins such as occudin2 and claudin-2 in response to a number of probiotic bacteria in several IECs[14]. Commensal flora also contribute to the production of mucus as the mucus layer is considerably reduced in the gut of germ-free mice, but recovers on exposure to bacterial products[15,16]. Given the influence of microbes on the integrity of the intestinal epithelium, this may be of relevance in the context of the compromised epithelial barrier and alterations in permeability observed in IBS[17,18]. The mechanisms underlying this increased permeability in IBS include alterations in tight junction protein expression, localisation or function, changes in the microbiota, presence of active inflammation and/or presence of pro-inflammatory cytokines and increased cell shedding[19]. In particular, reduction of the tight junction protein zonula ocludin-1 (ZO-1) and disruption of apical expression of claudin-1, occludin and ZO-1 have been observed in IBS[20,21]. In addition, single nucleotide polymorphisms in the gene encoding the tight junction protein E-cadherin (CDH1) are associated with an increased risk for the development of post-infectious IBS[22]. Of particular note, is the relationship between increased permeability and the severity of abdominal pain experienced by IBS patients[23]. Moreover, in IBS patients, Zeng and colleagues partially reversed changes in small intestinal permeability with a probiotic cocktail[24]. This increased permeability of the barrier seen in IBS patients may also contribute to the low-grade inflammation that characterises this syndrome, due to increased bacterial translocation[25].

COMMENSAL REGULATION OF IMMUNITY: RELEVANCE FOR IBS

The mucosal surface of the intestinal epithelium has evolved to allow the correct balance of responsiveness, being broadly unresponsive to the presence of the commensal bacteria in the gut lumen whilst still being able to mount an immune response to the presence of pathogenic bacteria[26]. How commensals and the immune system achieve this balance is an area of on-going investigation[27]. It seems likely that no single mechanism applies to all commensals; different strains or species employ different strategies. Nonetheless, a range of potential mechanisms have been identified[28,29]. For example, *Bifidobacterium infantis* prevents nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and interleukin (IL)-8 activation and also inhibits the secretion of the chemokine CCL20 in response to *Salmonella typhimurium*, *Clostridium difficile*, *Mycobacterium paratuberculosis* and, even, bacterial flagellin[30,31]. Some strains, indeed, appear to exert potent anti-inflammatory effects: in an experimental animal (IL-10 knockout) model of colitis, both a *Lactobacillus* and a *Bifidobacterium* suppressed the production of the pro-inflammatory cytokines interferon-γ, tumor necrosis factor-α, and IL-12, while levels of the anti-inflammatory cytokine transforming growth factor-β were maintained[32]. Similar effects have been demonstrated for the probiotic cocktail VSL#3 in experimental models of colitis[33,34]. What is very exciting is the observation, again in an animal model, of the ability of orally administered probiotics to exert anti-inflammatory effects at sites well distant from the gut[35]. These differential cytokine responses to commensals and pathogens have also been demonstrated in man[36].

Immunological alterations are increasingly being reported in IBS with the hypothesis that there is a low-grade inflammatory state associated with this condition. Investigation of the role of the microbiota in mediating these immune alterations in IBS are in their infancy, but further study may provide some insight into the pathogenesis of IBS. Accumulating data support the presence of an immune engagement between the microbiota and the host in IBS; an interaction that involves both systemic and mucosal immunity that could generate a low-grade inflammatory response.

Toll like receptors, mucosal immunity and IBS

A number of factors may allow the epithelium to tolerate commensal organisms with the innate immune system and pattern recognition receptors (PPRs) playing a critical role. PPRs, such as toll-like receptors (TLRs), mediate the interaction between the host and the microbiota and, in doing so, facilitate both inflammatory and homeostatic processes[37]. Indeed commensal-TLR interactions in the intestine have been predominantly implicated in homeostatic events[38]. Commensal signalling through TLRs results in the inhibition of the NF-κB inflammatory pathway[39] and also in the upregulation of TLR inhibitory proteins such as PPARγ. For one commensal, *Bacteroides fragilis*, symbiosis with the host has been shown to be mediated through the activation of TLR2 on Foxp3$^+$ regulatory T cells by a PSA, produced by the bacterium, resulting in immunological tolerance[40]. The intimacy of the interaction between the microbiota and these PPR's is also illustrated by the observation that the microbiota determines expression of TLR2 in the colon[41].

Expression of TLRs has also been recently reported to be altered in IBS. Increased levels of TLRs 4 and 5 and decreased levels of TLRs 7 and 8 have been shown in colonic biopsy tissue of IBS patients[42]. The work of Belmonte *et al*[43] further characterised these changes according to IBS subtype and showed that only the IBS-mixed subgroup showed upregulation of TLRs 2 and 4. These authors also showed the alterations in expression were confined to epithelial cells. Similar alterations in expression of TLRs have been shown in a rat model of stress-induced IBS[44]. Work performed by Tattoli *et al*[45] has further demonstrated that TLR ligands can directly affect gastrointestinal motility possibly implying that disruptions in the composition of the microbiota may result in changes in gut motility, as observed in IBS patients. The colonic mucosal tissue from IBS patients also displays an altered cytokine profile possibly reflecting the alterations in TLR expression[46]. And, whilst evidence has been advanced to indicate that alterations in the microbiota are present in IBS[47], how such changes might directly affect TLR expression and cytokine production in these patients remains unclear. Moreover, the microbiota may also have the capacity to influence expression of non-TLR receptors, such as μ-opioid and cannabinoid receptors, in IECs which may be equally relevant in the context of IBS[48].

Commensals, systemic immunity and IBS

In addition to the ability of the microbiota to modulate local mucosal immune responses, extensive clinical and experimental data have been generated to indicate that commensal bacteria can also modify systemic immune responses[49]. Commensals may promote the development of T helper cells, including T_H17 cells and result in a controlled inflammatory response which is protective against pathogens, in part, through the production of IL-17[50]. Commensals, such as *Bifidobacterium infantis* and *Faecalobacterium prasunitzii*, differentially induce regulatory T cells (Tregs) and result in the production of the anti-inflammatory cytokine, IL-10[51]. Similarly, colonization of mice with *Bifidobacterium fragilis* resulted in the expansion of IL-10 producing Tregs and amelioration of the disease experimental autoimmune encephalomyelitis in a mouse model[52]. The regulation of immunity by commensals is likely to occur, not only *via* TLRs, but also through a variety of commensal-derived substances, ranging from relatively nonspecific fatty acids and peroxides to highly specific bacteriocins[53,54], which can inhibit or kill other, potentially pathogenic, bacteria[28]; meanwhile certain strains produce proteases capable of denaturing bacterial

toxins[55].

Systemic immune alterations have also been observed in IBS. B cells isolated from the blood of IBS patients display an amplified activation level[56]. Similarly, T cells isolated from both blood and colonic biopsies showed increased activation levels in IBS patients compared to healthy controls; evidenced by increased expression of the activation markers CD69 and HLA-DR[57]. Increased levels of antibodies to bacterial flagellin[58,59] and elevated levels of beta-defensin-2 in the faeces have also been demonstrated in IBS[60]. In addition, the ratio of IL-10 to IL-12 cytokines from peripheral monocytes is decreased in IBS patients compared to healthy controls; this ratio was normalised following treatment with *Bifidobacterium infantis*[61].

COMMENSAL REGULATION OF THE GUT-BRAIN AXIS: RELEVANCE FOR IBS

The ability of gut microbiota to communicate with the brain and thus modulate behaviour is emerging as an exciting concept in health and disease. Indeed, it has been proposed that the microbiota can influence the development[62] and function[63] of the central nervous system (CNS), thereby, leading to the concept of the microbiota-gut-brain axis[64,65]. Studies focusing on the impact of enteric microbiota on the host and, in particular, on the CNS are essential to our understanding of how the gut-brain axis may influence the pathogenesis of IBS[64]. Moreover, functionally, an association between psychological stress, intestinal transit and "dysbacteriosis" has been reported[66].

Influence of commensals on the central nervous system

There is clear evidence of communication between commensals and the CNS facilitated through neuroendocrine, neuroimmune, the autonomic nervous system and the enteric nervous system (ENS), collectively forming complex networks. This communication functions bidirectionally with the microbiota influencing CNS function and *vice versa*[67]. For example, oral administration of *Bifidobacterium infantis* 35624 influences the concentrations of 5-hydroxyindole acetic acid and dihydroxyphenylacetic acid in the frontal cortex and amygdala, respectively[68]. Moreover, *Bifidobacterium infantis* 35624 has been shown, in an animal model of depression and visceral hypersensitivity (the maternally-separated rat), to normalise immune responses, reverse behavioural deficits and restore basal norepinephrine concentrations in the brainstem[68]. A more recent study, describing the effects of *Lactobacillus rhamnosus* (*JB-1*) on behaviour and central expression of gamma aminobutyric acid receptors, demonstrated these effects to be vagal-dependent thereby establishing the vagus nerve as a key pathway in transducing microbe-gut to brain signals[69]. Germ-free models have also proven to be a useful tool in interrogating the influence of the gut microbiota on central nervous system function. For example, germ free mice display altered central expression of the neurotropic factor; brain derived neurotropic factor, as well as serotonin. Moreover, the latter was resistant to restoration of the microbiota in adulthood[70], implicating a role for the microbiota in early-life development and its absence with persistent long-term effects on hippocampal gene expression. The first clinical study demonstrating the influence of commensal organisms on brain activity, using a probiotic cocktail, is of particular relevance to IBS. Healthy female subjects, who consumed the probiotic cocktail containing *Bifidobacterium animalis* subsp *Lactis*, *Streptococcus thermophiles*, *Lactobacillus bulgaricus*, and *Lactococcus lactis* subsp *Lactis* twice daily for four weeks, exhibited altered activity in brain regions that control central processing of emotion and sensation[71], areas of particular relevance in the context of IBS. Collectively, these latter observations could address some of the proposed pathophysiological mechanisms associated with symptom development in IBS, namely, disturbances in the brain-gut axis.

Influence of commensals on the enteric nervous system and neuromuscular function

The ENS and human smooth muscle cells, key regulators of intestinal motility, express the machinery necessary to respond directly to commensals[72,73]. Therefore, commensals have the capacity to influence neuromuscular function, indicating a role for this interaction in IBS. Direct influence on the ENS can be inferred by studies examining peripheral TLR expression. TLR-4 and TLRs-3 and -7 are expressed in the ENS of both the murine and human intestine and colon[72]. Moreover, studies on human smooth muscle cells suggest that a direct interaction between these and the microbiota is possible, as stimulation of TLR4 induced inhibition of smooth muscle contractility[73]. While other studies have demonstrated that commensal organisms may influence neurotransmitter release and production of gamma-aminobutyric acid[74]. Of more direct relevance to IBS, manipulating the host-microbiota interaction to improve neuromuscular function was demonstrated in a study using *Lactobacillus paracasei,* in which the bacterium attenuated gut muscle hypercontractility in an animal model of post-infectious IBS[75]. This effect was strain-dependent and appeared to be mediated, in part, through a modulation of the immunological response to the initial infection and, in part, through the direct effects of the organism, or a metabolite thereof, on gut muscle. Additionally, studies interrogating the effects of several microbes on intestinal motility in germ-free animals highlight the selective and divergent effects of individual strains on intestinal motor function, with some, but not all strains, influencing transit[76].

Indirect interactions are mediated through commensal-derived factors including methane (CH_4), hydrogen sulphide (H_2S) and short-chain fatty acids. Noteworthy, in the context of IBS, levels of *Methanobrevibacter smithii* in the stools of constipation-predominant IBS patients correlate with levels of CH_4 production[77], suggesting that, in a subgroup of constipation-predominant IBS patients,

bacterial-derived CH_4 contributes to the pathophysiology of the disorder. Moreover, CH_4, produced mainly by *Methanobrevibacter. smithii* in humans, has been associated with alterations in intestinal motility. In an animal model, CH_4 significantly reduces intestinal transit following *in vivo* infusion[78], and *in vitro* recordings suggesting that one of the mechanisms by which CH_4 influences the ileal contractile response is *via* regulatory control of sensory neurotransmission[79]. Like CH_4, H_2S also exerts an inhibitory effect on intestinal neuromuscular function[80,81]. Sulphate reducing bacteria, responsible for the disposal of H_2, and subsequent generation of H_2S, are also relevant in the context of IBS[82]. H_2S exerts an inhibitory effect on neuromuscular activity[83]. Further studies confirmed an inhibitory role for H_2S on motor complexes and also indicated that this effect was independent of the ENS[84]. Moreover, H_2S-induced inhibitory responses were sensitive to potassium (K) channel and, in particular K_{Ca}^+ channel, blockade in the presence of neural inhibition *in vitro*[84].

One of the principal roles of the colonic microflora is to salvage energy from carbohydrates that have not been digested in the upper gastrointestinal tract, the major end-products of which are short-chain fatty acids (SCFA), in addition to the gaseous end-products H_2, CO_2 and CH_4[85,86]. The SCFAs include acetate, propionate and butyrate, the latter of which has multiple effects in the gastrointestinal tract, including impacts on visceral perception, motility and secreto-motor function[87]. Noteworthy, faecal bacteria from diarrhoea predominant IBS patients produce less SCFA in an *in vitro* fermentation system. Differences in SCFA production by colonic bacterial flora in patients with diarrhoea predominant IBS may be related to the development of gastrointestinal symptoms and, in particular, neuromuscular dysfunction[88]. SCFAs may also influence ENS plasticity through monocarboxylate transporters[89]. However, these plastic changes in the ENS display a level of SCFA specificity, as neither acetate nor propionate alter the neurochemical make-up of the myenteric plexus[89]. Moreover, butyrate also appears to directly influence intracellular calcium concentrations in myenteric neurons[90] as well as activating the G protein coupled receptors, GPR41 and GPR43 which are widely expressed in rat and human colon[91]. However, altering the activity of the microbiota, with prebiotics for example, supports the concept that it is not only the presence or absence of the microbiota that is capable of regulating intestinal motor physiology, but that qualitative changes in the microbiota can alter neuromuscular function[92]. The issue of differentiating between direct effects of the microbiota or its products and the secondary consequences induced by components of the microbiota is one that bedevils the interpretation of many studies in this area.

CONCLUSION

It is clear that the microbiota is altered in IBS and that such alterations could well contribute to the pathogenesis of the disorder through, for example, increased permeability, an altered immune profile, effects on the CNS and modulation of gut neuromuscular function. To date, however, there is a paucity of clinical studies in IBS patients evaluating the effects of selectively manipulating the microbiota based on preclinical evidence leading to a causality dilemma; whether changes in the microbiota are cause or effect in disorders such as IBS. It is quite unlikely in the context of IBS, given its comorbidities and variability in symptom presentation, that a single microbial alteration will be identified as causative for all IBS pathogenesis, or that one microbial intervention will universally improve all symptoms. Rather, several interventions may prove efficacious in ameliorating various subgroups or individual symptoms. Moreover, focus has moved from the description of qualitative changes in the microbiota in IBS to their metabolic activity. Such an approach has only recently been applied in the context of IBS, where the activity of the microbiota was assessed in relation to symptom presentation[93]. This approach now needs to be expanded with the expectation that data from such studies which will not only determine which microbes may be protective, or causative in IBS, but will also identify which metabolites may be effective therapeutically.

REFERENCES

1 **Guarner F**, Malagelada JR. Gut flora in health and disease. *Lancet* 2003; **361**: 512-519 [PMID: 12583961 DOI: 10.1016/S0140-6736(03)12489-0]

2 **Codling C**, O'Mahony L, Shanahan F, Quigley EM, Marchesi JR. A molecular analysis of fecal and mucosal bacterial communities in irritable bowel syndrome. *Dig Dis Sci* 2010; **55**: 392-397 [PMID: 19693670 DOI: 10.1007/s10620-009-0934-x]

3 **Jeffery IB**, Quigley EM, Öhman L, Simrén M, O'Toole PW. The microbiota link to irritable bowel syndrome: an emerging story. *Gut Microbes* 2012; **3**: 572-576 [PMID: 22895081 DOI: 10.4161/gmic.21772]

4 **Quigley EM**. Therapies aimed at the gut microbiota and inflammation: antibiotics, prebiotics, probiotics, synbiotics, anti-inflammatory therapies. *Gastroenterol Clin North Am* 2011; **40**: 207-222 [PMID: 21333908 DOI: 10.1016/j.gtc.2010.12.009]

5 **Choung RS**, Locke GR. Epidemiology of IBS. *Gastroenterol Clin North Am* 2011; **40**: 1-10 [PMID: 21333897 DOI: 10.1016/j.gtc.2010.12.006]

6 **Clarke G**, Quigley EM, Cryan JF, Dinan TG. Irritable bowel syndrome: towards biomarker identification. *Trends Mol Med* 2009; **15**: 478-489 [PMID: 19811951 DOI: 10.1016/j.molmed.2009.08.001]

7 **Longstreth GF**, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology* 2006; **130**: 1480-1491 [PMID: 16678561 DOI: 10.1053/j.gastro.2005.11.061]

8 **Quigley EM**, Abdel-Hamid H, Barbara G, Bhatia SJ, Boeckxstaens G, De Giorgio R, Delvaux M, Drossman DA, Foxx-Orenstein AE, Guarner F, Gwee KA, Harris LA, Hungin AP, Hunt RH, Kellow JE, Khalif IL, Kruis W, Lindberg G, Olano C, Moraes-Filho JP, Schiller LR, Schmulson M, Simrén M, Tzeuton C. A global perspective on irritable bowel syndrome: a consensus statement of the World Gastroenterology Organisation Summit Task Force on irritable bowel syndrome. *J Clin Gastroenterol* 2012; **46**: 356-366 [PMID:

22499071 DOI: 10.1097/MCG.0b013e318247157c]
9 **Quigley EM**. Bugs on the brain; brain in the gut--seeking explanations for common gastrointestinal symptoms. *Ir J Med Sci* 2013; **182**: 1-6 [PMID: 23179664 DOI: 10.1007/s11845-012-0865-y]
10 **Thabane M**, Kottachchi DT, Marshall JK. Systematic review and meta-analysis: The incidence and prognosis of post-infectious irritable bowel syndrome. *Aliment Pharmacol Ther* 2007; **26**: 535-544 [PMID: 17661757 DOI: 10.1111/j.1365-2036.2007.03399.x]
11 **Farhadi A**, Banan A, Fields J, Keshavarzian A. Intestinal barrier: an interface between health and disease. *J Gastroenterol Hepatol* 2003; **18**: 479-497 [PMID: 12702039]
12 **Goto Y**, Ivanov II. Intestinal epithelial cells as mediators of the commensal-host immune crosstalk. *Immunol Cell Biol* 2013; **91**: 204-214 [PMID: 23318659 DOI: 10.1038/icb.2012.80]
13 **Zyrek AA**, Cichon C, Helms S, Enders C, Sonnenborn U, Schmidt MA. Molecular mechanisms underlying the probiotic effects of Escherichia coli Nissle 1917 involve ZO-2 and PKCzeta redistribution resulting in tight junction and epithelial barrier repair. *Cell Microbiol* 2007; **9**: 804-816 [PMID: 17087734 DOI: 10.1111/j.1462-5822.2006.00836.x]
14 **Ulluwishewa D**, Anderson RC, McNabb WC, Moughan PJ, Wells JM, Roy NC. Regulation of tight junction permeability by intestinal bacteria and dietary components. *J Nutr* 2011; **141**: 769-776 [PMID: 21430248 DOI: 10.3945/jn.110.135657]
15 **Petersson J**, Schreiber O, Hansson GC, Gendler SJ, Velcich A, Lundberg JO, Roos S, Holm L, Phillipson M. Importance and regulation of the colonic mucus barrier in a mouse model of colitis. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**: G327-G333 [PMID: 21109593 DOI: 10.1152/ajpgi.00422.2010]
16 **Ohland CL**, Macnaughton WK. Probiotic bacteria and intestinal epithelial barrier function. *Am J Physiol Gastrointest Liver Physiol* 2010; **298**: G807-G819 [PMID: 20299599 DOI: 10.1152/ajpgi.00243.2009]
17 **Natividad JM**, Verdu EF. Modulation of intestinal barrier by intestinal microbiota: pathological and therapeutic implications. *Pharmacol Res* 2013; **69**: 42-51 [PMID: 23089410 DOI: 10.1016/j.phrs.2012.10.007]
18 **Dunlop SP**, Hebden J, Campbell E, Naesdal J, Olbe L, Perkins AC, Spiller RC. Abnormal intestinal permeability in subgroups of diarrhea-predominant irritable bowel syndromes. *Am J Gastroenterol* 2006; **101**: 1288-1294 [PMID: 16771951 DOI: 10.1111/j.1572-0241.2006.00672.x]
19 **Frese SA**, Benson AK, Tannock GW, Loach DM, Kim J, Zhang M, Oh PL, Heng NC, Patil PB, Juge N, Mackenzie DA, Pearson BM, Lapidus A, Dalin E, Tice H, Goltsman E, Land M, Hauser L, Ivanova N, Kyrpides NC, Walter J. The evolution of host specialization in the vertebrate gut symbiont Lactobacillus reuteri. *PLoS Genet* 2011; **7**: e1001314 [PMID: 21379339 DOI: 10.1371/journal.pgen.1001314]
20 **Bertiaux-Vandaële N**, Youmba SB, Belmonte L, Lecleire S, Antonietti M, Gourcerol G, Leroi AM, Déchelotte P, Ménard JF, Ducrotté P, Coëffier M. The expression and the cellular distribution of the tight junction proteins are altered in irritable bowel syndrome patients with differences according to the disease subtype. *Am J Gastroenterol* 2011; **106**: 2165-2173 [PMID: 22008894 DOI: 10.1038/ajg.2011.257]
21 **Martínez C**, Lobo B, Pigrau M, Ramos L, González-Castro AM, Alonso C, Guilarte M, Guilá M, de Torres I, Azpiroz F, Santos J, Vicario M. Diarrhoea-predominant irritable bowel syndrome: an organic disorder with structural abnormalities in the jejunal epithelial barrier. *Gut* 2013; **62**: 1160-1168 [PMID: 22637702 DOI: 10.1136/gutjnl-2012-302093]
22 **Villani AC**, Lemire M, Thabane M, Belisle A, Geneau G, Garg AX, Clark WF, Moayyedi P, Collins SM, Franchimont D, Marshall JK. Genetic risk factors for post-infectious irritable bowel syndrome following a waterborne outbreak of gastroenteritis. *Gastroenterology* 2010; **138**: 1502-1513 [PMID: 20044998 DOI: 10.1053/j.gastro.2009.12.049]
23 **Nicholson JK**, Holmes E, Lindon JC, Wilson ID. The challenges of modeling mammalian biocomplexity. *Nat Biotechnol* 2004; **22**: 1268-1274 [PMID: 15470467 DOI: 10.1038/nbt1015]
24 **Zeng J**, Li YQ, Zuo XL, Zhen YB, Yang J, Liu CH. Clinical trial: effect of active lactic acid bacteria on mucosal barrier function in patients with diarrhoea-predominant irritable bowel syndrome. *Aliment Pharmacol Ther* 2008; **28**: 994-1002 [PMID: 18671775 DOI: 10.1111/j.1365-2036.2008.03818.x]
25 **Vivinus-Nébot M**, Frin-Mathy G, Bzioueche H, Dainese R, Bernard G, Anty R, Filippi J, Saint-Paul MC, Tulic MK, Verhasselt V, Hébuterne X, Piche T. Functional bowel symptoms in quiescent inflammatory bowel diseases: role of epithelial barrier disruption and low-grade inflammation. *Gut* 2014; **63**: 744-752 [PMID: 23878165 DOI: 10.1136/gutjnl-2012-304066]
26 **Magalhaes JG**, Tattoli I, Girardin SE. The intestinal epithelial barrier: how to distinguish between the microbial flora and pathogens. *Semin Immunol* 2007; **19**: 106-115 [PMID: 17324587 DOI: 10.1016/j.smim.2006.12.006]
27 **Medzhitov R**. Origin and physiological roles of inflammation. *Nature* 2008; **454**: 428-435 [PMID: 18650913 DOI: 10.1038/nature07201]
28 **O'Hara AM**, Shanahan F. Gut microbiota: mining for therapeutic potential. *Clin Gastroenterol Hepatol* 2007; **5**: 274-284 [PMID: 17368226 DOI: 10.1016/j.cgh.2006.12.009]
29 **Thomas CM**, Versalovic J. Probiotics-host communication: Modulation of signaling pathways in the intestine. *Gut Microbes* 2010; **1**: 148-163 [PMID: 20672012]
30 **Lyons A**, O'Mahony D, O'Brien F, MacSharry J, Sheil B, Ceddia M, Russell WM, Forsythe P, Bienenstock J, Kiely B, Shanahan F, O'Mahony L. Bacterial strain-specific induction of Foxp3+ T regulatory cells is protective in murine allergy models. *Clin Exp Allergy* 2010; **40**: 811-819 [PMID: 20067483 DOI: 10.1111/j.1365-2222.2009.03437.x]
31 **Sibartie S**, O'Hara AM, Ryan J, Fanning A, O'Mahony J, O'Neill S, Sheil B, O'Mahony L, Shanahan F. Modulation of pathogen-induced CCL20 secretion from HT-29 human intestinal epithelial cells by commensal bacteria. *BMC Immunol* 2009; **10**: 54 [PMID: 19814810 DOI: 10.1186/1471-2172-10-54]
32 **McCarthy J**, O'Mahony L, O'Callaghan L, Sheil B, Vaughan EE, Fitzsimons N, Fitzgibbon J, O'Sullivan GC, Kiely B, Collins JK, Shanahan F. Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance. *Gut* 2003; **52**: 975-980 [PMID: 12801954]
33 **Rachmilewitz D**, Katakura K, Karmeli F, Hayashi T, Reinus C, Rudensky B, Akira S, Takeda K, Lee J, Takabayashi K, Raz E. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology* 2004; **126**: 520-528 [PMID: 14762789]
34 **Jijon H**, Backer J, Diaz H, Yeung H, Thiel D, McKaigney C, De Simone C, Madsen K. DNA from probiotic bacteria modulates murine and human epithelial and immune function. *Gastroenterology* 2004; **126**: 1358-1373 [PMID: 15131797]
35 **Sheil B**, McCarthy J, O'Mahony L, Bennett MW, Ryan P, Fitzgibbon JJ, Kiely B, Collins JK, Shanahan F. Is the mucosal route of administration essential for probiotic function? Subcutaneous administration is associated with attenuation of murine colitis and arthritis. *Gut* 2004; **53**: 694-700 [PMID: 15082588]
36 **O'Mahony L**, O'Callaghan L, McCarthy J, Shilling D, Scully P, Sibartie S, Kavanagh E, Kirwan WO, Redmond HP, Collins JK, Shanahan F. Differential cytokine response from dendritic cells to commensal and pathogenic bacteria in different lymphoid compartments in humans. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G839-G845 [PMID: 16293657 DOI: 10.1152/ajpgi.00112.2005]
37 **Abreu MT**. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal func-

tion. *Nat Rev Immunol* 2010; **10**: 131-144 [PMID: 20098461 DOI: 10.1038/nri2707]
38 **Kamdar K**, Nguyen V, DePaolo RW. Toll-like receptor signaling and regulation of intestinal immunity. *Virulence* 2013; **4**: 207-212 [PMID: 23334153 DOI: 10.4161/viru.23354]
39 **Neish AS**, Gewirtz AT, Zeng H, Young AN, Hobert ME, Karmali V, Rao AS, Madara JL. Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. *Science* 2000; **289**: 1560-1563 [PMID: 10968793]
40 **Round JL**, Lee SM, Li J, Tran G, Jabri B, Chatila TA, Mazmanian SK. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science* 2011; **332**: 974-977 [PMID: 21512004 DOI: 10.1126/science.1206095]
41 **Wang Y**, Devkota S, Musch MW, Jabri B, Nagler C, Antonopoulos DA, Chervonsky A, Chang EB. Regional mucosa-associated microbiota determine physiological expression of TLR2 and TLR4 in murine colon. *PLoS One* 2010; **5**: e13607 [PMID: 21042588 DOI: 10.1371/journal.pone.0013607]
42 **Brint EK**, MacSharry J, Fanning A, Shanahan F, Quigley EM. Differential expression of toll-like receptors in patients with irritable bowel syndrome. *Am J Gastroenterol* 2011; **106**: 329-336 [PMID: 21102570 DOI: 10.1038/ajg.2010.438]
43 **Belmonte L**, Beutheu Youmba S, Bertiaux-Vandaële N, Antonietti M, Lecleire S, Zalar A, Gourcerol G, Leroi AM, Déchelotte P, Coëffier M, Ducrotté P. Role of toll like receptors in irritable bowel syndrome: differential mucosal immune activation according to the disease subtype. *PLoS One* 2012; **7**: e42777 [PMID: 23028414 DOI: 10.1371/journal.pone.0042777]
44 **McKernan DP**, Nolan A, Brint EK, O'Mahony SM, Hyland NP, Cryan JF, Dinan TG. Toll-like receptor mRNA expression is selectively increased in the colonic mucosa of two animal models relevant to irritable bowel syndrome. *PLoS One* 2009; **4**: e8226 [PMID: 20011045 DOI: 10.1371/journal.pone.0008226]
45 **Tattoli I**, Petitta C, Scirocco A, Ammoscato F, Cicenia A, Severi C. Microbiota, innate immune system, and gastrointestinal muscle: ongoing studies. *J Clin Gastroenterol* 2012; **46** Suppl: S6-S11 [PMID: 22955360 DOI: 10.1097/MCG.0b013e318265ea7d]
46 **Macsharry J**, O'Mahony L, Fanning A, Bairead E, Sherlock G, Tiesman J, Fulmer A, Kiely B, Dinan TG, Shanahan F, Quigley EM. Mucosal cytokine imbalance in irritable bowel syndrome. *Scand J Gastroenterol* 2008; **43**: 1467-1476 [PMID: 18752146 DOI: 10.1080/00365520802276127]
47 **Lee BJ**, Bak YT. Irritable bowel syndrome, gut microbiota and probiotics. *J Neurogastroenterol Motil* 2011; **17**: 252-266 [PMID: 21860817 DOI: 10.5056/jnm.2011.17.3.252]
48 **Rousseaux C**, Thuru X, Gelot A, Barnich N, Neut C, Dubuquoy L, Dubuquoy C, Merour E, Geboes K, Chamaillard M, Ouwehand A, Leyer G, Carcano D, Colombel JF, Ardid D, Desreumaux P. Lactobacillus acidophilus modulates intestinal pain and induces opioid and cannabinoid receptors. *Nat Med* 2007; **13**: 35-37 [PMID: 17159985 DOI: 10.1038/nm1521]
49 **Kuhn KA**, Stappenbeck TS. Peripheral education of the immune system by the colonic microbiota. *Semin Immunol* 2013; **25**: 364-369 [PMID: 24169518 DOI: 10.1016/j.smim.2013.10.002]
50 **Falk PG**, Hooper LV, Midtvedt T, Gordon JI. Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. *Microbiol Mol Biol Rev* 1998; **62**: 1157-1170 [PMID: 9841668]
51 **O'Mahony C**, Scully P, O'Mahony D, Murphy S, O'Brien F, Lyons A, Sherlock G, MacSharry J, Kiely B, Shanahan F, O'Mahony L. Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB activation. *PLoS Pathog* 2008; **4**: e1000112 [PMID: 18670628 DOI: 10.1371/journal.ppat.1000112]
52 **Ochoa-Repáraz J**, Mielcarz DW, Ditrio LE, Burroughs AR, Begum-Haque S, Dasgupta S, Kasper DL, Kasper LH. Central nervous system demyelinating disease protection by the human commensal Bacteroides fragilis depends on polysaccharide A expression. *J Immunol* 2010; **185**: 4101-4108 [PMID: 20817872 DOI: 10.4049/jimmunol.1001443]
53 **Corr SC**, Li Y, Riedel CU, O'Toole PW, Hill C, Gahan CG. Bacteriocin production as a mechanism for the antiinfective activity of Lactobacillus salivarius UCC118. *Proc Natl Acad Sci USA* 2007; **104**: 7617-7621 [PMID: 17456596 DOI: 10.1073/pnas.0700440104]
54 **Rea MC**, Sit CS, Clayton E, O'Connor PM, Whittal RM, Zheng J, Vederas JC, Ross RP, Hill C. Thuricin CD, a post-translationally modified bacteriocin with a narrow spectrum of activity against Clostridium difficile. *Proc Natl Acad Sci USA* 2010; **107**: 9352-9357 [PMID: 20435915 DOI: 10.1073/pnas.0913554107]
55 **Castagliuolo I**, Riegler MF, Valenick L, LaMont JT, Pothoulakis C. Saccharomyces boulardii protease inhibits the effects of Clostridium difficile toxins A and B in human colonic mucosa. *Infect Immun* 1999; **67**: 302-307 [PMID: 9864230]
56 **Ohman L**, Lindmark AC, Isaksson S, Posserud I, Strid H, Sjövall H, Simrén M. B-cell activation in patients with irritable bowel syndrome (IBS). *Neurogastroenterol Motil* 2009; **21**: 644-650, e27 [PMID: 19222763 DOI: 10.1111/j.1365-2982.2009.01272.x]
57 **Ohman L**, Isaksson S, Lindmark AC, Posserud I, Stotzer PO, Strid H, Sjövall H, Simrén M. T-cell activation in patients with irritable bowel syndrome. *Am J Gastroenterol* 2009; **104**: 1205-1212 [PMID: 19367268 DOI: 10.1038/ajg.2009.116]
58 **Cremon C**, Pallotti F, Bacchilega M, Stanghellini V, Corinaldesi R, Barbara G. Antiflagellin antibodies suggest infective participation in irritable bowel syndrome pathogenesis. *Expert Rev Gastroenterol Hepatol* 2008; **2**: 735-740 [PMID: 19090734 DOI: 10.1586/17474124.2.6.735]
59 **Schoepfer AM**, Schaffer T, Seibold-Schmid B, Müller S, Seibold F. Antibodies to flagellin indicate reactivity to bacterial antigens in IBS patients. *Neurogastroenterol Motil* 2008; **20**: 1110-1118 [PMID: 18694443 DOI: 10.1111/j.1365-2982.2008.01166.x]
60 **Langhorst J**, Junge A, Rueffer A, Wehkamp J, Foell D, Michalsen A, Musial F, Dobos GJ. Elevated human beta-defensin-2 levels indicate an activation of the innate immune system in patients with irritable bowel syndrome. *Am J Gastroenterol* 2009; **104**: 404-410 [PMID: 19174795 DOI: 10.1038/ajg.2008.86]
61 **O'Mahony L**, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, O'Sullivan GC, Kiely B, Collins JK, Shanahan F, Quigley EM. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. *Gastroenterology* 2005; **128**: 541-551 [PMID: 15765388]
62 **Diaz Heijtz R**, Wang S, Anuar F, Qian Y, Björkholm B, Samuelsson A, Hibberd ML, Forssberg H, Pettersson S. Normal gut microbiota modulates brain development and behavior. *Proc Natl Acad Sci USA* 2011; **108**: 3047-3052 [PMID: 21282636 DOI: 10.1073/pnas.1010529108]
63 **Neufeld KM**, Kang N, Bienenstock J, Foster JA. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterol Motil* 2011; **23**: 255-264, e119 [PMID: 21054680 DOI: 10.1111/j.1365-2982.2010.01620.x]
64 **Cryan JF**, O'Mahony SM. The microbiome-gut-brain axis: from bowel to behavior. *Neurogastroenterol Motil* 2011; **23**: 187-192 [PMID: 21303428 DOI: 10.1111/j.1365-2982.2010.01664.x]
65 **Fleshner M**. The gut microbiota: a new player in the innate immune stress response? *Brain Behav Immun* 2011; **25**: 395-396 [PMID: 21168480 DOI: 10.1016/j.bbi.2010.12.007]
66 **Wang SX**, Wu WC. Effects of psychological stress on small intestinal motility and bacteria and mucosa in mice. *World J Gastroenterol* 2005; **11**: 2016-2021 [PMID: 15800998]
67 **Cryan JF**, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci* 2012; **13**: 701-712 [PMID: 22968153 DOI: 10.1038/nrn3346]
68 **Desbonnet L**, Garrett L, Clarke G, Kiely B, Cryan JF, Dinan TG. Effects of the probiotic Bifidobacterium infantis in the maternal separation model of depression. *Neuroscience* 2010;

170: 1179-1188 [PMID: 20696216 DOI: 10.1016/j.neuroscience.2010.08.005]
69 **Bravo JA**, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, Bienenstock J, Cryan JF. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci USA* 2011; **108**: 16050-16055 [PMID: 21876150 DOI: 10.1073/pnas.1102999108]
70 **Clarke G**, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, Dinan TG, Cryan JF. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol Psychiatry* 2013; **18**: 666-673 [PMID: 22688187 DOI: 10.1038/mp.2012.77]
71 **Tillisch K**, Labus J, Kilpatrick L, Jiang Z, Stains J, Ebrat B, Guyonnet D, Legrain-Raspaud S, Trotin B, Naliboff B, Mayer EA. Consumption of fermented milk product with probiotic modulates brain activity. *Gastroenterology* 2013; **144**: 1394-1401, 1401.e1-4 [PMID: 23474283 DOI: 10.1053/j.gastro.2013.02.043]
72 **Barajon I**, Serrao G, Arnaboldi F, Opizzi E, Ripamonti G, Balsari A, Rumio C. Toll-like receptors 3, 4, and 7 are expressed in the enteric nervous system and dorsal root ganglia. *J Histochem Cytochem* 2009; **57**: 1013-1023 [PMID: 19546475 DOI: 10.1369/jhc.2009.953539]
73 **Scirocco A**, Matarrese P, Petitta C, Cicenia A, Ascione B, Mannironi C, Ammoscato F, Cardi M, Fanello G, Guarino MP, Malorni W, Severi C. Exposure of Toll-like receptors 4 to bacterial lipopolysaccharide (LPS) impairs human colonic smooth muscle cell function. *J Cell Physiol* 2010; **223**: 442-450 [PMID: 20112289 DOI: 10.1002/jcp.22053]
74 **Hooper LV**, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 2001; **291**: 881-884 [PMID: 11157169 DOI: 10.1126/science.291.5505.881]
75 **Verdú EF**, Bercík P, Bergonzelli GE, Huang XX, Blennerhasset P, Rochat F, Fiaux M, Mansourian R, Corthésy-Theulaz I, Collins SM. Lactobacillus paracasei normalizes muscle hypercontractility in a murine model of postinfective gut dysfunction. *Gastroenterology* 2004; **127**: 826-837 [PMID: 15362038]
76 **Husebye E**, Hellström PM, Sundler F, Chen J, Midtvedt T. Influence of microbial species on small intestinal myoelectric activity and transit in germ-free rats. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G368-G380 [PMID: 11171619]
77 **Kim G**, Deepinder F, Morales W, Hwang L, Weitsman S, Chang C, Gunsalus R, Pimentel M. Methanobrevibacter smithii is the predominant methanogen in patients with constipation-predominant IBS and methane on breath. *Dig Dis Sci* 2012; **57**: 3213-3218 [PMID: 22573345 DOI: 10.1007/s10620-012-2197-1]
78 **Pimentel M**, Lin HC, Enayati P, van den Burg B, Lee HR, Chen JH, Park S, Kong Y, Conklin J. Methane, a gas produced by enteric bacteria, slows intestinal transit and augments small intestinal contractile activity. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G1089-G1095 [PMID: 16293652 DOI: 10.1152/ajpgi.00574.2004]
79 **Lee HR**, Pimentel M. Bacteria and irritable bowel syndrome: the evidence for small intestinal bacterial overgrowth. *Curr Gastroenterol Rep* 2006; **8**: 305-311 [PMID: 16836942]
80 **Linden DR**, Levitt MD, Farrugia G, Szurszewski JH. Endogenous production of H2S in the gastrointestinal tract: still in search of a physiologic function. *Antioxid Redox Signal* 2010; **12**: 1135-1146 [PMID: 19769466 DOI: 10.1089/ars.2009.2885]
81 **Fiorucci S**, Distrutti E, Cirino G, Wallace JL. The emerging roles of hydrogen sulfide in the gastrointestinal tract and liver. *Gastroenterology* 2006; **131**: 259-271 [PMID: 16831608 DOI: 10.1053/j.gastro.2006.02.033]
82 **Carbonero F**, Benefiel AC, Gaskins HR. Contributions of the microbial hydrogen economy to colonic homeostasis. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 504-518 [PMID: 22585131 DOI: 10.1038/nrgastro.2012.85]
83 **Teague B**, Asiedu S, Moore PK. The smooth muscle relaxant effect of hydrogen sulphide in vitro: evidence for a physiological role to control intestinal contractility. *Br J Pharmacol* 2002; **137**: 139-145 [PMID: 12208769 DOI: 10.1038/sj.bjp.0704858]
84 **Gallego D**, Clavé P, Donovan J, Rahmati R, Grundy D, Jiménez M, Beyak MJ. The gaseous mediator, hydrogen sulphide, inhibits in vitro motor patterns in the human, rat and mouse colon and jejunum. *Neurogastroenterol Motil* 2008; **20**: 1306-1316 [PMID: 19019033 DOI: 10.1111/j.1365-2982.2008.01201.x]
85 **Salminen S**, Bouley C, Boutron-Ruault MC, Cummings JH, Franck A, Gibson GR, Isolauri E, Moreau MC, Roberfroid M, Rowland I. Functional food science and gastrointestinal physiology and function. *Br J Nutr* 1998; **80** Suppl 1: S147-S171 [PMID: 9849357]
86 **Thompson JS**, Quigley EM, Adrian TE. Qualitative changes in enteric flora and short-chain fatty acids after intestinal resection. *Dig Dis Sci* 1998; **43**: 624-631 [PMID: 9539660]
87 **Canani RB**, Costanzo MD, Leone L, Pedata M, Meli R, Calignano A. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World J Gastroenterol* 2011; **17**: 1519-1528 [PMID: 21472114 DOI: 10.3748/wjg.v17.i12.]
88 **Treem WR**, Ahsan N, Kastoff G, Hyams JS. Fecal short-chain fatty acids in patients with diarrhea-predominant irritable bowel syndrome: in vitro studies of carbohydrate fermentation. *J Pediatr Gastroenterol Nutr* 1996; **23**: 280-286 [PMID: 8890079]
89 **Soret R**, Chevalier J, De Coppet P, Poupeau G, Derkinderen P, Segain JP, Neunlist M. Short-chain fatty acids regulate the enteric neurons and control gastrointestinal motility in rats. *Gastroenterology* 2010; **138**: 1772-1782 [PMID: 20152836 DOI: 10.1053/j.gastro.2010.01.053]
90 **Haschke G**, Schafer H, Diener M. Effect of butyrate on membrane potential, ionic currents and intracellular Ca2+ concentration in cultured rat myenteric neurones. *Neurogastroenterol Motil* 2002; **14**: 133-142 [PMID: 11975713]
91 **Tazoe H**, Otomo Y, Kaji I, Tanaka R, Karaki SI, Kuwahara A. Roles of short-chain fatty acids receptors, GPR41 and GPR43 on colonic functions. *J Physiol Pharmacol* 2008; **59** Suppl 2: 251-262 [PMID: 18812643]
92 **Lesniewska V**, Rowland I, Laerke HN, Grant G, Naughton PJ. Relationship between dietary-induced changes in intestinal commensal microflora and duodenojejunal myoelectric activity monitored by radiotelemetry in the rat in vivo. *Exp Physiol* 2006; **91**: 229-237 [PMID: 16263800 DOI: 10.1113/expphysiol.2005.031708]
93 **Durbán A**, Abellán JJ, Jiménez-Hernández N, Artacho A, Garrigues V, Ortiz V, Ponce J, Latorre A, Moya A. Instability of the faecal microbiota in diarrhoea-predominant irritable bowel syndrome. *FEMS Microbiol Ecol* 2013; **86**: 581-589 [PMID: 23889283 DOI: 10.1111/1574-6941.12184]

P- Reviewers: Campo SMA, Economopoulos K
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8867

World J Gastroenterol 2014 July 21; 20(27): 8867-8885
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

Recent advances in pharmacological treatment of irritable bowel syndrome

Georgia Lazaraki, Grigoris Chatzimavroudis, Panagiotis Katsinelos

Georgia Lazaraki, Grigoris Chatzimavroudis, Panagiotis Katsinelos, Department of Endoscopy and Motility Unit, G. Gennimatas General Hospital, School of Medicine, Aristotle University of Thessaloniki, 54635 Thessaloniki, Greece
Author contributions: Lazaraki G mainly drafted and wrote the manuscript; Chatzimavroudis G is involved in writing and editing the manuscript; Katsinelos P is responsible for drafting and critical approval of the manuscript.
Correspondence to: Panagiotis Katsinelos, MD, PhD, Assistant Professor of Gastroenterology, Department of Endoscopy and Motility Unit, G. Gennimatas General Hospital, School of Medicine, Aristotle University of Thessaloniki, Ethnikis Aminis 41, 54635 Thessaloniki, Greece. gchatzimav@yahoo.gr
Telephone: +30-2310-963341 Fax: +30-2310-963341
Received: October 1, 2013 Revised: March 13, 2014
Accepted: June 20, 2014
Published online: July 21, 2014

Abstract

Irritable bowel syndrome (IBS) is a highly prevalent functional disorder that reduces patients' quality of life. It is a chronic disorder characterized by abdominal pain or discomfort associated with disordered defecation in the absence of identifiable structural or biochemical abnormalities. IBS imposes a significant economic burden to the healthcare system. Alteration in neurohumoral mechanisms and psychological factors, bacterial overgrowth, genetic factors, gut motility, visceral hypersensitivity, and immune system factors are currently believed to influence the pathogenesis of IBS. It is possible that there is an interaction of one or more of these etiologic factors leading to heterogeneous symptoms of IBS. IBS treatment is predicated upon the patient's most bothersome symptoms. Despite the wide range of medications and the high prevalence of the disease, to date no completely effective remedy is available. This article reviews the literature from January 2008 to July 2013 on the subject of IBS peripherally acting pharmacological treatment. Drugs are categorized according to their administration for IBS-C, IBS-D or abdominal pain predominant IBS.

Key words: Irritable bowel syndrome; Irritable bowel syndrome constipation; Irritable bowel syndrome-diarrhea; Constipation; Diarrhea; Irritable bowel syndrome treatment; Irritable bowel syndrome-pain

Core tip: Irritable bowel syndrome (IBS) is a highly prevalent functional disorder that reduces patients' quality of life and imposes a significant economic burden to the healthcare system. This article extensively reviews the literature from January 2008 to July 2013 on the subject of IBS peripherally acting pharmacological treatment. Pathophysiology background and mode of action in IBS of each substance are also discussed.

Lazaraki G, Chatzimavroudis G, Katsinelos P. Recent advances in pharmacological treatment of irritable bowel syndrome. *World J Gastroenterol* 2014; 20(27): 8867-8885 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8867.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8867

INTRODUCTION

Irritable bowel syndrome (IBS) is a highly prevalent (10%-20% of the United States adult population)[1] functional disorder that reduces patients' quality of life. IBS is defined in the Rome Ⅲ criteria as a chronic disorder characterized by abdominal pain or discomfort associated with disordered defecation [either constipation (IBS-C), diarrhea (IBS-D), or mixed/ alternating symptoms of constipation and diarrhea (IBS-M)][2]. Symptoms should begin at least 6 mo before and abdominal pain or discomfort should be present at least 3 d per month for 3

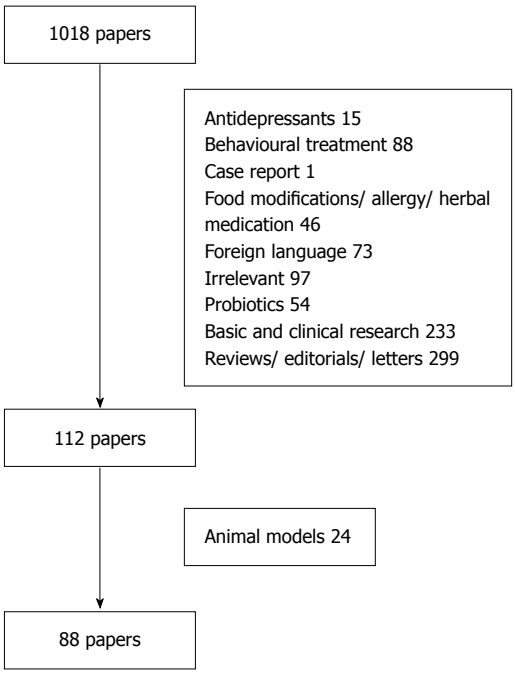


Figure 1 Flowgram of the selected studies for the review.

mo during last 6 mo and should be associated with two or more of the following: improvement with defecation, onset associated with a change in stool frequency and/or change in stool form. Bloating and abdominal distention are also frequently reported by IBS patients reflecting sensitivity to normal amounts of intestinal gas. By definition, no disease that could explain the symptoms should be present[2].

IBS represents important costs for the healthcare system. One should look carefully for alert signs [*i.e.*, anemia, unintentional weight loss, gastrointestinal (GI) bleeding, nausea/vomiting, family history of cancer] of a serious underlying disorder to differentiate functional symptoms from organic disorders. Thus, for younger patients who meet criteria for IBS with normal physical examination and no "red flags", an extensive laboratory work up should not be considered[3].

It is likely that the definition of IBS represent an auspice of different conditions/disease states for which we lack specific biomarkers. Alteration in neurohumoral mechanisms and psychological factors, bacterial overgrowth, genetic factors, gut motility, visceral hypersensitivity, and immune system factors are currently believed to influence the pathogenesis of IBS[4-6]. It is possible that there is an interaction of one or more of these etiologic factors leading to heterogeneous symptoms of IBS.

Since IBS is not a single disease entity, but rather likely consists of several different disease states, IBS treatment is predicated upon the patient's most bothersome symptoms. Specifically, our treatment strategy seems to target constipation, diarrhea, bloating or pain[7]. A wide range of medications (prokinetiks, antispasmodics, sedatives, tranquilizers, laxatives, fecal bulking agents, probiotics and antibiotics) along with life style and diet modifications have been proposed for this highly prevalent condition; however to date there is no definite effective cure for this state[7].

In the present review, we report the results of our search in PubMed, Scopus, and Google Scholar databases from January 2008 to July 2013 on the subject of IBS peripherally acting pharmacological treatment. MeSH terms "irritable bowel syndrome treatment" and "IBS treatment" were used as search terms. English-written articles only were included. Data from metanalysis and clinical studies were included. Abstracts, case reports, comments/reviews, *in vitro* studies, animal studies and pharmacogenetic studies were excluded from the review. The search resulted in 1018 papers after omission of duplicate articles; finally 86 papers were included after omission of non-relevant articles. Flowgram of the search is presented in Figure 1. Drugs are categorized according to their administration for IBS-C, IBS-D or abdominal pain predominant IBS.

IBS-C

The evaluated studies in each category are reported in Table 1. Below is a list of available treatment methods based on the findings.

Laxatives

Several clinical observations have reported a decrease in bowel motility and a prolonged transit time in patients with IBS-C compared with controls[8,9]. Also, some IBS-M patients report an alternation in bowel habits with extended periods with small, hard bowel movements or no bowel movement followed by periods with loose stools. Osmotic agents, stimulants, and stool softeners are all comprised in the category of laxatives. Polyethylene glycol (PEG) is the only laxative that has been evaluated in the treatment of IBS. The first study published in 2006 assessed the effects of PEG 3350 in patients with IBS-C (Rome Ⅱ criteria)[10]. Mean bowel movement frequency was significantly increased; however, there was no change in mean pain level for the group with the PEG therapy. In the last 5 years 2 new studies evaluated the efficacy of PEG in IBS-C. The first study[11], a randomized, double-blind, placebo-controlled trial used fasting and postprandial (PP) perception of rectal distension as measurements. Symptoms were also recorded. Forty two patients with IBS-C (Rome Ⅱ criteria) and with a pain threshold of < 32 mmHg participated. Patients received either oral PEG, 3.45 g t.i.d. orally for 30 d or placebo. PEG improved consistency of faeces. Both, PEG and placebo increased bowel movements per week ($P < 0.001$), and relieved symptoms without significant side-effects. However, there were not significant differences in fasting and PP rectal tone and thresholds for first sensation, gas sensation, urge to defecate, and pain between PEG and placebo. The investigators concluded that changes in rectal tone and sensation were not related to PEG 3350 and placebo effects. Patients with IBS-C gained some

Table 1 Pharmacological treatment irritable bowel syndrome-C studies and clinical efficacy during last 5 years

Category/No. of studies/ Ref.	No. of patients	*vs* Placebo	Abdominal distention/ pain	SBMs	Stool consistency	Recommendation *vs* placebo
Laxatives/2						
Awad *et al*[11] 2010		Yes	NS	NS	SS	Equal
Chapman *et al*[12] 2013		Yes	NS	SS	SS	Equal
Linaclotide/5						
Johnston *et al*[19] 2010		Yes	SS	SS	SS	Superior
Chey *et al*[20] 2012		Yes	SS	SS	-	Superior
Rao *et al*[21] 2012		Yes	SS	SS	-	Superior
Quigley *et al*[22] 2013		Yes	SS			Superior
Videlock *et al*[23] 2013		Yes	SS	SS	SS	Superior
5-HT4 agonists						
Renzapride/2						
Lembo *et al*[49] 2010		Yes	SS	SS	SS	Superior but AE
Ford *et al*[82] 2009	726	Yes	NS	NS	NS	Equal
Cisapride/1						
Ford *et al*[82] 2009	726	Yes	NS	NS	NS	Equal
Lubiprostone/4						
Johanson *et al*[57] 2008		Yes	SS (16/32/48 μg)	SS (16/32/48 μg)	SS (16/32/48 μg)	Superior
Fukudo *et al*[58] 2011		Yes	SS (48 μg)	SS (48 μg)	SS (48 μg)	Superior(48 μg)
Drossman *et al*[59] 2009		Yes	SS (16 μg)	SS (16 μg)	SS (16 μg)	Superior
Chey *et al*[60] 2012		No, extention study, comparison to inclusion	SS	SS	SS	Favourable profile of effectiveness, safety, tolerability
CDCA/1						
Rao *et al*[65] 2010		Yes	-	SS	SS	Superior

SBMs: Spontaneous bowel movements; SS: Statistically significant; NS: Not significant; 5-HT: 5-hydrodytryptamine; CDCA: Chenodeoxycholic acid.

relief from their symptoms both with PEG and placebo. In the second study[12], following a 14-d run-in period without study medication, 139 adult patients with IBS-C were randomized to receive PEG 3350+E or placebo for 28 d. The primary endpoint was the mean number of spontaneous bowel movements per day in the last treatment week. In both groups there was an increase in mean bowel movement frequency compared to run-in. The difference between the groups in week 4 from 4.40 (PEG 3350+E) to 3.11 (placebo) was statistically significant (95%Cl: 1.17- 1.95; $P < 0.0001$). However, although mean severity score for abdominal discomfort/pain was significantly reduced compared with run-in with PEG 3350+E, there was no difference *vs* placebo. Spontaneous bowel movements (SBMs), responder rates, stool consistency, and severity of straining also showed superior improvement in the PEG 3350+E group over placebo in the fourth week. The authors concluded that PEG 3350+E was superior to placebo for relief of constipation but resulted in no improvement to abdominal discomfort/pain compared to placebo in spite of the presence of a statistical significant improvement in abdominal discomfort/pain that was observed compared with baseline.

Guanylate cyclase-c receptor agonists

Linaclotide is a guanylin peptide. Guanylin peptides are a family of peptides with similar structure to the heat-stable enterotoxin produced by *Escherichia coli* and other enteric bacteria that cause secretory diarrhea. They have a conformation to bind with guanylate cyclase-c (GC-C) receptors. Binding of GC-C receptors, which are abundantly expressed on enterocytes lining the intestine, stimulates production of cyclic guanosine monophosphate[13]. This leads to a cascade of intracellular events resulting in the activation of the cystic fibrosis transmembrane conductance regulator (CFTR) and the subsequent transepithelial chloride (Cl^-) and potassium (K^+) ion efflux from enterocytes, with secondary passive water secretion into the intestinal lumen[14]. Linaclotide is minimally absorbed and therefore believed to act locally[15]. In animal models linaclotide has been shown to stimulate intestinal secretion, accelerate GI transit time and reduce visceral pain through GC-C dependent activation[13].

Clinical studies have investigated linaclotide in patients with IBS-C and chronic constipation (CC). In an earlier phase Ⅱa study[16] 36 women with IBS-C that received a 5-d course of linaclotide 1000 mg. The result was a significantly accelerated ascending colon ($P = 0.004$) and total colonic transit time at 48 h ($P = 0.01$). Linaclotide had no effect on gastric emptying or small bowel transit time; however, it accelerated the time to first bowel movement, decreased stool consistency, and enhanced ease of stool passage. Data from CC studies have demonstrated improvement of weekly SBMs and various other constipation-related clinical parameters, including stool consistency and straining in a dose-dependent fashion. In addition, patients treated with linaclotide experienced improvements in abdominal discomfort, bloating, and constipation severity. Constipation symptoms tended to return to baseline, without evidence of a rebound, after discontinuation of linaclotide[17,18]. The overall frequency of adverse events reported with linaclotide and placebo

were similar[18], with diarrhea the most common adverse event (AE) reported with linaclotide.

In the recent years 4 studies and 1 meta-analysis were published regarding linaclotide efficacy in IBS. Specifically, a phase Ⅱb study[19] published in 2010 the efficacy, safety, and dose response of linaclotide administered at 75, 150, 300, and 600 μg once daily for 12 wk. Four hundred twenty patients with IBS-C were assessed. The study recorded changes from baseline in daily bowel habits and daily abdominal symptoms. There were also weekly global assessments. All doses of linaclotide significantly improved the frequency of SBMs and complete spontaneous bowel movements (CSBM). They also improved the severity of straining, stool consistency and abdominal pain compared with placebo. Mean changes in abdominal pain (assessed on a 5-point scale) from baseline were -0.71, -0.71, -0.90, and -0.86 for linaclotide doses of 75, 150, 300, and 600 μg, respectively, compared with -0.49 for placebo. Other abdominal symptoms and global measures of IBS-C were also improved compared with placebo. The drug presented effect within the first week that sustained during the 12 wk of treatment. Diarrhea was the only dose-dependent adverse event and was usually of mild or moderate severity. Although all linaclotide doses were associated with a statistically significant improvement compared with placebo for most end points, the higher doses of linaclotide (*i.e.*, 300 and 600 μg) were generally more effective across most parameters. Because the 300 and 600 μg doses provided comparable efficacy and the higher dose was associated with an increase in side effects, a dosage of 300 μg per day was selected for continued evaluation in phase Ⅲ trials. In 2012, 2 studies were published together. The first, a phase Ⅲ trial[20] included 804 patients with IBS-C (Rome Ⅱ criteria). Participants were randomized to linaclotide 290 μg orally or placebo once daily for 26 wk. The study had the rigorous end point to be a "responder" as recommended for IBS-C in the Food and Drug Administration guidelines for IBS clinical trials (May 2012); the percentage of responders was 33.7% in the linaclotide group compared with 13.9% in the placebo group ($P < 0.0001$). Significant differences in favor of linaclotide ($P < 0.0001$) were also observed for an even more rigorous end point which required that patients meet the ⩾ 30 % of improvement in worst abdominal pain and both ⩾ 3 CSBMs/wk and an increase of ⩾ 1 CSBM/wk from baseline for a minimum of 9 out of 12 wk. The effects of linaclotide on abdominal and bowel symptoms were manifested within the first week of treatment and sustained over the entire 26-wk treatment period. The second study[21] randomized 800 patients with IBS-C to 290 μg linaclotide orally or placebo once daily, for 12 wk. This was followed by a 4-wk withdrawal period after randomization. The same FDA end points that were used in the former trial were used as primary end points. In the linaclotide group 33.6% of patients compared with 21% of patients in the placebo group ($P < 0.0001$) [number needed to treat (NNT) = 8.0, 95 %CI: 5.4-15.5] met the FDA end points. A statistically significant percentage of patients treated with linaclotide *vs* placebo met the rest of end points (primary and secondary, $P < 0.05$ and $P < 0.001$ respectively). During the withdrawal period, after randomization, patients remained improved as long as they were receiving linaclotide whereas those that were re-randomized to placebo presented relapse of symptoms. Symptoms did not become worse relative to baseline. In both studies AEs were generally comparable between linaclotide and placebo groups, with the exception of diarrhea, which occurred more commonly with linaclotide than with placebo, and was mostly mild or moderate in severity. Recently further analysis on the data of these 2 trials was performed[22]. Overall, 803 and 805 patients were randomized. A significantly greater proportion of patients in the linaclotide group *vs* placebo patients presented improvement in abdominal pain/discomfort during the 12 wk treatment period. Similarly, significantly more linaclotide-treated patients compared to placebo-treated patients were responders for ⩾ 13 wk (abdominal pain/discomfort: 53.6% *vs* 36.0%; IBS degree-of-relief: 37.2% *vs* 16.9%; $P < 0.0001$). The proportion of sustained responders was also significantly greater with linaclotide *vs* placebo in both trials ($P < 0.001$). In these trials, treatment-emergent AEs were reported by more than half of those receiving linaclotide, with the most noteworthy being a greater incidence of diarrhea in one of five subjects. These observations are obviously related to the secretagogue mechanism of the drug.

Finally a meta-analysis to determine the efficacy of linaclotide, compared with placebo, for patients with IBS-C or CC was published in 2013[23]. The search identified seven trials of linaclotide in patients with IBS-C or CC with six finally included in the analysis. The relative risk (RR) for the response to treatment with 290 mg linaclotide, compared with placebo, was 1.95 (95%CI: 1.3-2.9), and the NNT was 7 (95%CI: 5-11). Linaclotide also improved the stool form and reduced abdominal pain, bloating, and overall symptom severity in patients with IBS-C or CC.

Therefore, linaclotide has the potential to offer relief for the multiple symptoms from which patients with IBS-C suffer.

Serotonin receptor modulators

Serotonin (5-hydrodytryptamine; 5-HT) is predominantly (90%-95% of the body's 5-HT) produced in the enterochromaffin (EC) cells in the intestinal mucosa, and also by a subpopulation of enteric neurons[24]. Acting as a signaling molecule through the intrinsic and extrinsic afferent nervous system of the GI tract, 5-HT plays an important role in various aspects of GI sensory, secretory, absorptive, and motility function[24]. Abnormal levels have been shown in individuals with IBS. Several studies describe increased serotonergic activity in association with IBS-D[25-28]. Similarly, a decrease in serotonergic activity has been observed in IBS-C[26,27]. Pharmaceutical agents acting on 5-HT receptors have, therefore, evolved to ameliorate the smooth muscle spasm, abdominal

pain, and change in bowel habit that IBS patients experience. Of the identified serotonin-receptor subtypes, the 5-HT1p, 5-HT3, 5-HT4, and 5-HT7 receptors seem to play an important role in GI tract functioning[29]. Intraluminal distension of the intestine (translated to abdominal pain in IBS patients) stimulates 5-HT release from EC cells and activates 5-HT3 receptors of primary afferent neurons. 5-HT3 receptors activation results in the release of various neurotransmitters, such as acetylcholine. This induces colonic transit acceleration and abnormal water transport, which in turn leads to defecation abnormalities. Receptor antagonists of 5-HT3 have been reported to slow small bowel and colonic transit, decrease intestinal secretion and colonic tone[29]. Of great relevance to IBS-C and CC are the 5-HT4 receptors. In the gastrointestinal tract, 5-HT4 receptors are located on enteric neurons and smooth muscle cells, and their stimulation leads to acetylcholine release causing prokinetic effects. Based on biochemical structure, 5-HT4 agonists can be broadly categorized as benzamides (metoclopramide, cisapride, renzapride, mosapride, clebopride, and ATI-7505), carbazimidamides (tegaserod), benzofurancarboxamides (prucalopride), and other agonists such as velusetrag[15].

5-HT4 agonists

Tegaserod is a selective 5-HT4 receptor partial agonist with promotility effects in the small and large intestine[30-32] and modulation of visceral sensation[33,34]. The efficacy and tolerability of tegaserod in the treatment of women with IBS-C was initially reported in 2 multicenter, double-blind, placebo-controlled trials. More than 2000 patients from the Western hemisphere were involved[35,36]. These clinical trials consistently reported the superiority of tegaserod over placebo in improving IBS symptoms (abdominal pain, stool frequency, stool consistency, straining, and bloating). Later, other trials have confirmed the safety and tolerability of tegaserod[37-40]. Side effects included headache, abdominal pain and diarrhea. Although there were no reports of ischemic colitis in the clinical trials, 26 events of possible colonic ischemia were identified during postmarketing surveillance. This was translated to an estimated incidence of 7 cases of colonic ischemia per 100000 patient-years of tegaserod use[41]. Cardiovascular and cerebrovascular events in the group receiving tegaserod were also reported later[42] in a pooled analysis[13] cardiovascular ischemic events in 11614 patients receiving tegaserod compared with 1 out of 7031 patients in the placebo group (0.1% *vs* 0.01% respectively, $P = 0.02$)]. A pathogenetic mechanism that was proposed was that tegaserod may induce platelet aggregation through 5-HT4 receptors located on platelets[43]. Later retrospective studies found no relationship between tegaserod and cardiovascular events; however the drug was definitely withdrawn from the market in 2009.

Mosapride has stimulatory effects on gastric and colonic motility[44]. Unlike cisapride, mosapride does not bind to K1 channels or D2 dopaminergic receptors. Mosapride was primarily developed for upper GI tract conditions, such as functional dyspepsia, gastroesophageal reflux disease, and nausea and vomiting[15]. Data from animal models show that mosapride accelerates colonic transit time[45], augments motility in the proximal and distal colon in a dose-dependent manner[45] and has a stimulatory effect on the defecatory reflex[46,47]. In humans a study showed that mosapride changes rectosigmoid motility and perception in patients with IBS[48].

In 2010, the efficacy and safety of renzapride were assessed in a study of 1798 women with IBS-C. Patients were randomized to a 4 mg daily dosage of renzapride, 2 mg *b.i.d.* or placebo for 12 wk[49]. The primary end point was global relief of IBS symptoms. A subset of patients ($n = 971$) were enrolled in a 12-mo, open-label study of oral intake of renzapride 4 mg daily. Relief of overall IBS symptoms was achieved at (mean ± SD) 0.55 ± 0.04, 0.60 ± 0.04 and 0.44 ± 0.04 in the renzapride 4 mg daily, 2 mg b.i.d. and placebo groups ($P = 0.027$ and $P = 0.004$ respectively). Stool consistency and frequency were statistically significantly improved in the renzapride group, as well as bloating and abdominal distension. Three episodes of ischemic colitis were reported. The authors concluded that due to the limited benefit of renzapride over placebo and the reported cases of ischemic colitis, no further study with renzapride as possible treatment of IBS-C should be conducted.

Lubiprostone (chloride channel stimulators)

Lubiprostone is a bicyclic fatty acid derivative of prostaglandin E1. The underlying mechanism of lubiprostone is stimulation of electrogenic chloride secretion by activating chloride channel type-2 (ClC-2)[50] and CFTR[51] in the intestinal epithelial cells apical membrane. Primary functions of ClC-2 channels include maintenance of the membrane potential of the cell, regulation of pH and cell volume, and regulation of chloride ion channel transport and fluid secretion. Dose-dependent ClC-2 activation of ClC-2 channels or CFTR chloride channels in intestinal epithelial cells produces an active secretion of chloride ions from cells into the intestinal lumen followed by a passive secretion of electrolytes and water which increases the liquidity of the luminal contents. The luminal distension increased by intestinal fluid promotes the GI tract motility which in turn increases the intestinal and colonic transit[41]. Besides this mechanism, lubiprostone enhances and stimulates contraction in colonic as well as gastric muscles through prostaglandin E receptors (EP1 or EP4)[52], suggesting the modulatory effects of lubiprostone on GI motility through the activation of prostaglandin receptors.

Previous work has demonstrated that lubiprostone accelerates small bowel and colonic transit and increases the frequency of bowel movement in healthy adults[53]; however, the thresholds for pain do not seem to be affected by lubiprostone. Multiple randomized controlled trials (RCTs) have demonstrated the efficacy of lubiprostone in idiopathic CC[54-56]. In these trials, lubiprostone was consistently found to be superior to placebo at increasing

the number of weekly SBMs as well as improving stool consistency, straining, constipation severity, bloating, and treatment effectiveness. The most commonly reported side effects included nausea, headache, and diarrhea. A pooled analysis of 91 patients meeting diagnostic criteria for IBS-C from the 2 phase Ⅲ constipation trials revealed significant improvements in constipation symptoms as well as abdominal symptoms due to lubiprostone as compared to placebo. This observation led to further evaluation of lubiprostone in the treatment of IBS-C[41].

The efficacy and tolerability of lubiprostone have been assessed in several RCTs. First, 195 IBS-C patients received daily doses of 16 (8 μg twice daily), 32 (16 μg *b.i.d.*) or 48 μg (24 μg *b.i.d.*) lubiprostone or placebo for 3 mo[57]. In the lubiprostone group mean abdominal discomfort/pain scores were significantly improved compared to placebo after 1 and 2 mo ($P = 0.023$ and $P = 0.039$, respectively). All 3 doses of lubiprostone were superior to placebo with regard to frequency of SBM ($P = 0.0499$), constipation severity ($P = 0.0056$), stool consistency ($P < 0.0001$), and straining ($P = 0.0094$) in each of the 3 mo of treatment. Treatment with lubiprostone showed significantly higher rates of GI AEs ($P = 0.020$), especially diarrhea and nausea. The 16 μg/d dose demonstrated the optimal combination of efficacy and safety and was therefore the dose selected for further study in subsequent phase Ⅲ clinical trials. Another Japanese trial[58] studied adequate dosing of lubiprostone for the treatment of constipation in CC or IBS-C patients. One hundred seventy patients (128 without IBS and 42 with IBS) randomly received a placebo or 16 μg, 32 μg, or 48 μg of lubiprostone daily for two weeks. There was a dose-dependent increase in weekly average number of SBM compared to baseline in the first week (placebo: 1.5; 16 μg: 2.3, 32 μg: 3.5; and 48 μg: 6.8, per week, $P < 0.0001$). The 32 and 48 μg dosage treatments had a significantly higher primary efficacy endpoint than the placebo treatment ($P = 0.0017$, $P < 0.0001$, respectively). The 16 μg treatment showed no significant increase in change in SBMs during the first week over placebo. The primary endpoint was significantly better only in patients with IBS treated with 48 μg of lubiprostone than those treated with placebo ($P = 0.0086$).

There was a combined analysis of two phase-Ⅲ RCTs of lubiprostone 8 μg twice daily *vs* placebo for 12 wk that reported data of 1171 patients with IBS-C [Rome Ⅱ criteria][59]. Patients responded with respect to relief of IBS symptoms over the past week. Patients were characterized monthly responders (moderate relief in 4/4 wk or significant relief in 2/4 wk) or overall responders (a monthly responder in 2/3 mo of the trial). The primary efficacy endpoint was the percentage of overall responders. Significantly more patients in the lubiprostone group were considered overall responders compared with the placebo group (17.9% *vs* 10.1%, $P = 0.001$). Lubiprostone was also superior to placebo in improving individual IBS symptoms (abdominal discomfort/pain, stool consistency, straining, constipation severity), and quality of life (QOL). A similar incidence of AEs to those treated with placebo and lubiprostone was observed. Another recent study[60] evaluated the long-term safety, tolerability and patient outcomes of lubiprostone in patients with IBS-C. This was an extension study analyzing the data of 476 IBS-C patients who had completed one of two randomized phase Ⅲ studies. Patients received placebo or lubiprostone orally for 36-wk (8 μg, twice daily). Those receiving lubiprostone during the initial 12-wk phase Ⅲ trial experienced an increase in response from 15% to 37% and those initially receiving placebo experienced an increase in response from 8% to 31% at the conclusion of the 36-wk extension period. The overall safety profile of lubiprostone during this study was similar to that observed in the preceding phase Ⅲ studies. AEs were diarrhea (11.0%), nausea (11.0%), urinary tract infection (9.0%), sinusitis (9.0%) and abdominal distention (5.8%). Diarrhea and nausea were the most common treatment-related AEs.

An evidence-based systematic review was performed by the ACG IBS Task Force that evaluated lubiprostone in the treatment of IBS-C[61] concluding that "Lubiprostone in a dose of 8mg twice daily is more effective than placebo in relieving global IBS symptoms in women with IBS-C." Regarding men with IBS-C, the ACG task force suggested a need for further studies before a recommendation for use in this population. Lubiprostone is contraindicated in patients with mechanical bowel obstruction and should be avoided in patients with preexisting diarrhea; there have also been postmarketing reports of dyspnea (typically resolves over several hours but sometimes reoccurs with subsequent dosing)[41].

Bile acid modulators

Bile acids have been used in the treatment of patients with gallstones and cholestatic liver diseases. Longterm treatment is generally well tolerated other than the consistent side effect of diarrhea[62], which mimics the chronic loose stools observed in patients with a disrupted enterohepatic circulation from ileal disease resulting in spillage of bile acid into the colon[63]. In the setting of bile acid-related diarrhea after ileal resection or disease, high concentrations of bile acids decrease net colonic fluid and electrolyte absorption and induce secretion[64]. The mechanisms involved in promoting secretion include intracellular activation of adenylate cyclase, increased mucosal permeability, and inhibition of apical Cl^-/OH^- exchange[65]. Furthermore, instillation of bile acids directly into the colon increases intracolonic pressure and motility index[66].

Chenodeoxycholic acid (CDCA), a primary bile acid previously used for dissolution of gallstones, elicited diarrhea at dosages of 750 to 1000 mg/d[67]. CDCA (with hydroxyl groups in the 3α, 7α positions) promoted colonic secretion in comparison to its 3α, 7β epimer, ursodeoxycholic acid[68]. Previous studies in healthy volunteers[69] and in patients with gallstones who had CC receiving CDCA demonstrated a significant increase in the frequency of

Table 2 Pharmacological treatment irritable bowel syndrome-C studies and clinical efficacy during last 5 years

Category/No. of studies/Ref.	*n*	*vs* Placebo	Abdominal distention/pain	QOL/patient satisfaction/ global improvement	Stool consistency/ bowel habits	Recommendation *vs* placebo
5-HT3 antagonists						
Alosetron, cilansetron/4						
Cremonini *et al*[79] 2012	705	Yes	-	SS	-	Superior
Rahimi *et al*[80] 2008	4.17 Metanalysis	Yes	SS	SS	-	Superior
Andresen *et al*[81] 2008	7487 Metanalysis	Yes or mebeverine	SS	SS	-	Superior
Ford *et al*[82] 2009	7216 Metanalysis	Yes	SS	SS	-	Superior
Ramosetron/3						
Matsueda *et al*[85] 2008	418	Yes	SS (5/10 μg)	SS (5/10 μg)	-	Superior
Matsueda *et al*[86] 2008	539	Yes	SS (5 μg)	SS (5 μg)	SS (5 μg)	Superior
Lee *et al*[87] 2011	343	Mebeverine 135 mg t.i.d	NS(5 μg)	NS (5 μg)	NS(5 μg)	Equal
LX-1031/1						
Brown *et al*[92] 2011	155	Yes	SS only the 1st week (1000 mg 4 times/d)	-	SS (1000 mg 4 times/d)	Superior
Crofelemer/1						
Angel *et al*[94] 2008	246	Yes	SS 500 mg b.i.d	SS	SS	Superior
Antibiotics						
Rifaximin/2						
Pimentel *et al*[105] 2011	1260	Yes	SS	SS	SS	Superior
Menees *et al*[106] 2012	1803 Metanalysis	Yes	SS	SS	-	Superior
5ASA compounds, mesalazine/3						
Corinaldesi *et al*[108] 2009	20	Yes	NS	SS	NS	Equal
Andrews *et al*[109] 2011	12	No Comparison to baseline	SS	SS	-	-
Tuteja *et al*[110] 2012	17	Yes	NS	NS	NS	Equal

QOL: Quality of life; ASA: Aminosalicylic acid; SS: Statistically significant; NS: Not significant.

bowel movements and loosening of stools[70]. CDCA also accelerated colonic transit time resulting in ease of stool passage, and sense of complete evacuation[69].

Recently a double-blind placebo-controlled study[65] evaluated pharmacodynamics (colonic transit, bowel function) and pharmacogenetics of CDCA in 36 female patients with IBS-C. Participants were randomized to treatment with delayed-release oral formulations of placebo, 500 mg CDCA, or 1000 mg CDCA for 4 d. Colonic transit and ascending colon emptying were significantly accelerated in the CDCA group compared to the placebo group ($P = 0.005$ and $P = 0.028$, respectively). Looser stool consistency ($P = 0.003$), increased stool frequency ($P = 0.018$), and greater ease of passage ($P = 0.024$) were noted with CDCA compared with placebo. The investigators also found a correlation between fasting serum 7 alpha-hydroxy-4-cholesten-3-one (7aC4), a biomarker of bile acid synthesis, and colonic transit time in the placebo group: subjects with an increased 7aC4 showed a faster overall colonic transit time. In the CDC group, 7aC4 showed a modest influence on colonic transit at 24 h ($P = 0.055$) and 48 h ($P = 0.019$).

IBS-D

The evaluated studies in each category are reported in Table 2. Below is a list of available treatment methods based on the findings.

Antidiarrheals

As mentioned above alterations in bowel habits in IBS are in part a result of altered GI motility. Accelerated small bowel and colon transit times as well as exaggerated motility patterns have been demonstrated in those with IBS-D compared with controls[8,9]. Consequently, antidiarrheals remain among the more commonly used gut-acting agents used in the treatment of patients with IBS-D.

Among the class of antidiarrheals, loperamide is the only substance that has been evaluated in RCTs for the treatment of IBS. In total, four studies have been published[71-74] showing an improvement in the number of bowel movements and stool consistency compared to placebo in IBS-D patients; however results were rather disappointing regarding pain. The ACG Task Force recently performed a systematic review of antidiarrheals in the treatment of IBS and concluded that "The antidiarrheal agent loperamide is not more effective than placebo at reducing abdominal pain or global symptoms of IBS, but is an effective agent for treatment of diarrhea, improving stool frequency and stool consistency. RCTs with other antidiarrheal agents have not been performed.

Safety and tolerability data on loperamide are lacking"[61].

5-HT3 antagonist (alosetron, cilansetron, ramosetron)

As already mentioned receptor antagonists of 5-HT3 have been reported to slow colonic and small bowel transit and decrease intestinal secretion and colonic tone[29]. Early, rigorous, large clinical trials with alosetron 1 mg *b.i.d.* have all demonstrated the efficacy of alosetron in the global and individual symptoms of IBS-D in women. Alosetron decreases urgency, reduces stool frequency, and increases stool consistency. Improvement is seen within 1 wk of therapy, which persists throughout the treatment period[75,76]. The use of alosetron also demonstrated improvement in 3 QOL domains (including food/diet, social functioning, and role-physical on the validated generic QOL instrument, the SF-36 75)[77] and in the global IBS symptoms[78]. Recently a total of 705 women (severe IBS-D, Rome II criteria) were randomized to alosetron 0.5 mg *q.d.*, 1 mg *q.d.*, 1 mg *b.i.d.*, or placebo for 12 wk[79]. IBSQOL, treatment satisfaction, daily activities, and lost workplace productivity were evaluated. The authors concluded that in women with severe IBS-D, alosetron treatment, including 0.5 mg *q.d.,* resulted in statistically significant and clinically relevant improvements in health-related QOL, restriction of daily activities and treatment satisfaction over placebo.

During the last 5 years 3 metanalyses have been published on this subject. The first[80] included 8 multicenter, randomized, placebo-controlled, 12-wk clinical trials with 4170 patients with IBS randomized to receive either alosetron or placebo. Alosetron was significantly more effective in global improvement in symptoms than placebo (RR = 1.60; 95%CI: 1.44-1.76; $P < 0.001$), in adequate relief of IBS pain and discomfort (RR = 1.31; 95%CI: 1.20-1.43; $P < 0.001$). In the alosetron group, there were 4 cases of ischemic colitis (0.16%) and 2 cases of serious complications of constipation (0.08%). The second[81] trial collected data from 14 RCTs [alosetron (n = 3024) or cilansetron (n = 1116) *vs* placebo (n = 3043) or mebeverine (n = 304)]. 5-HT3 antagonists were more effective than mebeverine and placebo in achieving global IBS symptoms improvement (pooled RR = 1.60; 95%CI: 1.49-1.72), abdominal pain and discomfort relief (pooled RR = 1.30; 95%CI: 1.22-1.39). Superiority of both agents was demonstrated in patients of either sex. Nine patients (0.2%) in the 5-HT3 antagonists group were reported with possible ischemic colitis *vs* none in control groups. The third meta-analysis[82] pooled the data from eight clinical trials of alosetron and three clinical trials of cilansetron. This analysis, which included a total of 7216 patients with IBS, found 5-HT3 antagonists more effective than placebo in treating IBS-D. The RR of IBS symptoms persisting with 5-HT3 antagonists was 0.78 (95%CI: 0.71-0.86) compared to placebo.

Severe complications of constipation and ischemic colitis have emerged as significant side effects with alosetron use and this led to the drug's withdrawal from the United States marketplace in 2000. An expert panel reviewed the postmarketing data[83] reporting similar incidence rates for ischemic colitis and constipation (0.95 and 0.36 cases per 1000 patient-years, respectively) to rates during the postmarketing cycle before alosetron withdrawal. No mesenteric ischemia, surgeries, transfusions, or deaths occurred in patients with ischemic colitis and no cases of contipation were associated with toxic megacolon, perforation, surgeries, transfusions, or deaths. AEs were typically of short duration and all improved on prompt withdrawal of alosetron.

Ramosetron, is also a selective serotonin 5-HT3-receptor antagonist that possesses a specific three dimensional chemical conformation able to bind long lastingly to 5-HT3 receptors. Traditionally it has been used in oncology as a medication for hyperemesis due to chemotherapy[84]. The first double-blind, RCT[85] randomized 418 IBS-D patients to ramosetron 5 μg, 10 μg or placebo. Significantly higher rates of patients treated with both doses of ramosetron reported relief of IBS symptoms compared to placebo; the outcome measure was "global assessment of relief of IBS symptoms" in a monthly basis with similar benefits in men and women. The second study was also double-blind RCT. Five hundred thirty nine IBS-D patients received 5 μg ramosetron or placebo once daily. Ramosetron was shown effective for discomfort, altered bowel habits (44% *vs* 24%, for ramosetron vs placebo respectively, $P = 0.001$) and abdominal pain (46% *vs* 33%, for ramosetron vs placebo respectively, $P = 0.005$), without any serious AEs[86]. Overall 47% of individuals treated with ramosetron reported a positive response to treatment compared to 27% of placebo-treated patients ($P = 0.001$). Ramosetron was compared to mebeverine in another study with male IBS-D patients[87]. Patients (n = 343) were randomized to receive 5 μg ramosetron once daily or 135 mg mebeverine t.i.d for four weeks. Adequate relief of IBS symptoms at the last week of treatment was the primary end point and this was measured as the proportion of patients reporting relief in an intention to treat analysis. Both in the ramosetron and mebeverine groups, responder rates for global IBS symptoms, altered bowel habits and abdominal pain significantly increased during treatment. Although abdominal pain/discomfort and urgency (severity scores), the stool form score, and the stool frequency in both treatment arms significantly improved compared to baselines, statistical significance was not reached. Furthermore, in the comparison between ramosetron and mebeverine groups, the responder rates were similar (37% *vs* 38% on ITT analysis) as well as AEs. Events of severe constipation or ischemic colitis were not reported. When the oral administration of 5 μg ramosetron was prolon data analysis of the postmarketing survey[88]. Further RCTs studies ged for a minimum of 28 wk (up to 52 wk) the responder rate was increased as well as the overall improvement of IBS symptoms. The rate was further increased subsequently in the to evaluate ramosetron are needed.

LX-1031

As already mentioned 5-HT is an important neurotransmitter in the GI tract released from EC cells and inter-

neurons[24]. 5-HT is synthesized through the actions of the rate-limiting enzyme tryptophan hydroxylase (TPH), of which 2 different types, TPH1 and TPH2, are expressed by EC cells and neurons. After release of 5-HT from EC cells or neurons, it is inactivated by uptake into enterocytes or neurons through the 5-HT reuptake transporter, followed by metabolization to 5-hydroxyindole acetic acid (5-HIAA), which is excreted in the urine. Abnormalities of serotonergic signaling, including altered expression of TPH-1 and 5-HT reuptake transporter, and altered release of 5-HT, have been implicated in IBS pathogenesis[24,89]. Specifically, patients with IBS-D have increased platelet-depleted 5-HT concentrations during fasting and postprandial conditions compared with healthy volunteers and patients with IBS-C[27].

LX-1031 is an orally administrable, TPH inhibitor, with poor systemic absorption and low penetration through the blood-brain barrier that decreases serotonin synthesis[90,91]. Among healthy volunteers, LX-1031 was well tolerated and dose dependently inhibited 5-HIAA levels, supporting the potential of the drug to inhibit 5-HT synthesis in the human GI tract upon oral administration[91]. Brown *et al*[92] reported the results of a phase IIa study with LX-1031 in patients with non-constipating IBS. A total of 155 patients were randomized to a 4-wk treatment with placebo or 250 mg or 1000 mg LX-1031 *q.d.* After 1 wk, a significantly greater number of patients obtained adequate relief of IBS symptoms with the high dose of LX-1031 compared with placebo (48% *vs* 22%, $P = 0.02$). In weeks 2-4, the response to LX-1031 was higher compared with placebo, but no statistical significance was reached. As a result, the therapeutic gain (adequate relief) decreased from 25% to 10%. Stool consistency measured with the Bristol Stool Form Scale improved significantly with the high dose compared with placebo during weeks 1, 2, and 4. In a subset of patients, urinary 5-HIAA was measured as a marker of 5-HT synthesis before and after 4 wk of treatment with LX-1031. Overall, the high dosage decreased 5-HIAA excretion by approximately 25%. In this subgroup, a significant correlation was found between the percent decrease in urinary 5-HIAA excretion and the adequate relief response at the end of the treatment, indicating that decreased 5-HT synthesis is the mechanism underlying the symptomatic benefit. This is supported further by a post hoc analysis that showed a significantly higher symptomatic benefit in those who achieved a > 15% decrease in urinary 5-HIAA excretion during treatment. LX-1031 was well tolerated and no safety issues were observed; however, more studies are needed to establish fully the safety and tolerance profile of this drug[89].

Crofelemer

Crofelemer is a proanthocyanidin oligomer. Crofelemer acts through an antisecretory mechanism by reducing excess intestinal chloride ion secretion. It exerts an antisecretory action on two distinct chloride channel targets on the luminal membrane of intestinal epithelial cells, namely the CFTR and calcium-activated chloride channel[93]. The drug is being investigated for the treatment of acute infectious diarrhea, chronic diarrhea associated with human immunodeficiency virus/acquired immunodeficiency syndrome, and IBS-D.

A randomized, double-blind, placebo-controlled, phase Ⅱ a 12-wk treatment study evaluated crofelemer for IBS-D. A total of 246 patients with IBS-D received either placebo or crofelemer at dosages of 125, 250, or 500 mg twice daily[94]. The primary end point was improvement in stool consistency. The study found that none of the doses of crofelemer improve d stool consistency, stool frequency, or urgency, or provided adequate relief of IBS symptoms. However, the 500-mg twice-daily dosage of crofelemer significantly increased pain- and discomfort-free days especially in women with IBS-D. Large clinical trials are necessary to evaluate the effectiveness and safety of crofelemer.

Antibiotics

The potential utility of antibiotics in IBS treatment has been supported by a growing body of evidence demonstrating the important role of bacteria in IBS pathogenesis. It has been proposed that small intestinal bacterial overgrowth (SIBO) might explain the physiological hallmarks of altered gut motility, visceral hypersensitivity, abnormal brain-gut interaction and immune activation seen in IBS[95]. This is supported by multiple lines of evidence; first, gas analysis is abnormal in 10%-84% of IBS patients undergoing lactulose breath testing[96,97]; second, the distribution of inflammatory mediators and /or inflammatory cells have been shown to be disturbed in some patients with IBS[98]. It is thought that SIBO may contribute to many of the clinical manifestations of IBS through bacterial fermentation and stimulation of a gut immune response, characterized by release of inflammatory mediators, such as interleukins and tumour necrosis factor-α, which may affect motility, secretion and sensation[95,99]. Postinfectious IBS, which occurs in 4%-31% of individuals assessed up to 12 mo after an episode of acute gastroenteritis[100], also supports an aetiological role of bacteria in IBS.

In earlier studies[97,101] the systemic antibiotic neomycin has been evaluated and was found to improve global symptoms compared with placebo. The non-absorbed (< 0.4%), oral antibiotic rifaximin is the most thoroughly studied antibiotic for the treatment of IBS. Rifaximin appears to be well suited for the treatment of IBS because of its broad-spectrum bactericidal activity *in vitro*, its efficacy for SIBO *in vivo*, its favorable tolerability profile and its lack of association with clinically relevant resistance or Clostridium difficile colitis[99,102]. Rifaximin has demonstrated its efficacy in RCTs evaluating IBS patients[103,104]. IBS trials utilized high doses of rifaximin: 400 mg three times daily for 10 d[104], 400 mg twice daily for 10 d[103], and 550 mg twice daily for 14 d[105]. Rifaximin, at these high doses, demonstrated statistically significant improvement in symptoms whereas patients reported at signifi-

cantly greater rate global improvement in IBS symptoms and/or bloating compared to patients treated with placebo. Pimentel *et al*[105] evaluated rifaximin as treatment for IBS in TARGET 1 and TARGET 2 studies. These were phase Ⅲ, double-blind, placebo-controlled trials, identically designed. Patients who suffered from IBS without constipation were included in the studies and were randomized to receive for two weeks 550 mg rifaximin or placebo, three times daily. Patients were then followed for an additional period of 10 wk. The study measured (weekly assessments) the proportion of patients that responded reporting adequate relief of global IBS symptoms and IBS-related bloating. A significantly higher rate of patients in the rifaximin group reported adequate relief of global IBS symptoms and bloating during the first 4 wk after treatment compared to patients in the placebo group (40.7% *vs* 31.7%, $P < 0.001$ and 40.2% *vs* 30.3%, $P < 0.001$, respectively). AEs were similar between the two groups. A metanalysis[106] that included 5 trials reporting data from 1803 patients was published in 2012. Rifaximin was found to be more efficacious than placebo for global IBS symptom improvement (OR = 1.57; therapeutic gain = 9.8%; NNT = 10.2). Rifaximin was significantly more likely to improve bloating than placebo (OR = 1.55; therapeutic gain = 9.9 %; NNT = 10.1). The authors noticed that studies with older patients and more females demonstrated higher response rates, which was consistent regardless of treatment group. Although therapeutic gain offered by rifaximin is modest, it was similar to that yielded by other currently available therapies for IBS.

The American Task Force systematic review[61] concludes that rifaximin has shown improvement of global IBS symptoms and bloating in trials included in their analysis. Rifaximin has mostly been offered in patients with IBS-D; therefore it seems as a reasonable option for IBS patients with bloating and patients with IBS-D. The suggested dose is 400 mg three times a day for 10-14 d; however symptoms may recur over three to nine months.

5ASA compounds

Mesalamine is an anti-inflammatory agent, effective in the treatment of inflammatory bowel disease. It has been proposed for IBS-D on the basis of treatment of the underlying chronic inflammation. Bowel infections, bacterial overgrowth syndrome, antibiotics, stress and unfavorable dietary habits can precede visceral hypersensitivity and lead to a clinical manifestation of IBS. Although there is no specific morphologic correlate of IBS, these predictors can affect the colon microbiota and the local immune system, decrease the protective properties of the bowel mucosa, impair mucus production, and may be caused by only minimal alterations on the cellular level. The detection of minor lesions is often accompanied by a decrease of proliferation and enhanced apoptosis of colonocytes[107]. Progression of the disease leads to more pronounced morphological changes of the colon mucosa epithelium: reduced frequency of serotonin-producing cells and mast cells and increased frequency of secondary cells and increasing number of cellular infiltrations by eosinophils, neutrophils, lymphocytes, plasmocytes and fibroblasts of stroma[107]. These morphological criteria are signs of inflammatory processes and activation of immune mechanisms. In this context mesalazine has been evaluated in a RCT trial in 20 IBS patients[108]. Patients received 800 mg mesalazine or placebo three times daily for eight weeks. The primary outcome measure was changes in the number of colonic immune cells on biopsies obtained at baseline and at the end of treatment. Symptom severity, changes in subsets of immune cells and inflammatory mediators were also evaluated. In the group of mesalazine the total count of immune cells and specifical the mast cells were reduced as compared with placebo ($P = 0.0082$ and $P = 0.0014$, respectively). General well-being was also improved in the group of mesalazine ($P = 0.038$), but did not seem to have an impact on abdominal pain ($P = 0.084$), bowel habits or bloating ($P = 0.177$). The drug was well tolerated with no serious AEs reported. In another study[109] 12 women with diarrhoea-predominant IBS received oral mesalazine (1.5 g *b.i.d.*) for four weeks followed by a 4-wk washout phase. Molecular profiling of stool bacterial communities and IBS symptoms were assessed before, during and after mesalazine treatment. Qualitative and quantitative effects of mesalazine on stool microbiota, mucosal proteolytic activity and IBS symptoms were assessed. Faecal bacteria decreased by 46% on mesalazine treatment ($P = 0.014$), but returned to baseline during washout. Eight of 12 (67%) patients responded favorably to mesalazine based on a global relief questionnaire, with significant decreases in the number of days with discomfort and increases in bowel movement satisfaction. In a recent trial[110] 17 patients who developed IBS-D after gastroenteritis were randomized to receive mesalamine 1.6 gm *b.i.d.* or placebo for 12 wk. Mesalamine was not associated with significant improvement in global symptoms, abdominal pain, bloating, stool urgency, frequency, or consistency (all $P \geqslant 0.11$) or QOL ($P \geqslant 0.16$). At this point, data from all these studies seem inconclusive. Further study of the bacteriological and anti-inflammatory properties of mesalazine in IBS is necessary.

ABDOMINAL PAIN

Antispasmodics

Exaggerated motility response of the small bowel and colon to environmental stimuli may be responsible for the symptoms, especially pain, experienced in IBS[111-113]. For this reason antispasmodics have been used for the symptoms of IBS. Antispasmodics encompass several different drug classes (smooth-muscle relaxants, antimuscarinics, anticholinergics) and unique agents (pinaverium, trimebutine). Given their mechanism of action, these agents are directed at those subgroups of IBS, with a predominant symptom of abdominal pain and stool patterns that are either mixed or more diarrheal in nature. The propensity of these agents to promote constipation

makes them a less attractive option for patients with IBS-C. The anticholinergic properties of these agents restrict their usefulness in clinical practice. Common side effects that often limit these drugs usefulness in the treatment of IBS are dizziness, dry mouth, confusion (particularly in elderly patients), blurry vision, urinary retention, and constipation[41].

A systematic review and meta-analysis of antispasmodics as a class was performed by the ACG IBS Task Force[61]. The Task Force identified 22 studies suitable for inclusion in their systematic review. Most of these clinical trials are dated, with only 3 of the studies performed in the last 10 years. Studies evaluated hyoscine, hyoscyamine, otilonium, cimetropium, pinaverium, trimebutine, alverine, mebeverine, pirenzipine, prifinium, propinox, and a trimebutine/rociverine combination. The 22 trials collectively included data from 1778 patients with IBS. The pooled analysis of these studies revealed a RR of symptoms persisting with antispasmodics compared with placebo of 0.68 (95%CI: 0.57-0.81) and a NNT of 5. The pooled analysis that was performed on the 13 studies, included 1379 patients in whom AEs were reported. There was significant heterogeneity among these patients; moreover these clinical trials were collectively fraught with methodological flaws, including diagnostic criteria used, inclusion criteria used, dosing schedule used, duration of therapy studied, study end points used to assess response, and study size (only three studies enrolled more than 100 patients). The review concluded that some drugs in the antispasmodics class (cimetropium, hyoscine, pinaverium) may be an option for relief of abdominal discomfort and pain in IBS-patients. Older systematic reviews have yielded mixed results regarding the efficacy of antispasmodics for IBS[114,115].

Mebeverine is an antispasmodic that has been successfully used in the management of IBS for many years. Mebeverine is a musculotropic agent that has antispasmodic activity and regulatory effects on the bowel function[116]. During oral administration at doses of 135-270 mg *t.i.d.*, it shows no typical anticholinergic side effects. There is no indication that the incidence of side effects caused by mebeverine is higher than that of a placebo[114]. In 2010, a metanalysis was published on the efficacy and tolerability of mebeverine in IBS in its usual dosages[117]. Eight randomized trials including 555 patients with all IBS subtypes, randomized to receive either mebeverine or placebo, met the metanalysis criteria. The pooled RR for clinical improvement of mebeverine was 1.13 (P = 0.7) and 1.33 (P = 0.12) for relief of abdominal pain. The efficacy of mebeverine 200 mg compared to mebeverine 135 mg indicated RRs of 1.12 (P = 0.168) for clinical or global improvement and 1.08 (P = 0.463) for relief of abdominal pain. Thus, mebeverine was shown to be well tolerated with no significant AEs; however, its efficacy in global improvement of IBS did not reach statistical significance. Recently the results of an exploratory RCT of mebeverine, methylcellulose, placebo and a self-management online (website) treatment method (cognitive behavior treatment) were published[118]. One hundred thirty-five patients, with IBS symptoms fulfilling Rome Ⅲ criteria were randomized to over-encapsulated mebeverine, methylcellulose or placebo for six weeks and to 1 of 3 website conditions. Mean IBS SSS (symptom severity scale) decreased by 35 points from baseline to 12 wk of treatment. There was no significant difference in IBS SSS or IBS-QOL score between medication and website groups. However, IBS SSS at six weeks was lower in the No-website group than the website groups (P = 0.037). In the end of the study, the global relief of IBS symptoms was significantly improved in the website groups compared to the non-website group at 12 wk of treatment (Enablement and Subjects Global Assessment of relief P = 0.001 and P = 0.035 respectively).

Otilonium bromide (OB) has been shown to reduce the pain severity in IBS patients effectively[61]. OB is an ammonium derivative with spasmolytic activity in GI smooth muscle by inhibiting the calcium ion influx through L-type voltage operated calcium channels. OB pharmacologically has been demonstrated to inhibit central/peripheral tachykinin-2 receptor; in this way it reduces the sensory signals afferent transmission from the periphery to central nervous system[119]. Additionally, OB binds with high affinity to muscarinic receptor subtypes M1, M2, M3, M4 and M5[120,121]. M3 sub-receptor is located in human colonic crypt cells to mediate secretion coupled with calcium channels. Due to its potent muscarinic blockade of M3, OB exhibits its antisecretory properties, thus improving stool consistency[121]. Among researches on the OB efficacy on IBS patients, early studies indicated that OB is effective for abdominal pain and bloating but there was a difficulty in demonstrating efficacy over placebo[122,123]. A review based on four OB trials was eventually conducted in 2008. Various antispasmodics were studied, but OB (four trials, 435 patients, RR of persistent symptoms 0.55, 0.31 to 0.97) showed consistent evidence of efficacy over placebo[124]. Subsequently, two RCTs were published. The first multi-center phase Ⅳ double-blind study[125] randomized 356 patients with various IBS subtypes to receive OB (40 mg *t.d.s.*) or placebo for 15 weeks, and follow-up was extended 10 additional weeks. The effect of OB was significantly greater than placebo in the reduction of weekly frequency of episodes of abdominal pain at the end of treatment period (P = 0.03); similarly OB was superior to placebo in the reduction of abdominal bloating (P = 0.02) and in the global efficacy by patient assessment (P = 0.047). However, no difference between the effect of OB and placebo was found in the intensity of abdominal pain, the proportion of patient responders, and the safety and quality of life scores. During follow-up, the therapeutic effect of OB remained greater than placebo in terms of withdrawal rate due to symptom relapse (P = 0.009), global efficacy of treatment and relapse-free probability (P = 0.038). Therefore, the study demonstrated superiority of OB *vs* placebo in the reduction of pain and bloating, and in protection from relapse as a result of the long-lasting

effect. These symptoms improved progressively during the study. It should be pointed out that IBS trials are subjected to high placebo effect, typically between 30% and 60% thus making difficult to detect the therapeutic gain and interpretation of the results[126]. The second trial was an Asian study[127] which randomized 117 participants to receive 40 mg OB or 100 mg mebeverine, thrice daily for eight weeks. The abdominal pain/discomfort frequency score (APDFS) and safety profile were assessed. Compared to baselines, the APDFSs in OB and mebeverine were significantly reduced (0.55; $P = 0.011$ and 0.37; $P = 0.042$ respectively). However, when the improved results of the two treatments were compared between them, statistical significance was not reached). One hundred eighteen AEs were reported (OB = 65 and mebeverine = 53); these comprised mostly dry mouth in both arms, followed by nausea and dizziness (particularly in OB).

Similarly, solifenacin, a muscarinic type 3 receptor antagonist, that is used to treat overactive bladder in adults has been evaluated in a recent study for the symptomatic relief of diarrhea in 20 IBS-D patients[128]. After a 2-wk observation period, all participants received solifenacin for six weeks. Subsequently, the administration of solifenacin was discontinued and ramosetron, a serotonin 3 receptor antagonist, was administered for four weeks. Two weeks after initiation of solifenacin, an overall improvement was observed in 16 out of 20 participants (80%). The efficacy of solifenacin in the treatment of IBS with diarrhea was not inferior to that of ramosetron. However, the study had the limitation of not being placebo-controlled.

In recent years, increasing attention has been given to the role of the nonadrenergic and noncholinergic (NANC) nervous system for the regulation of colonic motility. Nitric oxide (NO) has been identified as an important component of the NANC nervous system and as an inhibitory neurotransmitter in the colon[129]. NO mediates the relaxation of smooth muscle cells in the GI tract by production of intracellular guanosine 3,5-cyclic monophosphate (cGMP)[129] and is also involved in nociception[130]. Sildenafil is an orally administered drug that has been used to augment NO activity and is widely used as a treatment for erectile dysfunction. In an earlier study[131] stimulation of the NO-cGMP pathway by sildenaifil administration decreased rectal tone but did not influence rectal distensibility. Relaxation of the rectum was accompanied by an increase in rectal volumes to reach perception thresholds in healthy subjects and in patients with IBS, but no direct effect on rectal perception could be demonstrated. Recently, another small study[132] evaluated the effects of sildenafil tone inhibition on rectal sensitivity. Eight control subjects and 21 IBS patients (Rome Ⅱ) were enrolled in a double-blinded study, after dosing with placebo or sildenafil (50 mg *p.o.*). Sildenafil increased the first desire to defecate and the pain in the hypersensitive IBS patients. It also increased rectal compliance, but only in diarrhea-IBS. No trials regarding the effectiveness of sildenafil on the relief of the IBS symptoms and the quality of life are available.

Opioid receptor agonists

Opioid receptors, including m, d, and k, are expressed along the GI tract and play a key role in regulating GI motility, secretion, and visceral sensation. Recently, exogenous opioids have been shown to reduce GI transit through activation of m-opioid receptor (MOR) and they can treat diarrhea in acute situations. Agents that simultaneously activate MOR and antagonize d-opioid receptor (DOR) have differential GI effects and can possess increased analgesic potency compared to pure MOR agonists[133]. Eluxadoline is a locally active, mixed MOR agonist/DOR antagonist with low oral bioavailability that is being developed for the treatment of IBS-D. In vitro, eluxadoline reduces contractility in intestinal tissue and inhibits neurogenically mediated secretion[134]. In a recent phase Ⅱ study[135] 807 patients were randomly assigned to groups receiving twice daily 5, 25, 100, or 200 mg oral eluxadoline or oral placebo for 12 wk. The primary end point was clinical response at week four, defined by a mean reduction in daily pain score of more than 30% from baseline and of at least 2 points on 0-10 scale, as well as a stool consistency score of 3 or 4 on the Bristol Stool Scale (1-7) for at least 66% of daily diary entries during that week. The authors concluded that patients given eluxadoline were significantly more likely to be clinical responders, based on a combination of improvement in abdominal pain and stool consistency. Another selective, potent k-opioid agonist, asimadoline, which has been shown to improve pain and abnormal bowel function, has been evaluated in a trial[136]. Asimadoline has low permeability through the blood-brain barrier. In this trial, 596 patients with varying IBS subtypes were randomized to receive 0.15, 0.5, 1.0 mg asimadoline or placebo *b.i.d* for twelve weeks. Asimadoline (0.5 mg) significantly prolonged the total time (number of mo) with adequate relief of IBS pain or discomfort (46.7% *vs* 20.0%), adequate relief of IBS symptoms (46.7% *vs* 23.0%). It also significantly reduced pain scores (week 12: -1.6 *vs* -0.7), increased pain free days (42.9% *vs* 18.0%), and improved urgency and stool frequency (-2.3 *vs* -0.3). These positive results were observed in IBS-D patients with at least moderate pain in baseline. However, no significant difference was observed in the percentage of months with adequate relief. Asimadoline failed to show a benefit in IBS-C.

Drugs acting through the endocannabinoid system have also been studied. Two types of G-protein- coupled cannabinoid receptors, CB1 and CB2, have been identified and cloned[137]. CB1-immunoreactivity is located on the normal colonic epithelium, smooth muscle, and the myenteric plexus. Dronabinol, a nonselective CB receptor agonist, has been shown to inhibit and colonic motility in healthy humans[138]. In a recent study[139], the effect of dronabinol on colonic sensory and motor functions in 75 patients with mixed IBS subtypes who were cannabinoid naïve was assessed. Patients were randomly assigned to

groups that were given a single dose of placebo or 2.5 mg or 5.0 mg dronabinol. Single nucleotide polymorphisms CNR1 rs806378, fatty acid amide hydrolase (FAAH) rs324420, and MGLL rs4881 were also studied. In all patients, dronabinol decreased fasting proximal left colonic motility index compared with placebo and increased the colonic compliance. The effects of dronabinol were greatest in IBS patients with diarrhea or IBS alternating. Dronabinol did not alter sensation or tone but it affected fasting distal motility index in patients, regardless of FAAH rs324420 variant (CA/AA *vs* CC) ($P = 0.046$)

GLP-1 (Rose-10)

GLP-1(glucagon-like peptide 1) is normally released after food intake. It stimulates insulin release and reduces gastric emptying and small intestinal motility[140]. GLP-1 has been reported to inhibits small intestinal motility in IBS patients[141] and to prolong colonic transit[142]. The initial use of GLP-1 analogues was to normalize blood glucose levels in patients with diabetes; however, based on the aforementioned observation,s are now being studied to treat abdominal pain attacks in patients with IBS. The GLP-1 analog ROSE-010 has been demonstrated to reduce acute IBS pain in a RCT involving 166 IBS patients[143]. Participants were assigned to receive single subcutaneous injections of ROSE-010 100 μg, 300 μg and placebo in a cross-over design. Patient-rated pain relief and intensity were evaluated with a visual-analog scale. The primary outcome measure was the proportion of patients with a minimum 50% pain reduction from 10 to 60 min after treatment. A significantly higher proportion of patients reported greater than 50% of the maximum total pain relief response after 100 and 300 μg of ROSE-010 treatments than after placebo (23% and 24% *vs* 12%; $P = 0.011$ and $P = 0.005$, respectively). Times to meaningful and total pain relief were shorter for both doses of active drug *vs* placebo. A second single-center RCT evaluated safety, pharmacodynamics, and pharmacokinetics in women with IBS-C[144]. Patients were administered once daily 30, 100, or 300 μg ROSE-010 subcutaneously or placebo for three consecutive days as well as a single repetitive dose after 2-10 d. Validated scintigraphy was used to measure GI and colonic transit. Single-photon emission computed tomography was used to measure gastric volumes. The primary outcome measures were gastric emptying of solids half time, the colonic transit geometric center at 24 h, and the gastric accommodation volume. Gastric emptying was significantly retarded at the doses of 100 and 300 μg ROSE-010. Gastric volumes, small bowel or colonic transit at 24 h and bowel functions were not significantly altered by ROSE-010. Colonic transit at 48 h was accelerated with the 30 and 100 μg ROSE-010 doses. AEs were vomiting ($P = 0.008$) and nausea nausea ($P < 0.001$). Based on the observation that at the doses of 30 and 100 μg the drug accelerated colonic transit time, the authors concluded that it could be a candidate for relief of constipation in IBS-C. More in-depth assessments of the IBS pain attack characteristics are ongoing and future clinical trials with ROSE-010 are being planned[15].

Ketotifen

Experimental studies have shown that mast cells play an important role in IBS through visceral hypersensitivity[145]. Patients with IBS excibit an increased number of mast cells in the small intestine[146], large intestine[147,148] and rectum[149]. The number of mucosal mast cells and their proximity to sensory nerves in colonic tissue has also been studied and found positively correlated to abdominal pain[148]. Mast cells activation results in degranulation; thus mediators pre-stored in vesicles such as tryptase, histamine and several cytokines are rapidly released inducing an inflammatory response. Sodium cromoglycate and ketotifen are well known membrane stabilizers that act by blocking mast cell degranulation[145]. Klooker *et al*[145] conducted a RCT to assess the effect of ketotifen on IBS. Sixty patients with various IBS subtypes (Rome Ⅱ criteria) were included in the study. The idea was to evaluate whether increased number of mast cells and/or increased spontaneous mucosal tryptase release is associated with visceral hypersensitivity and whether mast cell stabilization with ketotifen had an impact on visceral perception; this was estimated by measurements of rectal distension in hypersensitive patients with IBS. Abdominal symptoms were also monitored. The trial consisted of two weeks of screening/observation, then a treatment period of eight weeks and a follow-up period of another two weeks. Barostat measurements were performed at baseline and then after eight weeks of treatment with ketotifen or placebo. Rectal biopsies were also collected before and after treatment. Ketotifen was shown to be superior to placebo in increasing the threshold for discomfort in patients with IBS with visceral hypersensitivity; it also significantly improved abdominal pain and quality of life. Mast cells and spontaneous release of tryptase were lower in patients with IBS than in healthy volunteers. However, ketotifen did not histamine and tryptase release. Further studies are needed to confirm the beneficial effect of ketotifen in IBS symptoms and clarify its way of action.

CONCLUSION

IBS is a highly prevalent functional disorder that reduces patients' quality of life. IBS is not a single disease entity, but rather likely consists of several different disease states; currently, treatment is predicated upon the patient's most bothersome symptoms. Various drug categories (antispasmodics, laxatives, dopamine antagonists, 5-HT3 antagonists and/or 5-HT4 agonists, sedatives, antibiotics, probiotics), modifications in diet and lifestyle, and complementary and alternative therapies have been proposed as symptomatic treatment. It is difficult to draw conclusions from previous studies since IBS trials are subjected to high placebo effect, typically between 30% and 60% thus complicating the detection of the thera-

peutic gain and interpretation of the results. For IBS-C, linaclotide and lubiprostone seem promising for the relief of multiple symptoms from which patients with IBS-C suffer. Regarding IBS-D, although the 5-HT3 antagonist alosetron was shown to be superior than placebo at relieving global IBS symptoms in male and female with a high level of evidence, it was withdrawn from the market due to complications (ischemic colitis). Newer 5-HT3 antagonists (cilansetron, ramosetron) have emerged; however there is lack of consistent data demonstrating whether the drug is superior over placebo. In the category of antibiotics, rifaximin has been presented as efficacious in RCTs evaluating IBS patients. It has emerged as a strong option for the treatment of IBS because of its broad-spectrum bactericidal activity *in vitro*, its efficacy for SIBO *in vivo*, its favorable tolerability profile and the lack of association with clinically relevant resistance or Clostridium difficile colitis. Among the antispasmodics, OB showed consistent evidence of efficacy over placebo. Other molecules, *i.e.* NO donors, Opioid Receptor Agonists, ketotifen, as well as GLP-1 have been proposed for IBS treatment as well.

REFERENCES

1 **Longstreth GF**, Wilson A, Knight K, Wong J, Chiou CF, Barghout V, Frech F, Ofman JJ. Irritable bowel syndrome, health care use, and costs: a U.S. managed care perspective. *Am J Gastroenterol* 2003; **98**: 600-607 [PMID: 12650794 DOI: 10.1111/j.1572-0241.2003.07296.x]

2 **Longstreth GF**, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology* 2006; **130**: 1480-1491 [PMID: 16678561 DOI: 10.1053/j.gastro.2005.11.061]

3 **Lacy BE**, Weiser K, De Lee R. The treatment of irritable bowel syndrome. *Therap Adv Gastroenterol* 2009; **2**: 221-238 [PMID: 21180545 DOI: 10.1177/1756283X09104794]

4 **Crowell MD**, Harris L, Jones MP, Chang L. New insights into the pathophysiology of irritable bowel syndrome: implications for future treatments. *Curr Gastroenterol Rep* 2005; **7**: 272-279 [PMID: 16042910 DOI: 10.1007/s11894-005-0019-8]

5 **Hasler WL**. Traditional thoughts on the pathophysiology of irritable bowel syndrome. *Gastroenterol Clin North Am* 2011; **40**: 21-43 [PMID: 21333899 DOI: 10.1016/j.gtc.2010.12.004]

6 **Pimentel M**, Chang C. Inflammation and microflora. *Gastroenterol Clin North Am* 2011; **40**: 69-85 [PMID: 21333901 DOI: 10.1016/j.gtc.2010.12.010]

7 **Salari P**, Abdollahi M. Systematic review of modulators of benzodiazepine receptors in irritable bowel syndrome: is there hope? *World J Gastroenterol* 2011; **17**: 4251-4257 [PMID: 22090780 DOI: 10.3748/wjg.v17.i38.4251]

8 **Manabe N**, Wong BS, Camilleri M, Burton D, McKinzie S, Zinsmeister AR. Lower functional gastrointestinal disorders: evidence of abnormal colonic transit in a 287 patient cohort. *Neurogastroenterol Motil* 2010; **22**: 293-e82 [PMID: 20025692 DOI: 10.1111/j.1365-2982.2009.01442.x]

9 **Deiteren A**, Camilleri M, Burton D, McKinzie S, Rao A, Zinsmeister AR. Effect of meal ingestion on ileocolonic and colonic transit in health and irritable bowel syndrome. *Dig Dis Sci* 2010; **55**: 384-391 [PMID: 19949866 DOI: 10.1007/s10620-009-1041-8]

10 **Khoshoo V**, Armstead C, Landry L. Effect of a laxative with and without tegaserod in adolescents with constipation predominant irritable bowel syndrome. *Aliment Pharmacol Ther* 2006; **23**: 191-196 [PMID: 16393297 DOI: 10.1111/j.1365-2036.2006.02705.x]

11 **Awad RA**, Camacho S. A randomized, double-blind, placebo-controlled trial of polyethylene glycol effects on fasting and postprandial rectal sensitivity and symptoms in hypersensitive constipation-predominant irritable bowel syndrome. *Colorectal Dis* 2010; **12**: 1131-1138 [PMID: 19575740 DOI: 10.1111/j.1463-1318.2009.01990.x]

12 **Chapman RW**, Stanghellini V, Geraint M, Halphen M. Randomized clinical trial: macrogol/PEG 3350 plus electrolytes for treatment of patients with constipation associated with irritable bowel syndrome. *Am J Gastroenterol* 2013; **108**: 1508-1515 [PMID: 23835436 DOI: 10.1038/ajg.2013.197]

13 **Bryant AP**, Busby RW, Bartolini WP, Cordero EA, Hannig G, Kessler MM, Pierce CM, Solinga RM, Tobin JV, Mahajan-Miklos S, Cohen MB, Kurtz CB, Currie MG. Linaclotide is a potent and selective guanylate cyclase C agonist that elicits pharmacological effects locally in the gastrointestinal tract. *Life Sci* 2010; **86**: 760-765 [PMID: 20307554 DOI: 10.1016/j.lfs.2010.03.015]

14 **Shailubhai K**. Therapeutic applications of guanylate cyclase-C receptor agonists. *Curr Opin Drug Discov Devel* 2002; **5**: 261-268 [PMID: 11926132]

15 **Maneerattanaporn M**, Chang L, Chey WD. Emerging pharmacological therapies for the irritable bowel syndrome. *Gastroenterol Clin North Am* 2011; **40**: 223-243 [PMID: 21333909 DOI: 10.1016/j.gtc.2010.12.002]

16 **Andresen V**, Camilleri M, Busciglio IA, Grudell A, Burton D, McKinzie S, Foxx-Orenstein A, Kurtz CB, Sharma V, Johnston JM, Currie MG, Zinsmeister AR. Effect of 5 days linaclotide on transit and bowel function in females with constipation-predominant irritable bowel syndrome. *Gastroenterology* 2007; **133**: 761-768 [PMID: 17854590]

17 **Johnston JM**, Kurtz CB, Drossman DA, Lembo AJ, Jeglinski BI, MacDougall JE, Antonelli SM, Currie MG. Pilot study on the effect of linaclotide in patients with chronic constipation. *Am J Gastroenterol* 2009; **104**: 125-132 [PMID: 19098860 DOI: 10.1038/ajg.2008.59]

18 **Lembo AJ**, Kurtz CB, Macdougall JE, Lavins BJ, Currie MG, Fitch DA, Jeglinski BI, Johnston JM. Efficacy of linaclotide for patients with chronic constipation. *Gastroenterology* 2010; **138**: 886-895.e1 [PMID: 20045700 DOI: 10.1053/j.gastro.2009.12.050]

19 **Johnston JM**, Kurtz CB, Macdougall JE, Lavins BJ, Currie MG, Fitch DA, O'Dea C, Baird M, Lembo AJ. Linaclotide improves abdominal pain and bowel habits in a phase IIb study of patients with irritable bowel syndrome with constipation. *Gastroenterology* 2010; **139**: 1877-1886.e2 [PMID: 20801122 DOI: 10.1053/j.gastro.2010.08.041]

20 **Chey WD**, Lembo AJ, Lavins BJ, Shiff SJ, Kurtz CB, Currie MG, MacDougall JE, Jia XD, Shao JZ, Fitch DA, Baird MJ, Schneier HA, Johnston JM. Linaclotide for irritable bowel syndrome with constipation: a 26-week, randomized, double-blind, placebo-controlled trial to evaluate efficacy and safety. *Am J Gastroenterol* 2012; **107**: 1702-1712 [PMID: 22986437 DOI: 10.1038/ajg.2012.254]

21 **Rao S**, Lembo AJ, Shiff SJ, Lavins BJ, Currie MG, Jia XD, Shi K, MacDougall JE, Shao JZ, Eng P, Fox SM, Schneier HA, Kurtz CB, Johnston JM. A 12-week, randomized, controlled trial with a 4-week randomized withdrawal period to evaluate the efficacy and safety of linaclotide in irritable bowel syndrome with constipation. *Am J Gastroenterol* 2012; **107**: 1714-1724; quiz p.1725 [PMID: 22986440 DOI: 10.1038/ajg.2012.255]

22 **Quigley EM**, Tack J, Chey WD, Rao SS, Fortea J, Falques M, Diaz C, Shiff SJ, Currie MG, Johnston JM. Randomised clinical trials: linaclotide phase 3 studies in IBS-C - a prespecified further analysis based on European Medicines Agency-specified endpoints. *Aliment Pharmacol Ther* 2013; **37**: 49-61 [PMID: 23116208 DOI: 10.1111/apt.12123]

23 **Videlock EJ**, Cheng V, Cremonini F. Effects of linaclotide in patients with irritable bowel syndrome with constipation

or chronic constipation: a meta-analysis. *Clin Gastroenterol Hepatol* 2013; **11**: 1084-1092.e3; quiz e68 [PMID: 23644388 DOI: 10.1016/j.cgh.2013.04.032]
24 **Hasler WL**. Serotonin and the GI tract. *Curr Gastroenterol Rep* 2009; **11**: 383-391 [PMID: 19765366 DOI: 10.1007/s11894-009-0058-7]
25 **Houghton LA**, Atkinson W, Whitaker RP, Whorwell PJ, Rimmer MJ. Increased platelet depleted plasma 5-hydroxytryptamine concentration following meal ingestion in symptomatic female subjects with diarrhoea predominant irritable bowel syndrome. *Gut* 2003; **52**: 663-670 [PMID: 12692050 DOI: 10.1136/gut.52.5.663]
26 **Dunlop SP**, Coleman NS, Blackshaw E, Perkins AC, Singh G, Marsden CA, Spiller RC. Abnormalities of 5-hydroxytryptamine metabolism in irritable bowel syndrome. *Clin Gastroenterol Hepatol* 2005; **3**: 349-357 [PMID: 15822040]
27 **Atkinson W**, Lockhart S, Whorwell PJ, Keevil B, Houghton LA. Altered 5-hydroxytryptamine signaling in patients with constipation- and diarrhea-predominant irritable bowel syndrome. *Gastroenterology* 2006; **130**: 34-43 [PMID: 16401466 DOI: 10.1053/j.gastro.2005.09.031]
28 **Zuo XL**, Li YQ, Yang XZ, Guo M, Guo YT, Lu XF, Li JM, Desmond PV. Plasma and gastric mucosal 5-hydroxytryptamine concentrations following cold water intake in patients with diarrhea-predominant irritable bowel syndrome. *J Gastroenterol Hepatol* 2007; **22**: 2330-2337 [PMID: 18265445 DOI: 10.1111/j.1440-1746.2006.04772.x]
29 **Gershon MD**, Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. *Gastroenterology* 2007; **132**: 397-414 [PMID: 17241888 DOI: 10.1053/j.gastro.2006.11.002]
30 **Grider JR**, Foxx-Orenstein AE, Jin JG. 5-Hydroxytryptamine4 receptor agonists initiate the peristaltic reflex in human, rat, and guinea pig intestine. *Gastroenterology* 1998; **115**: 370-380 [PMID: 9679042 DOI: 10.1016/S0016-5085(98)70203-3]
31 **Degen L**, Matzinger D, Merz M, Appel-Dingemanse S, Osborne S, Lüchinger S, Bertold R, Maecke H, Beglinger C. Tegaserod, a 5-HT4 receptor partial agonist, accelerates gastric emptying and gastrointestinal transit in healthy male subjects. *Aliment Pharmacol Ther* 2001; **15**: 1745-1751 [PMID: 11683688 DOI: 10.1046/j.1365-2036.2001.01103.x]
32 **Prather CM**, Camilleri M, Zinsmeister AR, McKinzie S, Thomforde G. Tegaserod accelerates orocecal transit in patients with constipation-predominant irritable bowel syndrome. *Gastroenterology* 2000; **118**: 463-468 [PMID: 10702196 DOI: 10.1016/S0016-5085(00)70251-4]
33 **Jiao HM**, Xie PY. Tegaserod inhibits noxious rectal distention induced responses and limbic system c-Fos expression in rats with visceral hypersensitivity. *World J Gastroenterol* 2004; **10**: 2836-2841 [PMID: 15334681]
34 **Sabaté JM**, Bouhassira D, Poupardin C, Wagner A, Loria Y, Coffin B. Sensory signalling effects of tegaserod in patients with irritable bowel syndrome with constipation. *Neurogastroenterol Motil* 2008; **20**: 134-141 [PMID: 18004988 DOI: 10.1111/j.1365-2982.2007.01030.x]
35 **Müller-Lissner SA**, Fumagalli I, Bardhan KD, Pace F, Pecher E, Nault B, Rüegg P. Tegaserod, a 5-HT(4) receptor partial agonist, relieves symptoms in irritable bowel syndrome patients with abdominal pain, bloating and constipation. *Aliment Pharmacol Ther* 2001; **15**: 1655-1666 [PMID: 11564007 DOI: 10.1046/j.1365-2036.2001.01094.x]
36 **Novick J**, Miner P, Krause R, Glebas K, Bliesath H, Ligozio G, Rüegg P, Lefkowitz M. A randomized, double-blind, placebo-controlled trial of tegaserod in female patients suffering from irritable bowel syndrome with constipation. *Aliment Pharmacol Ther* 2002; **16**: 1877-1888 [PMID: 12390096 DOI: 10.1046/j.1365-2036.2002.01372.x]
37 **Chey WD**, Paré P, Viegas A, Ligozio G, Shetzline MA. Tegaserod for female patients suffering from IBS with mixed bowel habits or constipation: a randomized controlled trial. *Am J Gastroenterol* 2008; **103**: 1217-1225 [PMID: 18477346 DOI: 10.1111/j.1572-0241.2008.01808.x]
38 **Kellow J**, Lee OY, Chang FY, Thongsawat S, Mazlam MZ, Yuen H, Gwee KA, Bak YT, Jones J, Wagner A. An Asia-Pacific, double blind, placebo controlled, randomised study to evaluate the efficacy, safety, and tolerability of tegaserod in patients with irritable bowel syndrome. *Gut* 2003; **52**: 671-676 [PMID: 12692051 DOI: 10.1136/gut.52.5.671]
39 **Nyhlin H**, Bang C, Elsborg L, Silvennoinen J, Holme I, Rüegg P, Jones J, Wagner A. A double-blind, placebo-controlled, randomized study to evaluate the efficacy, safety and tolerability of tegaserod in patients with irritable bowel syndrome. *Scand J Gastroenterol* 2004; **39**: 119-126 [PMID: 15000272 DOI: 10.1080/00365520310006748]
40 **Tougas G**, Snape WJ, Otten MH, Earnest DL, Langaker KE, Pruitt RE, Pecher E, Nault B, Rojavin MA. Long-term safety of tegaserod in patients with constipation-predominant irritable bowel syndrome. *Aliment Pharmacol Ther* 2002; **16**: 1701-1708 [PMID: 12269961 DOI: 10.1046/j.1365-2036.2002.01347.x]
41 **Saad RJ**. Peripherally acting therapies for the treatment of irritable bowel syndrome. *Gastroenterol Clin North Am* 2011; **40**: 163-182 [PMID: 21333906 DOI: 10.1016/j.gtc.2010.12.008]
42 **Thompson CA**. Novartis suspends tegaserod sales at FDA' s request. *Am J Health Syst Pharm* 2007; **64**: 1020 [PMID: 17494895 DOI: 10.2146/news070044]
43 **Serebruany VL**, El Mouelhi M, Pfannkuche HJ, Rose K, Marro M, Angiolillo DJ. Investigations on 5-HT$_4$ receptor expression and effects of tegaserod on human platelet aggregation in vitro. *Am J Ther* 2010; **17**: 543-552 [PMID: 19797939 DOI: 10.1097/MJT.0b013e3181b63f21]
44 **Liu Z**, Sakakibara R, Odaka T, Uchiyama T, Uchiyama T, Yamamoto T, Ito T, Asahina M, Yamaguchi K, Yamaguchi T, Hattori T. Mosapride citrate, a novel 5-HT4 agonist and partial 5-HT3 antagonist, ameliorates constipation in parkinsonian patients. *Mov Disord* 2005; **20**: 680-686 [PMID: 15719424 DOI: 10.1002/mds.20387]
45 **Kim HS**, Choi EJ, Park H. The effect of mosapride citrate on proximal and distal colonic motor function in the guinea-pig in vitro. *Neurogastroenterol Motil* 2008; **20**: 169-176 [PMID: 17931343 DOI: 10.1111/j.1365-2982.2007.00996.x]
46 **Kojima Y**, Fujii H, Katsui R, Nakajima Y, Takaki M. Enhancement of the intrinsic defecation reflex by mosapride, a 5-HT4 agonist, in chronically lumbosacral denervated guinea pigs. *J Smooth Muscle Res* 2006; **42**: 139-147 [PMID: 17159330 DOI: 10.1540/jsmr.42.139]
47 **Kojima Y**, Nakagawa T, Katsui R, Fujii H, Nakajima Y, Takaki M. A 5-HT4 agonist, mosapride, enhances intrinsic rectorectal and rectoanal reflexes after removal of extrinsic nerves in guinea pigs. *Am J Physiol Gastrointest Liver Physiol* 2005; **289**: G351-G360 [PMID: 15817810 DOI: 10.1152/ajpgi.00532.2004]
48 **Kanazawa M**, Watanabe S, Tana C, Komuro H, Aoki M, Fukudo S. Effect of 5-HT4 receptor agonist mosapride citrate on rectosigmoid sensorimotor function in patients with irritable bowel syndrome. *Neurogastroenterol Motil* 2011; **23**: 754-e332 [PMID: 21615623 DOI: 10.1111/j.1365-2982.2011.01732.x]
49 **Lembo AJ**, Cremonini F, Meyers N, Hickling R. Clinical trial: renzapride treatment of women with irritable bowel syndrome and constipation - a double-blind, randomized, placebo-controlled, study. *Aliment Pharmacol Ther* 2010; **31**: 979-990 [PMID: 20163375 DOI: 10.1111/j.1365-2036.2010.04265.x]
50 **Lacy BE**, Levy LC. Lubiprostone: a chloride channel activator. *J Clin Gastroenterol* 2007; **41**: 345-351 [PMID: 17413599 DOI: 10.1097/01.mcg.0000225665.68920.df]
51 **Bijvelds MJ**, Bot AG, Escher JC, De Jonge HR. Activation of intestinal Cl- secretion by lubiprostone requires the cystic fibrosis transmembrane conductance regulator. *Gastroenterology* 2009; **137**: 976-985 [PMID: 19454284 DOI: 10.1053/j.gastro.2009.05.037]
52 **Chan WW**, Mashimo H. Lubiprostone Increases Small Intestinal Smooth Muscle Contractions Through a Prostaglandin

E Receptor 1 (EP1)-mediated Pathway. *J Neurogastroenterol Motil* 2013; **19**: 312-318 [PMID: 23875097 DOI: 10.5056/jnm.2013.19.3.312]
53 **Camilleri M**, Bharucha AE, Ueno R, Burton D, Thomforde GM, Baxter K, McKinzie S, Zinsmeister AR. Effect of a selective chloride channel activator, lubiprostone, on gastrointestinal transit, gastric sensory, and motor functions in healthy volunteers. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G942-G947 [PMID: 16603730 DOI: 10.1152/ajpgi.00264.2005]
54 **Johanson JF**, Ueno R. Lubiprostone, a locally acting chloride channel activator, in adult patients with chronic constipation: a double-blind, placebo-controlled, dose-ranging study to evaluate efficacy and safety. *Aliment Pharmacol Ther* 2007; **25**: 1351-1361 [PMID: 17509103 DOI: 10.1111/j.1365-2036.2007.03320.x]
55 **Johanson JF**, Morton D, Geenen J, Ueno R. Multicenter, 4-week, double-blind, randomized, placebo-controlled trial of lubiprostone, a locally-acting type-2 chloride channel activator, in patients with chronic constipation. *Am J Gastroenterol* 2008; **103**: 170-177 [PMID: 17916109 DOI: 10.1111/j.1572-0241.2007.01524.x]
56 **Barish CF**, Drossman D, Johanson JF, Ueno R. Efficacy and safety of lubiprostone in patients with chronic constipation. *Dig Dis Sci* 2010; **55**: 1090-1097 [PMID: 20012484]
57 **Johanson JF**, Drossman DA, Panas R, Wahle A, Ueno R. Clinical trial: phase 2 study of lubiprostone for irritable bowel syndrome with constipation. *Aliment Pharmacol Ther* 2008; **27**: 685-696 [PMID: 18248656 DOI: 10.1111/j.1365-2036.2008.03629.x]
58 **Fukudo S**, Hongo M, Kaneko H, Ueno R. Efficacy and safety of oral lubiprostone in constipated patients with or without irritable bowel syndrome: a randomized, placebo-controlled and dose-finding study. *Neurogastroenterol Motil* 2011; **23**: 544-e205 [PMID: 21303430 DOI: 10.1111/j.1365-2982.2011.01668.x]
59 **Drossman DA**, Chey WD, Johanson JF, Fass R, Scott C, Panas R, Ueno R. Clinical trial: lubiprostone in patients with constipation-associated irritable bowel syndrome--results of two randomized, placebo-controlled studies. *Aliment Pharmacol Ther* 2009; **29**: 329-341 [PMID: 19006537 DOI: 10.1111/j.1365-2036.2008.03881.x]
60 **Chey WD**, Drossman DA, Johanson JF, Scott C, Panas RM, Ueno R. Safety and patient outcomes with lubiprostone for up to 52 weeks in patients with irritable bowel syndrome with constipation. *Aliment Pharmacol Ther* 2012; **35**: 587-599 [PMID: 22251419 DOI: 10.1111/j.1365-2036.2011.04983.x]
61 **Brandt LJ**, Chey WD, Foxx-Orenstein AE, Schiller LR, Schoenfeld PS, Spiegel BM, Talley NJ, Quigley EM. An evidence-based position statement on the management of irritable bowel syndrome. *Am J Gastroenterol* 2009; **104** Suppl 1: S1-S35 [PMID: 19521341 DOI: 10.1038/ajg.2008.122]
62 **Iser JH**, Sali A. Chenodeoxycholic acid: a review of its pharmacological properties and therapeutic use. *Drugs* 1981; **21**: 90-119 [PMID: 7009140 DOI: 10.2165/00003495-198121020-00002]
63 **Mitchell WD**, Findlay JM, Prescott RJ, Eastwood MA, Horn DB. Bile acids in the diarrhoea of ileal resection. *Gut* 1973; **14**: 348-353 [PMID: 4736774 DOI: 10.1136/gut.14.5.348]
64 **Aldini R**, Roda A, Festi D, Sama C, Mazzella G, Bazzoli F, Morselli AM, Roda E, Barbara L. Bile acid malabsorption and bile acid diarrhea in intestinal resection. *Dig Dis Sci* 1982; **27**: 495-502 [PMID: 6806052 DOI: 10.1007/BF01296727]
65 **Rao AS**, Wong BS, Camilleri M, Odunsi-Shiyanbade ST, McKinzie S, Ryks M, Burton D, Carlson P, Lamsam J, Singh R, Zinsmeister AR. Chenodeoxycholate in females with irritable bowel syndrome-constipation: a pharmacodynamic and pharmacogenetic analysis. *Gastroenterology* 2010; **139**: 1549-1558, 1558.e1 [PMID: 20691689 DOI: 10.1053/j.gastro.2010.07.052]
66 **Kirwan WO**, Smith AN, Mitchell WD, Falconer JD, Eastwood MA. Bile acids and colonic motility in the rabbit and the human. *Gut* 1975; **16**: 894-902 [PMID: 1193418 DOI: 10.1136/gut.16.11.894]
67 **Mok HY**, Bell GD, Dowling RH. Effect of different doses of chenodeoxycholic acid on bile-lipid composition and on frequency of side-effects in patients with gallstones. *Lancet* 1974; **2**: 253-257 [PMID: 4135688 DOI: 10.1016/S0140-6736(74)91415-9]
68 **Caspary WF**, Meyne K. Effects of chenodeoxy- and ursodeoxycholic acid on absorption, secretion and permeability in rat colon and small intestine. *Digestion* 1980; **20**: 168-174 [PMID: 7390046]
69 **Odunsi-Shiyanbade ST**, Camilleri M, McKinzie S, Burton D, Carlson P, Busciglio IA, Lamsam J, Singh R, Zinsmeister AR. Effects of chenodeoxycholate and a bile acid sequestrant, colesevelam, on intestinal transit and bowel function. *Clin Gastroenterol Hepatol* 2010; **8**: 159-165 [PMID: 19879973 DOI: 10.1016/j.cgh.2009.10.020]
70 **Fromm H**, Roat JW, Gonzalez V, Sarva RP, Farivar S. Comparative efficacy and side effects of ursodeoxycholic and chenodeoxycholic acids in dissolving gallstones. A double-blind controlled study. *Gastroenterology* 1983; **85**: 1257-1264 [PMID: 6354826]
71 **Cann PA**, Read NW, Holdsworth CD, Barends D. Role of loperamide and placebo in management of irritable bowel syndrome (IBS). *Dig Dis Sci* 1984; **29**: 239-247 [PMID: 6365490 DOI: 10.1007/BF01296258]
72 **Hovdenak N**. Loperamide treatment of the irritable bowel syndrome. *Scand J Gastroenterol Suppl* 1987; **130**: 81-84 [PMID: 3306904 DOI: 10.3109/00365528709091004]
73 **Lavö B**, Stenstam M, Nielsen AL. Loperamide in treatment of irritable bowel syndrome--a double-blind placebo controlled study. *Scand J Gastroenterol Suppl* 1987; **130**: 77-80 [PMID: 3306903 DOI: 10.3109/00365528709091003]
74 **Efskind PS**, Bernklev T, Vatn MH. A double-blind placebo-controlled trial with loperamide in irritable bowel syndrome. *Scand J Gastroenterol* 1996; **31**: 463-468 [PMID: 8734343 DOI: 10.3109/00365529609006766]
75 **Camilleri M**, Northcutt AR, Kong S, Dukes GE, McSorley D, Mangel AW. Efficacy and safety of alosetron in women with irritable bowel syndrome: a randomised, placebo-controlled trial. *Lancet* 2000; **355**: 1035-1040 [PMID: 10744088 DOI: 10.1016/S0140-6736(00)02033-X]
76 **Camilleri M**, Chey WY, Mayer EA, Northcutt AR, Heath A, Dukes GE, McSorley D, Mangel AM. A randomized controlled clinical trial of the serotonin type 3 receptor antagonist alosetron in women with diarrhea-predominant irritable bowel syndrome. *Arch Intern Med* 2001; **161**: 1733-1740 [PMID: 11485506 DOI: 10.1001/archinte.161.14.1733]
77 **Watson ME**, Lacey L, Kong S, Northcutt AR, McSorley D, Hahn B, Mangel AW. Alosetron improves quality of life in women with diarrhea-predominant irritable bowel syndrome. *Am J Gastroenterol* 2001; **96**: 455-459 [PMID: 11232690 DOI: 10.1111/j.1572-0241.2001.03525.x]
78 **Lembo T**, Wright RA, Bagby B, Decker C, Gordon S, Jhingran P, Carter E. Alosetron controls bowel urgency and provides global symptom improvement in women with diarrhea-predominant irritable bowel syndrome. *Am J Gastroenterol* 2001; **96**: 2662-2670 [PMID: 11569692 DOI: 10.1111/j.1572-0241.2001.04128.x]
79 **Cremonini F**, Nicandro JP, Atkinson V, Shringarpure R, Chuang E, Lembo A. Randomised clinical trial: alosetron improves quality of life and reduces restriction of daily activities in women with severe diarrhoea-predominant IBS. *Aliment Pharmacol Ther* 2012; **36**: 437-448 [PMID: 22779693 DOI: 10.1111/j.1365-2036.2012.05208.x]
80 **Rahimi R**, Nikfar S, Abdollahi M. Efficacy and tolerability of alosetron for the treatment of irritable bowel syndrome in women and men: a meta-analysis of eight randomized, placebo-controlled, 12-week trials. *Clin Ther* 2008; **30**: 884-901 [PMID: 18555935 DOI: 10.1016/j.clinthera.2008.05.002]
81 **Andresen V**, Montori VM, Keller J, West CP, Layer P, Ca-

milleri M. Effects of 5-hydroxytryptamine (serotonin) type 3 antagonists on symptom relief and constipation in non-constipated irritable bowel syndrome: a systematic review and meta-analysis of randomized controlled trials. *Clin Gastroenterol Hepatol* 2008; **6**: 545-555 [PMID: 18242143 DOI: 10.1016/j.cgh.2007.12.015]

82 **Ford AC**, Brandt LJ, Young C, Chey WD, Foxx-Orenstein AE, Moayyedi P. Efficacy of 5-HT3 antagonists and 5-HT4 agonists in irritable bowel syndrome: systematic review and meta-analysis. *Am J Gastroenterol* 2009; **104**: 1831-1843; quiz 1844 [PMID: 19471254 DOI: 10.1038/ajg.2009.223]

83 **Chang L**, Tong K, Ameen V. Ischemic colitis and complications of constipation associated with the use of alosetron under a risk management plan: clinical characteristics, outcomes, and incidences. *Am J Gastroenterol* 2010; **105**: 866-875 [PMID: 20197759 DOI: 10.1038/ajg.2010.25]

84 **Ohta M**, Suzuki T, Nagashima S, Tokunaga T, Miyata K, Mase T. Novel 5-hydroxytryptamine (5-HT3) receptor antagonists. IV. Synthesis and pharmacological evaluation of the oxidation products of (-)-(R)-5-[(1-methyl-1H-indol-3-yl)carbonyl] -4,5,6,7-tetrahydro-1H-benzimidazole hydrochloride (YM060: ramosetron). *Chem Pharm Bull* (Tokyo) 1996; **44**: 1717-1722 [PMID: 8855366 DOI: 10.1248/cpb.44.1707]

85 **Matsueda K**, Harasawa S, Hongo M, Hiwatashi N, Sasaki D. A phase II trial of the novel serotonin type 3 receptor antagonist ramosetron in Japanese male and female patients with diarrhea-predominant irritable bowel syndrome. *Digestion* 2008; **77**: 225-235 [PMID: 18667823 DOI: 10.1159/000150632]

86 **Matsueda K**, Harasawa S, Hongo M, Hiwatashi N, Sasaki D. A randomized, double-blind, placebo-controlled clinical trial of the effectiveness of the novel serotonin type 3 receptor antagonist ramosetron in both male and female Japanese patients with diarrhea-predominant irritable bowel syndrome. *Scand J Gastroenterol* 2008; **43**: 1202-1211 [PMID: 18618371 DOI: 10.1080/00365520802240255]

87 **Lee KJ**, Kim NY, Kwon JK, Huh KC, Lee OY, Lee JS, Choi SC, Sohn CI, Myung SJ, Park HJ, Choi MK, Bak YT, Rhee PL. Efficacy of ramosetron in the treatment of male patients with irritable bowel syndrome with diarrhea: a multicenter, randomized clinical trial, compared with mebeverine. *Neurogastroenterol Motil* 2011; **23**: 1098-1104 [PMID: 21920001 DOI: 10.1111/j.1365-2982.2011.01771.x]

88 **Chiba T**, Yamamoto K, Sato S, Suzuki K. Long-term efficacy and safety of ramosetron in the treatment of diarrhea-predominant irritable bowel syndrome. *Clin Exp Gastroenterol* 2013; **6**: 123-128 [PMID: 23922505 DOI: 10.2147/CEG.S32721]

89 **Tack J**, Janssen P, Wouters M, Boeckxstaens G. Targeting serotonin synthesis to treat irritable bowel syndrome. *Gastroenterology* 2011; **141**: 420-422 [PMID: 21703266 DOI: 10.1053/j.gastro.2011.06.024]

90 **Liu Q**, Yang Q, Sun W, Vogel P, Heydorn W, Yu XQ, Hu Z, Yu W, Jonas B, Pineda R, Calderon-Gay V, Germann M, O' Neill E, Brommage R, Cullinan E, Platt K, Wilson A, Powell D, Sands A, Zambrowicz B, Shi ZC. Discovery and characterization of novel tryptophan hydroxylase inhibitors that selectively inhibit serotonin synthesis in the gastrointestinal tract. *J Pharmacol Exp Ther* 2008; **325**: 47-55 [PMID: 18192499 DOI: 10.1124/jpet.107.132670]

91 **Camilleri M**. LX-1031, a tryptophan 5-hydroxylase inhibitor, and its potential in chronic diarrhea associated with increased serotonin. *Neurogastroenterol Motil* 2011; **23**: 193-200 [PMID: 21159063 DOI: 10.1111/j.1365-2982.2010.01643.x]

92 **Brown PM**, Drossman DA, Wood AJ, Cline GA, Frazier KS, Jackson JI, Bronner J, Freiman J, Zambrowicz B, Sands A, Gershon MD. The tryptophan hydroxylase inhibitor LX1031 shows clinical benefit in patients with nonconstipating irritable bowel syndrome. *Gastroenterology* 2011; **141**: 507-516 [PMID: 21684281 DOI: 10.1053/j.gastro.2011.05.005]

93 **Tradtrantip L**, Namkung W, Verkman AS. Crofelemer, an antisecretory antidiarrheal proanthocyanidin oligomer extracted from Croton lechleri, targets two distinct intestinal chloride channels. *Mol Pharmacol* 2010; **77**: 69-78 [PMID: 19808995 DOI: 10.1124/mol.109.061051]

94 **Mangel AW**, Chaturvedi P. Evaluation of crofelemer in the treatment of diarrhea-predominant irritable bowel syndrome patients. *Digestion* 2008; **78**: 180-186 [PMID: 19092244 DOI: 10.1159/000185719]

95 **Lin HC**. Small intestinal bacterial overgrowth: a framework for understanding irritable bowel syndrome. *JAMA* 2004; **292**: 852-858 [PMID: 15316000 DOI: 10.1001/jama.292.7.852]

96 **Pimentel M**, Chow EJ, Lin HC. Eradication of small intestinal bacterial overgrowth reduces symptoms of irritable bowel syndrome. *Am J Gastroenterol* 2000; **95**: 3503-3506 [PMID: 11151884 DOI: 10.1111/j.1572-0241.2000.03368.x]

97 **Pimentel M**, Chow EJ, Lin HC. Normalization of lactulose breath testing correlates with symptom improvement in irritable bowel syndrome. a double-blind, randomized, placebo-controlled study. *Am J Gastroenterol* 2003; **98**: 412-419 [PMID: 12591062 DOI: 10.1111/j.1572-0241.2003.07234.x]

98 **O'Mahony L**, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, O'Sullivan GC, Kiely B, Collins JK, Shanahan F, Quigley EM. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. *Gastroenterology* 2005; **128**: 541-551 [PMID: 15765388 DOI: 10.1053/j.gastro.2004.11.050]

99 **Frissora CL**, Cash BD. Review article: the role of antibiotics vs. conventional pharmacotherapy in treating symptoms of irritable bowel syndrome. *Aliment Pharmacol Ther* 2007; **25**: 1271-1281 [PMID: 17509095 DOI: 10.1111/j.1365-2036.2007.03313.x]

100 **Connor BA**. Sequelae of traveler's diarrhea: focus on postinfectious irritable bowel syndrome. *Clin Infect Dis* 2005; **41** Suppl 8: S577-S586 [PMID: 16267722 DOI: 10.1086/432956]

101 **Pimentel M**, Chatterjee S, Chow EJ, Park S, Kong Y. Neomycin improves constipation-predominant irritable bowel syndrome in a fashion that is dependent on the presence of methane gas: subanalysis of a double-blind randomized controlled study. *Dig Dis Sci* 2006; **51**: 1297-1301 [PMID: 16832617 DOI: 10.1007/s10620-006-9104-6]

102 **Robins GW**, Wellington K. Rifaximin: a review of its use in the management of traveller's diarrhoea. *Drugs* 2005; **65**: 1697-1713 [PMID: 16060706 DOI: 10.2165/00003495-200565120-00011]

103 **Sharara AI**, Aoun E, Abdul-Baki H, Mounzer R, Sidani S, Elhajj I. A randomized double-blind placebo-controlled trial of rifaximin in patients with abdominal bloating and flatulence. *Am J Gastroenterol* 2006; **101**: 326-333 [PMID: 16454838 DOI: 10.1111/j.1572-0241.2006.00458.x]

104 **Pimentel M**, Park S, Mirocha J, Kane SV, Kong Y. The effect of a nonabsorbed oral antibiotic (rifaximin) on the symptoms of the irritable bowel syndrome: a randomized trial. *Ann Intern Med* 2006; **145**: 557-563 [PMID: 17043337 DOI: 10.7326/0003-4819-145-8-200610170-00004]

105 **Pimentel M**, Lembo A, Chey WD, Zakko S, Ringel Y, Yu J, Mareya SM, Shaw AL, Bortey E, Forbes WP. Rifaximin therapy for patients with irritable bowel syndrome without constipation. *N Engl J Med* 2011; **364**: 22-32 [PMID: 21208106 DOI: 10.1056/NEJMoa1004409]

106 **Menees SB**, Maneerattannaporn M, Kim HM, Chey WD. The efficacy and safety of rifaximin for the irritable bowel syndrome: a systematic review and meta-analysis. *Am J Gastroenterol* 2012; **107**: 28-35; quiz 36 [PMID: 22045120 DOI: 10.1038/ajg.2011.355]

107 **Clouse RE**, Alpers DH, Hockenbery DM, DeSchryver-Kecskemeti K. Pericrypt eosinophilic enterocolitis and chronic diarrhea. *Gastroenterology* 1992; **103**: 168-176 [PMID: 1612324]

108 **Corinaldesi R**, Stanghellini V, Cremon C, Gargano L, Cogliandro RF, De Giorgio R, Bartesaghi G, Canovi B, Barbara G. Effect of mesalazine on mucosal immune biomarkers in irritable bowel syndrome: a randomized controlled proof-

of-concept study. *Aliment Pharmacol Ther* 2009; **30**: 245-252 [PMID: 19438846 DOI: 10.1111/j.1365-2036.2009.04041.x]
109 **Andrews CN**, Griffiths TA, Kaufman J, Vergnolle N, Surette MG, Rioux KP. Mesalazine (5-aminosalicylic acid) alters faecal bacterial profiles, but not mucosal proteolytic activity in diarrhoea-predominant irritable bowel syndrome. *Aliment Pharmacol Ther* 2011; **34**: 374-383 [PMID: 21671966 DOI: 10.1111/j.1365-2036.2011.04732.x]
110 **Tuteja AK**, Fang JC, Al-Suqi M, Stoddard GJ, Hale DC. Double-blind placebo-controlled study of mesalamine in post-infective irritable bowel syndrome--a pilot study. *Scand J Gastroenterol* 2012; **47**: 1159-1164 [PMID: 22783919 DOI: 10.3109/00365521.2012.694903]
111 **Chey WY**, Jin HO, Lee MH, Sun SW, Lee KY. Colonic motility abnormality in patients with irritable bowel syndrome exhibiting abdominal pain and diarrhea. *Am J Gastroenterol* 2001; **96**: 1499-1506 [PMID: 11374689 DOI: 10.1111/j.1572-0241.2001.03804.x]
112 **Drossman DA**, Camilleri M, Mayer EA, Whitehead WE. AGA technical review on irritable bowel syndrome. *Gastroenterology* 2002; **123**: 2108-2131 [PMID: 12454866 DOI: 10.1053/gast.2002.37095]
113 **Clemens CH**, Samsom M, Roelofs JM, van Berge Henegouwen GP, Smout AJ. Association between pain episodes and high amplitude propagated pressure waves in patients with irritable bowel syndrome. *Am J Gastroenterol* 2003; **98**: 1838-1843 [PMID: 12907341 DOI: 10.1111/j.1572-0241.2003.07541.x]
114 **Poynard T**, Regimbeau C, Benhamou Y. Meta-analysis of smooth muscle relaxants in the treatment of irritable bowel syndrome. *Aliment Pharmacol Ther* 2001; **15**: 355-361 [PMID: 11207510 DOI: 10.1046/j.1365-2036.2001.00937.x]
115 **Jailwala J**, Imperiale TF, Kroenke K. Pharmacologic treatment of the irritable bowel syndrome: a systematic review of randomized, controlled trials. *Ann Intern Med* 2000; **133**: 136-147 [PMID: 10896640 DOI: 10.7326/0003-4819-133-2-200007180-00013]
116 **Talley NJ**. Drug therapy options for patients with irritable bowel syndrome. *Am J Manag Care* 2001; **7**: S261-S267 [PMID: 11474911]
117 **Darvish-Damavandi M**, Nikfar S, Abdollahi M. A systematic review of efficacy and tolerability of mebeverine in irritable bowel syndrome. *World J Gastroenterol* 2010; **16**: 547-553 [PMID: 20128021 DOI: 10.3748/wjg.v16.i5.547]
118 **Everitt H**, Moss-Morris R, Sibelli A, Tapp L, Coleman N, Yardley L, Smith P, Little P. Management of irritable bowel syndrome in primary care: the results of an exploratory randomised controlled trial of mebeverine, methylcellulose, placebo and a self-management website. *BMC Gastroenterol* 2013; **13**: 68 [PMID: 23602047 DOI: 10.1186/1471-230X-13-68]
119 **Evangelista S**. Quaternary ammonium derivatives as spasmolytics for irritable bowel syndrome. *Curr Pharm Des* 2004; **10**: 3561-3568 [PMID: 15579053 DOI: 10.2174/1381612043382972]
120 **Evangelista S**, Giachetti A, Chapelain B, Neliat G, Maggi CA. Receptor binding profile of Otilonium bromide. *Pharmacol Res* 1998; **38**: 111-117 [PMID: 9721598 DOI: 10.1006/phrs.1998.0340]
121 **Lindqvist S**, Hernon J, Sharp P, Johns N, Addison S, Watson M, Tighe R, Greer S, Mackay J, Rhodes M, Lewis M, Stebbings W, Speakman C, Evangelista S, Johnson I, Williams M. The colon-selective spasmolytic otilonium bromide inhibits muscarinic M(3) receptor-coupled calcium signals in isolated human colonic crypts. *Br J Pharmacol* 2002; **137**: 1134-1142 [PMID: 12429587 DOI: 10.1038/sj.bjp.0704942]
122 **Battaglia G**, Morselli-Labate AM, Camarri E, Francavilla A, De Marco F, Mastropaolo G, Naccarato R. Otilonium bromide in irritable bowel syndrome: a double-blind, placebo-controlled, 15-week study. *Aliment Pharmacol Ther* 1998; **12**: 1003-1010 [PMID: 9798806 DOI: 10.1046/j.1365-2036.1998.00397.x]
123 **Baldi F**, Longanesi A, Blasi A, Monello S, Cestari R, Missale G, Corazziari E, Badiali G, Pescatori M, Anastasio G. Clinical and functional evaluation of the efficacy of otilonium bromide: a multicenter study in Italy. *Ital J Gastroenterol* 1991; **23**: 60-63 [PMID: 1756285]
124 **Ford AC**, Talley NJ, Spiegel BM, Foxx-Orenstein AE, Schiller L, Quigley EM, Moayyedi P. Effect of fibre, antispasmodics, and peppermint oil in the treatment of irritable bowel syndrome: systematic review and meta-analysis. *BMJ* 2008; **337**: a2313 [PMID: 19008265 DOI: 10.1136/bmj.a2313]
125 **Clavé P**, Acalovschi M, Triantafillidis JK, Uspensky YP, Kalayci C, Shee V, Tack J. Randomised clinical trial: otilonium bromide improves frequency of abdominal pain, severity of distention and time to relapse in patients with irritable bowel syndrome. *Aliment Pharmacol Ther* 2011; **34**: 432-442 [PMID: 21679214 DOI: 10.1111/j.1365-2036.2011.04730.x]
126 **Ford AC**, Moayyedi P. Meta-analysis: factors affecting placebo response rate in the irritable bowel syndrome. *Aliment Pharmacol Ther* 2010; **32**: 144-158 [PMID: 20412064 DOI: 10.1111/j.1365-2036.2010.04328.x]
127 **Chang FY**, Lu CL, Luo JC, Chen TS, Chen MJ, Chang HJ. The evaluation of otilonium bromide treatment in asian patients with irritable bowel syndrome. *J Neurogastroenterol Motil* 2011; **17**: 402-410 [PMID: 22148110 DOI: 10.5056/jnm.2011.17.4.402]
128 **Fukushima Y**, Suzuki H, Matsuzaki J, Kiyosue A, Hibi T. Efficacy of Solifenacin on Irritable Bowel Syndrome With Diarrhea: Open-label Prospective Pilot Trial. *J Neurogastroenterol Motil* 2012; **18**: 317-323 [PMID: 22837880 DOI: 10.5056/jnm.2012.18.3.317]
129 **Mizuta Y**, Takahashi T, Owyang C. Nitrergic regulation of colonic transit in rats. *Am J Physiol* 1999; **277**: G275-G279 [PMID: 10444440]
130 **Luo ZD**, Cizkova D. The role of nitric oxide in nociception. *Curr Rev Pain* 2000; **4**: 459-466 [PMID: 11060592 DOI: 10.1007/s11916-000-0070-y]
131 **Fritz E**, Hammer J, Schmidt B, Eherer AJ, Hammer HF. Stimulation of the nitric oxide-guanosine 3', 5'-cyclic monophosphate pathway by sildenafil: effect on rectal muscle tone, distensibility, and perception in health and in irritable bowel syndrome. *Am J Gastroenterol* 2003; **98**: 2253-2260 [PMID: 14572576 DOI: 10.1111/j.1572-0241.2003.07661.x]
132 **Gonçalves de Medeiros MT**, de Oliveira RB, dos Santos AA, de Leopoldino DM, Lima MC, Nobre RA, Nobre e Souza MÂ. The effects of sildenafil on rectal sensitivity and tone in patients with the irritable bowel syndrome. *Aliment Pharmacol Ther* 2012; **35**: 577-586 [PMID: 22229478 DOI: 10.1111/j.1365-2036.2011.04977.x]
133 **Holzer P**. Opioid receptors in the gastrointestinal tract. *Regul Pept* 2009; **155**: 11-17 [PMID: 19345246 DOI: 10.1016/j.regpep.2009.03.012]
134 **Wade PR**, Palmer JM, McKenney S, Kenigs V, Chevalier K, Moore BA, Mabus JR, Saunders PR, Wallace NH, Schneider CR, Kimball ES, Breslin HJ, He W, Hornby PJ. Modulation of gastrointestinal function by MuDelta, a mixed μ opioid receptor agonist/ μ opioid receptor antagonist. *Br J Pharmacol* 2012; **167**: 1111-1125 [PMID: 22671931 DOI: 10.1111/j.1476-5381.2012.02068.x]
135 **Dove LS**, Lembo A, Randall CW, Fogel R, Andrae D, Davenport JM, McIntyre G, Almenoff JS, Covington PS. Eluxadoline benefits patients with irritable bowel syndrome with diarrhea in a phase 2 study. *Gastroenterology* 2013; **145**: 329-338.e1 [PMID: 23583433 DOI: 10.1053/j.gastro.2013.04.006]
136 **Mangel AW**, Bornstein JD, Hamm LR, Buda J, Wang J, Irish W, Urso D. Clinical trial: asimadoline in the treatment of patients with irritable bowel syndrome. *Aliment Pharmacol Ther* 2008; **28**: 239-249 [PMID: 18466359 DOI: 10.1111/j.1365-2036.2008.03730.x]
137 **Howlett AC**, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG. International Union of Pharma-

cology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* 2002; **54**: 161-202 [PMID: 12037135 DOI: 10.1124/pr.54.2.161]

138 **Esfandyari T**, Camilleri M, Busciglio I, Burton D, Baxter K, Zinsmeister AR. Effects of a cannabinoid receptor agonist on colonic motor and sensory functions in humans: a randomized, placebo-controlled study. *Am J Physiol Gastrointest Liver Physiol* 2007; **293**: G137-G145 [PMID: 17395895 DOI: 10.1152/ajpgi.00565.2006]

139 **Wong BS**, Camilleri M, Busciglio I, Carlson P, Szarka LA, Burton D, Zinsmeister AR. Pharmacogenetic trial of a cannabinoid agonist shows reduced fasting colonic motility in patients with nonconstipated irritable bowel syndrome. *Gastroenterology* 2011; **141**: 1638-1647.e1-7 [PMID: 21803011 DOI: 10.1053/j.gastro.2011.07.036]

140 **Hellström PM**, Grybäck P, Jacobsson H. The physiology of gastric emptying. *Best Pract Res Clin Anaesthesiol* 2006; **20**: 397-407 [PMID: 17080692 DOI: 10.1016/j.bpa.2006.02.002]

141 **Hellström PM**, Näslund E, Edholm T, Schmidt PT, Kristensen J, Theodorsson E, Holst JJ, Efendic S. GLP-1 suppresses gastrointestinal motility and inhibits the migrating motor complex in healthy subjects and patients with irritable bowel syndrome. *Neurogastroenterol Motil* 2008; **20**: 649-659 [PMID: 18298441 DOI: 10.1111/j.1365-2982.2007.01079.x]

142 **Brubaker PL**, Drucker DJ, Asa SL, Swallow C, Redston M, Greenberg GR. Prolonged gastrointestinal transit in a patient with a glucagon-like peptide (GLP)-1- and -2-producing neuroendocrine tumor. *J Clin Endocrinol Metab* 2002; **87**: 3078-3083 [PMID: 12107204 DOI: 10.1210/jcem.87.7.8584]

143 **Hellström PM**, Hein J, Bytzer P, Björnssön E, Kristensen J, Schambye H. Clinical trial: the glucagon-like peptide-1 analogue ROSE-010 for management of acute pain in patients with irritable bowel syndrome: a randomized, placebo-controlled, double-blind study. *Aliment Pharmacol Ther* 2009; **29**: 198-206 [PMID: 18945254 DOI: 10.1111/j.1365-2036.2008.03870.x]

144 **Camilleri M**, Vazquez-Roque M, Iturrino J, Boldingh A, Burton D, McKinzie S, Wong BS, Rao AS, Kenny E, Månsson M, Zinsmeister AR. Effect of a glucagon-like peptide 1 analog, ROSE-010, on GI motor functions in female patients with constipation-predominant irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* 2012; **303**: G120-G128 [PMID: 22517769 DOI: 10.1152/ajpgi.00076.2012]

145 **Klooker TK**, Braak B, Koopman KE, Welting O, Wouters MM, van der Heide S, Schemann M, Bischoff SC, van den Wijngaard RM, Boeckxstaens GE. The mast cell stabiliser ketotifen decreases visceral hypersensitivity and improves intestinal symptoms in patients with irritable bowel syndrome. *Gut* 2010; **59**: 1213-1221 [PMID: 20650926 DOI: 10.1136/gut.2010.213108]

146 **Santos J**, Alonso C, Guilarte M, Vicario M, Malagelada JR. Targeting mast cells in the treatment of functional gastrointestinal disorders. *Curr Opin Pharmacol* 2006; **6**: 541-546 [PMID: 16956793 DOI: 10.1016/j.coph.2006.08.001]

147 **Piche T**, Saint-Paul MC, Dainese R, Marine-Barjoan E, Iannelli A, Montoya ML, Peyron JF, Czerucka D, Cherikh F, Filippi J, Tran A, Hébuterne X. Mast cells and cellularity of the colonic mucosa correlated with fatigue and depression in irritable bowel syndrome. *Gut* 2008; **57**: 468-473 [PMID: 18194987 DOI: 10.1136/gut.2007.127068]

148 **Barbara G**, Stanghellini V, De Giorgio R, Cremon C, Cottrell GS, Santini D, Pasquinelli G, Morselli-Labate AM, Grady EF, Bunnett NW, Collins SM, Corinaldesi R. Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. *Gastroenterology* 2004; **126**: 693-702 [PMID: 14988823 DOI: 10.1053/j.gastro.2003.11.055]

149 **Park JH**, Rhee PL, Kim HS, Lee JH, Kim YH, Kim JJ, Rhee JC. Mucosal mast cell counts correlate with visceral hypersensitivity in patients with diarrhea predominant irritable bowel syndrome. *J Gastroenterol Hepatol* 2006; **21**: 71-78 [PMID: 16706815 DOI: 10.1111/j.1440-1746.2005.04143.x]

P- Reviewers: Kuo SM, Mansour-Ghanaei F, Marsh MN
S- Editor: Qi Y **L- Editor**: A **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8886

World J Gastroenterol 2014 July 21; 20(27): 8886-8897
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

Intestinal microbiota in pathophysiology and management of irritable bowel syndrome

Kang Nyeong Lee, Oh Young Lee

Kang Nyeong Lee, Oh Young Lee, Department of Internal Medicine, Hanyang University College of Medicine, Seoul 133-791, South Korea
Author contributions: Lee KN performed the literature review and drafted the paper; Lee OY performed the critical revision of the manuscript.
Correspondence to: Oh Young Lee, MD, PhD, Department of Internal Medicine, Hanyang University College of Medicine, 222 Wangsimni-ro, Seongdong-gu, Seoul 133-791, South Korea. leeoy@hanyang.ac.kr
Telephone: +82-2-22908343 Fax: +82-2-22988314
Received: February 9, 2014 Revised: April 2, 2014
Accepted: June 14, 2014
Published online: July 21, 2014

Abstract

Irritable bowel syndrome (IBS) is a functional bowel disorder without any structural or metabolic abnormalities that sufficiently explain the symptoms, which include abdominal pain and discomfort, and bowel habit changes such as diarrhea and constipation. Its pathogenesis is multifactorial: visceral hypersensitivity, dysmotility, psychosocial factors, genetic or environmental factors, dysregulation of the brain-gut axis, and altered intestinal microbiota have all been proposed as possible causes. The human intestinal microbiota are composed of more than 1000 different bacterial species and 10^{14} cells, and are essential for the development, function, and homeostasis of the intestine, and for individual health. The putative mechanisms that explain the role of microbiota in the development of IBS include altered composition or metabolic activity of the microbiota, mucosal immune activation and inflammation, increased intestinal permeability and impaired mucosal barrier function, sensory-motor disturbances provoked by the microbiota, and a disturbed gut-microbiota-brain axis. Therefore, modulation of the intestinal microbiota through dietary changes, and use of antibiotics, probiotics, and anti-inflammatory agents has been suggested as strategies for managing IBS symptoms. This review summarizes and discusses the accumulating evidence that intestinal microbiota play a role in the pathophysiology and management of IBS.

Key words: Immunity; Irritable bowel syndrome; Microbiota; Permeability; Probiotics

Core tip: Irritable bowel syndrome (IBS) is a functional bowel disorder with multiple pathophysiology, which is not fully understood. Intestinal microbiota has recently been postulated to be involved in the pathophysiology of IBS. Many studies of IBS focus on investigating the efficacy of modulating the microbiota by probiotics and antibiotics. However, the role of the intestinal microbiota in the pathophysiology and management of IBS is not clear. This review provides the accumulating evidence on it.

Lee KN, Lee OY. Intestinal microbiota in pathophysiology and management of irritable bowel syndrome. *World J Gastroenterol* 2014; 20(27): 8886-8897 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8886.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8886

INTRODUCTION

Irritable bowel syndrome (IBS) is a functional bowel disorder characterized by abdominal pain or discomfort relieved by defecation, and accompanied by changes in bowel habits such as diarrhea or constipation, which cannot be explained by structural, biochemical, or metabolic abnormalities[1]. The symptoms of IBS have been accounted for as resulting from visceral hypersensitivity, intestinal dysmotility, genetic or environmental factors,

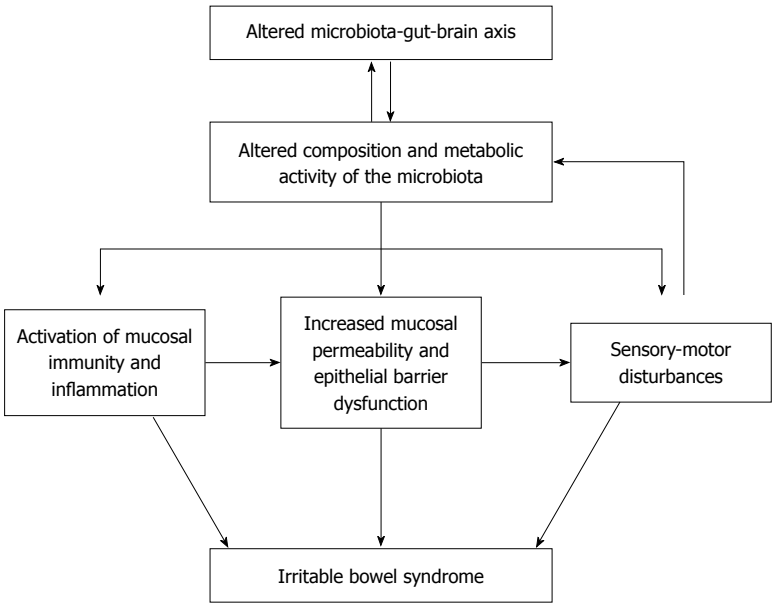


Figure 1 Putative pathophysiologic role of the microbiota in irritable bowel syndrome. Intestinal microbiota play a substantial role in irritable bowel syndrome (IBS). Although the microbiota may contribute directly to the symptoms of IBS, it is more likely that altered composition and metabolic activity of the microbiota caused by stress or other psychological disturbances indirectly activate mucosal immunity and inflammation, increase epithelial permeability, and reduce barrier function, thereby activating the sensory-motor dysfunction responsible for a variety of symptoms of patients with IBS.

psychological factors, or a dysregulated brain-gut axis[2]. In addition to these factors, bacterial infection, dysregulated intestinal immune function, and chronic low-grade mucosal inflammation have all been suggested as putative pathogenetic mechanisms, in which the intestinal microbiota might play an important role, but their role in IBS cannot be fully explained (Figure 1)[3,4].

Intestinal microbiota is a collective term for a complex ecosystem of microbes inhabiting the intestine[5]. In the human intestine, this ecosystem may include any one of over 1000 microbial species, and 10^{14} cells (*i.e.*, about 10 times more than the number of human cells in the body[6]), containing 150-fold more genes than the human genome[7]. The microbiota can be divided into mucosal and luminal subtypes[8], and it was previously thought to comprise three predominant enterotypes: *Bacteroides, Prevotella,* and *Ruminococcus*[9], although such a strict categorization is no longer widely accepted[10].

To evaluate the composition and metabolic activity of the intestinal microbiota, culture-dependent and -independent tests have been developed[11]. It has been shown that size and diversity of the microbiota increase distally from the upper to the lower gastrointestinal (GI) tract[12] and are modulated by gastric acid, intestinal motility, and the function of the ileocecal valve. Their distribution also varies according to the region of the GI tract with gram-positive facultative anaerobic bacteria in the proximal small intestine and gram-negative anaerobes in the distal small intestine. Although the composition and diversity of the microbiota are genetically controlled from birth and become stable after weaning and throughout life, qualitative and quantitative changes can occur over the longitudinal and cross-sectional axes of the intestine: changes in bacterial enzymes and metabolic activity, as well as in microbial populations. The composition and metabolic activity of the microbiota vary between, but also within, individuals due to many factors including mode of delivery at birth, diet, sanitation, antibiotics, and ageing[13]. At birth, contamination from the vaginal canal provides the intestine with the maternal microbiome, while during a delivery by cesarean-section, the gut comes into contact with commensals from the skin and the surgical environment[14]. The composition of the microbiota can also be altered by the feeding method: bifidobacteria increase in breast-fed babies (*i.e.*, babies receiving a high-carbohydrate and high-fiber diet), and *Bacteroides* spp. increase in formula-fed babies (babies receiving a high-fat diet)[15]. Lastly, it can vary across geographical regions, *e.g.*, between rural Africa and urban Europe[16].

The intestinal microbiota is essential for maintaining individual health, including normal GI function. In this context, its main functions are metabolic, protective, and trophic: it can help to digest and absorb nutrients, and produces a variety of beneficial compounds such as short-chain fatty acids (SCFA)[17], it can act as a barrier against pathogens by adhering to the mucosa, generating immune responses, and interacting with components of the epithelial layer, it can also influence the differentiation and proliferation of the intestinal epithelial cells and the development of the enteric immune system.

In parallel with the beneficial effects of microbial activity on the gut, bacterial fermentation may give rise to large amounts of gas and thus contribute to the symptoms of bloating, flatulence, and abdominal distension, which are commonly reported by patients with IBS[18]. An association between the microbiota and IBS has been supported by the evidence of modulation of mucosal immunity: IBS symptoms were found to be more frequent

after an episode of gastroenteritis, and some IBS symptoms were found to improve after antibiotic treatment targeting the intestinal microbiota[19]. This putative link was also demonstrated in studies of probiotics, which modulated the intestinal microbiota in IBS patients. Finally, mucosal immunity-gut microbiota-brain axis is being suggested as a possible pathway for the development of IBS due to altered intestinal microbiota. This review article explores the role of the microbiota in the pathophysiology and management of IBS, and provides a comprehensive summary of the evidence for the concept of IBS as a microbiota-related disorder.

Despite the large volume of studies of the intestinal microbiota, our understanding of its role in health and disease is still in its infancy. In studying the microbiota, culture-based methods are being replaced by advanced, culture-independent, molecular techniques. However, these two approaches are complementary: culture studies of fecal matter or colonic mucosa are valuable for identifying functional groups and for selective enumeration, whereas advanced molecular study are a powerful tool for monitoring changes in microbial composition. The molecular methodology includes sequencing of the small-subunit ribosomal RNA genes through amplification of nucleic acids extracted from fecal or mucosal samples, fingerprinting methods such as denaturing gradient gel electrophoresis, targeted methods such as fluorescence in situ hybridization and quantitative PCR, new high-throughput sequencing, and 16S rRNA-based microarraying[20].

PUTATIVE PATHOPHYSIOLOGIC ROLE OF INTESTINAL MICROBIOTA IN IBS

Alteration of the microbiota-gut-brain axis

The microbiota in the gut can be altered by brain function, and microbial alteration can, in turn, influence brain function. It is evidenced by the finding that patients with IBS frequently have accompanying psychological disorders, such as anxiety or depression, and those with psychological stress are more likely to develop post-infectious (PI)-IBS. This connection between the microbiota, the gut, and the brain in IBS postulates the existence of a bidirectional, homeostatic network, and it is an exciting area of ongoing research.

Animal studies have demonstrated the influence of the intestinal microbiota on brain development. Brain dysfunction in Germ-free (GF) mice was reported, including an exaggerated hypothalamic-pituitary response to mild stress[21], more exploratory and risk-taking behavior[22], and altered brain chemistry and memory, indicative of impaired hippocampal development[23]. Brain chemistry and behavior were also influenced by altered microbiota; a study showed that transient alteration of the microbial composition by diet provoked exploratory behavior, accompanied by changes of in the levels of brain-derived neurotrophic factor in the specific regions of the brain such as hippocampus and amygdala[24]. The gut microbiota and the brain may be communicated by neural, metabolic (bacterial and host), immunologic, or endocrine pathways[25]. The neural pathways was first suggested in animal models; anxiety-related behavior was reduced after probiotic treatment, provided vagus nerve integrity was maintained[26,27]. Metabolic pathways were revealed in a study that brain function and behavioral changes were closely associated with bacterial metabolites such as SCFAs (which comprise most of the circulating organic acids) and tryptophan metabolites[28,29]. A role of immunologic pathways was demonstrated in animal and human studies showing that certain psychological disorders were associated with pro-inflammatory cytokines, whose levels had been altered by manipulating the composition of the microbiota[30-32]. Endocrine pathways in microbiota-gut-brain axis were suggested in a study showing that the endocrine structure and function of the GI tract which secrets a variety of hormones such as cholecystokinin and serotonin [5-hydroxytryptamine (5-HT)] were reduced in GF rats[33].

Likewise, the intestinal microbiota can be affected by signals from the central nervous system produced in response to stress or psychological disturbances. Stress can change GI motility and secretions, which alter the microbial habitat. The microbial habitat may also be altered by changes in gene expression of some microbial species. Conversely, the intestinal microbiota can influence neurotransmitters like norepinephrine, dopamine, and serotonin in the brain, and activation of the hypothalamic-pituitary-adrenal axis is also thought to be involved in the microbiota-gut-brain axis.

As a result of alteration of the microbiota in this axis, mucosal immunity may be activate and thereby epithelial barrier function can be disrupted, which could contribute to the visceral hypersensitivity and dysmotility in IBS. Furthermore, the intestinal microbiota may not only release metabolites but also induce the formation of host-derived immune mediators, thereby affecting the enteric nervous system both directly and indirectly. However, much about the role of the microbiota-gut-brain axis in IBS remains poorly understood.

Dysbiosis: quantitative and qualitative changes in the microbiota

Altered composition of the intestinal microbiota: Intestinal microbiota can be grouped into luminal and mucosal microbiota. It is generally accepted that the composition of the luminal and mucosal microbiota differs between patients with IBS and healthy controls, and the composition may also vary according to the subtype of IBS[34], although studies of the intestinal microbiota have been as diverse and complex as the microbiota itself, with inconsistent and conflicting results[29,35-44] (Table 1). According to both the early culture-based and the more recent advanced molecular studies, it was found in IBS that the proportions of specific bacterial groups were altered, the diversity of microbial populations was reduced, and the degree of variability in the microbiota composition was different. The findings included decreased levels of

Table 1 Summary of studies of the intestinal microbiota in patients with irritable bowel syndrome

Ref.	Subject (*n*)	Method	Finding
Si *et al*[35]	IBS (25)	Culture	Decreased *Bifidobacterium*
	Control (25)		Increased *Enterobacteriaceae*
Malinen *et al*[36]	IBS (27)	qPCR	Decreased *Lactobacillus* in IBS-D
	Control (22)		Increased *Veillonella* in IBS-C
Mättö *et al*[37]	IBS (26)	Culture	Increased coliform and aerob to anaerob ratio
	Control (25)	PCR-DGGE	Temporal instability
Codling *et al*[38]	IBS (41)	PCR-DGGE	No difference in fecal/mucosal
	Control (33)		Temporal instability
Ponnusamy *et al*[39]	IBS (11)	DGGE	Increased diversity in *Bacteroidetes*, *Lactobacillus*
	Control (8)	qPCR-16sRNA	
Tana *et al*[29]	IBS (26)	Culture	Increased *Lactobacillus* and *Veillonella*
	Control (26)	q-PCR	
Lyra *et al*[40]	IBS (20)	qPCR	Increased *Ruminococcus* torques and decreased
	Control (15)		*Clostridium thermosuccinogenes* in IBS-D
Krogius-Kurikka *et al*[41]	IBS (10)	16S rRNA	Increased *Proteobacteria* and *Firmicutes*
	Control (23)	sequencing	Decreased *Actinobacteria* and *Bacteroidetes*
Kerckhoffs *et al*[42]	IBS (41)	FISH	Decreased *Bifidobacterium*
	Control (26)	qPCR	
Kassinen *et al*[43]	IBS (24)	16S rRNA	Decreased *Collinsella aerofaciens*, *Clostridium cocleatum*, and *Coprococcus*
	Control (23)	sequencing	*eutactus*
Maukonen *et al*[44]	IBS (24)	PCR-DGGE	Decreased *Clostridium coccoides*
	Control (16)		Temporal instability
Jeffery *et al*[46]	IBS (37)	16S rRNA	Increased ratio of *Firmicutes* to *Bacteroidetes*
	Control (20)	pyrosequencing	Clustering in IBS

DGGE: Denaturing gradient gel electrophoresis; FISH: Fluorescent in situ hybridization; IBS: Irritable bowel syndrome; qPCR: Quantitative polymerase chain reaction.

fecal lactobacilli and bifidobacteria, increased levels of facultative anaerobic bacteria dominated by streptococci and *Escherichia coli* (*E. coli*), increased ratios of *Firmicutes*: *Bacteroidetes* and higher counts of anaerobic organisms (such as clostridium)[44,45]. In addition, the microbiota of IBS patients reportedly belonged to entirely different enterotypes than those of healthy controls[34,46]. These inconsistent and sometimes conflicting results are thought to be due to the use of a single fecal sample irrespective of the fluctuating symptoms of IBS.

Altered metabolic activity of the intestinal microbiota: Intestinal microbiota may produce excessive amounts of gas by fermenting poorly absorbable carbohydrates (*e.g.*, the so-called FODMAPs, fermentable oligosaccharides, disaccharides, monosaccharides and polyols), which may cause abdominal pain, bloating, flatulence, and distension in IBS. Additionally, altered fermentation of poorly absorbable carbohydrates could increase the production of SCFAs, which would then lead to release of 5-HT from the intestinal mucosa[47]. In fact, increased numbers of acetic and propionic acid-producing bacteria (*Veionella* and *Lactobacillus* spp) were reported in patients with IBS[29]. It has been demonstrated that the release of 5-HT initiated high-amplitude, propagated colonic contractions, accelerated intestinal transit, and increased gut motility[47,48], all of which may contribute to IBS symptoms, suggesting that fermentation products play a potential role of in contributing IBS symptoms.

However, considering the large variability due to different methodologies of microbiota studies, and individual differences in relation to dietary, genetic and geographical factors, as well as heterogeneity of the disease, these results should be cautiously interpreted. Research on the luminal and mucosal microbiota is still in infancy, and further studies using advanced techniques such as 16s rRNA and DNA sequencing are needed to improve our understanding of the microbiota changes in IBS.

Activation of mucosal immunity and inflammation in IBS

The altered composition and metabolic activity of the intestinal microbiota found in IBS may be associated with activation of mucosal immunity and inflammation. Changes in the intestinal microbiota were observed after an episode of infective gastroenteritis with subsequent antibiotic use. In fact, some patients start to report IBS symptoms following such episodes[49], which suggests an association between IBS and activation of mucosal immunity and inflammation caused by altered microbiota. Chronic low-grade mucosal inflammation has been frequently observed in many studies of IBS patients and in animal models of IBS[50-56].

The intestinal microbiota plays an essential role in the development, functioning, and regulation of both intestinal and systemic immunities. By interacting with the microbiota, the intestinal (or enteric) immune system, composed of innate and adaptive immunity, helps to maintain normal GI function[57]. In IBS patients, however, the interactions between enteric immunity and commensal and/or pathogenic microbes were found to be dys-regulated. Under normal conditions, intestinal microbes are recognized via their ligands, identified by toll-

like receptors (TLRs) on intestinal immune cells. Expression of TLRs in the colonic mucosa of IBS patients was found to be increased[58], as was the level of circulating antibodies such as antiflagellin antibodies[59]. Together, these findings suggest that in IBS, bacterial components such as lipopolysaccharides (LPS) and flagellin are recognized more frequently due to the increased TLRs and circulating antibodies. In addition, one of the antibacterial proteins, β-defensin-2, was found to be elevated in IBS[60]. These increased interactions of immunologic components with the microbiota could eventually lead to the mucosal inflammation in IBS.

Mucosal inflammation provoked by dysregulated innate and adaptive enteric immunities has been observed in many studies of IBS[61,62]. The numbers of activated mast cells were shown to be increased in the colon of IBS patients, and also to be in close proximity to enteric nerves, which correlated well with IBS symptoms[63], although this increase was specific to diarrhea predominant IBS (IBS-D)[52], and varied according to the region of the intestine[64]. In addition to mast cells, lymphocytes ($CD4^+$ and $CD8^+$ T cells) were also found to be elevated, suggesting that they may play a role in IBS, although there are some inconsistencies[50,55,56,65]. Immune alterations associated with IBS were also found in IgA-producing B cells[66], IgG^+ B cells[67], and in the levels of pro- and anti-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin (IL)-10, IL-6, and interferon-γ in the intestinal mucosa of IBS patients[68]. Similarly, in the peripheral blood, levels of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α were higher in patients with IBS than in controls, but the levels varied according to IBS subtype[69]. It is thought that mucosal inflammation and activated immunity in IBS may lead to increased permeability of the intestinal mucosa, and may thus induce abnormal sensory and motor function, which could contribute to the symptoms of IBS. However, the association between activated immunity and the intestinal microbiota is not clearly established, and further studies in this area are warranted.

Altered mucosal permeability and the epithelial barrier in IBS

The activation of mucosal immunity and inflammation driven by the altered microbiota in IBS may increase mucosal permeability and impair epithelial barrier function. The intestinal epithelium functions not only as an exchanger, absorbing fluid and nutrients, but also as a protective barrier against pathogens. It is covered with a thick layer of mucus, composed of a complex mixture of glycoproteins, mucins, bactericidal enzymes, and secretory immunoglobulin A (IgA). Alterations to the epithelial barrier observed in IBS have included increased mucosal permeability, increased expression of specific proteins, *e.g.* MUC20 (gene involved in the production of mucin) and PARM1, and increased fecal excretion of the antibacterial protein β-defensin-2[60]. Increased mucosal permeability in the small intestine was observed in patients with IBS-D[70], and it was associated with the expression and distribution of tight junction proteins; lower levels of the protein zonula occludens (ZO)-1 were found in IBS patients than controls[71,72]. Elsewhere, increased permeability along with mast cell infiltration into the colon was found to be associated with the severity of IBS symptoms[73]. Both the increased permeability and symptoms of IBS were improved by lactic acid bacteria, suggesting that there may be an association between an altered epithelial barrier and IBS symptoms[74]. It is worth noting that one study of gut permeability found that the increase was limited to the colon[75], whereas another IBS study reported that the expression and distribution of ZO-1 was altered in the jejunum[76]. Generally, this increase in gut permeability was found to be associated with bacteria-related protease activity and its receptors in the intestinal epithelium[77]. It is also thought that a single-nucleotide polymorphism in the gene encoding the tight junction protein, E-cadherin, may increase the risk of developing PI-IBS[78]. On the other hand, some bacterial metabolites produced by the intestinal microbiota were found to improve epithelial barrier function[79]. It has also been suggested that the barrier dysfunction with increased mucosal permeability in IBS may also be associated with visceral hypersensitivity[80].

Sensory-motor disturbances caused by intestinal microbiota

In addition to the mucosal inflammation of the gut that may affect sensory-motor and secretory functions, neuronal structure, and neurotransmitter release in the gut[81], the intestinal microbiota can directly affect intestinal sensory-motor functions[82]. Alterations in the microbiota induced by antibiotic treatment were found to precipitate visceral hypersensitivity, which was restored by probiotic treatment[83]. Probiotic treatment was also found to reduce sensation of pain via the enteric nerve in a model of visceral pain induced by colorectal distension[84]. A similar level of pain modulation was also achieved by inducing the expression of opioid and cannabinoid receptors[85]. With respect to motor disturbances, it has been reported that colonic motor function was enhanced by supernatants from the *E. coli* strain Nissle 1917, and that this was mediated by stimulation of smooth muscle cells[86]. Also, probiotic treatment was found to increase small-intestinal motor function in rats[87]. Furthermore, transplantation of healthy human fecal microbiota into GF mice increased their colonic motility and shortened GI transit, which was closely associated with the type and amount of carbohydrates in the diet[88]. The beneficial effects of the microbiota on motility were shown to be region-specific with migrating motor complex velocity increased in the jejunum but decreased in the colon[89]. These interactions between intestinal microbiota and GI sensory-motor function may be related to IBS, although the exact mechanism of the interactions is not well understood.

It seems that normal GI motility relies on TLR4 sig-

naling stimulated by the microbiota. It was demonstrated that mice lacking TLR4, which is frequently stimulated by bacterial LPS, exhibited longer GI transit times and reduced abundance of colonic nitrergic neurons[90]. In addition to the microbiota itself, the metabolites from bacterial fermentation may also exert an effect on GI motility. One of the colonic metabolites, CH4, was shown to delay intestinal transit[91], H_2S was shown to inhibit the contraction of intestinal smooth muscle[92], SCFA, to stimulate colonic transit by triggering 5-HT release[47], and tryptamine from tryptophan, to increase intestinal contractions[93]. Other bacterial metabolites that may be related to GI motility include bile acid metabolites[94] and ligands of GABA receptors with a suppressive effect on GI motility[95]. While the microbiota may affect gut sensory-motor function, the reverse may also be true: the microbial ecosystem in the gut may be disturbed by accelerated or decelerated GI transit[88]. It is thought that the changes in GI transit may alter the flow rate of intestinal contents and thereby affect the environment for resident bacteria, which then impinges on both the organizational structure and the gene expressed in the microbiota.

MODULATION OF THE INTESTINAL MICROBIOTA FOR MANAGING IBS SYMPTOMS

Dietary modifications

An association between diet and symptom development in IBS is reported frequently but its mechanisms are not clearly defined. Some of the proposed causative factors include hypersensitivity and/or allergic reaction to specific foods, and alterations of the habitat and metabolic activity of the intestinal microbiota. Diet is thought to be a powerful factor influencing the composition and metabolic activity of the microbiota in an individual. The composition of the microbiota in babies change after weaning, and in adults it varies according to geographic regions due to differences in the food consumed, the type of meat consumed, and cooking methods (whether the food is fried, baked or boiled). Therefore, any dietary strategy aimed at modifying the microbiota should be matched to the individual because different microbial species are responsive to different kinds of dietary components.

However, whether a change in the diet can directly affect the microbiota in IBS is not clear. This is partly due to the lack of well-designed, controlled trials that investigate the effects of diet on IBS. Although specific diets, *e.g.*, the FODMAPs diet, have been shown to provoke IBS symptoms in some patients, not all studies regarding the effects of exclusion diets on the symptoms of IBS are completely reliable due to a variety of confounding factors, including a high placebo effect. Nevertheless, it can be speculated that in some IBS patients, intake of certain foods may provoke abnormal fermentation due to aspects of the composition of their intestinal microbiota and that the composition of the microbiota in those patients could be changed to normal by excluding the symptom-provoking foods.

Dietary fiber stimulates the production of SCFAs by mixing with microbes and enzymes. In a healthy gut, these by-products can improve the function and homeostasis of the GI tract. Although it has been suggested that some patients with IBS may benefit from dietary fiber, many patients report an increase in abdominal distension and bloating as a result of fermentation of the fiber. It may be that water holding properties of fiber and its ability to accelerate intestinal transit may alter the habitat for the microbiota and therefore indirectly affect its composition and metabolic activity.

It seems that individualized advice on dietary consumption of non-digestible carbohydrates in the management of IBS, as the inter-individual differences in the response of the microbiota lead to different responses to changes in diet[96].

Antibiotics

Antibiotic treatment in IBS assumes that small intestinal bacterial overgrowth (SIBO) plays an important role in the development of IBS. Despite the limited validity and lack of standardization of the methods used to evaluate SIBO, treatment with non-absorbable antibiotics such as rifaximin has yielded a therapeutic benefit. Double-blind, placebo-controlled trials of rifaximin in IBS yielded an improvement in IBS symptoms, which correlated well with the reduced excretion of hydrogen in the breath[97,98]. These findings together with the positive effects of other antibiotic treatments, suggest that a short course of poorly absorbable antibiotics may be of some use in the management of IBS symptoms in some patients. However, data on the long-term effects of antibiotics in IBS are limited. Furthermore, information on the optimal dose of antibiotics, and predictors of treatment success and failure are needed to confirm the benefit of this type of treatment[99].

Probiotics

Effects of probiotics: By adhering to intestinal epithelial cells and competing for nutrients and space, probiotics can protect against pathogens. This protective effect of probiotics has been demonstrated *in vitro* using intestinal cell lines with lactobacilli, bifidobacteria and *E. coli* subspecies[100-102]. In addition, probiotics can improve mucosal barrier function and thereby prevent pathogens from increasing intestinal permeability[103,104]. Intestinal permeability can also be increased by stress, which may facilitate the subsequent translocation of pathogenic bacteria. However, it was observed that the increase in intestinal permeability caused by stress was inhibited by lactobacilli[105-107]. In addition, lactobacilli increased levels of bacterial fermentation products such as SCFAs (acetic, propionic and butyric acids) and thereby acidifying the colon, which subsequently increased the numbers of *Bifidobacterium* and *Lactobacillus* species and decreased

clostridia[108]. In addition to these roles, probiotics were also shown to modulate immunity in animals with experimentally-induced colitis[109,110]. Furthermore, they were shown to reduce visceral hypersensitivity by increasing the expression of opioid and cannabinoid receptors in the intestinal mucosa[85].

However, regarding the effect of probiotics on IBS symptoms, the mechanism is not clearly defined. It is possible that probiotics may not only modulate gut dysmotility and hypersensitivity but also have anti-inflammatory properties. It was found that probiotic treatment attenuated intestinal dysmotility in a mouse model, induced intestinal cell mediators related to reduced hypersensitivity such as cannabinoid and opioid receptors, and normalized the ratio of cytokines IL-10/IL-12 in the systemic circulation.

Probiotic studies in IBS: A majority of studies of probiotics in IBS have been performed to evaluate their effect on either overall or specific IBS symptoms. Although most of them have used *Lactobacillus* or *Bifidobacterium* species, single strains or combinations of multiple strains have also been used with multiple doses (from 10^6/mL to 10^{10}/mL) and for variable durations. Similarly, primary and secondary outcomes in those studies were evaluated using variable factors such as abdominal pain, symptom severity, quality of life, and global IBS symptoms. On balance, these studies found a therapeutic benefit, *i.e.*, improvement in symptoms of bloating, flatulence, bowel frequency, and in global symptoms, although there are some inconsistencies between specific studies. In particular, beneficial effects of probiotics were reported in a well-designed study using bifidobacteria such as *Bifidobacterium infantis* 35624[30,111], *B. lactis*, *B. animalis* DN173010, and *B. bifidum* MIMBb75[112]. Symptom improvement was also reported in studies using probiotic mixtures such as *Escherichia coli* (DSM 17252) and *Enterococcus faecalis* (DSM 16440)[113], and *Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *Bifidobacterium breve* Bb99 and *Propionibacterium freudenreichii* ssp. *shermanii* JS[114,115]. By contrast, negative results were reported in studies using other probiotic combinations[116], such as *Lactobacillus paracasei* spp. *paracasei* F19, *L. acidophilus* La5 and *Bifidobacterium lactis* Bb12[117,118], and *Lactobacillus plantarum* MF1298[119].

In recent studies, it was found that 4-wk treatment with probiotics improved IBS symptoms and altered composition of the microbiota as well[120], and that probiotic treatment in IBS patients reduced the genus Bacteroides to the levels of healthy controls and also improved global IBS symptoms[121]. However, as indicated in several meta-analyses, the previous studies of probiotics in IBS fail to report whether symptom improvement was accompanied by a change in the microbiota or not. Furthermore, many systematic reviews pointed out several study limitations including heterogeneity, inadequate statistical methods, and possible publication bias. Examples of heterogeneity include differences in types, doses, and delivery of probiotics[122-125], which may have produced different outcomes. Therefore, despite the reported benefits of probiotics in IBS, there are many aspects of potential treatment regimens that are yet to be established, such as adequate dosage, treatment duration, choice of species for each individual or symptom of IBS, target symptoms for probiotics, and probiotic formulation. Future studies should aim to identify which species, strains, and doses of probiotics provide the optimal therapeutic benefit to individual patients with IBS, and which specific symptoms of IBS should be the target of probiotic treatment.

CONCLUSION

Intestinal microbiota can play a substantial role in IBS. Although the microbiota may contribute directly to the symptoms of IBS, it is more likely that altered composition and metabolic activity of the microbiota caused by stress or other psychological disturbances indirectly activate mucosal immunity and inflammation, increase epithelial permeability, and reduce barrier function, thereby activating the sensory-motor dysfunction responsible for a variety of IBS symptoms. Therefore, our knowledge of the link between the microbiota and IBS may enable us to treat focusing on the possible mechanism of this disorder; Dysbiosis may be restored by probiotic or antibiotic treatment and also by diet modification. Activation of mucosal immunity and inflammation can be treated by immune-modulating agents. Increased intestinal permeability and barrier dysfunction can be a potential therapeutic target of probiotics. However, the microbial pathophysiology of IBS is not clearly understood, as microbiota alterations in IBS might be either a cause of IBS or a consequence of intestinal secretion and motility changed by IBS. Furthermore, due to the heterogeneity of IBS studies as well as IBS itself, there has been variability in the results of studies. Therefore, objective diagnostic modalities in IBS are warranted, and further studies using advanced molecular techniques are needed.

REFERENCES

1 **Drossman DA**. The functional gastrointestinal disorders and the Rome III process. *Gastroenterology* 2006; **130**: 1377-1390 [PMID: 16678553 DOI: 10.1053/j.gastro.2006.03.008]

2 **Drossman DA**, Camilleri M, Mayer EA, Whitehead WE. AGA technical review on irritable bowel syndrome. *Gastroenterology* 2002; **123**: 2108-2131 [PMID: 12454866 DOI: 10.1053/gast.2002.37095]

3 **Simrén M**, Barbara G, Flint HJ, Spiegel BM, Spiller RC, Vanner S, Verdu EF, Whorwell PJ, Zoetendal EG. Intestinal microbiota in functional bowel disorders: a Rome foundation report. *Gut* 2013; **62**: 159-176 [PMID: 22730468 DOI: 10.1136/gutjnl-2012-302167]

4 **Parkes GC**, Brostoff J, Whelan K, Sanderson JD. Gastrointestinal microbiota in irritable bowel syndrome: their role in its pathogenesis and treatment. *Am J Gastroenterol* 2008; **103**: 1557-1567 [PMID: 18513268 DOI: 10.1111/j.1572-0241.2008.01869.x]

5 **Young VB**, Schmidt TM. Overview of the gastrointestinal microbiota. *Adv Exp Med Biol* 2008; **635**: 29-40 [PMID: 18841701 DOI: 10.1007/978-0-387-09550-9_3]

6 **Sekirov I**, Russell SL, Antunes LC, Finlay BB. Gut microbio-

ta in health and disease. *Physiol Rev* 2010; **90**: 859-904 [PMID: 20664075 DOI: 10.1152/physrev.00045.2009]
7 **Qin J**, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR, Li J, Xu J, Li S, Li D, Cao J, Wang B, Liang H, Zheng H, Xie Y, Tap J, Lepage P, Bertalan M, Batto JM, Hansen T, Le Paslier D, Linneberg A, Nielsen HB, Pelletier E, Renault P, Sicheritz-Ponten T, Turner K, Zhu H, Yu C, Li S, Jian M, Zhou Y, Li Y, Zhang X, Li S, Qin N, Yang H, Wang J, Brunak S, Doré J, Guarner F, Kristiansen K, Pedersen O, Parkhill J, Weissenbach J, Bork P, Ehrlich SD, Wang J. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 2010; **464**: 59-65 [PMID: 20203603 DOI: 10.1038/nature08821]
8 **Swidsinski A**, Loening-Baucke V, Lochs H, Hale LP. Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice. *World J Gastroenterol* 2005; **11**: 1131-1140 [PMID: 15754393]
9 **Arumugam M**, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, Bertalan M, Borruel N, Casellas F, Fernandez L, Gautier L, Hansen T, Hattori M, Hayashi T, Kleerebezem M, Kurokawa K, Leclerc M, Levenez F, Manichanh C, Nielsen HB, Nielsen T, Pons N, Poulain J, Qin J, Sicheritz-Ponten T, Tims S, Torrents D, Ugarte E, Zoetendal EG, Wang J, Guarner F, Pedersen O, de Vos WM, Brunak S, Doré J, Antolín M, Artiguenave F, Blottiere HM, Almeida M, Brechot C, Cara C, Chervaux C, Cultrone A, Delorme C, Denariaz G, Dervyn R, Foerstner KU, Friss C, van de Guchte M, Guedon E, Haimet F, Huber W, van Hylckama-Vlieg J, Jamet A, Juste C, Kaci G, Knol J, Lakhdari O, Layec S, Le Roux K, Maguin E, Mérieux A, Melo Minardi R, M'rini C, Muller J, Oozeer R, Parkhill J, Renault P, Rescigno M, Sanchez N, Sunagawa S, Torrejon A, Turner K, Vandemeulebrouck G, Varela E, Winogradsky Y, Zeller G, Weissenbach J, Ehrlich SD, Bork P. Enterotypes of the human gut microbiome. *Nature* 2011; **473**: 174-180 [PMID: 21508958 DOI: 10.1038/nature09944]
10 **Jeffery IB**, Claesson MJ, O'Toole PW, Shanahan F. Categorization of the gut microbiota: enterotypes or gradients? *Nat Rev Microbiol* 2012; **10**: 591-592 [PMID: 23066529]
11 **Martin FP**, Dumas ME, Wang Y, Legido-Quigley C, Yap IK, Tang H, Zirah S, Murphy GM, Cloarec O, Lindon JC, Sprenger N, Fay LB, Kochhar S, van Bladeren P, Holmes E, Nicholson JK. A top-down systems biology view of microbiome-mammalian metabolic interactions in a mouse model. *Mol Syst Biol* 2007; **3**: 112 [PMID: 17515922 DOI: 10.1038/msb4100153]
12 **O'Hara AM**, Shanahan F. The gut flora as a forgotten organ. *EMBO Rep* 2006; **7**: 688-693 [PMID: 16819463 DOI: 10.1038/sj.embor.7400731]
13 **Power SE**, O'Toole PW, Stanton C, Ross RP, Fitzgerald GF. Intestinal microbiota, diet and health. *Br J Nutr* 2014; **111**: 387-402 [PMID: 23931069 DOI: 10.1017/S0007114513002560]
14 **Lupp C**, Finlay BB. Intestinal microbiota. *Curr Biol* 2005; **15**: R235-R236 [PMID: 15823518 DOI: 10.1016/j.cub.2005.03.032]
15 **Bezirtzoglou E**, Tsiotsias A, Welling GW. Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH). *Anaerobe* 2011; **17**: 478-482 [PMID: 21497661 DOI: 10.1016/j.anaerobe.2011.03.009]
16 **De Filippo C**, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci USA* 2010; **107**: 14691-14696 [PMID: 20679230 DOI: 10.1073/pnas.1005963107]
17 **Brestoff JR**, Artis D. Commensal bacteria at the interface of host metabolism and the immune system. *Nat Immunol* 2013; **14**: 676-684 [PMID: 23778795 DOI: 10.1038/ni.2640]
18 **Jiang X**, Locke GR, Choung RS, Zinsmeister AR, Schleck CD, Talley NJ. Prevalence and risk factors for abdominal bloating and visible distention: a population-based study. *Gut* 2008; **57**: 756-763 [PMID: 18477677 DOI: 10.1136/gut.2007.142810]
19 **Spiller R**, Garsed K. Postinfectious irritable bowel syndrome. *Gastroenterology* 2009; **136**: 1979-1988 [PMID: 19457422 DOI: 10.1053/j.gastro.2009.02.074]
20 **Fraher MH**, O'Toole PW, Quigley EM. Techniques used to characterize the gut microbiota: a guide for the clinician. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 312-322 [PMID: 22450307 DOI: 10.1038/nrgastro.2012.44]
21 **Sudo N**, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, Kubo C, Koga Y. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *J Physiol* 2004; **558**: 263-275 [PMID: 15133062 DOI: 10.1113/jphysiol.2004.063388]
22 **Neufeld KM**, Kang N, Bienenstock J, Foster JA. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterol Motil* 2011; **23**: 255-264, e119 [PMID: 21054680 DOI: 10.1111/j.1365-2982.2010.01620.x]
23 **Gareau MG**, Wine E, Rodrigues DM, Cho JH, Whary MT, Philpott DJ, Macqueen G, Sherman PM. Bacterial infection causes stress-induced memory dysfunction in mice. *Gut* 2011; **60**: 307-317 [PMID: 20966022 DOI: 10.1136/gut.2009.202515]
24 **Bercik P**, Denou E, Collins J, Jackson W, Lu J, Jury J, Deng Y, Blennerhassett P, Macri J, McCoy KD, Verdu EF, Collins SM. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. *Gastroenterology* 2011; **141**: 599-609, 609.e1-3 [PMID: 21683077 DOI: 10.1053/j.gastro.2011.04.052]
25 **Collins SM**, Surette M, Bercik P. The interplay between the intestinal microbiota and the brain. *Nat Rev Microbiol* 2012; **10**: 735-742 [PMID: 23000955 DOI: 10.1038/nrmicro2876]
26 **Bercik P**, Park AJ, Sinclair D, Khoshdel A, Lu J, Huang X, Deng Y, Blennerhassett PA, Fahnestock M, Moine D, Berger B, Huizinga JD, Kunze W, McLean PG, Bergonzelli GE, Collins SM, Verdu EF. The anxiolytic effect of Bifidobacterium longum NCC3001 involves vagal pathways for gut-brain communication. *Neurogastroenterol Motil* 2011; **23**: 1132-1139 [PMID: 21988661 DOI: 10.1111/j.1365-2982.2011.01796.x]
27 **Bravo JA**, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, Bienenstock J, Cryan JF. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci USA* 2011; **108**: 16050-16055 [PMID: 21876150 DOI: 10.1073/pnas.1102999108]
28 **Ledochowski M**, Widner B, Sperner-Unterweger B, Propst T, Vogel W, Fuchs D. Carbohydrate malabsorption syndromes and early signs of mental depression in females. *Dig Dis Sci* 2000; **45**: 1255-1259 [PMID: 10961700]
29 **Tana C**, Umesaki Y, Imaoka A, Handa T, Kanazawa M, Fukudo S. Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome. *Neurogastroenterol Motil* 2010; **22**: 512-519, e114-5 [PMID: 19903265 DOI: 10.1111/j.1365-2982.2009.01427.x]
30 **O'Mahony L**, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, O'Sullivan GC, Kiely B, Collins JK, Shanahan F, Quigley EM. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. *Gastroenterology* 2005; **128**: 541-551 [PMID: 15765388 DOI: 10.1053/j.gastro.2004.11.050]
31 **Lotrich FE**, El-Gabalawy H, Guenther LC, Ware CF. The role of inflammation in the pathophysiology of depression: different treatments and their effects. *J Rheumatol Suppl* 2011; **88**: 48-54 [PMID: 22045979 DOI: 10.3899/jrheum.110903]
32 **Desbonnet L**, Garrett L, Clarke G, Kiely B, Cryan JF, Dinan TG. Effects of the probiotic Bifidobacterium infantis in the maternal separation model of depression. *Neuroscience* 2010; **170**: 1179-1188 [PMID: 20696216 DOI: 10.1016/j.neuroscienc

e.2010.08.005]
33 **Uribe A**, Alam M, Johansson O, Midtvedt T, Theodorsson E. Microflora modulates endocrine cells in the gastrointestinal mucosa of the rat. *Gastroenterology* 1994; **107**: 1259-1269 [PMID: 7926490]
34 **Rajilić-Stojanović M**, Biagi E, Heilig HG, Kajander K, Kekkonen RA, Tims S, de Vos WM. Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. *Gastroenterology* 2011; **141**: 1792-1801 [PMID: 21820992 DOI: 10.1053/j.gastro.2011.07.043]
35 **Si JM**, Yu YC, Fan YJ, Chen SJ. Intestinal microecology and quality of life in irritable bowel syndrome patients. *World J Gastroenterol* 2004; **10**: 1802-1805 [PMID: 15188510]
36 **Malinen E**, Rinttilä T, Kajander K, Mättö J, Kassinen A, Krogius L, Saarela M, Korpela R, Palva A. Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. *Am J Gastroenterol* 2005; **100**: 373-382 [PMID: 15667495 DOI: 10.1111/j.1572-0241.2005.40312.x]
37 **Mättö J**, Maunuksela L, Kajander K, Palva A, Korpela R, Kassinen A, Saarela M. Composition and temporal stability of gastrointestinal microbiota in irritable bowel syndrome--a longitudinal study in IBS and control subjects. *FEMS Immunol Med Microbiol* 2005; **43**: 213-222 [PMID: 15747442]
38 **Codling C**, O'Mahony L, Shanahan F, Quigley EM, Marchesi JR. A molecular analysis of fecal and mucosal bacterial communities in irritable bowel syndrome. *Dig Dis Sci* 2010; **55**: 392-397 [PMID: 19693670 DOI: 10.1007/s10620-009-0934-x]
39 **Ponnusamy K**, Choi JN, Kim J, Lee SY, Lee CH. Microbial community and metabolomic comparison of irritable bowel syndrome faeces. *J Med Microbiol* 2011; **60**: 817-827 [PMID: 21330412 DOI: 10.1099/jmm.0.028126-0]
40 **Lyra A**, Rinttilä T, Nikkilä J, Krogius-Kurikka L, Kajander K, Malinen E, Mättö J, Mäkelä L, Palva A. Diarrhoea-predominant irritable bowel syndrome distinguishable by 16S rRNA gene phylotype quantification. *World J Gastroenterol* 2009; **15**: 5936-5945 [PMID: 20014457]
41 **Krogius-Kurikka L**, Lyra A, Malinen E, Aarnikunnas J, Tuimala J, Paulin L, Mäkivuokko H, Kajander K, Palva A. Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers. *BMC Gastroenterol* 2009; **9**: 95 [PMID: 20015409 DOI: 10.1186/1471-230X-9-95]
42 **Kerckhoffs AP**, Samsom M, van der Rest ME, de Vogel J, Knol J, Ben-Amor K, Akkermans LM. Lower Bifidobacteria counts in both duodenal mucosa-associated and fecal microbiota in irritable bowel syndrome patients. *World J Gastroenterol* 2009; **15**: 2887-2892 [PMID: 19533811 DOI: 10.3748/Wjg.15.2887]
43 **Kassinen A**, Krogius-Kurikka L, Mäkivuokko H, Rinttilä T, Paulin L, Corander J, Malinen E, Apajalahti J, Palva A. The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. *Gastroenterology* 2007; **133**: 24-33 [PMID: 17631127 DOI: 10.1053/j.gastro.2007.04.005]
44 **Maukonen J**, Satokari R, Mättö J, Söderlund H, Mattila-Sandholm T, Saarela M. Prevalence and temporal stability of selected clostridial groups in irritable bowel syndrome in relation to predominant faecal bacteria. *J Med Microbiol* 2006; **55**: 625-633 [PMID: 16585652 DOI: 10.1099/jmm.0.46134-0]
45 **Balsari A**, Ceccarelli A, Dubini F, Fesce E, Poli G. The fecal microbial population in the irritable bowel syndrome. *Microbiologica* 1982; **5**: 185-194 [PMID: 7121297]
46 **Jeffery IB**, O'Toole PW, Öhman L, Claesson MJ, Deane J, Quigley EM, Simrén M. An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. *Gut* 2012; **61**: 997-1006 [PMID: 22180058 DOI: 10.1136/gutjnl-2011-301501]
47 **Fukumoto S**, Tatewaki M, Yamada T, Fujimiya M, Mantyh C, Voss M, Eubanks S, Harris M, Pappas TN, Takahashi T. Short-chain fatty acids stimulate colonic transit via intraluminal 5-HT release in rats. *Am J Physiol Regul Integr Comp Physiol* 2003; **284**: R1269-R1276 [PMID: 12676748 DOI: 10.1152/ajpregu.00442.2002]
48 **Kamath PS**, Hoepfner MT, Phillips SF. Short-chain fatty acids stimulate motility of the canine ileum. *Am J Physiol* 1987; **253**: G427-G433 [PMID: 3661705]
49 **Halvorson HA**, Schlett CD, Riddle MS. Postinfectious irritable bowel syndrome--a meta-analysis. *Am J Gastroenterol* 2006; **101**: 1894-1899; quiz 1942 [PMID: 16928253 DOI: 10.1111/j.1572-0241.2006.00654.x]
50 **Cremon C**, Gargano L, Morselli-Labate AM, Santini D, Cogliandro RF, De Giorgio R, Stanghellini V, Corinaldesi R, Barbara G. Mucosal immune activation in irritable bowel syndrome: gender-dependence and association with digestive symptoms. *Am J Gastroenterol* 2009; **104**: 392-400 [PMID: 19174797 DOI: 10.1038/ajg.2008.94]
51 **Gwee KA**, Collins SM, Read NW, Rajnakova A, Deng Y, Graham JC, McKendrick MW, Moochhala SM. Increased rectal mucosal expression of interleukin 1beta in recently acquired post-infectious irritable bowel syndrome. *Gut* 2003; **52**: 523-526 [PMID: 12631663]
52 **Lee KJ**, Kim YB, Kim JH, Kwon HC, Kim DK, Cho SW. The alteration of enterochromaffin cell, mast cell, and lamina propria T lymphocyte numbers in irritable bowel syndrome and its relationship with psychological factors. *J Gastroenterol Hepatol* 2008; **23**: 1689-1694 [PMID: 19120860 DOI: 10.1111/j.1440-1746.2008.05574.x]
53 **Liebregts T**, Adam B, Bredack C, Röth A, Heinzel S, Lester S, Downie-Doyle S, Smith E, Drew P, Talley NJ, Holtmann G. Immune activation in patients with irritable bowel syndrome. *Gastroenterology* 2007; **132**: 913-920 [PMID: 17383420 DOI: 10.1053/j.gastro.2007.01.046]
54 **Macsharry J**, O'Mahony L, Fanning A, Bairead E, Sherlock G, Tiesman J, Fulmer A, Kiely B, Dinan TG, Shanahan F, Quigley EM. Mucosal cytokine imbalance in irritable bowel syndrome. *Scand J Gastroenterol* 2008; **43**: 1467-1476 [PMID: 18752146 DOI: 10.1080/00365520802276127]
55 **Spiller RC**, Jenkins D, Thornley JP, Hebden JM, Wright T, Skinner M, Neal KR. Increased rectal mucosal enteroendocrine cells, T lymphocytes, and increased gut permeability following acute Campylobacter enteritis and in post-dysenteric irritable bowel syndrome. *Gut* 2000; **47**: 804-811 [PMID: 11076879 DOI: 10.1136/gut.47.6.804]
56 **Törnblom H**, Lindberg G, Nyberg B, Veress B. Full-thickness biopsy of the jejunum reveals inflammation and enteric neuropathy in irritable bowel syndrome. *Gastroenterology* 2002; **123**: 1972-1979 [PMID: 12454854 DOI: 10.1053/gast.2002.37059]
57 **Lee YK**, Mazmanian SK. Has the microbiota played a critical role in the evolution of the adaptive immune system? *Science* 2010; **330**: 1768-1773 [PMID: 21205662 DOI: 10.1126/science.1195568]
58 **Brint EK**, MacSharry J, Fanning A, Shanahan F, Quigley EM. Differential expression of toll-like receptors in patients with irritable bowel syndrome. *Am J Gastroenterol* 2011; **106**: 329-336 [PMID: 21102570 DOI: 10.1038/ajg.2010.438]
59 **Schoepfer AM**, Schaffer T, Seibold-Schmid B, Müller S, Seibold F. Antibodies to flagellin indicate reactivity to bacterial antigens in IBS patients. *Neurogastroenterol Motil* 2008; **20**: 1110-1118 [PMID: 18694443 DOI: 10.1111/j.1365-2982.2008.01166.x]
60 **Langhorst J**, Junge A, Rueffer A, Wehkamp J, Foell D, Michalsen A, Musial F, Dobos GJ. Elevated human beta-defensin-2 levels indicate an activation of the innate immune system in patients with irritable bowel syndrome. *Am J Gastroenterol* 2009; **104**: 404-410 [PMID: 19174795 DOI: 10.1038/ajg.2008.86]
61 **Ohman L**, Simrén M. Pathogenesis of IBS: role of inflam-

mation, immunity and neuroimmune interactions. *Nat Rev Gastroenterol Hepatol* 2010; **7**: 163-173 [PMID: 20101257 DOI: 10.1038/nrgastro.2010.4]
62 **Barbara G**, Cremon C, Carini G, Bellacosa L, Zecchi L, De Giorgio R, Corinaldesi R, Stanghellini V. The immune system in irritable bowel syndrome. *J Neurogastroenterol Motil* 2011; **17**: 349-359 [PMID: 22148103 DOI: 10.5056/jnm.2011.17.4.349]
63 **Barbara G**, Stanghellini V, De Giorgio R, Cremon C, Cottrell GS, Santini D, Pasquinelli G, Morselli-Labate AM, Grady EF, Bunnett NW, Collins SM, Corinaldesi R. Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. *Gastroenterology* 2004; **126**: 693-702 [PMID: 14988823]
64 **O'Sullivan M**, Clayton N, Breslin NP, Harman I, Bountra C, McLaren A, O'Morain CA. Increased mast cells in the irritable bowel syndrome. *Neurogastroenterol Motil* 2000; **12**: 449-457 [PMID: 11012945 DOI: 10.1046/j.1365-2982.2000.00221.x]
65 **Chadwick VS**, Chen W, Shu D, Paulus B, Bethwaite P, Tie A, Wilson I. Activation of the mucosal immune system in irritable bowel syndrome. *Gastroenterology* 2002; **122**: 1778-1783 [PMID: 12055584]
66 **Forshammar J**, Isaksson S, Strid H, Stotzer PO, Sjövall H, Simrén M, Ohman L. A pilot study of colonic B cell pattern in irritable bowel syndrome. *Scand J Gastroenterol* 2008; **43**: 1461-1466 [PMID: 18663666 DOI: 10.1080/00365520802272126]
67 **Ohman L**, Lindmark AC, Isaksson S, Posserud I, Strid H, Sjövall H, Simrén M. B-cell activation in patients with irritable bowel syndrome (IBS). *Neurogastroenterol Motil* 2009; **21**: 644-650, e27 [PMID: 19222763 DOI: 10.1111/j.1365-2982.2009.01272.x]
68 **Dinan TG**, Clarke G, Quigley EM, Scott LV, Shanahan F, Cryan J, Cooney J, Keeling PW. Enhanced cholinergic-mediated increase in the pro-inflammatory cytokine IL-6 in irritable bowel syndrome: role of muscarinic receptors. *Am J Gastroenterol* 2008; **103**: 2570-2576 [PMID: 18785949 DOI: 10.1111/j.1572-0241.2008.01871.x]
69 **Dinan TG**, Quigley EM, Ahmed SM, Scully P, O'Brien S, O'Mahony L, O'Mahony S, Shanahan F, Keeling PW. Hypothalamic-pituitary-gut axis dysregulation in irritable bowel syndrome: plasma cytokines as a potential biomarker? *Gastroenterology* 2006; **130**: 304-311 [PMID: 16472586 DOI: 10.1053/j.gastro.2005.11.033]
70 **Dunlop SP**, Hebden J, Campbell E, Naesdal J, Olbe L, Perkins AC, Spiller RC. Abnormal intestinal permeability in subgroups of diarrhea-predominant irritable bowel syndromes. *Am J Gastroenterol* 2006; **101**: 1288-1294 [PMID: 16771951 DOI: 10.1111/j.1572-0241.2006.00672.x]
71 **Piche T**, Barbara G, Aubert P, Bruley des Varannes S, Dainese R, Nano JL, Cremon C, Stanghellini V, De Giorgio R, Galmiche JP, Neunlist M. Impaired intestinal barrier integrity in the colon of patients with irritable bowel syndrome: involvement of soluble mediators. *Gut* 2009; **58**: 196-201 [PMID: 18824556 DOI: 10.1136/gut.2007.140806]
72 **Bertiaux-Vandaële N**, Youmba SB, Belmonte L, Lecleire S, Antonietti M, Gourcerol G, Leroi AM, Déchelotte P, Ménard JF, Ducrotté P, Coëffier M. The expression and the cellular distribution of the tight junction proteins are altered in irritable bowel syndrome patients with differences according to the disease subtype. *Am J Gastroenterol* 2011; **106**: 2165-2173 [PMID: 22008894 DOI: 10.1038/ajg.2011.257]
73 **Vivinus-Nébot M**, Dainese R, Anty R, Saint-Paul MC, Nano JL, Gonthier N, Marjoux S, Frin-Mathy G, Bernard G, Hébuterne X, Tran A, Theodorou V, Piche T. Combination of allergic factors can worsen diarrheic irritable bowel syndrome: role of barrier defects and mast cells. *Am J Gastroenterol* 2012; **107**: 75-81 [PMID: 21931380 DOI: 10.1038/ajg.2011.315]
74 **Zeng J**, Li YQ, Zuo XL, Zhen YB, Yang J, Liu CH. Clinical trial: effect of active lactic acid bacteria on mucosal barrier function in patients with diarrhoea-predominant irritable bowel syndrome. *Aliment Pharmacol Ther* 2008; **28**: 994-1002 [PMID: 18671775 DOI: 10.1111/j.1365-2036.2008.03818.x]
75 **Gecse K**, Róka R, Séra T, Rosztóczy A, Annaházi A, Izbéki F, Nagy F, Molnár T, Szepes Z, Pávics L, Bueno L, Wittmann T. Leaky gut in patients with diarrhea-predominant irritable bowel syndrome and inactive ulcerative colitis. *Digestion* 2012; **85**: 40-46 [PMID: 22179430 DOI: 10.1159/000333083]
76 **Martínez C**, Vicario M, Ramos L, Lobo B, Mosquera JL, Alonso C, Sánchez A, Guilarte M, Antolín M, de Torres I, González-Castro AM, Pigrau M, Saperas E, Azpiroz F, Santos J. The jejunum of diarrhea-predominant irritable bowel syndrome shows molecular alterations in the tight junction signaling pathway that are associated with mucosal pathobiology and clinical manifestations. *Am J Gastroenterol* 2012; **107**: 736-746 [PMID: 22415197 DOI: 10.1038/ajg.2011.472]
77 **Gecse K**, Róka R, Ferrier L, Leveque M, Eutamene H, Cartier C, Ait-Belgnaoui A, Rosztóczy A, Izbéki F, Fioramonti J, Wittmann T, Bueno L. Increased faecal serine protease activity in diarrhoeic IBS patients: a colonic lumenal factor impairing colonic permeability and sensitivity. *Gut* 2008; **57**: 591-599 [PMID: 18194983 DOI: 10.1136/gut.2007.140210]
78 **Villani AC**, Lemire M, Thabane M, Belisle A, Geneau G, Garg AX, Clark WF, Moayyedi P, Collins SM, Franchimont D, Marshall JK. Genetic risk factors for post-infectious irritable bowel syndrome following a waterborne outbreak of gastroenteritis. *Gastroenterology* 2010; **138**: 1502-1513 [PMID: 20044998 DOI: 10.1053/j.gastro.2009.12.049]
79 **Fukuda S**, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K, Tobe T, Clarke JM, Topping DL, Suzuki T, Taylor TD, Itoh K, Kikuchi J, Morita H, Hattori M, Ohno H. Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature* 2011; **469**: 543-547 [PMID: 21270894 DOI: 10.1038/nature09646]
80 **Zhou Q**, Zhang B, Verne GN. Intestinal membrane permeability and hypersensitivity in the irritable bowel syndrome. *Pain* 2009; **146**: 41-46 [PMID: 19595511 DOI: 10.1016/j.pain.2009.06.017]
81 **Vasina V**, Barbara G, Talamonti L, Stanghellini V, Corinaldesi R, Tonini M, De Ponti F, De Giorgio R. Enteric neuroplasticity evoked by inflammation. *Auton Neurosci* 2006; **126-127**: 264-272 [PMID: 16624634 DOI: 10.1016/j.autneu.2006.02.025]
82 **Barbara G**, Stanghellini V, Brandi G, Cremon C, Di Nardo G, De Giorgio R, Corinaldesi R. Interactions between commensal bacteria and gut sensorimotor function in health and disease. *Am J Gastroenterol* 2005; **100**: 2560-2568 [PMID: 16279914 DOI: 10.1111/j.1572-0241.2005.00230.x]
83 **Verdú EF**, Bercik P, Verma-Gandhu M, Huang XX, Blennerhassett P, Jackson W, Mao Y, Wang L, Rochat F, Collins SM. Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. *Gut* 2006; **55**: 182-190 [PMID: 16105890 DOI: 10.1136/gut.2005.066100]
84 **Kamiya T**, Wang L, Forsythe P, Goettsche G, Mao Y, Wang Y, Tougas G, Bienenstock J. Inhibitory effects of Lactobacillus reuteri on visceral pain induced by colorectal distension in Sprague-Dawley rats. *Gut* 2006; **55**: 191-196 [PMID: 16361309 DOI: 10.1136/gut.2005.070987]
85 **Rousseaux C**, Thuru X, Gelot A, Barnich N, Neut C, Dubuquoy L, Dubuquoy C, Merour E, Geboes K, Chamaillard M, Ouwehand A, Leyer G, Carcano D, Colombel JF, Ardid D, Desreumaux P. Lactobacillus acidophilus modulates intestinal pain and induces opioid and cannabinoid receptors. *Nat Med* 2007; **13**: 35-37 [PMID: 17159985 DOI: 10.1038/nm1521]
86 **Bär F**, Von Koschitzky H, Roblick U, Bruch HP, Schulze L, Sonnenborn U, Böttner M, Wedel T. Cell-free supernatants of Escherichia coli Nissle 1917 modulate human colonic motility: evidence from an in vitro organ bath study. *Neurogastroenterol Motil* 2009; **21**: 559-566, e16-7 [PMID: 19220758 DOI: 10.1111/j.1365-2982.2008.01258.x]
87 **Lesniewska V**, Rowland I, Laerke HN, Grant G, Naughton PJ. Relationship between dietary-induced changes in intestinal commensal microflora and duodenojejunal myoelectric

activity monitored by radiotelemetry in the rat in vivo. *Exp Physiol* 2006; **91**: 229-237 [PMID: 16263800 DOI: 10.1113/expphysiol.2005.031708]

88 **Kashyap PC**, Marcobal A, Ursell LK, Larauche M, Duboc H, Earle KA, Sonnenburg ED, Ferreyra JA, Higginbottom SK, Million M, Tache Y, Pasricha PJ, Knight R, Farrugia G, Sonnenburg JL. Complex interactions among diet, gastrointestinal transit, and gut microbiota in humanized mice. *Gastroenterology* 2013; **144**: 967-977 [PMID: 23380084 DOI: 10.1053/j.gastro.2013.01.047]

89 **Wu RY**, Pasyk M, Wang B, Forsythe P, Bienenstock J, Mao YK, Sharma P, Stanisz AM, Kunze WA. Spatiotemporal maps reveal regional differences in the effects on gut motility for Lactobacillus reuteri and rhamnosus strains. *Neurogastroenterol Motil* 2013; **25**: e205-e214 [PMID: 23316914 DOI: 10.1111/nmo.12072]

90 **Carvalho FA**, Aitken JD, Vijay-Kumar M, Gewirtz AT. Toll-like receptor-gut microbiota interactions: perturb at your own risk! *Annu Rev Physiol* 2012; **74**: 177-198 [PMID: 22035346 DOI: 10.1146/annurev-physiol-020911-153330]

91 **Pimentel M**, Lin HC, Enayati P, van den Burg B, Lee HR, Chen JH, Park S, Kong Y, Conklin J. Methane, a gas produced by enteric bacteria, slows intestinal transit and augments small intestinal contractile activity. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G1089-G1095 [PMID: 16293652 DOI: 10.1152/ajpgi.00574.2004]

92 **Dhaese I**, Van Colen I, Lefebvre RA. Mechanisms of action of hydrogen sulfide in relaxation of mouse distal colonic smooth muscle. *Eur J Pharmacol* 2010; **628**: 179-186 [PMID: 19919833 DOI: 10.1016/j.ejphar.2009.11.024]

93 **Takaki M**, Mawe GM, Barasch JM, Gershon MD, Gershon MD. Physiological responses of guinea-pig myenteric neurons secondary to the release of endogenous serotonin by tryptamine. *Neuroscience* 1985; **16**: 223-240 [PMID: 2940472]

94 **Sayin SI**, Wahlström A, Felin J, Jäntti S, Marschall HU, Bamberg K, Angelin B, Hyötyläinen T, Orešič M, Bäckhed F. Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. *Cell Metab* 2013; **17**: 225-235 [PMID: 23395169 DOI: 10.1016/j.cmet.2013.01.003]

95 **Yurdaydin C**, Walsh TJ, Engler HD, Ha JH, Li Y, Jones EA, Basile AS. Gut bacteria provide precursors of benzodiazepine receptor ligands in a rat model of hepatic encephalopathy. *Brain Res* 1995; **679**: 42-48 [PMID: 7648264]

96 **Walker AW**, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X, Brown D, Stares MD, Scott P, Bergerat A, Louis P, McIntosh F, Johnstone AM, Lobley GE, Parkhill J, Flint HJ. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *ISME J* 2011; **5**: 220-230 [PMID: 20686513 DOI: 10.1038/ismej.2010.118]

97 **Pimentel M**, Chow EJ, Lin HC. Normalization of lactulose breath testing correlates with symptom improvement in irritable bowel syndrome. a double-blind, randomized, placebo-controlled study. *Am J Gastroenterol* 2003; **98**: 412-419 [PMID: 12591062 DOI: 10.1111/j.1572-0241.2003.07234.x]

98 **Pimentel M**, Lembo A, Chey WD, Zakko S, Ringel Y, Yu J, Mareya SM, Shaw AL, Bortey E, Forbes WP. Rifaximin therapy for patients with irritable bowel syndrome without constipation. *N Engl J Med* 2011; **364**: 22-32 [PMID: 21208106 DOI: 10.1056/Nejmoa1004409]

99 **Tack J**. Antibiotic therapy for the irritable bowel syndrome. *N Engl J Med* 2011; **364**: 81-82 [PMID: 21208112 DOI: 10.1056/NEJMe1011211]

100 **Gopal PK**, Prasad J, Smart J, Gill HS. In vitro adherence properties of Lactobacillus rhamnosus DR20 and Bifidobacterium lactis DR10 strains and their antagonistic activity against an enterotoxigenic Escherichia coli. *Int J Food Microbiol* 2001; **67**: 207-216 [PMID: 11518430]

101 **Fujiwara S**, Hashiba H, Hirota T, Forstner JF. Purification and characterization of a novel protein produced by Bifidobacterium longum SBT2928 that inhibits the binding of enterotoxigenic Escherichia coli Pb176 (CFA/II) to ganglio-tetraosylceramide. *J Appl Microbiol* 1999; **86**: 615-621 [PMID: 10212407]

102 **Boudeau J**, Glasser AL, Julien S, Colombel JF, Darfeuille-Michaud A. Inhibitory effect of probiotic Escherichia coli strain Nissle 1917 on adhesion to and invasion of intestinal epithelial cells by adherent-invasive E. coli strains isolated from patients with Crohn's disease. *Aliment Pharmacol Ther* 2003; **18**: 45-56 [PMID: 12848625]

103 **Madsen K**, Cornish A, Soper P, McKaigney C, Jijon H, Yachimec C, Doyle J, Jewell L, De Simone C. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterology* 2001; **121**: 580-591 [PMID: 11522742]

104 **Mangell P**, Nejdfors P, Wang M, Ahrné S, Weström B, Thorlacius H, Jeppsson B. Lactobacillus plantarum 299v inhibits Escherichia coli-induced intestinal permeability. *Dig Dis Sci* 2002; **47**: 511-516 [PMID: 11911334]

105 **Eutamene H**, Lamine F, Chabo C, Theodorou V, Rochat F, Bergonzelli GE, Corthésy-Theulaz I, Fioramonti J, Bueno L. Synergy between Lactobacillus paracasei and its bacterial products to counteract stress-induced gut permeability and sensitivity increase in rats. *J Nutr* 2007; **137**: 1901-1907 [PMID: 17634262]

106 **Zareie M**, Johnson-Henry K, Jury J, Yang PC, Ngan BY, McKay DM, Soderholm JD, Perdue MH, Sherman PM. Probiotics prevent bacterial translocation and improve intestinal barrier function in rats following chronic psychological stress. *Gut* 2006; **55**: 1553-1560 [PMID: 16638791 DOI: 10.1136/gut.2005.080739]

107 **Ait-Belgnaoui A**, Han W, Lamine F, Eutamene H, Fioramonti J, Bueno L, Theodorou V. Lactobacillus farciminis treatment suppresses stress induced visceral hypersensitivity: a possible action through interaction with epithelial cell cytoskeleton contraction. *Gut* 2006; **55**: 1090-1094 [PMID: 16507583 DOI: 10.1136/gut.2005.084194]

108 **Johansson ML**, Nobaek S, Berggren A, Nyman M, Björck I, Ahrné S, Jeppsson B, Molin G. Survival of Lactobacillus plantarum DSM 9843 (299v), and effect on the short-chain fatty acid content of faeces after ingestion of a rose-hip drink with fermented oats. *Int J Food Microbiol* 1998; **42**: 29-38 [PMID: 9706795]

109 **McCarthy J**, O'Mahony L, O'Callaghan L, Sheil B, Vaughan EE, Fitzsimons N, Fitzgibbon J, O'Sullivan GC, Kiely B, Collins JK, Shanahan F. Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance. *Gut* 2003; **52**: 975-980 [PMID: 12801954]

110 **Rachmilewitz D**, Katakura K, Karmeli F, Hayashi T, Reinus C, Rudensky B, Akira S, Takeda K, Lee J, Takabayashi K, Raz E. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology* 2004; **126**: 520-528 [PMID: 14762789]

111 **Whorwell PJ**, Altringer L, Morel J, Bond Y, Charbonneau D, O'Mahony L, Kiely B, Shanahan F, Quigley EM. Efficacy of an encapsulated probiotic Bifidobacterium infantis 35624 in women with irritable bowel syndrome. *Am J Gastroenterol* 2006; **101**: 1581-1590 [PMID: 16863564 DOI: 10.1111/j.1572-0241.2006.00734.x]

112 **Guglielmetti S**, Mora D, Gschwender M, Popp K. Randomised clinical trial: Bifidobacterium bifidum MIMBb75 significantly alleviates irritable bowel syndrome and improves quality of life--a double-blind, placebo-controlled study. *Aliment Pharmacol Ther* 2011; **33**: 1123-1132 [PMID: 21418261 DOI: 10.1111/j.1365-2036.2011.04633.x]

113 **Enck P**, Zimmermann K, Menke G, Müller-Lissner S, Martens U, Klosterhalfen S. A mixture of Escherichia coli (DSM 17252) and Enterococcus faecalis (DSM 16440) for treatment of the irritable bowel syndrome--a randomized controlled trial with primary care physicians. *Neurogastroenterol*

Motil 2008; **20**: 1103-1109 [PMID: 18565142 DOI: 10.1111/j.1365-2982.2008.01156.x]
114 **Kajander K**, Hatakka K, Poussa T, Färkkilä M, Korpela R. A probiotic mixture alleviates symptoms in irritable bowel syndrome patients: a controlled 6-month intervention. *Aliment Pharmacol Ther* 2005; **22**: 387-394 [PMID: 16128676 DOI: 10.1111/j.1365-2036.2005.02579.x]
115 **Kajander K**, Krogius-Kurikka L, Rinttilä T, Karjalainen H, Palva A, Korpela R. Effects of multispecies probiotic supplementation on intestinal microbiota in irritable bowel syndrome. *Aliment Pharmacol Ther* 2007; **26**: 463-473 [PMID: 17635381 DOI: 10.1111/j.1365-2036.2007.03391.x]
116 **Drouault-Holowacz S**, Bieuvelet S, Burckel A, Cazaubiel M, Dray X, Marteau P. A double blind randomized controlled trial of a probiotic combination in 100 patients with irritable bowel syndrome. *Gastroenterol Clin Biol* 2008; **32**: 147-152 [PMID: 18387426 DOI: 10.1016/j.gcb.2007.06.001]
117 **Søndergaard B**, Olsson J, Ohlson K, Svensson U, Bytzer P, Ekesbo R. Effects of probiotic fermented milk on symptoms and intestinal flora in patients with irritable bowel syndrome: a randomized, placebo-controlled trial. *Scand J Gastroenterol* 2011; **46**: 663-672 [PMID: 21443416 DOI: 10.3109/00365521.2011.565066]
118 **Simrén M**, Ohman L, Olsson J, Svensson U, Ohlson K, Posserud I, Strid H. Clinical trial: the effects of a fermented milk containing three probiotic bacteria in patients with irritable bowel syndrome - a randomized, double-blind, controlled study. *Aliment Pharmacol Ther* 2010; **31**: 218-227 [PMID: 19863495 DOI: 10.1111/j.1365-2036.2009.04183.x]
119 **Ligaarden SC**, Axelsson L, Naterstad K, Lydersen S, Farup PG. A candidate probiotic with unfavourable effects in subjects with irritable bowel syndrome: a randomised controlled trial. *BMC Gastroenterol* 2010; **10**: 16 [PMID: 20144246 DOI: 10.1186/1471-230X-10-16]
120 **Yoon JS**, Sohn W, Lee OY, Lee SP, Lee KN, Jun DW, Lee HL, Yoon BC, Choi HS, Chung WS, Seo JG. Effect of multispecies probiotics on irritable bowel syndrome: a randomized, double-blind, placebo-controlled trial. *J Gastroenterol Hepatol* 2014; **29**: 52-59 [PMID: 23829297 DOI: 10.1111/jgh.12322]
121 **Ng SC**, Lam EF, Lam TT, Chan Y, Law W, Tse PC, Kamm MA, Sung JJ, Chan FK, Wu JC. Effect of probiotic bacteria on the intestinal microbiota in irritable bowel syndrome. *J Gastroenterol Hepatol* 2013; **28**: 1624-1631 [PMID: 23800182 DOI: 10.1111/jgh.12306]
122 **Brenner DM**, Moeller MJ, Chey WD, Schoenfeld PS. The utility of probiotics in the treatment of irritable bowel syndrome: a systematic review. *Am J Gastroenterol* 2009; **104**: 1033-149; quiz 1050 [PMID: 19277023 DOI: 10.1038/ajg.2009.25]
123 **Hoveyda N**, Heneghan C, Mahtani KR, Perera R, Roberts N, Glasziou P. A systematic review and meta-analysis: probiotics in the treatment of irritable bowel syndrome. *BMC Gastroenterol* 2009; **9**: 15 [PMID: 19220890 DOI: 10.1186/1471-230X-9-15]
124 **McFarland LV**, Dublin S. Meta-analysis of probiotics for the treatment of irritable bowel syndrome. *World J Gastroenterol* 2008; **14**: 2650-2661 [PMID: 18461650]
125 **Moayyedi P**, Ford AC, Talley NJ, Cremonini F, Foxx-Orenstein AE, Brandt LJ, Quigley EM. The efficacy of probiotics in the treatment of irritable bowel syndrome: a systematic review. *Gut* 2010; **59**: 325-332 [PMID: 19091823 DOI: 10.1136/gut.2008.167270]

P- Reviewers: Camara NOS, Jin B, Pehl C, Riccardi C
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8898

World J Gastroenterol 2014 July 21; 20(27): 8898-8909
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

Constipation-predominant irritable bowel syndrome: A review of current and emerging drug therapies

Khaled A Jadallah, Susan M Kullab, David S Sanders

Khaled A Jadallah, Susan M Kullab, Department of Internal Medicine, King Abdullah University Hospital, Faculty of Medicine, Jordan University of Science and Technology, Irbid 22110, Jordan
David S Sanders, Department of Gastroenterology and Liver Unit, Royal Hallamshire Hospital, Sheffield, South Yorkshire S102JF, United Kingdom
Author contributions: Jadallah KA and Sanders DS conceived and designed the study; Jadallah KA and Kullab SM performed the literature search and wrote the manuscript; all authors revised and approved the final version.
Correspondence to: Khaled A Jadallah, MD, Assistant Professor of Medicine, Department of Internal Medicine, King Abdullah University Hospital, Faculty of Medicine, Jordan University of Science and Technology, Irbid 22110, Jordan. khaled-j@just.edu.jo
Telephone: +962-79-6673884 Fax: +962-2-7200624
Received: January 15, 2014 Revised: March 23, 2014
Accepted: June 2, 2014
Published online: July 21, 2014

Abstract

Irritable bowel syndrome (IBS) is a highly prevalent medical condition that adversely affects patient quality of life and constitutes a significant economic burden on healthcare resources. A large proportion of patients suffer from the constipation subtype of IBS (IBS-C), most commonly afflicting older individuals and those with a lower socioeconomic status. Conventional pharmacologic and nonpharmacologic treatment options have limited efficacies and/or significant adverse events, which lead to increased long-term health care expenditures. Failure to effectively treat IBS-C patients over the past decades has largely been due to a poor understanding of disease pathophysiology, lack of a global view of the patient, and an inappropriate selection of patients and treatment endpoints in clinical trials. In recent years, however, more effective and safer drugs have been developed for the treatment of IBS-C. The advancement in the area of pharmacologic treatment is based on new knowledge of the pathophysiologic basis of IBS-C and the development of drugs with increased selectivity within pharmacologic classes with recognized efficacies. This narrative review covers the spectrum of available drugs and their mechanisms of action, as well as the efficacy and safety profiles of each as determined in relevant clinical trials that have investigated treatment options for IBS-C and chronic constipation. A brief summary of laxative-based treatment options is presented, followed by up-to-date assessments for three classes of drugs: prokinetics, prosecretory agents, and bile acid modulators.

Key words: Constipation; Irritable bowel syndrome; Drug therapy; Serotonergic agents; Prokinetics; 5-hydroxytryptamine type 4 agonists; Secretagogues; Prosecretory agents; Bile acid modulators

Core tip: Constipation-predominant irritable bowel syndrome (IBS-C) is one of the most common disorders seen by gastroenterologists worldwide, and is associated with a substantial burden on health care resources. Pharmacologic treatments for IBS-C have largely been unsatisfactory, mainly due to the multifaceted and poorly understood pathophysiology of this disorder. Recently approved drugs and novel investigational compounds are expected to streamline the management of IBS-C. This narrative review covers the mechanisms, clinical trial efficacies, and safety profiles of these pharmacologic agents, in order to help practicing physicians keep up with the rapidly developing field of IBS-C therapy.

Jadallah KA, Kullab SM, Sanders DS. Constipation-predominant irritable bowel syndrome: A review of current and emerging drug therapies. *World J Gastroenterol* 2014; 20(27): 8898-8909 Avail-

able from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8898.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8898

INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal (GI) disorders across all ages and ethnicities, with a worldwide prevalence ranging between 5% and 20%[1-4]. The majority of individuals with IBS experience impairments to their performance of daily activities and decreased health-related quality of life, for which conventional treatments provide limited resolutions[5,6]. For some IBS sufferers, substantial psychologic and psychiatric disturbances develop over time, leading to polypharmacy accompanied by the inherent risk of drug interactions, further deterioration of health status, and increased health care expenditures[6,7].

The constipation-predominant subtype of IBS (IBS-C), defined by constipation associated with abdominal pain that is generally relieved by defecation[8], affects about 34% of the IBS population[9], of which a substantial fraction are of older age and lower socioeconomic status[3]. Recent evidence suggests that IBS-C is associated with higher rates of functional impairment, as compared to other subtypes of IBS[10-12]. Conventional laxative-based pharmacologic treatment of IBS-C, which is mostly symptom-based, is largely unsatisfactory[13,14]. Yet, despite the substantial burden of IBS-C-associated ailments and the well-recognized need for more efficacious and safer treatments, few novel treatment compounds have been approved for clinical use. The need for a drug therapy that effectively treats all of the symptoms of IBS-C (abdominal pain, constipation, and secondary symptoms of constipation), improves the patient's health-related quality of life, and can be used safely on a chronic basis remains unfulfilled.

Advancement in the treatment of IBS-C requires a greater focus on the pathophysiologic abnormalities underlying each of the symptoms of this complex disorder[15], which is the scientific basis for the development of new pharmaceutical compounds. The present article reviews the current pharmacologic agents for the treatment of IBS-C, in terms of their clinical trial efficacy, tolerability, and safety. A brief description of the broad spectrum of laxative-based treatment options is also presented. In general, this review focuses on the main classes of drugs that have been the subject of active research in recent years (prokinetics, prosecretory agents or secretagogues, and bile acid modulators). Furthermore, in addition to the well-established drugs (tegaserod and lubiprostone), newly-approved drugs (prucalopride, velusetrag, linaclotide, plecanatide, chenodeoxycholate (CDC) and elobixibat) as well as drugs currently in development for the treatment of IBS-C are discussed. As there is significant overlap between IBS-C and chronic constipation (CC)[16], drugs that are currently approved or being investigated for the treatment of CC are also included in this review, according to their potential for use in the management of IBS-C; for instance, lubiprostone, which was initially developed and approved for CC, has subsequently received approval for the treatment of IBS-C. Nonpharmacologic remedies, such as fiber supplements and probiotics, however, are not discussed.

Studies included in this review were collected from a PubMed search for English-language articles published between 1980 and December 2013 using the following keywords alone or in combination: irritable bowel syndrome, constipation, constipation-predominant irritable bowel syndrome, drug therapy, laxatives, prokinetics, serotonergic agents, 5-HT$_4$ agonists, secretagogues, prosecretory agents, bile acid modulators, randomized controlled trials (RCTs), meta-analysis. Governmental websites [www.clinicaltrials.gov (United States), www.clinicaltrialsregister.eu (European Union)] were searched for data concerning ongoing clinical trials. Only high quality studies were cited and discussed in the present review.

LAXATIVE-BASED PHRARMACOLOGIC AGENTS

Conventional laxatives and stool softeners have been used for decades for the treatment of CC, and have also been used by IBS-C patients to improve their bowel habits[13,14]. Clinical experience and, to a lesser extent, evidence from the literature indicate that about half of the patients treated with laxatives are disappointed by the lack of long-term efficacy[17-19]. Despite the high prevalence and the remarkable socioeconomic burden associated with IBS-C and CC, concrete evidence from high-quality RCTs on laxative efficacy and safety is very limited[20]. In fact, only recently have well-conducted studies provided evidence for the use of bisacodyl in CC and polyethylene glycol in IBS-C[21,22].

Although laxative-based treatments provide short-term relief of constipation in many CC and IBS-C patients, there is a lack of high quality evidence to support their regular use. However, laxatives remain a suitable therapeutic option for many patients because of their relative safety, low cost, and over-the-counter availability. Well-conducted RCTs comparing the most commonly used laxatives and newer pharmacologic agents will help to identify the safest and most effective therapy for regular use. The mechanisms and most common adverse events of different types of laxatives are summarized in Table 1.

PROKINETICS

Slow colonic transit is recognized as one of the most important mechanisms underlying constipation. Prokinetics have been designed to stimulate muscle activity to counter the underlying hypomotility that is linked with slow-transit constipation[23,24]. A crucial role for 5-hydroxytryptamine (5-HT, serotonin) in normal enteric nervous system function has been documented[25-27], and the ex-

Table 1 Main types of pharmacologic laxatives

Type	Agents	Mechanism of action	Most common adverse events
Bulking agents	Psyllium Methylcellulose Calcium polycarbophil	Increase in stool bulk and reduction in consistency by luminal water binding	Bloating Flatulence
Stool softeners (surfactants)	Docusate potassium Docusate sodium Docusate calcium	Softening and lubrication of stools by increasing water secretion	Nausea Vomiting Abdominal pain/cramps Rectal urgency
Osmotic laxatives	Milk of Magnesia (magnesium hydroxide) Magnesium citrate Magnesium sulphate Sodium picosulphate/magnesium citrate (Picoprep®) Lactulose/lactilol Sorbitol Polyethylene glycol (macrogol)	Osmotic water retention, decreased stool consistency, and increase fecal volume and peristalsis	Sweet taste Nausea Bloating Flatulence Abdominal pain/cramps Electrolyte disturbances (?)
Stimulant laxatives	Anthraquinones Senna Cascara Bisacodyl Phenolphthalein	Luminal water retention through activation of CAMP, and induction of colonic contractions by acting on enteric nerves	Abdominal pain/cramps Dehydration Electrolyte disturbances Muscle cramps Melanosis coli/colonic inertia (?)

CAMP: Cyclic adenosine monophosphate.

Table 2 Chemical and clinical characteristics of discontinued/failed prokinetics

	Cisapride	Renzapride	Tegaserod
Chemical structure	Piperidinyl benzamide	Benzamide derivative	Indole carboxaldehyde derivative
Target receptors	Nonselective 5-HT$_4$ agonist and 5-HT$_3$ antagonist	Full 5-HT$_4$ agonist and antagonist of 5-HT$_3$ and 5-HT$_{2b}$	5-HT$_4$ and 5-HT$_1$ partial agonist
Mechanism of action/ pharmacodynamic effects	Local acetylcholine release; Acceleration of GI transit	Local acetylcholine release; Acceleration of GI transit	Augmentation of the peristaltic reflex; Enhanced intestinal secretion; Reduced sensitivity to rectal distension
Most common adverse events	Diarrhea Abdominal pain	Diarrhea Abdominal pain Headache Flatulence	Diarrhea Abdominal pain Headache Flatulence
Safety	Prolongation of QTc interval and fatal arrhythmias	No prolongation of QTc interval	Increased risk of serious ischemic cardiac events
Approval status	Approved in 1993; Withdrawn in 2000	Phase 3 RCTs terminated due to insufficient efficacy	Approved in 2002 for IBS-C (not in EU) and in 2004 for CC; Withdrawn in 2007

CC: Chronic constipation; EU: European Union; GI: Gastrointestinal; IBS-C: Constipation predominant-irritable bowel syndrome; QTc: Corrected QT interval; RCT: Randomized controlled trial; 5-HT: 5-hydroxytryptamine.

pression of the 5-HT type 4 (5-HT$_4$) receptor in the GI tract has been associated with intestinal motility[23,28]. In the past two decades, several prokinetic agonists of the 5-HT$_4$ receptor have been introduced in clinical practice. Table 2 presents the chemical and clinical characteristics of the older prokinetics, whereas Table 3 summarizes the characteristics of the newer prokinetics.

Cisapride

Cisapride, a non-selective 5-HT$_4$ agonist, was originally developed for the treatment of functional upper GI disorders, and later found to be efficacious for treating constipation[29]. However, its interaction with human ether-a-go-go-related gene (hERG) potassium channels leads to cardiac arrhythmias, which caused the drug to be withdrawn from the global market[29]. This "rise and fall" of cisapride underscores the importance of longitudinal safety studies for newer drugs, as well as the need for post-market monitoring.

Tegaserod

Tegaserod, a partial 5-HT$_4$ agonist devoid of the arrhythmogenic effect elicited by cisapride, was demonstrated in RCTs to be an efficacious and well-tolerated promotility agent in IBS-C patients[30,31]. The drug received approval for the treatment of women with IBS-C in July 2002 in the United States and a few other countries, but not in the European Union. In August 2004, the United States's Food and Drug Administration (FDA) also approved tegaserod for the treatment of patients with CC, and a subsequent multinational high-quality randomized controlled trial demonstrated its efficacy and tolerability in these patients[32]. Nevertheless, due to ensuing reports of ischemic cardiac events, tegaserod was withdrawn from the

Table 3 Chemical and clinical characteristics of novel prokinetic agents

	Prucalopride	Naronapride	Velusetrag	ROSE-010
Chemical structure	Dihydrobenzofuran carboxamide	Benzamide	Dihydroxyquinoline-carboxamide	Glucagon-related peptide
Target receptor/affinity	High selectivity and affinity for 5-HT$_4$ (> 150-fold)	5-HT$_4$ full agonist in the GI tract; partial agonist in the heart	Potent selective agonist of 5-HT$_4$ with high affinity (500-fold)	GLP-1 analogue
Pharmacodynamic effects	Accelerated colonic transit in health and CC	Accelerated colonic transit in health	Dose-dependent acceleration of colonic transit in health	Acceleration of colonic transit; antinociceptive effect in IBS-C
Most common adverse events	Diarrhea Nausea Headache Abdominal pain	Diarrhea Headache	Diarrhea Nausea Headache Vomiting	Nausea Headache
Approval status/stage of development	Approved for CC in EU in 2009 and in Canada in 2011	Phase 2 RCTs in CC completed	Phase 2 RCTs in CC completed	Phase 2 RCTs in IBS-C completed

CC: Chronic constipation; EU: European Union; GI: Gastrointestinal; GLP-1: Glucagon like peptide-1; IBS-C: Constipation-predominant irritable bowel syndrome; RCT: Randomized controlled trial; 5HT: 5-hydroxytryptamine.

market in March 2007, and since 2009, its use has been limited to emergency situations[33]. Although tegaserod was eventually removed from the worldwide market, it is still considered to represent an important step in the development of novel serotonergic drugs for the management of IBS-C and CC.

Prucalopride

In recent years, three highly selective 5-HT$_4$ agonists, namely prucalopride, velusetrag, and naronapride, have been investigated mainly for the treatment of CC (Table 2). In contrast to nonselective 5-HT$_4$ agonists, these pharmacologic compounds have not been associated with adverse cardiovascular events[34]. Large, multicenter RCTs have shown that prucalopride, the most extensively investigated drug of this class, is efficacious and safe for treating patients with CC[34-36]. In October 2009 the European Medicines Agency (EMA) approved prucalopride (Resolor®, 2 mg once daily) for the treatment of CC in women for whom laxative-based approaches failed to grant adequate relief[36]. In November 2011 the drug received approval in Canada (Resotran®, 1 or 2 mg once daily) for the same indication; although, to date, the drug remains unapproved by the United States FDA.

Recently, a large phase 3 RCT conducted in 46 sites from five countries of the Asia-Pacific region evaluated the efficacy and safety of a 12-wk treatment with daily prucalopride (2 mg) in CC patients[37]. In that study, significantly more patients responded to prucalopride than placebo (33.3% *vs* 10.3%), with responding patients having a weekly average of $\geqslant$ 3 spontaneous complete bowel movements (SCBMs). The most frequently reported adverse events were diarrhea, nausea, abdominal pain, and headache, all of which mainly occurred during the first and second day of drug administration. Thus, the authors concluded that daily 2 mg prucalopride was effective and well tolerated, with a favorable safety profile. Although no studies have yet addressed the efficacy of prucalopride in IBS-C, it is expected that it will also be efficacious for the disease symptoms, even though worsening of abdominal pain would limit its use in clinical practice.

Velusetrag

The second highly selective 5-HT$_4$ agonist, velusetrag (TD5108), has demonstrated stimulatory effects on colonic motility and transit in a phase 1 RCT[38]. In that trial, 60 healthy volunteers received one of four doses of velusetrag (5, 15, 30 or 50 mg) as a single dose or once daily for six days. A significant increase in the colonic transit and bowel emptying time of the descending colon was observed in participants receiving the single dose, and accelerated gastric emptying occurred in participants receiving multiple doses, with no serious adverse events. A four-week phase 2 RCT in 401 patients evaluated the efficacy, safety and tolerability of different velusetrag doses (15, 30 or 50 mg/d) in CC patients[39]. Patients treated in that study showed significant improvement in SCBMs, stool consistency, and time to achieve the first bowel movement, with adverse events, such as diarrhea, headache, nausea and vomiting, mostly occurring in the first two days of treatment. The adverse events-related discontinuation rate was 5%, and no manifestations of cardiac toxicity were noted. The results of these RCTs indicate that velusetrag is a safe drug and efficacious for the treatment of CC, though larger and longer phase 3 trials are required before robust conclusions are drawn. Furthermore, treatment of IBS-C patients with velusetrag has yet to be evaluated.

Naronapride

A third drug, naronapride (ATI-7505), is a full agonist of 5-HT$_4$ receptors in the GI tract and partial agonist of these receptors in the heart. It is structurally similar to cisapride, but without affinity for 5-HT$_3$ receptors and negligible hERG potassium channel activity[40,41]. The drug is currently being investigated for the treatment of upper and lower GI functional disorders, but only limited

Table 4 Chemical and clinical characteristics of prosecretory agents

Drug	Lubiprostone	Linaclotide	Plecanatide
Chemical structure	A prostone, bicyclic fatty acid (metabolite of prostaglandin E1)	14-amino acid peptide, analogue of guanylin	Analogue of uroguanylin
Target receptor/ mechanism of action	Activation of ClC-2 by direct action on epithelial cells provoking intestinal fluid secretion, also mediated by CFTR	Binding to GC-C with stimulation of cGMP and CFTR-mediated secretion; desensitization of afferent pain fibers mediated by production of extracellular cGMP	GC-C receptor activation with CFTR-mediated secretion
Pharmacodynamic effects	Accelerated small bowel and colonic transit	Dose-related acceleration of colonic transit	Probable acceleration of colonic transit
Most common adverse events	Nausea Diarrhea Abdominal pain	Dose-dependent diarrhea	Dose-independent diarrhea Nausea
Potential other beneficial effects	Mucosal protection	Antineoplastic	-
Cost	AWP is $296 for one month supply	AWP is $255 for 30 capsules	-
Approval status/stage of development	United States FDA-approved for women with IBS-C and men and women with CC	United States FDA-approved for both IBS-C and CC EMA-approved for IBS-C only	Phase 2b RCT in CC completed; Phase 3 RCT in CC recruiting patients; Phase 2 RCT in IBS-C recruiting patients

AWP: Average wholesale price; CC: Chronic constipation; CFTR: Cystic fibrosis transmembrane conduction regulator; cGMP: Cyclic guanosine monophosphate; ClC-2: Chloride channel-2; EMA: European Medicines Agency; FDA: Food and Drug Administration; GC-C: Guanylate cyclase-C; IBS-C: Constipation-predominant irritable bowel syndrome; RCT: Randomized controlled trial.

data are available in the literature thus far.

Renzapride, clebopride, and mosapride

Renzapride, clebopride, and mosapride are nonselective 5-HT$_4$ agonists that are no longer considered for the treatment of patients with IBS-C or CC. Though they were shown to be safe from a cardiovascular standpoint[33], they did not show significant efficacy in IBS-C clinical trials and were therefore abandoned[42,43].

ROSE-010

ROSE-010 is an experimental glucagon-like peptide-1 (GLP-1) analogue that affects the motility of and nociception in the GI tract[44]. In one RCT investigating the effect on acute abdominal pain in IBS, ROSE-010 was favored over a placebo for patient-rated pain relief[45]. More recently, a phase 2 RCT investigating the effect of ROSE-010 on GI motor functions in women with IBS-C found that although gastric emptying was delayed, colonic transit was significantly accelerated after 48 h, providing relief of constipation in these patients[46]. Although these results are encouraging, phase 3 RCTs are needed to confirm the efficacy and safety of ROSE-010.

PROSECRETORY AGENTS (SECRETAGOGUES)

In the last decade, intestinal secretion has been the subject of active research for the development of treatments for CC and IBS-C. The chemical and clinical characteristics of prosecretory agents, drugs that augment intestinal secretion, thus acting as a stool lubricant and facilitating its evacuation, are summarized in Table 4.

Lubiprostone

Lubiprostone, a chloride channel activator, was the first secretagogue to be investigated and approved for treatment of CC and IBS-C. Chloride channels have been recognized as the major effectors of fluid transport and secretion in the intestinal lumen[47]. In particular, type-2 chloride channels (ClC-2) have been explored with regard to their role in CC and IBS-C[48,49]. Lubiprostone is a highly specific activator of ClC-2 channels that leads to increased intestinal secretion[50,51], an effect that requires the cystic fibrosis transmembrane conductance regulator (CFTR)[52]. A phase 2, 12-wk double-blind RCT demonstrated that lubiprostone [8, 16 and 24 μg, twice daily (BID)] reduced abdominal pain in IBS-C patients, though higher doses were associated with more adverse events, namely nausea and diarrhea[53]. Schey and Rao demonstrated that 8 μg lubiprostone BID offered the best risk-benefit ratio for IBS-C patients[54].

The positive results from the phase 2 studies led to two phase 3, multicenter RCTs involving 1171 IBS-C patients treated for three months with 8 μg lubiprostone BID[55]. The primary efficacy endpoint was the percentage of overall responders that were at least moderately relieved for all four weeks of the month or significantly relieved for at least two weeks of the month. Patient-rated symptoms were significantly improved with lubiprostone treatment, with no increase in adverse events compared to the placebo. As the lubiprostone regimen was effective, well tolerated and safe, the long-term (up to 52 wk) efficacy, safety, and tolerability was evaluated in an extension study including 522 of these same IBS-C patients[56]. The results of this extended trial confirmed the efficacy of lubiprostone, with a favorable safety and tolerability profile for up to 13 mo. However, the absence

of a placebo arm raises some questions about the statistical validity of the data gathered.

Lubiprostone was approved by the United States FDA in April 2006 for the treatment of CC in men and women, and in April 2008 for the treatment of IBS-C in women. The recommended dose is 24 μg BID for CC and 8 μg BID for IBS-C. A four-week phase 3 RCT evaluated the efficacy and safety of 24 μg lubiprostone BID in 237 patients with CC and demonstrated significant improvement in the number of SCBMs, stool consistency, straining effort, and global bowel satisfaction[57]. Thus, lubiprostone was considered to be the "ideal" drug for IBS-C, as it was shown to be effective on all symptoms of IBS-C, including abdominal pain. However, recent data has suggested that lubiprostone may not have an anti-nociceptive effect in IBS-C. In fact, Whitehead *et al*[58] demonstrated that lubiprostone has no effect on visceral sensory thresholds in 62 IBS-C patients who completed a barostat test of pain and urge sensory thresholds. The authors concluded that lubiprostone did not relieve abdominal pain directly, but that the reduction in clinical pain in patients appeared to be secondary to changes in stool consistency.

Linaclotide

Linaclotide, a minimally absorbed first-in-class peptide agonist of guanylate cyclase C (GC-C), was recently approved for the treatment of IBS-C and CC. GC-C mediates intestinal secretion in response to heat-stable enterotoxins, the major cause of *Escherichia coli*-induced secretory diarrhea[59]. Linaclotide binds to GC-C, which is richly present on the luminal surface of the intestinal enterocytes[60], and ultimately activates CFTR, resulting in the secretion of chloride and bicarbonate into the intestinal lumen. Consequently, intestinal fluid secretion is increased, stools are softened, and colonic transit may be accelerated. The effect of linaclotide on ascending colonic transit has been demonstrated in a phase 2 RCT involving 36 women with IBS-C[61]. Additionally, unlike lubiprostone, linaclotide has been also shown to reduce visceral nociception in laboratory rodents[62]. More recently, this visceral antihyperalgesic effect has been replicated in healthy mice and those with chronic visceral hypersensitivity[63]. The dual action of linaclotide on both constipation and abdominal pain in IBS-C is likely related to its approval by both the United States FDA and the EMA.

The efficacy and safety of linaclotide for the treatment of IBS-C patients have been demonstrated in four well-conducted RCTs[61,64-66]. In a 12-wk RCT study of 420 IBS-C patients, Johnston *et al*[64] found that various doses of linaclotide (75, 150, 300 and 600 μg, once daily) were effective in improving all symptoms of IBS-C. The only observed adverse event in that trial was a dose-dependent diarrhea, whereas other adverse events were comparable between the treatment and placebo groups. A phase 3, 26-wk RCT[65] was recently conducted with linaclotide (290 μg daily) in 804 IBS-C patients according to the recommended United States FDA primary endpoints (responder: a patient who reported (1) ⩾ 30% improvement in an average daily worst abdominal pain score; and (2) an increase of ⩾ 1 average weekly SCBMs for at least half of the trial duration)[67]. The results of that trial showed that 33.7% of treated patients were United States FDA endpoint responders, compared to only 13.9% of those receiving a placebo. Specifically, 48.9% of treated patients met the criterion for pain responder, and 47.6% met the SCBM responder criterion, compared to 34.5% and 22.6% respectively of placebo-treated patients. In terms of safety and tolerability, diarrhea was the most common adverse event, occurring most often within the first four weeks of therapy, while the discontinuation rates were 10.2% and 2.5% for linaclotide and placebo, respectively. Another phase 3 RCT included a 12-wk treatment period followed by a four-week randomized withdrawal period[66]. The outcome measures of that study were the United States FDA endpoints for IBS-C and three other endpoints based on improvement in abdominal pain and SCBMs. The results of this trial also indicated that linaclotide was safe and effective in relieving IBS-C symptoms, with diarrhea being the most common adverse event and no worsening of symptoms in the withdrawal period.

Linaclotide (145 μg, once daily) was also shown by four well-conducted RCTs to be safe and effective for the treatment of CC[68-70]. Moreover, the safety and efficacy of linaclotide for the treatment of patients with IBS-C and CC has been confirmed by a recent meta-analysis study[71]. In August 2012, linaclotide (Linzess®; Ironwood Pharmaceuticals, Inc., Cambridge, MA, United States) was approved by the United States FDA for the treatment of IBS-C at a dose of 290 μg once daily and CC at a dose of 145 μg once daily[72]. In the European Union, the drug received approval for IBS-C patients but not for CC patients. The approval of linaclotide represented an important development in the treatment of IBS-C and CC, especially for those patients with poor tolerance or response to lubiprostone.

In summary, there is evidence showing that linaclotide is an effective, well tolerated, and safe therapeutic option for patients with IBS-C and CC, though the long-term safety and efficacy of linaclotide as well as a direct comparison with lubiprostone need to be investigated. Importantly, this drug has the advantage of improving both bowel symptoms and abdominal pain. However, the high cost of linaclotide and lubiprostone may limit their use in clinical practice, especially because a large proportion of IBS-C and CC patients belong to lower socioeconomic groups.

Plecanatide

Similar to linaclotide, plecanatide is a minimally absorbed GC-C agonist believed to act on both intestinal secretion and nociception. A phase 1 RCT was conduced in 72 healthy volunteers to assess the safety, tolerability, pharmacokinetics, and pharmacodynamics of various doses

Table 5 Chemical and clinical characteristics of bile acid modulators

	Chenodeoxycholate	Elobixibat
Chemical structure	Sodium chenodeoxycholic acid (primary bile acid)	Enantiomer of 1,5-benzothiazepine
Mechanism of action	Deconjugation to secondary bile acids, thus inducing colonic secretion and propulsive contractions	IBAT inhibition resulting in delivery of endogenous bile acids to the colon, thus inducing colonic secretion and propulsive contractions
Pharmacodynamic effects	Accelerated colonic transit	Dose-dependent acceleration of colonic transit
Most common adverse events	Diarrhea Abdominal cramping/pain Nausea	Diarrhea Abdominal cramping/pain
Potential other beneficial effects	Probable lowering of LDL	Lowering of LDL and cholesterol
Stage of development	Phase 3 RCT in IBS-C completed	Phase 3 RCTs in CC, completed; extended safety and tolerability RCTs enrolling

CC: Chronic constipation; IBAT: Ileal bile acid transporter; IBS-C: Constipation-predominant irritable bowel syndrome; LDL: Low-density lipoprotein; RCT: Randomized controlled trial.

(ranging from 0.1 to 48.6 mg) of oral plecanatide[73]. The study found no measurable systemic absorption of plecanatide, with adverse events similar to the placebo; thus, it was concluded that the drug acts locally in the intestine and is well tolerated and safe. However, low statistical power prevented the authors from making any conclusions with respect to the pharmacodynamic parameters. Preliminary results from a phase 2a RCT that is underway in patients with CC have suggested that plecanatide is effective, well tolerated, and safe at doses up to 9 mg[74]. Moreover, plecanatide-treated patients showed significant improvement in bowel symptoms without any observed serious adverse events. Other phase 2 RCTs using plecanatide in CC and IBS-C patients are still recruiting patients, and no results have been reported thus far.

BILE ACID MODULATORS

Bile acid modulators have been used to treat constipation disorders based on the observation of increased incidence of diarrhea in patients taking bile acids for gallstones or cholestatic liver diseases[75], and in patients with terminal ileum disease or resection[76]. The enhancement of colonic secretion and motility is caused mainly by the deconjugation of bile acids in the colon to secondary bile acids[77,78]. Thus far, two drugs, CDC and elobixibat, have been investigated for the treatment of IBS-C and CC. Their chemical and clinical characteristics are shown in Table 5.

CDC

CDC is a primary biliary acid that has been in use for many years for the dissolution of gallstones. In clinical studies, the main adverse event of CDC (Chenodal®; Manchester Pharmaceuticals, Fort Collins, CO, United States) was a dose-dependent diarrhea[77] that is of the secretory type, due mainly to intracellular activation of adenylate cyclase and increased intestinal permeability[77,79,80]. In a four-week placebo-controlled RCT of 20 gallstone patients with CC, Bazzoli *et al*[81] found that CDC significant improved bowel frequency and stool consistency. In a recent four-day double-blind RCT of 36 women with IBS-C, CDC (500 or 1000 mg, once daily) increased stool frequency, softened stools and improved straining, with lower abdominal cramping as the most commonly reported adverse event[82]. The authors concluded that the effect in these female patients was dependent on specific genetic variations in the negative feedback inhibition of bile acid synthesis. Therefore, CDC has the potential to be used as a "physiologic laxative" for the treatment of both IBS-C and CC; although, its use in IBS-C may be limited by the concern for worsening of abdominal pain.

Elobixibat

Elobixibat (formerly A3309) is a first-in-class ileal bile acid transporter inhibitor that is currently being investigated for the treatment of CC. Elobixibat has some potential advantages over currently approved drugs (prucalopride, lubiprostone, linaclotide). First, given its negligible systemic absorption, it is unlikely to induce cardiovascular toxicity, a theoretical effect of prucalopride. Second, it has a positive effect on both secretion and motility of the colon, while lubiprostone and linaclotide are only secretagogues, without any direct effect on colonic motility[77,78].

In the first human study of the pharmacokinetic and pharmacodynamic actions of elobixibat, Simrén *et al*[83] assessed the safety and tolerability of the drug in 30 patients with CC. The efficacy and metabolic parameters of patients receiving one of five elobixibat doses (from 0.1 to 10 mg, once daily) were favorable, with no significant adverse events. Two phase 2 RCTs focusing on the efficacy of elobixibat in CC patients with doses ranging from 5 to 20 mg once daily demonstrated significant improvement of all constipation parameters[84,85]. Furthermore, safety and tolerability analyses showed no serious adverse events, with lower abdominal cramping being the most common. Based on the results of these studies, elobixibat appears to be a promising pharmacologic option for patients with CC. The efficacy of elobixibat for the treatment of IBS-C has not yet been investigated, though the abdominal pain that is commonly observed might limit

Table 6 Chemical and clinical characteristics of drugs approved for other gastrointestinal indications and currently investigated for constipation-predominant irritable bowel syndrome

	Itopride	Neomycin/Rifaximin
Brand name	Ganaton®	Neomycin: Neo-Fradin® Rifaximin: Xifaxan®
Chemical structure	Benzamide derivative	Neomycin: aminoglycoside Rifaximin: semisynthetic antibiotic based on rifampicin
Mechanism of action	Dopamine D2 antagonist and acetylcholinesterase inhibitor	Neomycin: inhibition of protein synthesis Rifaximin: inhibition of bacterial RNA synthesis
Pharmacodynamic effects	Gastrokinetic; Acceleration of intestinal transit (?)	Eradication of methane; accelerated intestinal transit (?)
Most common adverse events	Diarrhea Headache Hyperprolactinemia	Neomycin: Neurotoxicity Ototoxicity Nephrotoxicity Rifaximin: Headache Nausea Dizziness Fatigue
Approval status/stage of development	Approved in Japan for functional dyspepsia; Phase 2 RCT in IBS-C completed in the United States	FDA-approved for hepatic encephalopathy and traveler's diarrhea; Phase 2 efficacy RCT in methane + IBS-C patients, comparing neomycin *vs* combination rifaximin and neomycin (completed)

FDA: Food and Drug Administration; IBS-C: Constipation-predominant irritable bowel syndrome; RCT: Randomized controlled trial.

Table 7 Quality of evidence supporting different pharmacologic agents for constipation-predominant irritable bowel syndrome and chronic constipation

Pharmacologic agent	Quality of evidence for IBS-C	Quality of evidence for CC
Laxatives		
Psyllium	No RCTs	Moderate
Docusate sodium	No RCTs	Low
Lactulose	No RCTs	Moderate
PEG	Moderate	High
Senna	No RCTs	Low
Bisacodyl	No RCTs	Moderate
Prokinetics		
Prucalopride	No RCTs	High
Naronapride	No RCTs	Low
Velusetrag	Low	Low
Rose-010	Moderate	No RCTs
Secretagogues		
Lubiprostone	High	High
Linaclotide	High	High
Plecanatide	Low	Low
Bile acid modulators		
CDC	Low	Low
Elobixibat	No RCTs	Moderate

The quality of evidence was assessed according to the Grading of Recommendations, Assessment, Development and Evaluation (GRADE) system[86], which defines study quality as high (further research is very unlikely to change confidence in the estimated effect); moderate (further research is likely to have an important impact on confidence in the estimated effect and may change the estimate); low (further research is very likely to have an important impact on confidence in the estimated effect and is likely to change the estimate); or very low (any estimate of effect is very uncertain). CC: Chronic constipation; CDC: Chenodeoxycholate; IBS-C: Constipation-predominant irritable bowel syndrome; PEG: Polyethylene glycol; RCT: Randomized controlled trials.

its use in clinical practice.

OTHER INVESTIGATIONAL AGENTS

The search for safer and more effective drugs for the treatment of IBS-C is ongoing, with phase 1 and phase 2 clinical trials underway to evaluate various pharmacologic options, including drugs already approved for other gastrointestinal indications [Ganaton® (Abbott India Ltd., Mumbai, India), Neo-Fradin® (X-Gen Pharmaceuticals Inc., Horseheads, NY, United States), Xifaxan® (Salix Pharmaceuticals Inc., Raleigh, NC, United States)] (Table 6), as well as novel molecules (DA6886, AZD1722, RDX5791, TC6499). Thus far, no results from completed studies are available, and other studies are still recruiting patients.

PERSPECTIVES AND CONCLUSION

IBS-C has been, and probably will remain for some time, a troubling disease for many sufferers and an enormous challenge for the treating physician. The multifactorial pathogenesis of the disease and the ill-defined drug targets make the goal of manufacturing a "universal drug" for IBS-C a hard one to attain. In recent years, new drug therapies have been added to the armamentarium for the treatment of IBS-C. The current available evidence indicates that linaclotide is the "ideal" treatment option for IBS-C patients at this time, but other investigational agents are showing promise as well. However, large scale, high quality longitudinal studies of such agents and post-

market monitoring of approved drugs are needed to confirm the efficacy, tolerability and safety of these treatments. The quality of current evidence in support of different drug classes is summarized in Table 7. However, drug choice is dictated not only by the supporting evidence, but also by the patients' and societal perspectives.

Patient-relevant symptoms in conjunction with a better understanding of the pathophysiologic mechanisms underlying IBS-C should drive the development of novel pharmacologic agents for this complex disorder. Novel drug therapies are expected to streamline the management of IBS-C, thus increasing patient satisfaction and ultimately reducing the use of healthcare resources. This could indeed compensate for the high cost of these drugs, which is one of the major concerns for many patients and insurers. Finally, since IBS-C is a spectrum disorder resulting in a broad range of responses to different drug regimens, the treatment of most IBS-C patients should be individualized. It is anticipated that in the near future, a multitude of pharmacologic agents with divergent mechanisms of action will be effective for diverse subsets of IBS-C patients, and the reconciliation of past pharmacologic treatment successes and failures will ultimately improve future management of IBS-C.

REFERENCES

1 **Lovell RM**, Ford AC. Global prevalence of and risk factors for irritable bowel syndrome: a meta-analysis. *Clin Gastroenterol Hepatol* 2012; **10**: 712-721.e4 [PMID: 22426087 DOI: 10.1016/j.cgh.2012.02.029]

2 **Saito YA**, Schoenfeld P, Locke GR. The epidemiology of irritable bowel syndrome in North America: a systematic review. *Am J Gastroenterol* 2002; **97**: 1910-1915 [PMID: 12190153 DOI: 10.1111/j.1572-0241.2002.05913.x]

3 **Brandt LJ**, Chey WD, Foxx-Orenstein AE, Schiller LR, Schoenfeld PS, Spiegel BM, Talley NJ, Quigley EM. An evidence-based position statement on the management of irritable bowel syndrome. *Am J Gastroenterol* 2009; **104** Suppl 1: S1-S35 [PMID: 19521341 DOI: 10.1038/ajg.2008.122]

4 **Dong YY**, Zuo XL, Li CQ, Yu YB, Zhao QJ, Li YQ. Prevalence of irritable bowel syndrome in Chinese college and university students assessed using Rome III criteria. *World J Gastroenterol* 2010; **16**: 4221-4226 [PMID: 20806442 DOI: 10.3748/wjg.v16.i33.4221]

5 **Leong SA**, Barghout V, Birnbaum HG, Thibeault CE, Ben-Hamadi R, Frech F, Ofman JJ. The economic consequences of irritable bowel syndrome: a US employer perspective. *Arch Intern Med* 2003; **163**: 929-935 [PMID: 12719202 DOI: 10.1001/archinte.163.8.929]

6 **Reilly MC**, Barghout V, McBurney CR, Niecko TE. Effect of tegaserod on work and daily activity in irritable bowel syndrome with constipation. *Aliment Pharmacol Ther* 2005; **22**: 373-380 [PMID: 16128674 DOI: 10.1111/j.1365-2036.2005.02577.x]

7 **Spiegel BM**. The burden of IBS: looking at metrics. *Curr Gastroenterol Rep* 2009; **11**: 265-269 [PMID: 19615301 DOI: 10.1007/s11894-009-0039-x]

8 **Longstreth GF**, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology* 2006; **130**: 1480-1491 [PMID: 16678561]

9 **Hungin AP**, Whorwell PJ, Tack J, Mearin F. The prevalence, patterns and impact of irritable bowel syndrome: an international survey of 40,000 subjects. *Aliment Pharmacol Ther* 2003; **17**: 643-650 [PMID: 12641512 DOI: 10.1046/j.1365-2036.2003.01456.x]

10 **DiBonaventura M**, Sun SX, Bolge SC, Wagner JS, Mody R. Health-related quality of life, work productivity and health care resource use associated with constipation predominant irritable bowel syndrome. *Curr Med Res Opin* 2011; **27**: 2213-2222 [PMID: 21951105 DOI: 10.1185/03007995.2011.623157]

11 **Dibonaventura MD**, Prior M, Prieto P, Fortea J. Burden of constipation-predominant irritable bowel syndrome (IBS-C) in France, Italy, and the United Kingdom. *Clin Exp Gastroenterol* 2012; **5**: 203-212 [PMID: 23162373 DOI: 10.2147/CEG.S35568]

12 **Fortea J**, Prior M. Irritable bowel syndrome with constipation: a European-focused systematic literature review of disease burden. *J Med Econ* 2013; **16**: 329-341 [PMID: 23216014 DOI: 10.3111/13696998.2012.756397]

13 **Camilleri M**, Tack JF. Current medical treatments of dyspepsia and irritable bowel syndrome. *Gastroenterol Clin North Am* 2010; **39**: 481-493 [PMID: 20951913 DOI: 10.1016/j.gtc.2010.08.005]

14 **Hulisz D**. The burden of illness of irritable bowel syndrome: current challenges and hope for the future. *J Manag Care Pharm* 2004; **10**: 299-309 [PMID: 15298528]

15 **Camilleri M**, Andresen V. Current and novel therapeutic options for irritable bowel syndrome management. *Dig Liver Dis* 2009; **41**: 854-862 [PMID: 19665953 DOI: 10.1016/j.dld.2009.07.009]

16 **Wong RK**, Palsson OS, Turner MJ, Levy RL, Feld AD, von Korff M, Whitehead WE. Inability of the Rome III criteria to distinguish functional constipation from constipation-subtype irritable bowel syndrome. *Am J Gastroenterol* 2010; **105**: 2228-2234 [PMID: 20502449 DOI: 10.1038/ajg.2010.200]

17 **Johanson JF**, Kralstein J. Chronic constipation: a survey of the patient perspective. *Aliment Pharmacol Ther* 2007; **25**: 599-608 [PMID: 17305761 DOI: 10.1111/j.1365-2036.2006.03238.x]

18 **Wald A**, Scarpignato C, Mueller-Lissner S, Kamm MA, Hinkel U, Helfrich I, Schuijt C, Mandel KG. A multinational survey of prevalence and patterns of laxative use among adults with self-defined constipation. *Aliment Pharmacol Ther* 2008; **28**: 917-930 [PMID: 18644012 DOI: 10.1111/j.1365-2036.2008.03806.x]

19 **Wald A**, Mueller-Lissner S, Kamm MA, Hinkel U, Richter E, Schuijt C, Mandel KG. Survey of laxative use by adults with self-defined constipation in South America and Asia: a comparison of six countries. *Aliment Pharmacol Ther* 2010; **31**: 274-284 [PMID: 19832728 DOI: 10.1111/j.1365-2036.2009.04169.x]

20 **Ford AC**, Suares NC. Effect of laxatives and pharmacological therapies in chronic idiopathic constipation: systematic review and meta-analysis. *Gut* 2011; **60**: 209-218 [PMID: 21205879 DOI: 10.1136/gut.2010.227132]

21 **Kamm MA**, Mueller-Lissner S, Wald A, Richter E, Swallow R, Gessner U. Oral bisacodyl is effective and well-tolerated in patients with chronic constipation. *Clin Gastroenterol Hepatol* 2011; **9**: 577-583 [PMID: 21440672 DOI: 10.1016/j.cgh.2011.03.026]

22 **Awad RA**, Camacho S. A randomized, double-blind, placebo-controlled trial of polyethylene glycol effects on fasting and postprandial rectal sensitivity and symptoms in hypersensitive constipation-predominant irritable bowel syndrome. *Colorectal Dis* 2010; **12**: 1131-1138 [PMID: 19575740 DOI: 10.1111/j.1463-1318.2009.01990.x]

23 **Sanger GJ**, Alpers DH. Development of drugs for gastrointestinal motor disorders: translating science to clinical need. *Neurogastroenterol Motil* 2008; **20**: 177-184 [PMID: 18257767 DOI: 10.1111/j.1365-2982.2008.01084.x]

24 **Crowell MD**, Shetzline MA, Moses PL, Mawe GM, Talley NJ. Enterochromaffin cells and 5-HT signaling in the pathophysiology of disorders of gastrointestinal function. *Curr Opin Investig Drugs* 2004; **5**: 55-60 [PMID: 14983974]

25 **Kim DY**, Camilleri M. Serotonin: a mediator of the brain-gut connection. *Am J Gastroenterol* 2000; **95**: 2698-2709 [PMID: 11051338 DOI: 10.1111/j.1572-0241.2000.03177.x]

26 **Hoyer D**, Hannon JP, Martin GR. Molecular, pharmacological and functional diversity of 5-HT receptors. *Pharmacol Biochem Behav* 2002; **71**: 533-554 [PMID: 11888546]

27 **Read NW**, Gwee KA. The importance of 5-hydroxytryptamine receptors in the gut. *Pharmacol Ther* 1994; **62**: 159-173 [PMID: 7991641]

28 **Emmanuel AV**, Tack J, Quigley EM, Talley NJ. Pharmacological management of constipation. *Neurogastroenterol Motil* 2009; **21** Suppl 2: 41-54 [PMID: 19824937 DOI: 10.1111/j.1365-2982.2009.01403.x]

29 **Quigley EM**. Cisapride: what can we learn from the rise and fall of a prokinetic? *J Dig Dis* 2011; **12**: 147-156 [PMID: 21615867 DOI: 10.1111/j.1751-2980.2011.00491.x]

30 **Müller-Lissner SA**, Fumagalli I, Bardhan KD, Pace F, Pecher E, Nault B, Rüegg P. Tegaserod, a 5-HT(4) receptor partial agonist, relieves symptoms in irritable bowel syndrome patients with abdominal pain, bloating and constipation. *Aliment Pharmacol Ther* 2001; **15**: 1655-1666 [PMID: 11564007]

31 **Novick J**, Miner P, Krause R, Glebas K, Bliesath H, Ligozio G, Rüegg P, Lefkowitz M. A randomized, double-blind, placebo-controlled trial of tegaserod in female patients suffering from irritable bowel syndrome with constipation. *Aliment Pharmacol Ther* 2002; **16**: 1877-1888 [PMID: 12390096 DOI: 10.1046/j.1365-2036.2002.01372.x]

32 **Kamm MA**, Müller-Lissner S, Talley NJ, Tack J, Boeckxstaens G, Minushkin ON, Kalinin A, Dzieniszewski J, Haeck P, Fordham F, Hugot-Cournez S, Nault B. Tegaserod for the treatment of chronic constipation: a randomized, double-blind, placebo-controlled multinational study. *Am J Gastroenterol* 2005; **100**: 362-372 [PMID: 15667494 DOI: 10.1111/j.1572-0241.2005.40749.x]

33 **Tack J**, Camilleri M, Chang L, Chey WD, Galligan JJ, Lacy BE, Müller-Lissner S, Quigley EM, Schuurkes J, De Maeyer JH, Stanghellini V. Systematic review: cardiovascular safety profile of 5-HT(4) agonists developed for gastrointestinal disorders. *Aliment Pharmacol Ther* 2012; **35**: 745-767 [PMID: 22356640 DOI: 10.1111/j.1365-2036.2012.05011.x]

34 **Camilleri M**, Kerstens R, Rykx A, Vandeplassche L. A placebo-controlled trial of prucalopride for severe chronic constipation. *N Engl J Med* 2008; **358**: 2344-2354 [PMID: 18509121 DOI: 10.1056/NEJMoa0800670]

35 **Quigley EM**, Vandeplassche L, Kerstens R, Ausma J. Clinical trial: the efficacy, impact on quality of life, and safety and tolerability of prucalopride in severe chronic constipation--a 12-week, randomized, double-blind, placebo-controlled study. *Aliment Pharmacol Ther* 2009; **29**: 315-328 [PMID: 19035970 DOI: 10.1111/j.1365-2036.2008.03884.x]

36 **Tack J**, van Outryve M, Beyens G, Kerstens R, Vandeplassche L. Prucalopride (Resolor) in the treatment of severe chronic constipation in patients dissatisfied with laxatives. *Gut* 2009; **58**: 357-365 [PMID: 18987031 DOI: 10.1136/gut.2008.162404]

37 **Ke M**, Zou D, Yuan Y, Li Y, Lin L, Hao J, Hou X, Kim HJ. Prucalopride in the treatment of chronic constipation in patients from the Asia-Pacific region: a randomized, double-blind, placebo-controlled study. *Neurogastroenterol Motil* 2012; **24**: 999-e541 [PMID: 22882724 DOI: 10.1111/j.1365-2982.2012.01983.x]

38 **Manini ML**, Camilleri M, Goldberg M, Sweetser S, McKinzie S, Burton D, Wong S, Kitt MM, Li YP, Zinsmeister AR. Effects of Velusetrag (TD-5108) on gastrointestinal transit and bowel function in health and pharmacokinetics in health and constipation. *Neurogastroenterol Motil* 2010; **22**: 42-49, e7-8 [PMID: 19691492 DOI: 10.1111/j.1365-2982.2009.01378.x]

39 **Goldberg M**, Li YP, Johanson JF, Mangel AW, Kitt M, Beattie DT, Kersey K, Daniels O. Clinical trial: the efficacy and tolerability of velusetrag, a selective 5-HT4 agonist with high intrinsic activity, in chronic idiopathic constipation - a 4-week, randomized, double-blind, placebo-controlled, dose-response study. *Aliment Pharmacol Ther* 2010; **32**: 1102-1112 [PMID: 21039672 DOI: 10.1111/j.1365-2036.2010.04456.x]

40 **Camilleri M**, Vazquez-Roque MI, Burton D, Ford T, McKinzie S, Zinsmeister AR, Druzgala P. Pharmacodynamic effects of a novel prokinetic 5-HT receptor agonist, ATI-7505, in humans. *Neurogastroenterol Motil* 2007; **19**: 30-38 [PMID: 17187586]

41 **Bowersox SS**, Lightning LK, Rao S, Palme M, Ellis D, Coleman R, Davies AM, Kumaraswamy P, Druzgala P. Metabolism and pharmacokinetics of naronapride (ATI-7505), a serotonin 5-HT(4) receptor agonist for gastrointestinal motility disorders. *Drug Metab Dispos* 2011; **39**: 1170-1180 [PMID: 21447732 DOI: 10.1124/dmd.110.037564]

42 **Ford AC**, Brandt LJ, Young C, Chey WD, Foxx-Orenstein AE, Moayyedi P. Efficacy of 5-HT3 antagonists and 5-HT4 agonists in irritable bowel syndrome: systematic review and meta-analysis. *Am J Gastroenterol* 2009; **104**: 1831-1843; quiz 1844 [PMID: 19471254 DOI: 10.1038/ajg.2009.223]

43 **Lembo AJ**, Cremonini F, Meyers N, Hickling R. Clinical trial: renzapride treatment of women with irritable bowel syndrome and constipation - a double-blind, randomized, placebo-controlled, study. *Aliment Pharmacol Ther* 2010; **31**: 979-990 [PMID: 20163375 DOI: 10.1111/j.1365-2036.2010.04265.x]

44 **Hellström PM**, Näslund E, Edholm T, Schmidt PT, Kristensen J, Theodorsson E, Holst JJ, Efendic S. GLP-1 suppresses gastrointestinal motility and inhibits the migrating motor complex in healthy subjects and patients with irritable bowel syndrome. *Neurogastroenterol Motil* 2008; **20**: 649-659 [PMID: 18298441 DOI: 10.1111/j.1365-2982.2007.01079.x]

45 **Hellström PM**, Hein J, Bytzer P, Björnssön E, Kristensen J, Schambye H. Clinical trial: the glucagon-like peptide-1 analogue ROSE-010 for management of acute pain in patients with irritable bowel syndrome: a randomized, placebo-controlled, double-blind study. *Aliment Pharmacol Ther* 2009; **29**: 198-206 [PMID: 18945254 DOI: 10.1111/j.1365-2036.2008.03870.x]

46 **Camilleri M**, Vazquez-Roque M, Iturrino J, Boldingh A, Burton D, McKinzie S, Wong BS, Rao AS, Kenny E, Månsson M, Zinsmeister AR. Effect of a glucagon-like peptide 1 analog, ROSE-010, on GI motor functions in female patients with constipation-predominant irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* 2012; **303**: G120-G128 [PMID: 22517769 DOI: 10.1152/ajpgi.00076.2012]

47 **Suzuki M**, Morita T, Iwamoto T. Diversity of Cl(-) channels. *Cell Mol Life Sci* 2006; **63**: 12-24 [PMID: 16314923 DOI: 10.1007/s00018-005-5336-4]

48 **Lacy BE**, Chey WD. Lubiprostone: chronic constipation and irritable bowel syndrome with constipation. *Expert Opin Pharmacother* 2009; **10**: 143-152 [PMID: 19236188 DOI: 10.1517/14656560802631319]

49 **Lipecka J**, Bali M, Thomas A, Fanen P, Edelman A, Fritsch J. Distribution of ClC-2 chloride channel in rat and human epithelial tissues. *Am J Physiol Cell Physiol* 2002; **282**: C805-C816 [PMID: 11880269 DOI: 10.1152/ajpcell.00291.2001]

50 **Camilleri M**, Bharucha AE, Ueno R, Burton D, Thomforde GM, Baxter K, McKinzie S, Zinsmeister AR. Effect of a selective chloride channel activator, lubiprostone, on gastrointestinal transit, gastric sensory, and motor functions in healthy volunteers. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G942-G947 [PMID: 16603730]

51 **Ginzburg R**, Ambizas EM. Clinical pharmacology of lubiprostone, a chloride channel activator in defecation disorders. *Expert Opin Drug Metab Toxicol* 2008; **4**: 1091-1097 [PMID: 18680443 DOI: 10.1517/17425255.4.8.1091]

52 **Bijvelds MJ**, Bot AG, Escher JC, De Jonge HR. Activation of intestinal Cl- secretion by lubiprostone requires the cystic fibrosis transmembrane conductance regulator. *Gastroenterology* 2009; **137**: 976-985 [PMID: 19454284 DOI: 10.1053/

j.gastro.2009.05.037]
53 **Johanson JF**, Drossman DA, Panas R, Wahle A, Ueno R. Clinical trial: phase 2 study of lubiprostone for irritable bowel syndrome with constipation. *Aliment Pharmacol Ther* 2008; **27**: 685-696 [PMID: 18248656 DOI: 10.1111/j.1365-2036.2008.03629.x]
54 **Schey R**, Rao SS. Lubiprostone for the treatment of adults with constipation and irritable bowel syndrome. *Dig Dis Sci* 2011; **56**: 1619-1625 [PMID: 21523369 DOI: 10.1007/s10620-011-1702-2]
55 **Drossman DA**, Chey WD, Johanson JF, Fass R, Scott C, Panas R, Ueno R. Clinical trial: lubiprostone in patients with constipation-associated irritable bowel syndrome--results of two randomized, placebo-controlled studies. *Aliment Pharmacol Ther* 2009; **29**: 329-341 [PMID: 19006537 DOI: 10.1111/j.1365-2036.2008.03881.x]
56 **Chey WD**, Drossman DA, Johanson JF, Scott C, Panas RM, Ueno R. Safety and patient outcomes with lubiprostone for up to 52 weeks in patients with irritable bowel syndrome with constipation. *Aliment Pharmacol Ther* 2012; **35**: 587-599 [PMID: 22251419 DOI: 10.1111/j.1365-2036.2011.04983.x]
57 **Barish CF**, Drossman D, Johanson JF, Ueno R. Efficacy and safety of lubiprostone in patients with chronic constipation. *Dig Dis Sci* 2010; **55**: 1090-1097 [PMID: 20012484 DOI: 10.1007/s10620-009-1068-x]
58 **Whitehead WE**, Palsson OS, Gangarosa L, Turner M, Tucker J. Lubiprostone does not influence visceral pain thresholds in patients with irritable bowel syndrome. *Neurogastroenterol Motil* 2011; **23**: 944-e400 [PMID: 21914041 DOI: 10.1111/j.1365-2982.2011.01776.x]
59 **Schulz S**, Green CK, Yuen PS, Garbers DL. Guanylyl cyclase is a heat-stable enterotoxin receptor. *Cell* 1990; **63**: 941-948 [PMID: 1701694 DOI: 10.1016/0092-8674(90)90497-3]
60 **Busby RW**, Bryant AP, Bartolini WP, Cordero EA, Hannig G, Kessler MM, Mahajan-Miklos S, Pierce CM, Solinga RM, Sun LJ, Tobin JV, Kurtz CB, Currie MG. Linaclotide, through activation of guanylate cyclase C, acts locally in the gastrointestinal tract to elicit enhanced intestinal secretion and transit. *Eur J Pharmacol* 2010; **649**: 328-335 [PMID: 20863829 DOI: 10.1016/j.ejphar.2010.09.019]
61 **Andresen V**, Camilleri M, Busciglio IA, Grudell A, Burton D, McKinzie S, Foxx-Orenstein A, Kurtz CB, Sharma V, Johnston JM, Currie MG, Zinsmeister AR. Effect of 5 days linaclotide on transit and bowel function in females with constipation-predominant irritable bowel syndrome. *Gastroenterology* 2007; **133**: 761-768 [PMID: 17854590 DOI: 10.1053/j.gastro.2007.06.067]
62 **Eutamene H**, Bradesi S, Larauche M, Theodorou V, Beaufrand C, Ohning G, Fioramonti J, Cohen M, Bryant AP, Kurtz C, Currie MG, Mayer EA, Bueno L. Guanylate cyclase C-mediated antinociceptive effects of linaclotide in rodent models of visceral pain. *Neurogastroenterol Motil* 2010; **22**: 312-e84 [PMID: 19706070 DOI: 10.1111/j.1365-2982.2009.01385.x]
63 **Castro J**, Harrington AM, Hughes PA, Martin CM, Ge P, Shea CM, Jin H, Jacobson S, Hannig G, Mann E, Cohen MB, MacDougall JE, Lavins BJ, Kurtz CB, Silos-Santiago I, Johnston JM, Currie MG, Blackshaw LA, Brierley SM. Linaclotide inhibits colonic nociceptors and relieves abdominal pain via guanylate cyclase-C and extracellular cyclic guanosine 3′,5′-monophosphate. *Gastroenterology* 2013; **145**: 1334-1346.e1-11 [PMID: 23958540 DOI: 10.1053/j.gastro.2013.08.017]
64 **Johnston JM**, Kurtz CB, Macdougall JE, Lavins BJ, Currie MG, Fitch DA, O'Dea C, Baird M, Lembo AJ. Linaclotide improves abdominal pain and bowel habits in a phase IIb study of patients with irritable bowel syndrome with constipation. *Gastroenterology* 2010; **139**: 1877-1886.e2 [PMID: 20801122 DOI: 10.1053/j.gastro.2010.08.041]
65 **Chey WD**, Lembo AJ, Lavins BJ, Shiff SJ, Kurtz CB, Currie MG, MacDougall JE, Jia XD, Shao JZ, Fitch DA, Baird MJ, Schneier HA, Johnston JM. Linaclotide for irritable bowel syndrome with constipation: a 26-week, randomized, double-blind, placebo-controlled trial to evaluate efficacy and safety. *Am J Gastroenterol* 2012; **107**: 1702-1712 [PMID: 22986437 DOI: 10.1038/ajg.2012.254]
66 **Rao S**, Lembo AJ, Shiff SJ, Lavins BJ, Currie MG, Jia XD, Shi K, MacDougall JE, Shao JZ, Eng P, Fox SM, Schneier HA, Kurtz CB, Johnston JM. A 12-week, randomized, controlled trial with a 4-week randomized withdrawal period to evaluate the efficacy and safety of linaclotide in irritable bowel syndrome with constipation. *Am J Gastroenterol* 2012; **107**: 1714-1724; quiz p.1725 [PMID: 22986440 DOI: 10.1038/ajg.2012.255]
67 FDA, Center for Drug Evaluation and Research. Guidance for Industry: Irritable Bowel Syndrome. Clinical Evaluation of Drugs for Treatment. May 2012. Accessed December 10, 2013. Available from: URL: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatory Information/Guidances/UCM205269.pdf
68 **Lembo AJ**, Schneier HA, Shiff SJ, Kurtz CB, MacDougall JE, Jia XD, Shao JZ, Lavins BJ, Currie MG, Fitch DA, Jeglinski BI, Eng P, Fox SM, Johnston JM. Two randomized trials of linaclotide for chronic constipation. *N Engl J Med* 2011; **365**: 527-536 [PMID: 21830967 DOI: 10.1056/NEJMoa1010863]
69 **Johnston JM**, Kurtz CB, Drossman DA, Lembo AJ, Jeglinski BI, MacDougall JE, Antonelli SM, Currie MG. Pilot study on the effect of linaclotide in patients with chronic constipation. *Am J Gastroenterol* 2009; **104**: 125-132 [PMID: 19098860 DOI: 10.1038/ajg.2008.59]
70 **Lembo AJ**, Kurtz CB, Macdougall JE, Lavins BJ, Currie MG, Fitch DA, Jeglinski BI, Johnston JM. Efficacy of linaclotide for patients with chronic constipation. *Gastroenterology* 2010; **138**: 886-895.e1 [PMID: 20045700 DOI: 10.1053/j.gastro.2009.12.050]
71 **Videlock EJ**, Cheng V, Cremonini F. Effects of linaclotide in patients with irritable bowel syndrome with constipation or chronic constipation: a meta-analysis. *Clin Gastroenterol Hepatol* 2013; **11**: 1084-1092.e3; quiz e68 [PMID: 23644388 DOI: 10.1016/j.cgh.2013.04.032]
72 FDA approves Linzess to treat certain cases of irritable bowel syndrome and constipation, August 30, 2012. Accessed September 20, 2013. Available from: URL: http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm317505.htm#
73 **Shailubhai K**, Comiskey S, Foss JA, Feng R, Barrow L, Comer GM, Jacob GS. Plecanatide, an oral guanylate cyclase C agonist acting locally in the gastrointestinal tract, is safe and well-tolerated in single doses. *Dig Dis Sci* 2013; **58**: 2580-2586 [PMID: 23625291 DOI: 10.1007/s10620-013-2684-z]
74 **Shailubhai K**, Talluto C, Comiskey S, Foss J, Joslyn A, Jacob G. A Phase IIa randomized, double-blind, placebo-controlled, 14-day repeat, oral, dose-ranging study to assess the safety, pharmacokinetic and pharmacodynamic effects of plecanatide (SP- 304) in patients with chronic idiopathic constipation (Protocol No. SPSP304201- 09). Accessed 10th Dec, 2013. Available from: URL: http://www.sec.gov/Archives/edgar/data/1347613/000110465910052588/a10-19351_3ex99d1.htm
75 **Iser JH**, Sali A. Chenodeoxycholic acid: a review of its pharmacological properties and therapeutic use. *Drugs* 1981; **21**: 90-119 [PMID: 7009140 DOI: 10.2165/00003495-198121020-00002]
76 **Mitchell WD**, Findlay JM, Prescott RJ, Eastwood MA, Horn DB. Bile acids in the diarrhoea of ileal resection. *Gut* 1973; **14**: 348-353 [PMID: 4736774 DOI: 10.1136/gut.14.5.348]
77 **Chadwick VS**, Gaginella TS, Carlson GL, Debongnie JC, Phillips SF, Hofmann AF. Effect of molecular structure on bile acid-induced alterations in absorptive function, permeability, and morphology in the perfused rabbit colon. *J Lab Clin Med* 1979; **94**: 661-674 [PMID: 501195]
78 **Bampton PA**, Dinning PG, Kennedy ML, Lubowski DZ,

Cook IJ. The proximal colonic motor response to rectal mechanical and chemical stimulation. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G443-G449 [PMID: 11841994]

79 **Conley DR**, Coyne MJ, Bonorris GG, Chung A, Schoenfield LJ. Bile acid stimulation of colonic adenylate cyclase and secretion in the rabbit. *Am J Dig Dis* 1976; **21**: 453-458 [PMID: 183496 DOI: 10.1007/BF01072128]

80 **Caspary WF**, Meyne K. Effects of chenodeoxy- and ursodeoxycholic acid on absorption, secretion and permeability in rat colon and small intestine. *Digestion* 1980; **20**: 168-174 [PMID: 7390046 DOI: 10.1159/000198436]

81 **Bazzoli F**, Malavolti M, Petronelli A, Barbara L, Roda E. Treatment of constipation with chenodeoxycholic acid. *J Int Med Res* 1983; **11**: 120-123 [PMID: 6852359]

82 **Rao AS**, Wong BS, Camilleri M, Odunsi-Shiyanbade ST, McKinzie S, Ryks M, Burton D, Carlson P, Lamsam J, Singh R, Zinsmeister AR. Chenodeoxycholate in females with irritable bowel syndrome-constipation: a pharmacodynamic and pharmacogenetic analysis. *Gastroenterology* 2010; **139**: 1549-1558, 1558.e1 [PMID: 20691689 DOI: 10.1053/j.gastro.2010.07.052]

83 **Simrén M**, Bajor A, Gillberg PG, Rudling M, Abrahamsson H. Randomised clinical trial: The ileal bile acid transporter inhibitor A3309 vs. placebo in patients with chronic idiopathic constipation--a double-blind study. *Aliment Pharmacol Ther* 2011; **34**: 41-50 [PMID: 21545606 DOI: 10.1111/j.1365-2036.2011.04675.x]

84 **Wong BS**, Camilleri M, McKinzie S, Burton D, Graffner H, Zinsmeister AR. Effects of A3309, an ileal bile acid transporter inhibitor, on colonic transit and symptoms in females with functional constipation. *Am J Gastroenterol* 2011; **106**: 2154-2164 [PMID: 21876564 DOI: 10.1038/ajg.2011.285]

85 **Chey WD**, Camilleri M, Chang L, Rikner L, Graffner H. A randomized placebo-controlled phase IIb trial of a3309, a bile acid transporter inhibitor, for chronic idiopathic constipation. *Am J Gastroenterol* 2011; **106**: 1803-1812 [PMID: 21606974 DOI: 10.1038/ajg.2011.162]

86 **Guyatt GH**, Oxman AD, Vist GE, Kunz R, Falck-Ytter Y, Alonso-Coello P, Schünemann HJ. GRADE: an emerging consensus on rating quality of evidence and strength of recommendations. *BMJ* 2008; **336**: 924-926 [PMID: 18436948 DOI: 10.1136/bmj.39489.470347.AD]

P- Reviewers: Bian ZX, Ma XP, Ojetti V **S- Editor**: Ma YJ
L- Editor: A **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8910

World J Gastroenterol 2014 July 21; 20(27): 8910-8920
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Possible biological and translational significance of mast cells density in colorectal cancer

Ilaria Marech, Michele Ammendola, Claudia Gadaleta, Nicola Zizzo, Caroline Oakley, Cosmo Damiano Gadaleta, Girolamo Ranieri

Ilaria Marech, Caroline Oakley, Cosmo Damiano Gadaleta, Girolamo Ranieri, Interventional Radiology Unit with Integrated Section of Translational Medical Oncology, National Cancer Research Centre Istituto Tumori "Giovanni Paolo Ⅱ", 70124 Bari, Italy
Michele Ammendola, Department of Clinical Surgery, University of Catanzaro "Magna Graecia" Medical School, 88100 Catanzaro, Italy
Claudia Gadaleta, Nicola Zizzo, Department of Pathology, Veterinary Medical School, University of Bari, Valenzano, 70010 Bari, Italy
Author contributions: Marech I, Gadaleta CD and Ranieri G ideated the manuscript and performed a critical review of the literature; Ammendola M, Gadaleta C and Zizzo N contributed to literature research and data analysis; Oakley C edited the manuscript; all authors wrote the manuscript.
Correspondence to: Girolamo Ranieri, MD, Interventional Radiology Unit with Integrated Section of Translational Medical Oncology, National Cancer Research Centre Istituto Tumori "Giovanni Paolo Ⅱ", Via Orazio Flacco 65, 70124 Bari, Italy. giroran@tiscalinet.it
Telephone: +39-80-5555561 Fax: +39-80-5555563
Received: September 23, 2013 Revised: January 24, 2014
Accepted: April 21, 2014
Published online: July 21, 2014

Abstract

Mast cells (MCs), located ubiquitously near blood vessels, are descended from $CD34^+$ hematopoietic stem cells. Initially, although their role has been well defined in hypersensitivity reactions, the discovery of their sharing in both innate and adaptive immunity has allowed to redefine their crucial interplay on the regulatory function between inflammatory and tumor cells through the release of mediators granule-associated (mainly tryptase and vascular endothelial growth factor). In particular, in several animal and human malignancies it has been well demonstrated that activated c-Kit receptor (c-KitR) and tryptase (an agonist of the proteinase-activated receptor-2) take pivotal part in tumor angiogenesis after the MCs activation, contributing to tumor cells invasion and metastasis. In this review, we focused on crucial MCs density (MCD) role in colorectal cancer (CRC) development and progression angiogenesis-mediated; then, we will analyze the principal studies that have focused on MCD as possible prognostic factor. Finally, we will consider a possible role of MCD as novel therapeutic target mainly by c-KitR tyrosine kinase inhibitors (imatinib, masitinib) and tryptase inhibitors (gabexate and nafamostat mesylate) with the aim to prevent CRC progression.

Key words: Tryptase; Mast cell density; Proteinase-activated receptor-2; c-Kit receptor; Vascular endothelial growth factor; Angiogenesis; Colorectal cancer; Tumor progression; Tryptase inhibitors; c-Kit receptor tyrosine kinase inhibitors

Core tip: In several malignancies it has been well demonstrated that mast cell (MC), activated c-Kit receptor (c-KitR) and tryptase secreted after MC degranulation play a pivotal role in tumor angiogenesis, helping tumor cell invasion and metastasis. The close relationship between MC density, angiogenesis and tumor progression could suggest a role for MCs as a possible prognostic factor in colorectal cancer (CRC). Moreover, considering MC-mediated CRC development, c-KitR tyrosine kinase inhibitors (imatinib, masitinib) and tryptase inhibitors (gabexate and nafamostat mesylate) could be used to block MC activation/degranulation and the tryptase/proteinase-activated receptor-2 axis respectively, and may be evaluated in future clinical trials in CRC patients.

Marech I, Ammendola M, Gadaleta C, Zizzo N, Oakley C, Gadaleta CD, Ranieri G. Possible biological and translational

significance of mast cells density in colorectal cancer. *World J Gastroenterol* 2014; 20(27): 8910-8920 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8910.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8910

INTRODUCTION

In 1869 Nettleship and Tay[1] described a particular form of pigmented rash ("urticaria pigmentosa"), which presented a dermographism entirely similar to some urticaria forms. Mast cells (MCs) were identified by Ehrlich[2] in 1879 and named "mastzellen"(from the German mast = well-fed) because it was believed that they were particularly numerous in overfed animals. It was subsequently shown that cutaneous lesions observed in these animals were characterized by a focal accumulation of some of these mast cells[2]. In 1949 Ellis[3] described a form of systemic mastocytosis characterized by an abnormal infiltration of MCs into extracutaneous organs. Historically, "mastocytosis" is a morbid condition characterized by a marked increase (usually about ten times compared to normal) of the density of tissue MCs in specific anatomical sites[4]. Currently, "mastocytosis" includes a wide spectrum of clinical disorders (with an extremely heterogeneous clinical course and prognosis) sharing particular tyrosine kinase c-Kit receptor (c-KitR) mutations that confer its increased activation, determining stem cell factor (SCF)-independent MC proliferation[5,6].

MCs are the progeny of $CD34^+$ hematopoietic stem cells and require SCF for their differentiation, activation and proliferation[7]. MCs are located throughout the body; on the epithelial surface, in blood vessels, nerves and glands[8]. Classically, MCs are divided into three subgroups according to the protease expression in their granules: the first type of MC contains only tryptase, the second only chymase, and the third tryptase, chymase and other proteases[8,9].

Although the role of mast cells has long been well defined in hypersensitivity reactions, since 1990[10,11] it has been discovered that they also have a role in both innate and adaptive immunity. This has allowed us to redefine their crucial interplay on the regulatory function between inflammatory and tumor cells[12-15] by means of the release of various granule-associated mediators [histamine, serotonin, heparin, tryptase, chymase, thymidine phosphorylase, tumour necrosis factor, vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), platelet-derived growth factor-β (PDGF-β), epidermal growth factor (EGF)]; lipid-derived mediators (leukotrienes, prostaglandins, platelet-activating factor); cytokines (transforming growth factor-β, interleukins, IL-6); and chemokines[16-19].

MCs express many types of receptors allowing them to recognize different stimuli and to respond accordingly[8,9]. For the fragment crystallisable portion of Immunoglobulin (Ig)G and IgE, MCs express various receptors, and in response to several antigens they release preformed (*e.g.*, histamine, tryptase) and synthesized *de novo* mediators (*i.e.*, leukotrienes, prostaglandins)[10,20]. Regarding innate immunity, MCs express some receptors for components of complement (CR3, CR4, CR5), and others belonging to the Nod-like receptors family. The recognition of pathogens by the innate immune cells and the link between innate and adaptive immunity however are *via* toll-like receptors (TLR type 1, 2, 3, 4, 6, 7 and 9)[21].

Many experimental studies have assessed MCs as protagonists both in inflammation and angiogenesis[20,22,23], processes closely interconnected and related to tumor development and progression[24-27]. Following the above-mentioned synthetic review of the various functions of MCs, in the upcoming sections we focus on the crucial role of MCs in angiogenesis-mediated tumor development and progression and illustrate the most common identification methods of MCs. In particular, as well as playing a role in tumor angiogenesis, it has been demonstrated that the number of MCs, so-called MC density (MCD), increases in several human and animal malignancies, and this increased MCD correlates with increased angiogenesis. On this basis, we analyze the principal studies that have focused on MCD as a possible prognostic factor, considering the MC as a possible novel therapeutic target in colorectal cancer (CRC).

INVOLVEMENT OF MAST CELLS IN ANGIOGENESIS-MEDIATED TUMOR DEVELOPMENT AND PROGRESSION

During inflammatory reactions, immune cells (MCs, macrophages, neutrophils, and lymphocytes) synthesize pro-angiogenic factors that induce first neovascularization, then the further migration of inflammatory cells to the site of inflammation, amplifying the process[25,28]. At the same time, there is well-established evidence that tumor cells are surrounded by an infiltrate of inflammatory cells, which synergize with stromal cells and malignant cells in a paracrine manner[29-31]. As a consequence, there is a stimulation of endothelial cell proliferation and blood vessel formation[32-34]. It is important to underline that MCs are located near blood vessels and regulate many functions of endothelial cells[35-37].

In particular, the c-KitR activated by SCF and tryptase after MC degranulation play pivotal part in tumor angiogenesis[38,39].

The increased activation of the c-KitR pathway leads to MC activation, which induces pro-angiogenic cytokines (such as VEGF, PDGF, FGF-2) and tryptase degranulation[38,39]. MC c-KitR activation induces cross-talk between MCs and the tumor cell microenvironment (endothelial and other cells), leading consequentially to the strengthening of pro-angiogenic signaling[6].

Tryptase is also an agonist of proteinase-activated receptor-2 (PAR-2)[40], which is expressed in epithelial and endothelial cells with proteolytic activities. It belongs to the unique superfamily of G-protein-coupled receptors

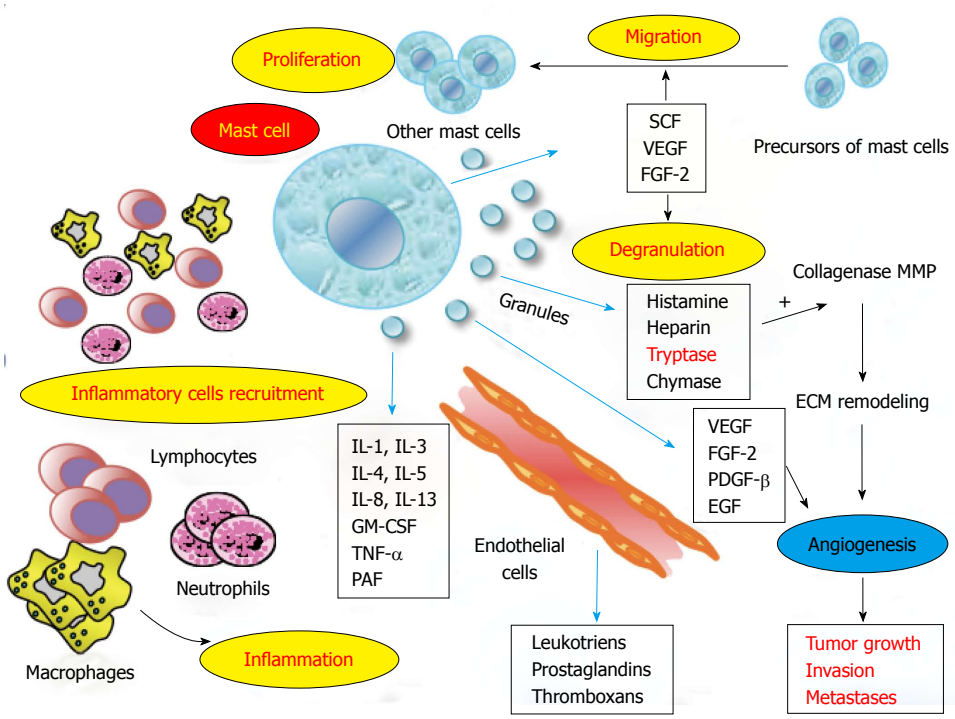


Figure 1 Close relationship between mast cells and angiogenesis-mediated tumor progression. FGF-2: Fibroblast growth factor-2; VEGF: Vascular endothelial growth factor; PDGF-β: Platelet-derived growth factor-β; EGF: Epidermal growth factor; IL: Interleukin; GM-CSF: Granulocyte/macrophage colony stimulating factor; TNF-α: Tumor necrosis factor-α; ECM: Extracellular matrix; MMP: Matrix metalloproteinase.

and is activated by tryptase. Tryptase activation leads to cell proliferation and the release of IL-6 and granulocyte-macrophage colony-stimulating factor, which act as pro-angiogenic molecules[41]. Moreover, tryptase degrades extracellular matrix components[42], activating in its stored matrix metalloproteinases[43] and plasminogen activators that together help the invasion and metastasis of tumor cells[44] (Figure 1). *In vitro* studies on matrigel and *in vivo* studies on the chick embryo chorioallantoic membrane displayed the capillary growth induced by tryptase and, conversely, suppressed by tryptase inhibitors[45,46].

Apart from the above biological background, the role of MCs in tumor development has emerged from observation of a strong correlation between an increase of MCD and an increase of microvascular density (MVD) in many human and animal malignancies such as oral squamous carcinoma[13,47], breast cancer[11,12,16], gastrointestinal cancer[26,48-50], hepatocarcinoma[51], pancreatic adenocarcinoma[52], renal cell carcinoma[53], non-small cell lung cancer[54,55], melanoma[56], endometrial carcinoma[27,57], non-Hodgkin's lymphomas[58], and multiple myeloma[59]. With particular reference to hematological disorders, some evidence suggest that high MCD infiltration is directly correlated with tumor progression and worse disease outcome[60-62].

Conversely, a few studies have shown that high MCD is linked to good prognosis[63,64].

To further emphasize that MC activation plays a pivotal role in tumor progression, it was shown in breast cancer that degranulated MCs (MCs-Try) are mainly present in peri-tumoral tissue (to strengthen the hypothesis that they are tumor-reactive), unlike those rich in granules MCs (MCs-TB) which are especially present in tumor infiltration and contribute to stromal remodeling and differentiation of myofibroblasts (through tryptase released in stromal microenvironment)[11].

The close relationship between MCD, angiogenesis and tumor progression could suggest a role for MCs and the pro-angiogenic factors released from them as novel therapeutic targets in cancer. In particular, it is possible to block MC activation/degranulation by means of c-KitR tyrosine kinase inhibitors (TKI) such as imatinib and masitinib, and also to block the tryptase released from MCs by means of tryptase inhibitors (gabexate and nafamostat mesylate)[12,65-67].

PRINCIPAL METHODS FOR IDENTIFICATION OF TISSUE MAST CELLS

MCs can be classically or conventionally identified by means of histochemical methods. Among these, Toluidine blue histochemistry (Undritz Stain) metachromatically stains MC granules, making them appear red or blue-red due to the presence of sulphated proteoglycans (heparin)[68]. With the above histochemistry, MCs appear as rather large oval or elongated cells (diameter of 20-30 μm) containing numerous basophilic granules in their cytoplasm that can hide the nucleus[12,69].

By immunohistochemistry MCs can be stained with antibodies towards c-KitR (*e.g.*, human-specific mono-

Table 1 Principal studies correlating mast cell density with survival/stage in colorectal cancer patients

Ref.	Disease stage/main stages	Neoadjuvant therapy	Patients (*n*)/site	Methods of MCs identification	Correlation with overall survival/stage	*P* value
Xia *et al*[83]	All TNM stages (mainly Ⅱ-Ⅲ)	No	155 CC	Immunohistochemistry primary anti-tryptase and anti-chymase abs	No with OS	NS
Xia *et al*[84]	Stage ⅢB	No	93 CC	Immunohistochemistry primary anti-tryptase ab	No with OS	NS
Nielsen *et al*[85]	All Dukes' stage (mainly B-C)	No	584 CRC	Immunohistochemistry primary anti-tryptase ab	Yes, high MCD with high OS	0.02
Tan *et al*[86]	All TNM stages	NR	60 CRC	Immunohistochemistry primary anti-tryptase and anti-chymase abs	Yes, high MCD with high OS	< 0.01
Fisher *et al*[88]	All Dukes' stage (mainly B-C)	No	331 RC	Giemsa method	Yes, high MCD with low OS	NE
Yodavudh *et al*[89]	All TNM stages (mainly Ⅱ-Ⅲ)	No	130 CRC	Immunohistochemistry primary anti-tryptase ab	Yes, high MCD with low OS	< 0.0001
Elezoğlu *et al*[87]	All TNM stages (mainly Ⅱ-Ⅲ)	NR	204 CRC	Toluidine blue histochemistry	Yes, high MCD with high OS	0.035
Acikalin *et al*[49]	All TNM stages (mainly Ⅱ-Ⅲ)	No	60 CRC	Giemsa method	Yes, high MCD with low OS	0.0013
Gulubova *et al*[50]	All TNM stages (mainly Ⅱ)	No	106 CRC	Immunohistochemistry primary anti-tryptase ab; toluidine blue histochemistry	Yes, high MCD with low OS	0.038

CC: Colon cancer; OS: Overall survival; NS: Not significant; CRC: Colorectal cancer; MCD: Mast cell density; NR: Not reported; MCs: Minimal consistent set.

clonal antibodies anti-CD117), towards the content of their granules, *i.e.*, tryptase or chymase[68]. With a primary anti-c-KitR antibody, a membrane, cytoplasmic or mixed staining is observed[68]. With primary anti-chymase and anti-tryptase antibodies a diffuse cytoplasmic staining is observed[68].

Under the electron microscope MCs present a small, round nucleus, few mitochondria, some meandering tanks of rough endoplasmic reticulum and a small Golgi complex. The numerous specific granules (some hundreds) measure 0.3-0.8 μm in diameter and appear bordered by a membrane showing a variable fine granular or lamellar structure[70,71].

Following their activation, MCs degranulate and exocytose the content into the surroundings. Piecemeal degranulation is typified by variable losses of the granule content[71-73].

MAST CELL DENSITY INVOLVEMENT IN COLORECTAL CANCER AND ITS POSSIBLE ROLE AS PROGNOSTIC FACTOR

Normally, MCs are present in the mucosa and submucosa of the gastrointestinal tract in humans and mice[74].

In a preclinical study in mice, MCs played a crucial role in epithelial tumorigenesis, appearing in early dysplastic tissue and expanding in polyps[75]. However, when analysing the potential role of MCs in tumor development in several mice studies, Heijmans *et al*[76] were unable to draw certain conclusions due to a lack of a suitable animal model to study CRC. In fact, in IL-10-deficient mice with MCs Chichlowski *et al*[77] showed a reduced risk of development of inflammatory bowel disease (IBD) compared to in that of IL-10-deficient mice without MCs. Thus, this result emphasizes the protective role of MCs within the colonic microenvironment by enhancing the efficacy of the mucosal barrier. In reality, these data suggest that MCs can play a dual and opposite function, and this is probably due to the presence in the intestinal tract of different types of MCs, each with a specific role, with specific granules, and expressing various receptors[74].

It is noted that patients affected by IBD have an increased cumulative incidence of CRC than the general population and that this incidence increases with the duration of the bowel disease[78]. In particular, it was found that high MCD in intestinal adenomatous polyps[75,79-81] could drive a cascade of events to boost the progressive growth of adenomatous polyps, the immediate precursors of CRC[75].

In this regard, Taweevisit, considering 192 CRC patients, displayed a direct correlation between MCD, tumor development and grading[82].

With the aim to find a correlation between MCD and stage/prognosis in CRC patients, many studies (summarized in Table 1) have been conducted with mixed results. One Author showed no correlation between MCD and prognosis[83,84]. Other Authors have shown a direct and significant correlation between high MCD and improved prognosis[85-87]. The majority of studies however have shown that high MCD is related to tumor aggressiveness[48-50] and reduced survival[88-90].

Xia *et al*[83] studied MCD in 39 patients with colon adenoma and in 155 colon cancer (CC) patients of all TNM stages, evaluating a relationship between MCD (positive to both tryptase and chymase) and tumor progression. Interestingly, a significant increase of MCD localized in adjacent normal colon mucosa in CC patients was noted compared to those with colon adenomas ($P < 0.05$)[83]. Moreover, MCD located in adjacent normal colon mu-

cosa in CC patients was significantly related to pathologic classification (*i.e.*, papillary plus tubular or other), depth of penetration (*i.e.*, high T according to TNM), distant metastases (*i.e.*, M1 according to TNM), and hepatic metastases ($P = 0.029$, $P = 0.054$, $P = 0.008$, $P = 0.027$)[83]. Instead, there is no correlation between MCD located in the invasive margin or in adjacent normal colon mucosa and survival ($P = 0.092$ and $P = 0.003$)[83]. Similarly, in 93 CC patients only in stage ⅢB (according to TNM staging), the same Author observed a higher MCD positive to tryptase in non-metastatic regional-draining lymph nodes than in metastatic lymph nodes ($P = 0.000$)[84].

In 1999, Nielsen *et al*[85] analysis in a large cohort of CRC patients ($n = 584$) of all Dukes' stages displayed a significant correlation between high MCD positive to tryptase and good prognosis ($P = 0.02$); 50% of all patients with high MCD positive to tryptase were still alive at 3 years.

Subsequently, Tan *et al*[86] observed that high MCD (positive to tryptase and chymase) is also related to a significantly higher 5-year survival rate (SR). In their study on 60 CRC patients of all TNM stages, a 59% SR was recorded for patients with high MCD compared to 33.3% in those with low MCD ($P < 0.01$). Curiously, low MCD was significantly related to deeper depth of invasion, but also to low rates of lymph node and distant metastases[86].

Recently, Elezoğlu and Tolunay[87] displayed a significant correlation between MCD positive to tryptase, MVD, and survival in 204 CRC patients of all TNM stages. In the MC group, for values < 10, the five-year SR was 48%, whereas for values > 10 it rose to 58% ($P = 0.035$). In the MVD arm for values < 10, the five-year SR was 46%, while for values ⩾ 10 it was 58% ($P = 0.042$)[87].

In 1989 Fisher *et al*[88] was one of the first researchers to identify high MCD as an unfavorable prognostic factor independent from disease stage or lymph nodal status in 331 rectal cancer patients of all Dukes' stages.

In 60 patients with CRC of all TNM stages Acikalin *et al*[49] showed that MCD (evaluated by means of the Giemsa stain) was higher in patients with disease recurrence compared to those patients who had been disease free for at least 24 mo ($P < 0.001$), and that it was correlated to short disease-free survival ($P = 0.0013$), vascular invasion ($P = 0.06$), depth of penetration ($P = 0.05$), lymph nodes metastases ($P = 0.05$), liver metastases ($P = 0.05$) and high TNM stage ($P = 0.05$).

Yodavudh *et al*[89] confirmed Elezoğlu and Tolunay[87], s report of a strong correlation between MCD positive to tryptase, MVD, and survival in 130 CRC patients of all TNM stages. Contrarily however, they showed that low MVD (hypovascular tumor tissue) and low MCD are related to significantly longer survival rates ($P < 0.0001$).

Gulubova and Vlaykova[50] also confirmed a significant correlation between MCD positive to tryptase, MVD, and survival in 106 CRC patients of all TNM stages. Patients with low MCD had a significantly better prognosis compared to those with high MCD ($P = 0.038$)[50]. In the same way, hypovascular tumor tissue was related to highly significantly longer survival than hypervascular tumor tissue ($P < 0.0001$)[50].

In a recent series of 41 gastrointestinal cancer patients (of whom 22 had CRC of TNM stage ⅢC), Ammendola *et al*[30] showed a significant correlation between MCD positive to tryptase and the number of metastatic lymph nodes harvested ($P = 0.01$), and between MCD in primary tumor tissue and in metastatic lymph node tissue ($P = 0.02$). These data suggest that MCD in primary tumor tissue could be a useful prognostic marker[30,49], surrogating the number of postoperative metastatic lymph nodes after surgical treatment in gastrointestinal cancer patients[91-94].

Even more recently, Malfettone *et al*[90] showed in 115 CRC patients of all TNM stages that high MCD positive to tryptase correlates with the advanced stages of CRC ($P = 0.025$). In particular, the expression of PAR-2 (especially at the sites most infiltrated by MCs) is related to MCD expression[90]. Due to the pro-angiogenic activity of tryptase, which stimulates PAR-2 on endothelial cells, it is possible to suggest an involvement of tryptase in CRC angiogenesis[90].

MAST CELLS, c-KIT RECEPTOR AND PRO-ANGIOGENIC FACTORS FROM MAST CELLS RELEASED AS POSSIBLE THERAPEUTIC TARGETS IN COLORECTAL CANCER

Ducroc *et al*[95] demonstrated a pivotal role of MC tryptase in inducing PAR-2 activation in several human CC cell lines (T84, Caco-2, HT-29, Cl.19A), promoting their proliferation.

Yoshii *et al*[96] investigated the distribution of MCD (positive to tryptase) in 30 human CC, showing the prevalence of MCD in the invasive front rather than in either the central tumor part or the normal tissue. In addition, the Authors showed a higher density of PAR-2 in the tumor tissue compared to the normal tissue[96].

Interestingly, two Authors explored the tryptase/PAR-2 axis in one human colon carcinoma cell line (DLD-1)[96,97]. Specifically, the proliferation signal induced by tryptase on DLD-1 cells is mediated by PAR-2, that in turn leads to the increase of calcium[98] and transient phosphorylation of mitogen-activated protein kinase/extracellular signal-related kinase (MEKK) and the mitogen-activated protein kinase (MAPK) pathway[96]. In addition, the increase of calcium PAR-2/Phospholipase C-mediated led to the activation of CycloOXygenase-2 (COX-2) and prostaglandin E2 (PGE2) synthesis, suggesting that the MEKK and MAPK pathway activation and PGE2 synthesis were together essential for DLD-1 proliferation[96] (Figure 2).

Sodium-hydrogen antiporter 3 regulator 1 (NHERF-1) is a cytoplasmic adaptor protein present in various cel-

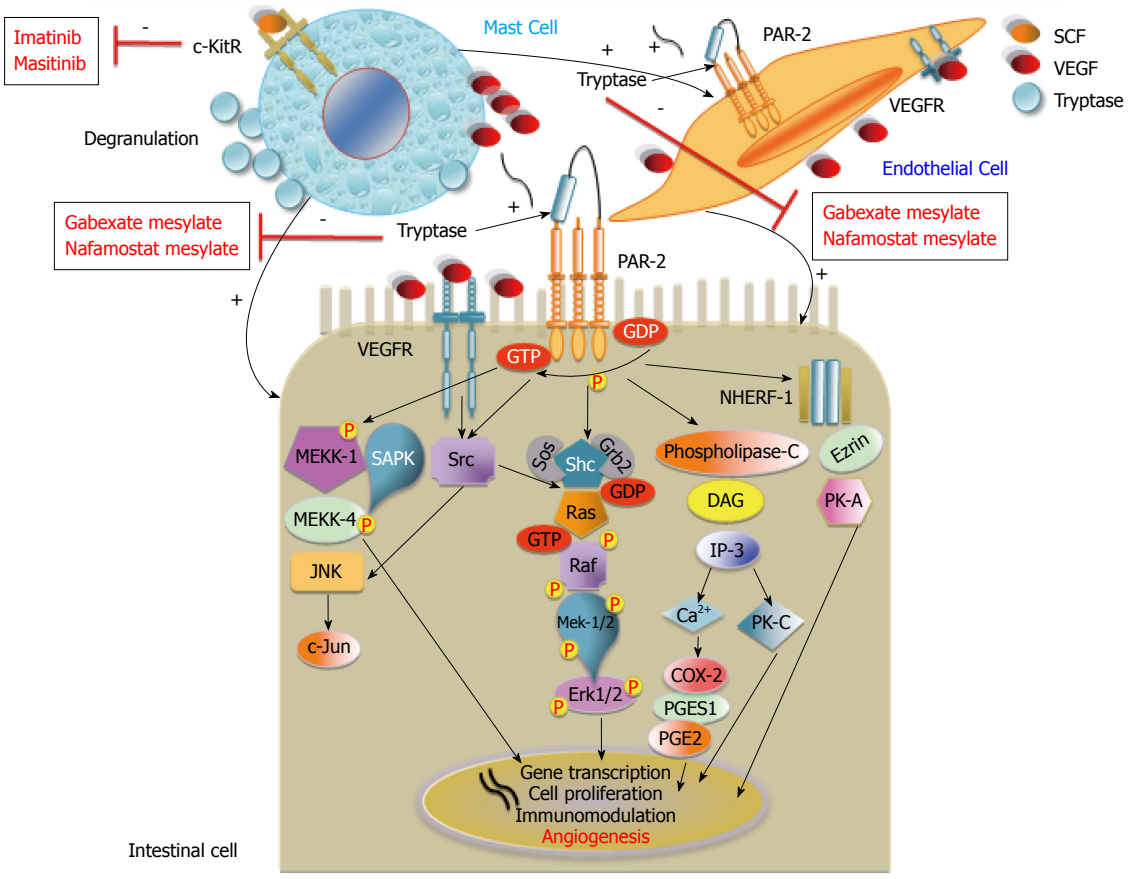


Figure 2 In both intestinal and endothelial cells, the tryptase/proteinase-activated receptor-2 and vascular endothelial growth factor/vascular endothelial growth factor receptor axes, induced by mast cells, lead to tumor angiogenesis and intestinal cell growth. Note that targeting mast cells with molecular agents (c-KitR tyrosine kinase and tryptase inhibitors) could prevent angiogenesis-mediated colorectal cancer progression. c-KitR: c-Kit receptor; PAR-2: Proteinase-activated receptor-2; VEGFR: Vascular endothelial growth factor receptor; SCF: Stem cell factor: VEGF: Vascular endothelial growth factor; NHERF-1: Na^+/H^+ exchanger regulatory factor-1; MEKK-1: Mitogen-activated protein kinase/extracellular signal-related kinase-1; MEKK-4: Mitogen-activated protein kinase/ extracellular signal-related kinase-4; JNK: c-Jun N-terminal kinase; c-Jun: Jun proto-oncogene; SAPK: Mitogen-activated protein kinase-9; GEF: Rho/rac guanine nucleotide exchange factor; Rho: Rhodopsin transcription termination factor; SOS: Son of sevenless protein; Grb2: Growth factor receptor-bound protein 2; Shc: Shc transforming protein kinase; Ras: Ras protein kinase; Raf: Raf protein kinase; Mitogen-activated protein kinase/extracellular signal-related kinase-1/2; Erk: Elk-related tyrosine kinase; DAG: Diacylglycerol; IP-3: Inositol triphosphate; PK-C: Protein kinase-C; COX-2: Cyclooxygenase-2; PGE2: Prostaglandin E2; PGES-1: Prostaglandin E synthase-1; PK-A: Protein kinase-A.

lular types (including intestinal cells). NHERF-1 regulates several transmembrane receptors, transporters and other proteins localized near the plasma membrane, and *via* the Ezrin/protein kinase-A- mediated network seems to lead to CRC progression[99,100].

Interestingly, Malfettone *et al*[90], having confirmed the close interplay between MCD and PAR-2 in tumour progression and invasiveness, showed that the PAR-2(+)/cytoplasmic NHERF-1(+) expression immunophenotype is an unfavourable prognostic factor in CRC patients, as it is associated with the presence of lymph nodal and distant metastasis, poor differentiation grade and lymphovascular invasion. If further studies conducted in stage II CRC patients should confirm the role of the PAR-2(+)/cytoplasmic NHERF-1(+) expression immunophenotype as a negative prognostic biomarker, it will become a prerequisite to the treatment of patients with adjuvant chemotherapy.

Finally, if future studies demonstrate that high MCD positive to tryptase is an independent unfavourable prognostic factor[30,49,50,88,89] related to a significant and increased risk of tumor progression, this parameter could be considered in the decision to give chemotherapy associated with tryptase inhibitors (gabexate and nafamostat mesylate).

Clearly, before being able to use MC targeted agents, a more in-depth knowledge of MC-mediated angiogenic mechanisms and the complex hierarchical relationships between the various angiogenesis signaling pathways will be necessary[101-104].

In this regard, tryptase may induce angiogenesis mainly by the increase of VEGF expression mediated *via* PAR-2, which is expressed also on endothelial cells as well as intestinal cells[12,27,45,54]. Moreover, VEGF and its receptors are widely expressed in intestinal carcinoma cells, and VEGF stimulates VEGFR-2-positive tumor, mast and endothelial cells directly, leading to tumor growth and angiogenesis by paracrine and autocrine stimulation signals[26,105,106].

Considering the central role of MCs in the activation of gastrointestinal and endothelial cells which contribute to tumor angiogenesis and progression, c-KitR could

also be a potential therapeutic target for inhibiting their pro-angiogenic cytokine degranulation (VEGF, PDGF, FGF, tryptase) and activation[6,38,67,107]. In fact, MC c-KitR activation potentiates the cross-talk between MCs and endothelial cells (Figure 2), leading to the strengthening of pro-angiogenic signaling. Therefore, MCs could represent a possible therapeutic target through tryptase inhibitors (gabexate and nafamostat mesylate) and c-KitR inhibitors (imatinib, masitinib) to arrest angiogenesis-mediated tumor growth in gastrointestinal cancer[108-110].

CONCLUSION

Although the role of MCs was well defined in hypersensitivity reactions, the discovery of their regulatory function in innate and adaptive immunity has allowed us to understand their complex interplay between inflammatory and tumor cells. In fact, much evidence obtained from *in vitro* and *in vivo* studies has demonstrated that common MCs phenotypes, if adequately stimulated by various factors (histamine, heparin, tryptase, chymase, VEGF, FGF-2, PDGF-β, EGF), are able to interfere with tumor cells and the tumor microenvironment inducing tumor angiogenesis and progression[10,12].

Although the majority of studies have reported that several malignancies are associated with an increase of MC infiltration, controversial data about the relationship between MCD and prognosis in CRC have been reported. Considering these studies, conflicting conclusions[48-50], may in part depend on considerable *bias* related to CRC disease (radical surgical treatment with relative lymph node collection, type of resection, histology or stage tumor, colon plus rectal cancer, small sample size)[83,85,86,88], and different methods of MC evaluation (histochemistry with Toluidine blue, Giemsa stain, primary antibody anti-tryptase or anti-chymase for immunohistochemistry, standardization of MC counts with reference to magnification, MC location, microscopic field of evaluation)[76,84,87,90]. Despite these *biases*, the majority of the published studies suggest that high MCD in tumors may play a role as an unfavourable prognostic marker. Should this prognostic marker be validated in expected future studies it would be intriguing to conduct clinical trials employing chemotherapy plus tryptase inhibitors or TK inhibitors MC c-KitR.

REFERENCES

1 **Nettleship T**, Tay W. Rare forms of urticaria. *Brit Med J* 1869; **2**: 323-330
2 **Ehrlich P**. Beiträge zur Kenntniss der granulirten Bindegewebszellen und der eosinophilen Leukocythen. *Arch Anat Physiol* (Leipzig) 1879; **3**: 166-169
3 **Ellis JM**. Urticaria pigmentosa; a report of a case with autopsy. *Arch Pathol* (Chic) 1949; **48**: 426-435 [PMID: 18149230]
4 **Marone G**, Spadaro G, Genovese A. Biology, diagnosis and therapy of mastocytosis. *Chem Immunol* 1995; **62**: 1-21 [PMID: 7546277 DOI: 10.1159/000319293]
5 **Horny HP**, Sotlar K, Valent P. Mastocytosis: state of the art. *Pathobiology* 2007; **74**: 121-132 [PMID: 17587883 DOI: 10.1159/000101711]
6 **Orfao A**, Garcia-Montero AC, Sanchez L, Escribano L. Recent advances in the understanding of mastocytosis: the role of KIT mutations. *Br J Haematol* 2007; **138**: 12-30 [PMID: 17555444 DOI: 10.1111/j.1365-2141.2007.06619.x]
7 **Shea-Donohue T**, Stiltz J, Zhao A, Notari L. Mast cells. *Curr Gastroenterol Rep* 2010; **12**: 349-357 [PMID: 20711694 DOI: 10.1007/s11894-010-0132-1]
8 **Irani AM**, Schwartz LB. Human mast cell heterogeneity. *Allergy Proc* 1994; **15**: 303-308 [PMID: 7721078 DOI: 10.2500/108854194778816472]
9 **Irani AA**, Schechter NM, Craig SS, DeBlois G, Schwartz LB. Two types of human mast cells that have distinct neutral protease compositions. *Proc Natl Acad Sci USA* 1986; **83**: 4464-4468 [PMID: 3520574 DOI: 10.1073/pnas.83.12.4464]
10 **Marshall JS**. Mast-cell responses to pathogens. *Nat Rev Immunol* 2004; **4**: 787-799 [PMID: 15459670 DOI: 10.1038/nri1460]
11 **Mangia A**, Malfettone A, Rossi R, Paradiso A, Ranieri G, Simone G, Resta L. Tissue remodelling in breast cancer: human mast cell tryptase as an initiator of myofibroblast differentiation. *Histopathology* 2011; **58**: 1096-1106 [PMID: 21707711 DOI: 10.1111/j.1365-2559.2011.03842.x]
12 **Ranieri G**, Ammendola M, Patruno R, Celano G, Zito FA, Montemurro S, Rella A, Di Lecce V, Gadaleta CD, Battista De Sarro G, Ribatti D. Tryptase-positive mast cells correlate with angiogenesis in early breast cancer patients. *Int J Oncol* 2009; **35**: 115-120 [PMID: 19513558 DOI: 10.3892/ijo_00000319]
13 **Ranieri G**, Labriola A, Achille G, Florio G, Zito AF, Grammatica L, Paradiso A. Microvessel density, mast cell density and thymidine phosphorylase expression in oral squamous carcinoma. *Int J Oncol* 2002; **21**: 1317-1323 [PMID: 12429983]
14 **Ranieri G**, Roccaro AM, Vacca A, Ribatti D. Thymidine phosphorylase (platelet-derived endothelial cell growth factor) as a target for capecitabine: from biology to the bedside. *Recent Pat Anticancer Drug Discov* 2006; **1**: 171-183 [PMID: 18221035 DOI: 10.2174/157489206777442241]
15 **Passantino L**, Patruno R, Valerio P, Penna A, Mazzone F, Zito AF, Catalano V, Pellecchia A, Jirillo E, Ranieri G. Thymidine phosphorylase profiles in nonmalignant and malignant pancreatic tissue. Potential therapeutic role of capecitabine on tumoral and endothelial cells and tumor-infiltrating macrophages. *Immunopharmacol Immunotoxicol* 2005; **27**: 95-107 [PMID: 15803863 DOI: 10.1081/IPH-51753]
16 **Raica M**, Cimpean AM, Ceausu R, Ribatti D, Gaje P. Interplay between mast cells and lymphatic vessels in different molecular types of breast cancer. *Anticancer Res* 2013; **33**: 957-963 [PMID: 23482767]
17 **Ribatti D**, Nico B, Finato N, Crivellato E. Tryptase-positive mast cells and CD8-positive T cells in human endometrial cancer. *Pathol Int* 2011; **61**: 442-444 [PMID: 21707849]
18 **Nagata M**, Shijubo N, Walls AF, Ichimiya S, Abe S, Sato N. Chymase-positive mast cells in small sized adenocarcinoma of the lung. *Virchows Arch* 2003; **443**: 565-573 [PMID: 12827514 DOI: 10.1007/s00428-003-0842-y]
19 **Horny HP**, Greschniok A, Jordan JH, Menke DM, Valent P. Chymase expressing bone marrow mast cells in mastocytosis and myelodysplastic syndromes: an immunohistochemical and morphometric study. *J Clin Pathol* 2003; **56**: 103-106 [PMID: 12560387 DOI: 10.1136/jcp.56.2.103]
20 **Galli SJ**, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CM, Tsai M. Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annu Rev Immunol* 2005; **23**: 749-786 [PMID: 15771585 DOI: 10.1146/annurev.immunol.21.120601.141025]
21 **Oda K**, Kitano H. A comprehensive map of the toll-like receptor signaling network. *Mol Syst Biol* 2006; **2**: 2006.0015 [PMID: 16738560]
22 **Coussens LM**, Raymond WW, Bergers G, Laig-Webster M, Behrendtsen O, Werb Z, Caughey GH, Hanahan D. Inflammatory mast cells up-regulate angiogenesis during squa-

mous epithelial carcinogenesis. *Genes Dev* 1999; **13**: 1382-1397 [PMID: 10364156 DOI: 10.1101/gad.13.11.1382]
23 **Nakayama T**, Yao L, Tosato G. Mast cell-derived angiopoietin-1 plays a critical role in the growth of plasma cell tumors. *J Clin Invest* 2004; **114**: 1317-1325 [PMID: 15520864 DOI: 10.1172/JCI22089]
24 **Theoharides TC**, Conti P. Mast cells: the Jekyll and Hyde of tumor growth. *Trends Immunol* 2004; **25**: 235-241 [PMID: 15099563 DOI: 10.1016/j.it.2004.02.013]
25 **Ribatti D**, Crivellato E. Mast cells, angiogenesis and cancer. *Adv Exp Med Biol* 2011; **716**: 270-288 [PMID: 21713661 DOI: 10.1007/978-1-4419-9533-9_14]
26 **Ribatti D**, Guidolin D, Marzullo A, Nico B, Annese T, Benagiano V, Crivellato E. Mast cells and angiogenesis in gastric carcinoma. *Int J Exp Pathol* 2010; **91**: 350-356 [PMID: 20412338 DOI: 10.1111/j.1365-2613.2010.00714.x]
27 **Ribatti D**, Finato N, Crivellato E, Marzullo A, Mangieri D, Nico B, Vacca A, Beltrami CA. Neovascularization and mast cells with tryptase activity increase simultaneously with pathologic progression in human endometrial cancer. *Am J Obstet Gynecol* 2005; **193**: 1961-1965 [PMID: 16325597 DOI: 10.1016/j.ajog.2005.04.055]
28 **Ranieri G**. Hot topic: targeting tumor angiogenesis: an update. *Curr Med Chem* 2012; **19**: 937 [PMID: 22214460 DOI: 10.2174/092986712799320718]
29 **Saponaro C**, Malfettone A, Ranieri G, Danza K, Simone G, Paradiso A, Mangia A. VEGF, HIF-1α expression and MVD as an angiogenic network in familial breast cancer. *PLoS One* 2013; **8**: e53070 [PMID: 23326384 DOI: 10.1371/journal.pone.0053070]
30 **Ammendola M**, Zuccalà V, Patruno R, Russo E, Luposella M, Amorosi A, Vescio G, Sammarco G, Montemurro S, De Sarro G, Sacco R, Ranieri G. Tryptase-positive mast cells and angiogenesis in keloids: a new possible post-surgical target for prevention. *Updates Surg* 2013; **65**: 53-57 [PMID: 23117746 DOI: 10.1007/s13304-012-0183-y]
31 **Ranieri G**, Coviello M, Chiriatti A, Stea B, Montemurro S, Quaranta M, Dittadi R, Paradiso A. Vascular endothelial growth factor assessment in different blood fractions of gastrointestinal cancer patients and healthy controls. *Oncol Rep* 2004; **11**: 435-439 [PMID: 14719080]
32 **Ranieri G**, Coviello M, Patruno R, Valerio P, Martino D, Milella P, Catalano V, Scotto F, De Ceglie A, Quaranta M, Ribatti D, Pellecchia A. Vascular endothelial growth factor concentrations in the plasma-activated platelets rich (P-APR) of healthy controls and colorectal cancer patients. *Oncol Rep* 2004; **12**: 817-820 [PMID: 15375505]
33 **Mangia A**, Chiriatti A, Ranieri G, Abbate I, Coviello M, Simone G, Zito FA, Montemurro S, Rucci A, Di Leo A, Tommasi S, Berloco P, Xu JM, Paradiso A. H pylori status and angiogenesis factors in human gastric carcinoma. *World J Gastroenterol* 2006; **12**: 5465-5472 [PMID: 17006982]
34 **Ranieri G**, Patruno R, Ruggieri E, Montemurro S, Valerio P, Ribatti D. Vascular endothelial growth factor (VEGF) as a target of bevacizumab in cancer: from the biology to the clinic. *Curr Med Chem* 2006; **13**: 1845-1857 [PMID: 16842197 DOI: 10.2174/092986706777585059]
35 **Miyazaki T**, Okada N, Ishibashi K, Ogata K, Ohsawa T, Ishiguro T, Nakada H, Yokoyama M, Matsuki M, Kato H, Kuwano H, Ishida H. Clinical significance of plasma level of vascular endothelial growth factor-C in patients with colorectal cancer. *Jpn J Clin Oncol* 2008; **38**: 839-843 [PMID: 18923001 DOI: 10.1093/jjco/hyn106]
36 **Werther K**, Christensen IJ, Nielsen HJ. The association between preoperative concentration of soluble vascular endothelial growth factor, perioperative blood transfusion, and survival in patients with primary colorectal cancer. *Eur J Surg* 2001; **167**: 287-292 [PMID: 11354321 DOI: 10.1080/11024150130009148O]
37 **Ranieri G**, Ruggieri E, Falco G, Zizzo N, Mattioli E, Zito AF, Patruno R, Gasparini G. Drug targets to pro-angiogenetic factors with special reference to primary peritoneal mesothelioma. *Endocr Metab Immune Disord Drug Targets* 2006; **6**: 271-277 [PMID: 17017978 DOI: 10.2174/187153006778250028]
38 **Hassan S**, Kinoshita Y, Kawanami C, Kishi K, Matsushima Y, Ohashi A, Funasaka Y, Okada A, Maekawa T, He-Yao W, Chiba T. Expression of protooncogene c-kit and its ligand stem cell factor (SCF) in gastric carcinoma cell lines. *Dig Dis Sci* 1998; **43**: 8-14 [PMID: 9508539 DOI: 10.1023/A:1018851415704]
39 **Ribatti D**, Ranieri G, Basile A, Azzariti A, Paradiso A, Vacca A. Tumor endothelial markers as a target in cancer. *Expert Opin Ther Targets* 2012; **16**: 1215-1225 [PMID: 22978444 DOI: 10.1517/14728222.2012.725047]
40 **Macfarlane SR**, Seatter MJ, Kanke T, Hunter GD, Plevin R. Proteinase-activated receptors. *Pharmacol Rev* 2001; **53**: 245-282 [PMID: 11356985]
41 **Liu Y**, Mueller BM. Protease-activated receptor-2 regulates vascular endothelial growth factor expression in MDA-MB-231 cells via MAPK pathways. *Biochem Biophys Res Commun* 2006; **344**: 1263-1270 [PMID: 16650817 DOI: 10.1016/j.bbrc.2006.04.005]
42 **Taipale J**, Lohi J, Saarinen J, Kovanen PT, Keski-Oja J. Human mast cell chymase and leukocyte elastase release latent transforming growth factor-beta 1 from the extracellular matrix of cultured human epithelial and endothelial cells. *J Biol Chem* 1995; **270**: 4689-4696 [PMID: 7876240 DOI: 10.1074/jbc.270.9.4689]
43 **Gruber BL**, Marchese MJ, Suzuki K, Schwartz LB, Okada Y, Nagase H, Ramamurthy NS. Synovial procollagenase activation by human mast cell tryptase dependence upon matrix metalloproteinase 3 activation. *J Clin Invest* 1989; **84**: 1657-1662 [PMID: 2553780 DOI: 10.1172/JCI114344]
44 **Stack MS**, Johnson DA. Human mast cell tryptase activates single-chain urinary-type plasminogen activator (pro-urokinase). *J Biol Chem* 1994; **269**: 9416-9419 [PMID: 8144524]
45 **Blair RJ**, Meng H, Marchese MJ, Ren S, Schwartz LB, Tonnesen MG, Gruber BL. Human mast cells stimulate vascular tube formation. Tryptase is a novel, potent angiogenic factor. *J Clin Invest* 1997; **99**: 2691-2700 [PMID: 9169499 DOI: 10.1172/JCI119458]
46 **Ribatti D**, Ranieri G, Nico B, Benagiano V, Crivellato E. Tryptase and chymase are angiogenic in vivo in the chorioallantoic membrane assay. *Int J Dev Biol* 2011; **55**: 99-102 [PMID: 21425085 DOI: 10.1387/ijdb.103138dr]
47 **Tomita M**, Matsuzaki Y, Edagawa M, Shimizu T, Hara M, Sekiya R, Onitsuka T. Association of mast cells with tumor angiogenesis in esophageal squamous cell carcinoma. *Dis Esophagus* 2001; **14**: 135-138 [PMID: 11553224 DOI: 10.1046/j.1442-2050.2001.00171.x]
48 **Ammendola M**, Sacco R, Donato G, Zuccalà V, Russo E, Luposella M, Vescio G, Rizzuto A, Patruno R, De Sarro G, Montemurro S, Sammarco G, Ranieri G. Mast cell positivity to tryptase correlates with metastatic lymph nodes in gastrointestinal cancer patients treated surgically. *Oncology* 2013; **85**: 111-116 [PMID: 23887206 DOI: 10.1159/000351145]
49 **Acikalin MF**, Oner U, Topçu I, Yaşar B, Kiper H, Colak E. Tumour angiogenesis and mast cell density in the prognostic assessment of colorectal carcinomas. *Dig Liver Dis* 2005; **37**: 162-169 [PMID: 15888280 DOI: 10.1016/j.dld.2004.09.028]
50 **Gulubova M**, Vlaykova T. Prognostic significance of mast cell number and microvascular density for the survival of patients with primary colorectal cancer. *J Gastroenterol Hepatol* 2009; **24**: 1265-1275 [PMID: 17645466 DOI: 10.1111/j.1440-1746.2007.05009.x]
51 **Peng SH**, Deng H, Yang JF, Xie PP, Li C, Li H, Feng DY. Significance and relationship between infiltrating inflammatory cell and tumor angiogenesis in hepatocellular carcinoma tissues. *World J Gastroenterol* 2005; **11**: 6521-6524 [PMID: 16425427]
52 **Esposito I**, Menicagli M, Funel N, Bergmann F, Boggi U,

Mosca F, Bevilacqua G, Campani D. Inflammatory cells contribute to the generation of an angiogenic phenotype in pancreatic ductal adenocarcinoma. *J Clin Pathol* 2004; **57**: 630-636 [PMID: 15166270 DOI: 10.1136/jcp.2003.014498]

53 **Tuna B**, Yorukoglu K, Unlu M, Mungan MU, Kirkali Z. Association of mast cells with microvessel density in renal cell carcinomas. *Eur Urol* 2006; **50**: 530-534 [PMID: 16426730 DOI: 10.1016/j.eururo.2005.12.040]

54 **Ibaraki T**, Muramatsu M, Takai S, Jin D, Maruyama H, Orino T, Katsumata T, Miyazaki M. The relationship of tryptase- and chymase-positive mast cells to angiogenesis in stage I non-small cell lung cancer. *Eur J Cardiothorac Surg* 2005; **28**: 617-621 [PMID: 16125954 DOI: 10.1016/j.ejcts.2005.06.020]

55 **Carlini MJ**, Dalurzo MC, Lastiri JM, Smith DE, Vasallo BC, Puricelli LI, Lauría de Cidre LS. Mast cell phenotypes and microvessels in non-small cell lung cancer and its prognostic significance. *Hum Pathol* 2010; **41**: 697-705 [PMID: 20040391 DOI: 10.1016/j.humpath.2009.04.029]

56 **Ribatti D**, Ennas MG, Vacca A, Ferreli F, Nico B, Orru S, Sirigu P. Tumor vascularity and tryptase-positive mast cells correlate with a poor prognosis in melanoma. *Eur J Clin Invest* 2003; **33**: 420-425 [PMID: 12760367 DOI: 10.1046/j.1365-2362.2003.01152.x]

57 **Benítez-Bribiesca L**, Wong A, Utrera D, Castellanos E. The role of mast cell tryptase in neoangiogenesis of premalignant and malignant lesions of the uterine cervix. *J Histochem Cytochem* 2001; **49**: 1061-1062 [PMID: 11457936 DOI: 10.1177/002215540104900816]

58 **Ranieri G**, Patruno R, Lionetti A, Di Summa A, Mattioli E, Bufo P, Pellecchia A, Ribatti D, Zizzo N. Endothelial area and microvascular density in a canine non-Hodgkin's lymphoma: an interspecies model of tumor angiogenesis. *Leuk Lymphoma* 2005; **46**: 1639-1643 [PMID: 16236617 DOI: 10.1080/10428190500205150]

59 **Nico B**, Mangieri D, Crivellato E, Vacca A, Ribatti D. Mast cells contribute to vasculogenic mimicry in multiple myeloma. *Stem Cells Dev* 2008; **17**: 19-22 [PMID: 18205547 DOI: 10.1089/scd.2007.0132]

60 **Ribatti D**, Molica S, Vacca A, Nico B, Crivellato E, Roccaro AM, Dammacco F. Tryptase-positive mast cells correlate positively with bone marrow angiogenesis in B-cell chronic lymphocytic leukemia. *Leukemia* 2003; **17**: 1428-1430 [PMID: 12835741 DOI: 10.1038/sj.leu.2402970]

61 **Ribatti D**, Polimeno G, Vacca A, Marzullo A, Crivellato E, Nico B, Lucarelli G, Dammacco F. Correlation of bone marrow angiogenesis and mast cells with tryptase activity in myelodysplastic syndromes. *Leukemia* 2002; **16**: 1680-1684 [PMID: 12200681 DOI: 10.1038/sj.leu.2402586]

62 **Molica S**, Vacca A, Crivellato E, Cuneo A, Ribatti D. Tryptase-positive mast cells predict clinical outcome of patients with early B-cell chronic lymphocytic leukemia. *Eur J Haematol* 2003; **71**: 137-139 [PMID: 12890156 DOI: 10.1034/j.1600-0609.2003.00110.x]

63 **Tomita M**, Matsuzaki Y, Onitsuka T. Correlation between mast cells and survival rates in patients with pulmonary adenocarcinoma. *Lung Cancer* 1999; **26**: 103-108 [PMID: 10568681 DOI: 10.1016/S0169-5002(99)00076-8]

64 **Norrby K**. Mast cells and angiogenesis. *APMIS* 2002; **110**: 355-371 [PMID: 12076253 DOI: 10.1034/j.1600-0463.2002.100501.x]

65 **Erba F**, Fiorucci L, Pascarella S, Menegatti E, Ascenzi P, Ascoli F. Selective inhibition of human mast cell tryptase by gabexate mesylate, an antiproteinase drug. *Biochem Pharmacol* 2001; **61**: 271-276 [PMID: 11172730 DOI: 10.1016/S0006-2952(00)00550-5]

66 **Mori S**, Itoh Y, Shinohata R, Sendo T, Oishi R, Nishibori M. Nafamostat mesilate is an extremely potent inhibitor of human tryptase. *J Pharmacol Sci* 2003; **92**: 420-423 [PMID: 12939527 DOI: 10.1254/jphs.92.420]

67 **Bai Y**, Bandara G, Ching Chan E, Maric I, Simakova O, Bandara SN, Lu WP, Wise SC, Flynn DL, Metcalfe DD, Gilfillan AM, Wilson TM. Targeting the KIT activating switch control pocket: a novel mechanism to inhibit neoplastic mast cell proliferation and mast cell activation. *Leukemia* 2013; **27**: 278-285 [PMID: 22907049 DOI: 10.1038/leu.2012.218]

68 **Patruno R**, Zizzo N, Zito AF, Catalano V, Valerio P, Pellecchia V, D'Errico E, Mazzone F, Ribatti D, Ranieri G. Microvascular density and endothelial area correlate with Ki-67 proliferative rate in the canine non-Hodgkin's lymphoma spontaneous model. *Leuk Lymphoma* 2006; **47**: 1138-1143 [PMID: 16840207 DOI: 10.1080/10428190600565859]

69 **Bloom HJ**, Richardson WW. Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 years. *Br J Cancer* 1957; **11**: 359-377 [PMID: 13499785 DOI: 10.1038/bjc.1957.43]

70 **Crivellato E**, Nico B, Vacca A, Ribatti D. Ultrastructural analysis of mast cell recovery after secretion by piecemeal degranulation in B-cell non-Hodgkin's lymphoma. *Leuk Lymphoma* 2003; **44**: 517-521 [PMID: 12688324 DOI: 10.1080/1042819021000047001]

71 **Crivellato E**, Ribatti D, Mallardi F, Beltrami CA. Granule changes of human and murine endocrine cells in the gastrointestinal epithelia are characteristic of piecemeal degranulation. *Anat Rec* 2002; **268**: 353-359 [PMID: 12420282 DOI: 10.1002/ar.10149]

72 **Ward SM**, McLaren GJ, Sanders KM. Interstitial cells of Cajal in the deep muscular plexus mediate enteric motor neurotransmission in the mouse small intestine. *J Physiol* 2006; **573**: 147-159 [PMID: 16513671 DOI: 10.1113/jphysiol.2006.105189]

73 **Mikkelsen HB**. Interstitial cells of Cajal, macrophages and mast cells in the gut musculature: morphology, distribution, spatial and possible functional interactions. *J Cell Mol Med* 2010; **14**: 818-832 [PMID: 20132411 DOI: 10.1111/j.1582-4934.2010.01025.x]

74 **Hiromatsu Y**, Toda S. Mast cells and angiogenesis. *Microsc Res Tech* 2003; **60**: 64-69 [PMID: 12500262 DOI: 10.1002/jemt.10244]

75 **Gounaris E**, Erdman SE, Restaino C, Gurish MF, Friend DS, Gounari F, Lee DM, Zhang G, Glickman JN, Shin K, Rao VP, Poutahidis T, Weissleder R, McNagny KM, Khazaie K. Mast cells are an essential hematopoietic component for polyp development. *Proc Natl Acad Sci USA* 2007; **104**: 19977-19982 [PMID: 18077429 DOI: 10.1073/pnas.0704620104]

76 **Heijmans J**, Büller NV, Muncan V, van den Brink GR. Role of mast cells in colorectal cancer development, the jury is still out. *Biochim Biophys Acta* 2012; **1822**: 9-13 [PMID: 21146606 DOI: 10.1016/j.bbadis.2010.12.001]

77 **Chichlowski M**, Westwood GS, Abraham SN, Hale LP. Role of mast cells in inflammatory bowel disease and inflammation-associated colorectal neoplasia in IL-10-deficient mice. *PLoS One* 2010; **5**: e12220 [PMID: 20808919 DOI: 10.1371/journal.pone.0012220]

78 **Eaden JA**, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 2001; **48**: 526-535 [PMID: 11247898 DOI: 10.1136/gut.48.4.526]

79 **Lachter J**, Stein M, Lichtig C, Eidelman S, Munichor M. Mast cells in colorectal neoplasias and premalignant disorders. *Dis Colon Rectum* 1995; **38**: 290-293 [PMID: 7882795 DOI: 10.1007/BF02055605]

80 **Ranieri G**, Ria R, Roccaro AM, Vacca A, Ribatti D. Development of vasculature targeting strategies for the treatment of chronic inflammatory diseases. *Curr Drug Targets Inflamm Allergy* 2005; **4**: 13-22 [PMID: 15720230 DOI: 10.2174/1568010053622966]

81 **Kashiwase Y**, Inamura H, Morioka J, Igarashi Y, Kawai-Kowase K, Kurosawa M. Quantitative analysis of mast cells in benign and malignant colonic lesions: immunohistochemical study on formalin-fixed, paraffin-embedded tissues. *Allergol Immunopathol* (Madr) 2008; **36**: 271-276 [PMID: 19080799 DOI: 10.1016/S0301-0546(08)75222-4]

82 **Taweevisit M**. The association of stromal mast cell response and tumor cell differentiation in colorectal cancer. *J Med Assoc Thai* 2006; **89** Suppl 3: S69-S73 [PMID: 17718271]

83 **Xia Q**, Ding Y, Wu XJ, Peng RQ, Zhou Q, Zeng J, Hou JH, Zhang X, Zeng YX, Zhang XS, Chen YB. Mast Cells in Adjacent Normal Colon Mucosa rather than Those in Invasive Margin are Related to Progression of Colon Cancer. *Chin J Cancer Res* 2011; **23**: 276-282 [PMID: 23358806 DOI: 10.1007/s11670-011-0276-z]

84 **Xia Q**, Wu XJ, Zhou Q, Jing-Zeng JH, Pan ZZ, Zhang XS. No relationship between the distribution of mast cells and the survival of stage IIIB colon cancer patients. *J Transl Med* 2011; **9**: 88 [PMID: 21651824 DOI: 10.1186/1479-5876-9-88]

85 **Nielsen HJ**, Hansen U, Christensen IJ, Reimert CM, Brünner N, Moesgaard F. Independent prognostic value of eosinophil and mast cell infiltration in colorectal cancer tissue. *J Pathol* 1999; **189**: 487-495 [PMID: 10629548]

86 **Tan SY**, Fan Y, Luo HS, Shen ZX, Guo Y, Zhao LJ. Prognostic significance of cell infiltrations of immunosurveillance in colorectal cancer. *World J Gastroenterol* 2005; **11**: 1210-1214 [PMID: 15754407]

87 **Elezoğlu B**, Tolunay S. The relationship between the stromal mast cell number, microvessel density, c-erbB-2 staining and survival and prognostic factors in colorectal carcinoma. *Turk Patoloji Derg* 2012; **28**: 110-118 [PMID: 22627628 DOI: 10.5146/tjpath.2012.01109]

88 **Fisher ER**, Paik SM, Rockette H, Jones J, Caplan R, Fisher B. Prognostic significance of eosinophils and mast cells in rectal cancer: findings from the National Surgical Adjuvant Breast and Bowel Project (protocol R-01). *Hum Pathol* 1989; **20**: 159-163 [PMID: 2562788 DOI: 10.1016/0046-8177(89)90180-9]

89 **Yodavudh S**, Tangjitgamol S, Puangsa-art S. Prognostic significance of microvessel density and mast cell density for the survival of Thai patients with primary colorectal cancer. *J Med Assoc Thai* 2008; **91**: 723-732 [PMID: 18672639]

90 **Malfettone A**, Silvestris N, Saponaro C, Ranieri G, Russo A, Caruso S, Popescu O, Simone G, Paradiso A, Mangia A. High density of tryptase-positive mast cells in human colorectal cancer: a poor prognostic factor related to protease-activated receptor 2 expression. *J Cell Mol Med* 2013; **17**: 1025-1037 [PMID: 23991686 DOI: 10.1111/jcmm.12073]

91 **Song YX**, Gao P, Wang ZN, Liang JW, Sun Z, Wang MX, Dong YL, Wang XF, Xu HM. Can the tumor deposits be counted as metastatic lymph nodes in the UICC TNM staging system for colorectal cancer? *PLoS One* 2012; **7**: e34087 [PMID: 22461900 DOI: 10.1371/journal.pone.0034087]

92 **Morikawa T**, Tanaka N, Kuchiba A, Nosho K, Yamauchi M, Hornick JL, Swanson RS, Chan AT, Meyerhardt JA, Huttenhower C, Schrag D, Fuchs CS, Ogino S. Predictors of lymph node count in colorectal cancer resections: data from US nationwide prospective cohort studies. *Arch Surg* 2012; **147**: 715-723 [PMID: 22508672 DOI: 10.1001/archsurg.2012.353]

93 **Liu X**, Cai H, Shi Y, Wang Y. Prognostic factors in patients with node-negative gastric cancer: a single center experience from China. *J Gastrointest Surg* 2012; **16**: 1123-1127 [PMID: 22488657 DOI: 10.1007/s11605-012-1881-y]

94 **Sjo OH**, Merok MA, Svindland A, Nesbakken A. Prognostic impact of lymph node harvest and lymph node ratio in patients with colon cancer. *Dis Colon Rectum* 2012; **55**: 307-315 [PMID: 22469798 DOI: 10.1097/DCR.0b013e3182423f62]

95 **Ducroc R**, Bontemps C, Marazova K, Devaud H, Darmoul D, Laburthe M. Trypsin is produced by and activates protease-activated receptor-2 in human cancer colon cells: evidence for new autocrine loop. *Life Sci* 2002; **70**: 1359-1367 [PMID: 11883712 DOI: 10.1016/S0024-3205(01)01519-3]

96 **Yoshii M**, Jikuhara A, Mori S, Iwagaki H, Takahashi HK, Nishibori M, Tanaka N. Mast cell tryptase stimulates DLD-1 carcinoma through prostaglandin- and MAP kinase-dependent manners. *J Pharmacol Sci* 2005; **98**: 450-458 [PMID: 16093613 DOI: 10.1254/jphs.FPJ05002X]

97 **Jikuhara A**, Yoshii M, Iwagaki H, Mori S, Nishibori M, Tanaka N. MAP kinase-mediated proliferation of DLD-1 carcinoma by the stimulation of protease-activated receptor 2. *Life Sci* 2003; **73**: 2817-2829 [PMID: 14511767 DOI: 10.1016/S0024-3205(03)00702-1]

98 **Sendo T**, Sumimura T, Itoh Y, Goromaru T, Aki K, Yano T, Oike M, Ito Y, Mori S, Nishibori M, Oishi R. Involvement of proteinase-activated receptor-2 in mast cell tryptase-induced barrier dysfunction in bovine aortic endothelial cells. *Cell Signal* 2003; **15**: 773-781 [PMID: 12781870 DOI: 10.1016/S0898-6568(03)00014-7]

99 **Voltz JW**, Weinman EJ, Shenolikar S. Expanding the role of NHERF, a PDZ-domain containing protein adapter, to growth regulation. *Oncogene* 2001; **20**: 6309-6314 [PMID: 11607833 DOI: 10.1038/sj.onc.1204774]

100 **Mangia A**, Saponaro C, Malfettone A, Bisceglie D, Bellizzi A, Asselti M, Popescu O, Reshkin SJ, Paradiso A, Simone G. Involvement of nuclear NHERF1 in colorectal cancer progression. *Oncol Rep* 2012; **28**: 889-894 [PMID: 22766563 DOI: 10.3892/or.2012.1895]

101 **De Luisi A**, Ferrucci A, Coluccia AM, Ria R, Moschetta M, de Luca E, Pieroni L, Maffia M, Urbani A, Di Pietro G, Guarini A, Ranieri G, Ditonno P, Berardi S, Caivano A, Basile A, Cascavilla N, Capalbo S, Quarta G, Dammacco F, Ribatti D, Vacca A. Lenalidomide restrains motility and overangiogenic potential of bone marrow endothelial cells in patients with active multiple myeloma. *Clin Cancer Res* 2011; **17**: 1935-1946 [PMID: 21307145 DOI: 10.1158/1078-0432.CCR-10-2381]

102 **Basile A**, Moschetta M, Ditonno P, Ria R, Marech I, De Luisi A, Berardi S, Frassanito MA, Angelucci E, Derudas D, Specchia G, Curci P, Pavone V, Rossini B, Ribatti D, Bottazzi B, Mantovani A, Presta M, Dammacco F, Vacca A. Pentraxin 3 (PTX3) inhibits plasma cell/stromal cell cross-talk in the bone marrow of multiple myeloma patients. *J Pathol* 2013; **229**: 87-98 [PMID: 22847671 DOI: 10.1002/path.4081]

103 **Ranieri G**, Mammì M, Donato Di Paola E, Russo E, Gallelli L, Citraro R, Gadaleta CD, Marech I, Ammendola M, De Sarro G. Pazopanib a tyrosine kinase inhibitor with strong anti-angiogenetic activity: a new treatment for metastatic soft tissue sarcoma. *Crit Rev Oncol Hematol* 2014; **89**: 322-329 [PMID: 24041629]

104 **Gnoni A**, Marech I, Silvestris N, Vacca A, Lorusso V. Dasatinib: an anti-tumour agent via Src inhibition. *Curr Drug Targets* 2011; **12**: 563-578 [PMID: 21226671 DOI: 10.2174/138945011794751591]

105 **Yang XP**, Li Y, Wang Y, Wang Y, Wang P. beta-Tryptase upregulates vascular endothelial growth factor expression via proteinase-activated receptor-2 and mitogen-activated protein kinase pathways in bone marrow stromal cells in acute myeloid leukemia. *Leuk Lymphoma* 2010; **51**: 1550-1558 [PMID: 20578818 DOI: 10.3109/10428194.2010.496013]

106 **Zhang H**, Wu J, Meng L, Shou CC. Expression of vascular endothelial growth factor and its receptors KDR and Flt-1 in gastric cancer cells. *World J Gastroenterol* 2002; **8**: 994-998 [PMID: 12439912]

107 **Passantino L**, Passantino G, Cianciotta A, Ribaud MR, Lo Presti G, Ranieri G, Perillo A. Expression of proto-oncogene C-kit and correlation with morphological evaluations in canine cutaneous mast cell tumors. *Immunopharmacol Immunotoxicol* 2008; **30**: 609-621 [PMID: 18608529 DOI: 10.1080/08923970801949265]

108 **Ranieri G**, Gadaleta CD, Patruno R, Zizzo N, Daidone MG, Hansson MG, Paradiso A, Ribatti D. A model of study for human cancer: Spontaneous occurring tumors in dogs. Biological features and translation for new anticancer therapies. *Crit Rev Oncol Hematol* 2013; **88**: 187-197 [PMID: 23561333 DOI: 10.1016/j.critrevonc.2013.03.005]

109 **Ranieri G**, Passantino L, Patruno R, Passantino G, Jirillo F, Catino A, Mattioli V, Gadaleta C, Ribatti D. The dog mast

cell tumour as a model to study the relationship between angiogenesis, mast cell density and tumour malignancy. *Oncol Rep* 2003; **10**: 1189-1193 [PMID: 12883679]
110 **Ranieri G**, Pantaleo M, Piccinno M, Roncetti M, Mutinati M, Marech I, Patruno R, Rizzo A, Sciorsci RL. Tyrosine kinase inhibitors (TKIs) in human and pet tumours with special reference to breast cancer: a comparative review. *Crit Rev Oncol Hematol* 2013; **88**: 293-308 [PMID: 23768779]

P- Reviewers: Chen JL, Huang ZH, Stanojevic GZ
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8921

World J Gastroenterol 2014 July 21; 20(27): 8921-8927
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Which strategy after first-line therapy in advanced colorectal cancer?

Coinu Andrea, Petrelli Fausto, Borgonovo Karen Francesca, Cabiddu Mary, Ghilardi Mara, Lonati Veronica, Barni Sandro

Coinu Andrea, Petrelli Fausto, Borgonovo Karen Francesca, Cabiddu Mary, Ghilardi Mara, Lonati Veronica, Barni Sandro, Medical Oncology Unit, Oncology Department, Azienda Ospedaliera di Treviglio, Treviglio, 24047 BG, Italy

Author contributions: All authors contributed equally to the acquisition of data, drafting of the article and final approval of the version to be published.

Correspondence to: Coinu Andrea, MD, Medical Oncology Unit, Oncology Department, Azienda Ospedaliera di Treviglio, Piazzale Ospedale 1, Treviglio, 24047 BG, Italy. artefice2003@hotmail.com
Telephone: +39-363-424303 Fax: +39-363-424380
Received: September 29, 2013 Revised: December 31, 2013
Accepted: April 1, 2014
Published online: July 21, 2014

Abstract

Second-line therapy for advanced colorectal cancer is an integral part of the treatment strategy that needs to be set from the beginning for each patient, bearing in mind the expected toxicities of chosen treatments, the patient's clinical condition, comorbidities, preferences, the aims of the treatment and the molecular status. Furthermore, the distinction between lines of therapy is no longer absolute. The perspective of "continuum of care" includes switching chemotherapy prior to disease progression, maintenance therapy, drug "holidays" if needed, surgical resection of metastases in selected patients, and seems to allow a tailored treatment, in which patients are more likely to benefit from exposure to all active agents, which is known to correlate with overall survival. The scenario of second-line treatment has changed dramatically over the years and could currently benefit from several options including chemotherapy with a single agent or in combination and the addition of molecular-targeted agents developed in the last decade, such as epidermal growth factor receptor antibodies (cetuximab, panitumumab) and vascular endothelial growth factor-targeting agents (bevacizumab, aflibercept), with the possibility of bevacizumab use even beyond first progression. The purpose of this review is to summarize the most important scientific data supporting the use of chemotherapy and the new biologic agents in the second-line setting in advanced colorectal cancer.

Key words: Advanced colorectal cancer; Second-line; Targeted agents; Polychemotherapy; Overall survival

Core tip: This is a review of the current literature on second-line options in advanced colorectal cancer. This review was performed to analyse the different possible choices in this setting and the best strategies to treat patients with all available active drugs.

Andrea C, Fausto P, Francesca BK, Mary C, Mara G, Veronica L, Sandro B. Which strategy after first-line therapy in advanced colorectal cancer? *World J Gastroenterol* 2014; 20(27): 8921-8927 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8921.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8921

INTRODUCTION

Second-line therapy is an integral part of the treatment strategy for advanced colorectal cancer (aCRC). The availability of all active agents has been beneficial in patients with this disease, with an overall survival (OS) rate directly correlated to the number of agents the patients are exposed to[1]. Recently, the introduction of agents targeting the angiogenic and epidermal growth factor receptor (EGFR) pathways, including bevacizumab, aflibercept, regorafenib, cetuximab and panitumumab,

have expanded treatment options, particularly for pretreated diseases and upfront treatment[2-8]. The correct course of treatment, from the first-line to later lines of treatment, has yet to be validated, but the prudent application of all active drugs in the treatment strategy should be designed for each patient with advanced CRC. The strategy should be modified according to the site of disease, endpoint of cure, age, performance status and comorbidities.

Treatment options in pretreated patients depend on different parameters. First, the choice of second-line therapy is linked to the first-line agents the patients have been exposed to. If irinotecan was the agent of choice (*e.g.* FOLEIRI alone or in combination), oxaliplatin + 5-fluorouracil (*e.g.* FOLFOX) will be the cornerstone of treatment for the second-line.

Toxicity parameters, patient condition and preferences are alternative modes of choosing treatments. In the presence of disseminated disease, in poor performance status patients, and where palliation of symptoms and prolongation of progression-free survival (PFS) are the main endpoints, a sequence of single agents is a concrete alternative to second-line poly-chemotherapy. Two trials addressed this issue: the FOCUS and CAIRO trials. In both trials, the OS was similar in both study groups (upfront and second-line combinations *vs* single agents in both the first- and second-lines). However, in both studies, the median OS in the single agent groups was lower than expected (13.9 and 16.3 mo, respectively) with the modern agents now available. Conversely, a more intensive schedule, including biologic agents, has shown a benefit when adopted in combination with standard doublets. For example, the addition of panitumumab and aflibercept to FOLFIRI improved the outcomes in the oxaliplatin-pretreated population[3,7]. In second- or further lines of treatment, the influence of subsequent therapies is less pronounced; therefore, an OS benefit is more likely to be demonstrated. This is the reason why first-line agents confer little or nonsignificant gains in median OS. In this trial, the post-progression survival, that is the survival after first progression until death, has an enormous influence on the OS with first-line treatment[9]. This confirms that second-lines and beyond have increasing relevance on the overall treatment strategy.

With the current state-of-the-art CRC treatments, the advent of new agents, and the different treatment options that are now available, we have performed a review of the current literature to discuss the different options for patients with metastatic CRC who have failed first-line treatment.

SECOND-LINE TREATMENT: CHOICE OF CHEMOTHERAPY

The treatment strategy in advanced colorectal cancer has changed over the years, due (especially) to the availability of several new drugs which are able to double the average median survival rate, compared to the era in which 5-fluorouracil (5-FU) was the only active agent. Furthermore, the model of distinct lines of chemotherapy, in which regimens containing non cross-resistant drugs are used in progressive disease, is debated. A continuum of care approach has been proposed, and seems to allow a more tailored treatment. This approach includes switching chemotherapy prior to disease progression, maintenance therapy, drug holidays, and surgical resection of metastases in selected patients. With this approach, the distinction between lines of therapy is no longer absolute. This model emphasizes the importance of an individualized treatment strategy in aCRC, in which patients are given the opportunity to benefit from exposure to all active agents that correlate with OS[10].

Regardless of age, patients with a poor performance status (PS) (*e.g.* Eastern Cooperative Oncology Group PS $\geqslant$ 2 or Karnofsky PS $<$ 60) usually tolerate chemotherapy poorly and have a poor short-term prognosis.

Some patients, especially those whose PS decline is not cancer related, will be treated with monotherapy, often with LV-modulated 5-FU or capecitabine.

As a single agent, irinotecan has shown clinical benefits after 5-FU failure in patients with aCRC. In a randomized trial, 279 patients with 5-FU-refractory disease were randomly assigned, with a 2:1 ratio, to irinotecan with best supportive care (BSC) (189 patients) or to BSC alone (90 patients)[11]. The OS rate was significantly better in the irinotecan group (P = 0.0001), with a 1-year survival of 36.2% *vs* 13.8% in the BSC group. The quality-of-life analysis (except the diarrhoea score) also favoured irinotecan.

Conversely, oxaliplatin alone in the second-line setting has shown a low level of activity. In a randomized phase Ⅲ trial, 423 patients with metastatic colorectal cancer who progressed after IFL (irinotecan, fluorouracil, and leucovorin) therapy were randomly assigned to bolus and infusional FU and leucovorin (LV5FU2), single-agent oxaliplatin, or the combination (FOLFOX4). FOLFOX4 proved to be superior to LV5FU2 in all measures of clinical efficacy (objective response rate, time to tumour progression, and alleviation of tumour-related symptoms); however, single-agent oxaliplatin was not superior to LV5FU2 in any measure of efficacy, with an objective response rate of 0%[12].

The sequential use of active single agents, rather than combination regimens, has been proposed to reduce the overall toxicity of therapy, maintaining the same outcome in terms of survival.

The European FOCUS and CAIRO trials addressed the issue of initial combination *vs* single agent therapy.

In the FOCUS trial, 2135 non-pretreated patients were randomly assigned to three treatment strategies in the ratio of 1:1:1. Strategy A was single-agent fluorouracil until failure, then single-agent irinotecan. Strategy B was fluorouracil until failure, then combination chemotherapy (FOLFOX or FOLFIRI). Strategy C was upfront combination therapy. The OS (primary endpoint) of the patients allocated to Strategy A was 13.9 mo. The

median survival for each of the other groups was longer (15 mo for Strategy B, 16.4 mo for Strategy C). Only the comparison of initial FOLFIRI *vs* sequential single agent therapy was statistically significant (median survival: 16.7 *vs* 13.9 mo). This trial showed that sequential single agent therapy did not compromise overall survival, and initial single-agent treatment upgraded to combination when required was not worse than the first-line combination, and could be an option to consider.

The trial allowed the use of FOLFOX or FOLFIRI as the third-line treatment, but only 23% of all patients received all three active agents, with a higher rate for patients allocated to Strategy C than for Strategy A (33% *vs* 16%)[13].

In the CAIRO trial, 820 patients with aCRC were randomized to receive either first-line treatment with capecitabine, second-line irinotecan, or third-line capecitabine plus oxaliplatin (sequential treatment; n = 410), or first-line treatment with capecitabine plus irinotecan and second-line treatment with capecitabine plus oxaliplatin (combination treatment; n = 410). The median OS (primary endpoint) was similar for the sequential *vs* initial combination therapy (16.3 *vs* 17.4 mo) and PFS was superior with combination therapy. The XELIRI regimen was affected by a higher rate of grade 3-4 diarrhoea, and almost half of the patients starting with this combination did not receive second-line chemotherapy[14].

As in the FOCUS trial, in the CAIRO study the proportion of patients treated with a sequential strategy who eventually received all three drugs (19% in FOCUS and 36% in CAIRO) was lower when compared with patients treated with a combination regimen (33% in FOCUS and 55% in CAIRO). These data support the hypothesis that patients receiving first-line combination therapy are more likely to receive all three active agents during the course of their disease than those who initiate treatment with a single agent[15].

Thus, in fit patients, a reasonable first-line with a combination doublet (FOLFOX, XELOX, or FOLFIRI), and the choice of chemotherapy regimen, will be driven by the expected toxicity profile and by the patient' s preference. The second-line treatment should be linked with the first-line choice, and FOLFIRI (or irinotecan alone) will follow a first-line with an oxaliplatin-based regimen. Conversely, it seems logical to use FOLFOX in the second-line treatment if the irinotecan-based regimen has been previously chosen.

Anti-EGFR in the second-line setting

Cetuximab is a mouse/human chimeric monoclonal antibody, which binds the EGFR, competitively inhibiting ligand binding, and inducing receptor dimerization and internalization. In combination with irinotecan, after first-line fluoropyrimidine and oxaliplatin treatment failure, it was shown to improve the PFS and response rates in the multicentre, open-label, phase Ⅲ EPIC trial. In this study, 1298 patients, previously treated during first-line therapy with a fluoropyrimidine and oxaliplatin, were randomly assigned to cetuximab plus irinotecan, or irinotecan alone. The median OS (primary endpoint) was similar between the two arms of the study: 10.7 mo (95%CI: 9.6-11.3) with cetuximab/irinotecan and 10.0 mo (95%CI: 9.1-11.3) with irinotecan alone (HR = 0.975; 95%CI: 0.854-1.114; P = 0.71).

However, cetuximab combined with irinotecan significantly improved the PFS (median, 4.0 *vs* 2.6 mo; HR = 0.692; 95%CI: 0.617-0.776; $P \leqslant 0.0001$) and RR (16.4% *vs* 4.2%; $P < 0.0001$), and was associated with better scores in the QOL analysis of global health status (P = 0.047). The lack of difference in terms of survival could be explained by the fact that almost 47% of the patients assigned to irinotecan eventually received cetuximab. The addition of cetuximab to irinotecan did not result in meaningful increases in toxicity, with the exception of acneiform rash, diarrhoea, and electrolyte imbalances[16].

The KRAS mutation status was retrospectively obtained in only 23% of the randomized patients. In the small subset of patients with wild-type KRAS tumours (15% of the whole population), the PFS was longer when cetuximab was added to irinotecan, but the RR and OS were similar[17].

The activity of cetuximab + chemotherapy has been reported in a pooled analysis by Barni *et al*[18], which included 1712 KRAS wild-type patients. The overall response rate was 31.9%, with similar response rates of 28.7% for the second-line treatment and 31.1% for the third- or further lines. The overall weighted median OS and PFS were 12.5 and 6 mo, with a weighted OS of 11.56 and 12.2 mo for the second- and further line CRC settings, respectively.

Panitumumab is a fully human monoclonal antibody directed against the EGFR gene. As a single agent, it has been shown to prolong PFS in patients who had progressed after standard chemotherapy (5-FU, irinotecan and oxaliplatin)[2].

In the second-line setting, the efficacy of the combination panitumumab-FOLFIRI was evaluated in a phase Ⅲ trial, in which 1186 patients were randomly assigned to receive panitumumab plus FOLFIRI, *vs* FOLFIRI alone. The patient subgroups on the basis of KRAS status were considered. In the wild-type KRAS subpopulation, the combination FOLFIRI-panitumumab resulted in a significant improvement in PFS (5.9 mo for panitumumab-FOLFIRI *vs* 3.9 mo for FOLFIRI, HR = 0.73; 95%CI: 0.59-0.90; P = 0.004). A non-significant trend toward increased OS was observed, and the median OS was 14.5 mo *vs* 12.5 mo, respectively (HR = 0.85, 95%CI: 0.70-1.04; P = 0.12). One acceptable hypothesis to explain the discrepancy between the PFS and OS is crossover in the chemotherapy alone study groups. The response rate was improved from 10% to 35% with the addition of panitumumab. In patients with mutated KRAS, there was no difference in efficacy. In terms of safety, there were differences in the incidence of skin toxicity and hypomagnesaemia between the panitumumab and the control arms of the study (37% *vs* 2% and

3% *vs* < 1%, respectively), and a 4% increase in grade 3 to 4 events such as diarrhoea, due to the overlapping toxicities between the EGFR inhibitor and irinotecan regimen[3].

The phase Ⅲ PICCOLO trial investigated the potential benefits of adding panitumumab to irinotecan in patients with aCRC, progressing after fluoropyrimidine treatment with or without oxaliplatin. Four hundred and sixty patients, including KRAS wild-type and those who had not been treated previously with EGFR targeting agents, were randomized to irinotecan alone or irinotecan-panitumumab (IrPan group). There was no difference in OS between the groups (HR = 1.01; 95%CI: 0.83-1.23; P = 0.91), but the patients in the IrPan group had longer PFS rates (HR = 0.78; 95%CI: 0.64-0.95; P = 0.015) and a greater response rate (34% *vs* 12%; P < 0.0001) than patients in the irinotecan group[19].

In KRAS wild-type patients who had not previously been exposed to a first-line treatment with a combination containing an anti-EGFR, or who had been treated with chemotherapy plus bevacizumab, an option is to add an anti-EGFR to chemotherapy (with panitumumab, in second-line, which is only approved in combination with FOLFIRI).

Anti-VEGF in the second-line setting

The efficacy of bevacizumab, in combination with the FOLFOX4 regimen in previously treated aCRC patients, was proven in a randomized phase Ⅲ trial in which 829 patients pretreated with fluoropyrimidine and irinotecan were randomly assigned to receive oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) with or without bevacizumab, or bevacizumab alone.

The median OS (primary endpoint) for the group treated with FOLFOX4 and bevacizumab was 12.9 mo, compared with 10.8 mo for the group treated with FOLFOX4 alone (HR for death = 0.75; P < 0.0011), and 10.2 mo for those treated with bevacizumab alone. Furthermore, the combination of bevacizumab and FOLFOX4 resulted in a statistically significant improvement in PFS, when compared with those treated with chemotherapy alone (7.3 *vs* 4.7 mo; HR for progression = 0.61, P < 0.0001). FOLFOX4 plus bevacizumab was associated with a higher incidence of hypertension, bleeding, vomiting and neuropathy when compared with the doublet alone[8].

There are no randomized trials comparing irinotecan-based chemotherapy (FOLFIRI), with or without bevacizumab, as a second-line therapy in patients pretreated with first-line FOLFOX. A pooled analysis of published trials (11 publications, 435 patients) showed a pooled response rate of 26%, with median PFS and OS of 8.3 and 17.2 mo, respectively. This analysis shows that FOLFIRI-bevacizumab is a reasonable and effective option for aCRC pretreated with oxaliplatin, and not exposed to bevacizumab in first-line therapy[20].

Patients treated in the first-line with a chemotherapy regimen including an anti-EGFR, or patients with a KRAS mutation could, therefore, benefit from bevacizumab in second-line therapy.

For patients treated with a first-line bevacizumab-containing chemotherapy regimen, the continuation of bevacizumab beyond the first progression with a second-line fluoropyrimidine-based chemotherapy can be considered, particularly for those with a KRAS mutation, who would not benefit from the use of an EGFR-targeted therapy.

The Bevacizumab Regimens' Investigation of Treatment Effects (BRiTE) study was a large, observational, bevacizumab treatment study in which baseline characteristics, bevacizumab-related adverse events, and effectiveness data were collected from 1953 metastatic colorectal cancer (mCRC) patients who were receiving first-line treatment including bevacizumab. One-thousand four-hundred and forty-five of the 1953 patients with mCRC who were enrolled in the BRiTE study, and who experienced disease progression, were classified into three groups: no post-progression treatment (n = 253), post-progression treatment without bevacizumab (n = 531), and post-progression treatment with bevacizumab (n = 642). The median OS was 25.1 mo (95%CI: 23.4-27.5 mo), and median PFS was 10.0 mo in the overall BRiTE population. In multivariate analyses, the use of bevacizumab beyond progression, compared with a second-line treatment not containing bevacizumab, was strongly and independently associated with improved survival (HR = 0.48; P < .001) with a median OS of 31.8 mo. Hypertension that required medication was the only bevacizumab-related side effect that occurred more frequently in the group of patients treated with a bevacizumab-containing regimen beyond progression (24.6% *vs* 19.2%)[21].

Other data supporting the use of bevacizumab beyond progression came from the ARIES study, a community-based observational cohort study that evaluated the effectiveness and safety of first-line treatment patterns. An analysis of 1074 patients, who progressed after first-line bevacizumab-containing treatments, evaluated 390 patients who were treated in second-line with an irinotecan-based regimen ± bevacizumab, and 114 patients treated with an oxaliplatin-based therapy, (always as second-line) ± bevacizumab. In patients receiving second-line irinotecan or oxaliplatin-based chemotherapy, the post-progression survival in the bevacizumab-treated patients was longer when compared to those not on bevacizumab (HR = 0.52, 0.40-0.67, for irinotecan-based + bevacizumab; HR = 0.5, 0.23-1.14, for oxaliplatin-based + bevacizumab)[22].

In a more recent, open-label phase 3 study, 409 patients affected by mCRC, progressing up to 3 mo after discontinuing first-line bevacizumab plus chemotherapy, were randomly assigned in a 1:1 ratio to second-line chemotherapy with or without bevacizumab at 2.5 mg/kg per week. The median OS was 11.2 mo (95%CI: 10.4-12.2) for bevacizumab plus chemotherapy and 9.8 mo (8.9-10.7) for chemotherapy alone (HR = 0.81; 95%CI: 0.69-0.94; unstratified log-rank test, P = 0.0062),

showing that continuing VEGF inhibition with bevacizumab, plus standard second-line chemotherapy beyond disease progression, has clinical benefits in patients with mCRC[23].

Additional data supporting the use of bevacizumab beyond progression comes from the phase Ⅲ BEBYP trial by the Gruppo Oncologico Nord Ovest. In this study (results presented at ASCO 2013), 184 patients with aCRC treated in the first-line with bevacizumab plus fluoropyrimidine, FOLFIRI, FOLFOX or FOLFOXIRI were randomized to receive FOLFOX6 or FOLFIRI in the second-line, with or without bevacizumab. After a median follow-up of 22 mo, an improvement in the PFS for the study arm with bevacizumab was confirmed (5.2 mo *vs* 6.7 mo; HR = 0.66; 95%CI: 0.49-0.90; unstratified $P = 0.0072$). The benefit in terms of PFS was consistent in all subgroups considered[24].

An alternative option in the second-line for patients with mCRC, which is resistant to or has progressed following an oxaliplatin-containing regimen, is the combination of aflibercept and FOLFIRI. Aflibercept is a fusion protein with key domains for human VEGF receptors 1 and 2 with human IgG Fc[25] that blocks all human VEGF-A isoforms, VEGF-B and placental growth factor[26].

The approval of aflibercept in combination with FOLFIRI in this setting of patients was based on the placebo-controlled VELOUR trial, in which 1226 patients with mCRC that had progressed during or within six mo of receiving oxaliplatin-containing chemotherapy, with or without bevacizumab, were randomized to FOLFIRI with aflibercept (4 mg/kg Ⅳ) or placebo every two weeks until progression. Median OS was significantly longer in patients treated with aflibercept (13.5 *vs* 12.1 mo) as was the median PFS (6.9 *vs* 4.7 mo)[7]. The improvement in OS was consistent, regardless of prior treatments with bevacizumab.

CONCLUSION

In recent years, the introduction of new active drugs, and the development of new strategies in the management of advanced colorectal cancer, have enriched the second-line setting with several options that allow a more personalized treatment, with the perspective of treating the patient with all active agents available (obviously considering expected toxicities).

Fit patients seem more likely to be treated with all active drugs, starting in the first-line with a combination therapy (irinotecan or oxaliplatin-based). Regarding the chemotherapy backbone, a switch to an irinotecan-based treatment (in cases where the patient has been treated with an oxaliplatin-based regimen in the first-line) is, in general, the best choice; however, following FOLFOX failure, irinotecan and FOLFIRI are appropriate options. In the context of anti-angiogenic drugs, bevacizumab is approved in combination with a fluoropyrimidine-based regimen, and is an option to consider in the second-line, even beyond the first progression following a previous bevacizumab-containing therapy; additionally, aflibercept is a new option in combination with FOLFIRI. In KRAS patients, panitumumab can be used in the second-line in combination with FOLFIRI with patients who have received first-line fluoropyrimidine-based chemotherapy (excluding irinotecan). Cetuximab is indicated in combination with irinotecan-based chemotherapy, and in combination with irinotecan alone in those patients who have failed a previous treatment with an irinotecan-based regimen. No clear superiority between targeted agents in the second-line has been shown, as reported by the SPIRITT trial. This was a randomized phase Ⅱ study, evaluating panitumumab + FOLFIRI *vs* bevacizumab + FOLFIRI in patients with wild-type KRAS mCRC previously treated with a bevacizumab + oxaliplatin-based chemotherapy in the first-line. In this trial there was no difference in the OS and PFS between the 2 treatment arms[27].

With so many options to consider, one cannot definitively state which is the best second-line treatment choice. The ongoing COMETS study, a GISCAD trial, is attempting to clarify this point by comparing two different sequences of therapy, irinotecan/cetuximab followed by FOLFOX-4 *vs* FOLFOX-4 followed by irinotecan/cetuximab, in mCRC patients treated with FOLFIRI/bevacizumab as the first-line chemotherapy.

The best strategy is presently unknown. A tailored sequence of treatments should be planned and proposed to patients by the beginning of the first-line therapy. With this in mind, the COMETS trial was designed. From the discussions between patients and physicians, bearing in mind the objective of the treatment and patient performance status and preference, the choice of second-line therapy will be decided, as in other phases of the disease history.

REFERENCES

1 **Grothey A**, Sargent D, Goldberg RM, Schmoll HJ. Survival of patients with advanced colorectal cancer improves with the availability of fluorouracil-leucovorin, irinotecan, and oxaliplatin in the course of treatment. *J Clin Oncol* 2004; **22**: 1209-1214 [PMID: 15051767 DOI: 10.1200/JCO.2004.11.037]

2 **Van Cutsem E**, Peeters M, Siena S, Humblet Y, Hendlisz A, Neyns B, Canon JL, Van Laethem JL, Maurel J, Richardson G, Wolf M, Amado RG. Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. *J Clin Oncol* 2007; **25**: 1658-1664 [PMID: 17470858 DOI: 10.1200/JCO.2006.08.1620]

3 **Peeters M**, Price TJ, Cervantes A, Sobrero AF, Ducreux M, Hotko Y, André T, Chan E, Lordick F, Punt CJ, Strickland AH, Wilson G, Ciuleanu TE, Roman L, Van Cutsem E, Tzekova V, Collins S, Oliner KS, Rong A, Gansert J. Randomized phase III study of panitumumab with fluorouracil, leucovorin, and irinotecan (FOLFIRI) compared with FOLFIRI alone as second-line treatment in patients with metastatic colorectal cancer. *J Clin Oncol* 2010; **28**: 4706-4713 [PMID: 20921462 DOI: 10.1200/JCO.2009.27.6055]

4 **Cunningham D**, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C,

Chau I, Van Cutsem E. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* 2004; **351**: 337-345 [PMID: 15269313]

5 **Jonker DJ**, O'Callaghan CJ, Karapetis CS, Zalcberg JR, Tu D, Au HJ, Berry SR, Krahn M, Price T, Simes RJ, Tebbutt NC, van Hazel G, Wierzbicki R, Langer C, Moore MJ. Cetuximab for the treatment of colorectal cancer. *N Engl J Med* 2007; **357**: 2040-2048 [PMID: 18003960 DOI: 10.1056/NEJMoa033025]

6 **Grothey A**, Van Cutsem E, Sobrero A, Siena S, Falcone A, Ychou M, Humblet Y, Bouché O, Mineur L, Barone C, Adenis A, Tabernero J, Yoshino T, Lenz HJ, Goldberg RM, Sargent DJ, Cihon F, Cupit L, Wagner A, Laurent D. Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT): an international, multicentre, randomised, placebo-controlled, phase 3 trial. *Lancet* 2013; **381**: 303-312 [PMID: 23177514 DOI: 10.1016/S0140-6736(12)61900-X]

7 **Van Cutsem E**, Tabernero J, Lakomy R, Prenen H, Prausová J, Macarulla T, Ruff P, van Hazel GA, Moiseyenko V, Ferry D, McKendrick J, Polikoff J, Tellier A, Castan R, Allegra C. Addition of aflibercept to fluorouracil, leucovorin, and irinotecan improves survival in a phase III randomized trial in patients with metastatic colorectal cancer previously treated with an oxaliplatin-based regimen. *J Clin Oncol* 2012; **30**: 3499-3506 [PMID: 22949147 DOI: 10.1200/JCO.2012.42.8201]

8 **Giantonio BJ**, Catalano PJ, Meropol NJ, O'Dwyer PJ, Mitchell EP, Alberts SR, Schwartz MA, Benson AB, Eastern Cooperative Oncology Group Study E3200. Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. *J Clin Oncol* 2007; **25**: 1539-1544 [PMID: 17442997 DOI: 10.1200/JCO.2006.09.6305]

9 **Petrelli F**, Barni S. Correlation of progression-free and post-progression survival with overall survival in advanced colorectal cancer. *Ann Oncol* 2013; **24**: 186-192 [PMID: 22898038 DOI: 10.1093/annonc/mds289]

10 **Goldberg RM**, Rothenberg ML, Van Cutsem E, Benson AB, Blanke CD, Diasio RB, Grothey A, Lenz HJ, Meropol NJ, Ramanathan RK, Becerra CH, Wickham R, Armstrong D, Viele C. The continuum of care: a paradigm for the management of metastatic colorectal cancer. *Oncologist* 2007; **12**: 38-50 [PMID: 17227899 DOI: 10.1634/theoncologist.12-1-38]

11 **Cunningham D**, Pyrhönen S, James RD, Punt CJ, Hickish TF, Heikkila R, Johannesen TB, Starkhammar H, Topham CA, Awad L, Jacques C, Herait P. Randomised trial of irinotecan plus supportive care versus supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer. *Lancet* 1998; **352**: 1413-1418 [PMID: 9807987 DOI: 10.1016/S0140-6736(98)02309-5]

12 **Rothenberg ML**, Oza AM, Bigelow RH, Berlin JD, Marshall JL, Ramanathan RK, Hart LL, Gupta S, Garay CA, Burger BG, Le Bail N, Haller DG. Superiority of oxaliplatin and fluorouracil-leucovorin compared with either therapy alone in patients with progressive colorectal cancer after irinotecan and fluorouracil-leucovorin: interim results of a phase III trial. *J Clin Oncol* 2003; **21**: 2059-2069 [PMID: 12775730 DOI: 10.1200/JCO.2003.11.126]

13 **Seymour MT**, Maughan TS, Ledermann JA, Topham C, James R, Gwyther SJ, Smith DB, Shepherd S, Maraveyas A, Ferry DR, Meade AM, Thompson L, Griffiths GO, Parmar MK, Stephens RJ. Different strategies of sequential and combination chemotherapy for patients with poor prognosis advanced colorectal cancer (MRC FOCUS): a randomised controlled trial. *Lancet* 2007; **370**: 143-152 [PMID: 17630037 DOI: 10.1016/S0140-6736(07)61087-3]

14 **Koopman M**, Antonini NF, Douma J, Wals J, Honkoop AH, Erdkamp FL, de Jong RS, Rodenburg CJ, Vreugdenhil G, Loosveld OJ, van Bochove A, Sinnige HA, Creemers GJ, Tesselaar ME, Slee PH, Werter MJ, Mol L, Dalesio O, Punt CJ. Sequential versus combination chemotherapy with capecitabine, irinotecan, and oxaliplatin in advanced colorectal cancer (CAIRO): a phase III randomised controlled trial. *Lancet* 2007; **370**: 135-142 [PMID: 17630036]

15 **Braun MS**, Seymour MT. Balancing the efficacy and toxicity of chemotherapy in colorectal cancer. *Ther Adv Med Oncol* 2011; **3**: 43-52 [PMID: 21789155 DOI: 10.1177/1758834010388342]

16 **Sobrero AF**, Maurel J, Fehrenbacher L, Scheithauer W, Abubakr YA, Lutz MP, Vega-Villegas ME, Eng C, Steinhauer EU, Prausova J, Lenz HJ, Borg C, Middleton G, Kröning H, Luppi G, Kisker O, Zubel A, Langer C, Kopit J, Burris HA. EPIC: phase III trial of cetuximab plus irinotecan after fluoropyrimidine and oxaliplatin failure in patients with metastatic colorectal cancer. *J Clin Oncol* 2008; **26**: 2311-2319 [PMID: 18390971 DOI: 10.1200/JCO.2007.13.1193]

17 **Langer C**, Kopit J, Awad M, Williams K, Teegarden P, Xu L. Mutations in patients with metastatic colorectal cancer receiving cetuximab in combination with irinotecan: results from the EPIC trial. ESMO 2008. *Ann Oncol* 2008; **19** (Suppl 8): Abstract 385P, viii133

18 **Barni S**, Ghilardi M, Borgonovo K, Cabiddu M, Zaniboni A, Petrelli F. Cetuximab/irinotecan-chemotherapy in KRAS wild-type pretreated metastatic colorectal cancer: a pooled analysis and review of literature. *Rev Recent Clin Trials* 2013; **8**: 101-109 [PMID: 23859115]

19 **Seymour MT**, Brown SR, Middleton G, Maughan T, Richman S, Gwyther S, Lowe C, Seligmann JF, Wadsley J, Maisey N, Chau I, Hill M, Dawson L, Falk S, O'Callaghan A, Benstead K, Chambers P, Oliver A, Marshall H, Napp V, Quirke P. Panitumumab and irinotecan versus irinotecan alone for patients with KRAS wild-type, fluorouracil-resistant advanced colorectal cancer (PICCOLO): a prospectively stratified randomised trial. *Lancet Oncol* 2013; **14**: 749-759 [PMID: 23725851 DOI: 10.1016/S1470-2045(13)70163-3]

20 **Beretta GD**, Petrelli F, Stinco S, Cabiddu M, Ghilardi M, Squadroni M, Borgonovo K, Barni S. FOLFIRI + bevacizumab as second-line therapy for metastatic colorectal cancer pretreated with oxaliplatin: a pooled analysis of published trials. *Med Oncol* 2013; **30**: 486 [PMID: 23400961 DOI: 10.1007/s12032-013-0486-y]

21 **Grothey A**, Sugrue MM, Purdie DM, Dong W, Sargent D, Hedrick E, Kozloff M. Bevacizumab beyond first progression is associated with prolonged overall survival in metastatic colorectal cancer: results from a large observational cohort study (BRiTE). *J Clin Oncol* 2008; **26**: 5326-5334 [PMID: 18854571 DOI: 10.1200/JCO.2008.16.3212]

22 **Bekaii-Saab TS**, Grothey A, Bendell JC, Kozloff M, Cohn AL, Mun Y, Fish S, Flick ED, Dalal D, Hurwitz H. Effectiveness and safety of second-line (2L) irinotecan- and oxaliplatin-based regimens after first-line (1L) bevacizumab (BV)-containing treatment (tx) for metastatic colorectal cancer (mCRC): Results from the ARIES observational cohort study. *J Clin Oncol* 2012; **30** (4_suppl): 535

23 **Bennouna J**, Sastre J, Arnold D, Österlund P, Greil R, Van Cutsem E, von Moos R, Viéitez JM, Bouché O, Borg C, Steffens CC, Alonso-Orduña V, Schlichting C, Reyes-Rivera I, Bendahmane B, André T, Kubicka S. Continuation of bevacizumab after first progression in metastatic colorectal cancer (ML18147): a randomised phase 3 trial. *Lancet Oncol* 2013; **14**: 29-37 [PMID: 23168366 DOI: 10.1016/S1470-2045(12)70477-1]

24 **Masi G**, Loupakis F, Salvatore L, Cremolini C, Fornaro L, Schirripa M, Granetto C, Miraglio E, Di Costanzo F, Antonuzzo L, Marcucci L, Barbara C, Boni C, Banzi M, Chiara S, Garbarino D, Valsuani C, Bonetti A, Boni L, Falcone A. Second-line chemotherapy (CT) with or without bevacizumab (BV) in metastatic colorectal cancer (mCRC) patients (pts) who progressed to a first-line treatment containing BV: Updated

results of the phase III "BEBYP" trial by the Gruppo Oncologico Nord Ovest (GONO). *J Clin Oncol* 2013; **31**: (suppl; abstr 3615)

25 **Holash J**, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, Ioffe E, Huang T, Radziejewski C, Bailey K, Fandl JP, Daly T, Wiegand SJ, Yancopoulos GD, Rudge JS. VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc Natl Acad Sci USA* 2002; **99**: 11393-11398 [PMID: 12177445]

26 **Tew WP**, Gordon M, Murren J, Dupont J, Pezzulli S, Aghajanian C, Sabbatini P, Mendelson D, Schwartz L, Gettinger S, Psyrri A, Cedarbaum JM, Spriggs DR. Phase 1 study of aflibercept administered subcutaneously to patients with advanced solid tumors. *Clin Cancer Res* 2010; **16**: 358-366 [PMID: 20028764 DOI: 10.1158/1078-0432.CCR-09-2103]

27 **Hecht JR**, Cohn AL, Dakhil SR, Saleh MN, Piperdi B, Cline-Burkhardt VJM, Ying Tian, Go WY; Jonsson SPIRITT (study 20060141). A randomized phase II study of FOLFIRI with either panitumumab (pmab) or bevacizumab (bev) as second-line treatment (tx) in patients (pts) with wild-type (WT) KRAS metastatic colorectal cancer (mCRC). *J Clin Oncol* 2012; **30**: (suppl 34; abstr 454)

P- Reviewers: Pescatori M, Shi C, Steele SR
S- Editor: Ma YJ **L- Editor**: Webster JR **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8928

World J Gastroenterol 2014 July 21; 20(27): 8928-8938
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Molecular biomarkers for the detection of metastatic colorectal cancer cells

Hidenori Kamiyama, Hiroshi Noda, Fumio Konishi, Toshiki Rikiyama

Hidenori Kamiyama, Hiroshi Noda, Fumio Konishi, Toshiki Rikiyama, Department of Surgery, Saitama Medical Center, Jichi Medical University, Saitama 330-8503, Japan
Hidenori Kamiyama, Department of Surgery, Chichibu Municipal Hospital, Saitama 368-0025, Japan
Author contributions: Kamiyama H and Noda H contributed to the article design, writing, and revision; Konishi F and Rikiyama T contributed to sample collection and provided advice concerning the study; all authors have approved the version to be published.
Correspondence to: Hiroshi Noda, MD, Department of Surgery, Saitama Medical Center, Jichi Medical University, 1-847 Amanuma-cho, Omiya-ku, Saitama 330-8503, Japan. noda164@hotmail.co.jp
Telephone: +81-48-6472111 Fax: +81-48-6485188
Received: September 27, 2013 Revised: January 29, 2014
Accepted: April 1, 2014
Published online: July 21, 2014

Abstract

Approximately half of all patients with colorectal cancer develop local recurrence or distant metastasis during the course of their illness. Recently, the molecular detection of metastatic cancer cells in various types of clinical samples, such as lymph nodes, bone marrow, peripheral blood, and peritoneal lavage fluid, has been investigated as a potential prognostic marker. The prognostic value of molecular tumor cell detection was independent of the type of detection method used. As assays become more sensitive and quantitative, a more thorough assessment of the cancer status of patients will be based on molecular markers alone. At present, it is difficult to conclude that one specific molecular marker is superior to others. Comparative analyses are recommended to assess the prognostic impact of molecular analyses in the same patient and determine the biomarkers that provide the most accurate prognostic information.

Key words: Molecular biomarker; Metastatic colorectal cancer cell; Circulating tumor cell; Disseminated tumor cell; Peritoneal lavage fluid; Colorectal cancer

Core tip: We focus on methods of the detection of molecular changes in metastatic colorectal cancer cells, and describe the characteristics for the methods, such as DNA methylation, mRNA, microRNA, immunomagnetic separation, protein and cancer-associated mutations. Moreover, we review the clinical significance according to the type of samples, such as blood, lymph node, bone marrow and peritoneal lavage fluid. At present, it is difficult to conclude that one specific molecular marker is superior to others. Comparative analyses are recommended to assess the prognostic impact of molecular analyses in the same patient and determine the biomarkers that provide the most accurate prognostic information.

Kamiyama H, Noda H, Konishi F, Rikiyama T. Molecular biomarkers for the detection of metastatic colorectal cancer cells. *World J Gastroenterol* 2014; 20(27): 8928-8938 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8928.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8928

INTRODUCTION

Colorectal cancer (CRC) is a common cause of morbidity and mortality[1]. The 5-year survival rate of this disease is approximately 90% for patients with localized disease and approximately 66% for patients with regional disease, as determined at diagnosis[2,3]. The incidence of disease recurrence is 25% in the absence of regional node involvement, suggesting that conventional pathology may fail to detect occult nodal metastasis[2,4]. Most deaths from cancer are caused by metastatic disease, and the prevention of subsequent metastasis is now the focus of clinical research in this field[5]. The early spread of tumor

Table 1 Sensitivities and specificities of various studies that took blood samples *n* (%)

Source	Method	Marker	Sensitivity	Specificity	Ref.
Serum	MSP	CDKN2A (p16)	14/52 (27)	44/44 (100)	[32]
Serum	RT-PCR	CEA	51/121 (42)	27/33 (82)	[56]
Serum	RT-PCR	CK20	22/99 (22)	150/150 (100)	[38]
Serum	qPCR	miR-92	80/90 (89)	35/50 (70)	[84]
Serum	CellSearch	EpCAM	20/74 (27)	228/246 (93)	[88]
Serum	CLIA	CEA	188/429 (44)	184/201 (92)	[112]

MSP: Methylation-specific polymerase chain reaction; qPCR: Quantitative reverse transcription polymerase chain reaction (RT-PCR); CLIA: Chemiluminescence immunoassay; CEA: Carcinoembryonic antigen; CK20: Cytokeratin 20; EpCAM: Epithelial cell adhesion molecule.

cells is usually not detected by the imaging technologies currently available. Metastasis-specific markers are also urgently required to help in delineating the spread of disease to neighboring tissues, lymph nodes (LNs), and distant parts of the body[6].

Molecular biomarkers have also been used to detect metastatic cancer cells in peripheral blood samples, bone marrow (BM), LNs, or peritoneal fluid[6,7]. Tumor cells detected in the peripheral blood are called circulating tumor cells (CTCs). The precise role of CTCs detected in patients with metastases remains unknown. Some of these CTCs may be transiting from the primary tumor to sites of future metastasis, indicating that metastasis is in progress. Alternatively, the detected CTCs may primarily be innocent bystanders that simply reflect a high metastatic burden or aggressive disease. The latter theory would explain the fact that the detection of CTCs is associated with poor outcome in patients with metastatic CRC[8]. Tumor cells located in the BM are termed disseminated tumor cells (DTCs). Evidence indicates that the BM is the common organ to which tumor cells from many types of carcinomas migrate. It can be speculated that the BM also forms an important reservoir of tumor cells, from which these cells may recirculate into other distant organs where better growth conditions may exist (such as the liver or lungs)[9,10]. The pivotal roles of biomarkers of LNs may help in identifying patients with node-negative CRC who are at a high risk of tumor recurrence and who may benefit from adjuvant therapy[11]. Peritoneal dissemination in patients with CRC is less frequent; therefore, from a prognostic perspective, it is considered less important compared with LN and liver metastasis[12]. However, the incidence of peritoneal seeding during potentially curative surgery for primary CRC reported in a series of 12 patients varied widely from 3% to 28%, which may be explained by differences in the methods used for tumor cell detection[13,14].

In this review, we will focus on the different types of molecular biomarkers of CRC that can be used for the detection of metastatic cancer cells and discuss their potential as prognostic markers of CRC.

MOLECULAR BIOMARKERS OF CANCER

Alteration in gene sequence and expression levels and protein structure or function can be used as molecular biomarkers to detect cancers at an early stage, determine prognosis, and monitor disease progression or therapeutic response[6]. Molecular biomarkers are defined as markers detected using molecular detection techniques such as immunohistochemistry or polymerase chain reaction (PCR).

Molecular tests usually begin with the preparation of a DNA, RNA, or protein extract from a clinical sample. The ratio of neoplastic cells to normal cells varies considerably from one clinical sample to another. It is difficult to isolate specifically neoplastic cells on the basis of clinical sample analysis, considering that samples are often composed primarily of cellular debris and free substrates (such as DNA, RNA, and protein). Therefore, clinical samples are frequently a heterogeneous mix of normal and cancer cells, DNA, RNA, and protein[15]. Two of the most important factors in determining the efficiency of a molecular marker assay are sensitivity (*i.e.*, the minimal amount of the substrate that can be detected) and specificity (*i.e.*, the percentage of assays that correctly distinguish normal from cancer-containing samples). The sensitivities and specificities reported in various studies that used molecular methods to analyze blood samples are shown in Table 1. In general, there is a trade-off between sensitivity and specificity[6,15].

METHODS OF DETECTION OF MOLECULAR CHANGES IN METASTATIC CRC CELLS AND THEIR CLINICAL SIGNIFICANCE

DNA methylation

One of the most promising types of markers is based on the detection of hypermethylation of promoter regions of cancer-associated genes[6,16-18]. Many types of cancer cells use this mechanism to inactivate tumor suppressor genes[16,19-22]. This assay can detect approximately one cancer cell among 1000 normal cells, a sensitivity that is sufficient to detect tumor DNA in most body fluids[23,24]. DNA methylation profiles represent a more chemically and biologically stable source of molecular diagnostic information compared with RNA or most proteins[16,25]. Cancer-specific DNA methylation patterns can be found in detached tumor cells in bodily fluids and biopsies and in free-floating DNA released from cancer cells[6,16]. The methylation biomarker studies performed till date varied in methylation targets, source of DNA, and type of tumor[15-17]. If a patient with CRC tests negative for a methylation marker in a tumor as well as in a remote sample, the result is classified as false-negative for disease detection. Therefore, researchers need to choose multiple target genes that are reportedly methylated with different frequencies in tumors and rarely in normal tissues[18,26].

Serum (peripheral blood): Cancer patients exhibit elevated levels of free DNA in their blood because of a high

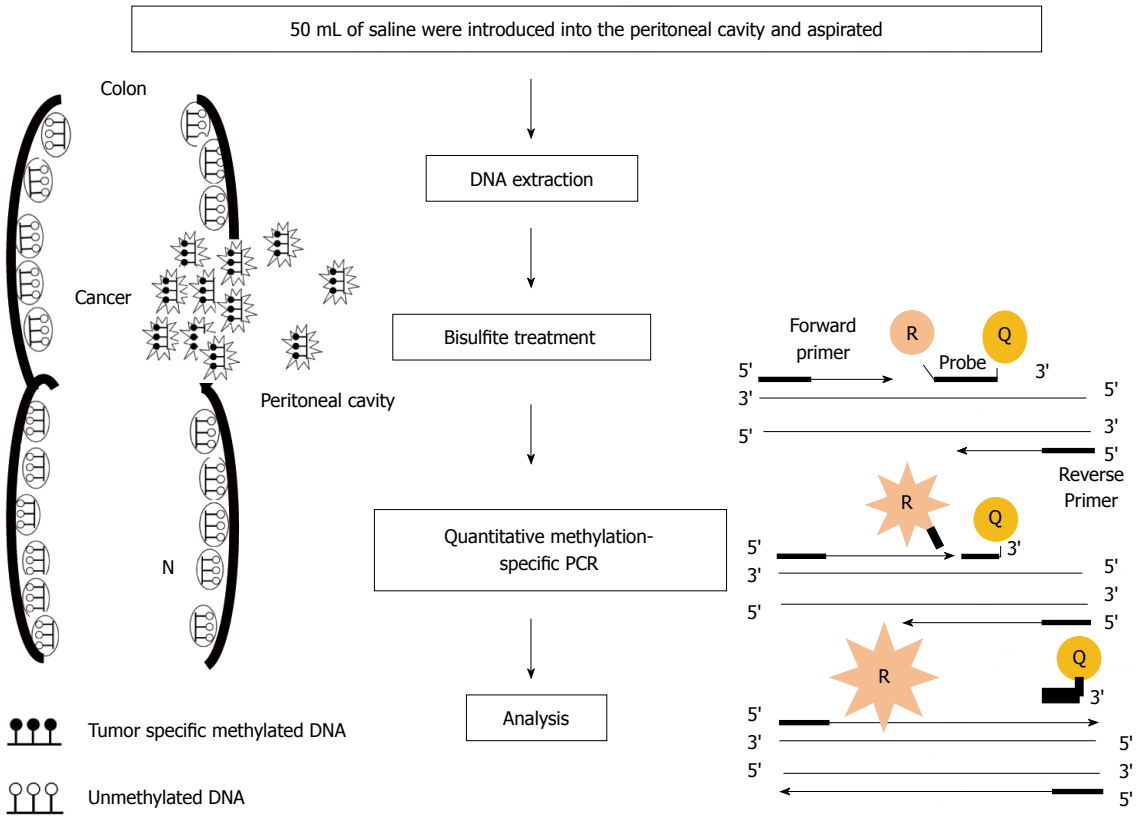


Figure 1 Outline of quantitative methylation-specific polymerase chain reaction analysis in peritoneal lavage. The depth of invasion and subsequent increased likelihood of tumor cells exfoliated from colonic serosa is reflected in the presence of tumor-related methylated DNA in peritoneal cavity. In quantitative methylation-specific polymerase chain reaction (PCR) step, the DNA polymerase cleaves only probes that are hybridized to the target. Cleavage separates the reporter dye (R) from the quencher dye (Q); resulting in increased fluorescence by the reporter. The increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is amplified during PCR.

cellular turnover[25,27]. The circulatory DNA from blood or other body fluids can be captured easily, and the status of DNA methylation at various gene promoters can be determined using various methods[16,21,25]. Nakayama *et al*[28] demonstrated that methylated *p16* is detectable in the serum of 69% patients with recurrent CRC. Several studies have addressed whether gene promoter methylation can be used to detect prevalent CRC using DNA recovered from the plasma or serum[19,29-32]. The presence of detectable tumor DNA in the plasma or serum is generally associated with a poor prognosis[26,28,29,32]. Wallner *et al*[26] identified *HPP1*, *HLTF*, and *hMLH1* as promising methylation markers in the serum of patients with CRC because these genes are not methylated in the serum of healthy controls and are methylated more frequently in metastatic disease than in local disease.

Peritoneal lavage fluid: Few studies have addressed gene methylation for the detection of micrometastasis to the peritoneal fluid in patients with gastrointestinal cancer[33]. We first reported the prognostic relevance of the detection of methylation of tumor-related genes in the peritoneal lavage fluid (PLF) of patients undergoing resection of CRC (Figure 1). The methylation pattern of the promoter of four target genes, *CDH1*, *CDKN2A* (*p16*), *MGMT*, and *APC*, was examined in 51 primary CRCs and the corresponding matched PLF DNA. The relative methylation levels of these genes in primary CRC tissues and paired PLF samples were assessed by quantitative methylation-specific PCR. An aberrant methylation of at least one gene was found in 45 out of the 51 (88%) primary tumors. In the PLF, the frequencies of aberrant promoter methylation were 16% for *CDH1*, 2% for *p16*, 4% for *MGMT*, and 24% for *APC*. Patients with PLF samples that exhibited methylation of more than one of these four target genes had a significantly shorter relapse-free survival[34].

mRNA (cDNA)

CRC cells show marked changes in the expression of many genes at the mRNA level[6]. One of the most common approaches used to identify and quantify mRNA levels in clinical samples is reverse transcription PCR (RT-PCR)[7,35]. Cytokeratin (CK) mRNA is a common marker of epithelial cells[36]. Many target genes have been previously used to detect micrometastasis from CRC, including carcinoembryonic antigen (CEA), MUC1, CK-8, CK18, CK19, and CK20. There is no specific marker of CRC, and the detection of disseminated neoplastic cells is based on epithelial markers such as CK20 and CEA. CK20 mRNA is considered to be a reliable target for the detection of disseminated CRC cells, and the frequency

of false-positive results is reportedly lower (0%-8%) for CK20 mRNA than for CEA mRNA (0%-33%)[37]. It is important to remember that the altered expression of some of these genes has also been reported in normal cells, leading to false-positive results[2,7]. To solve this problem, more quantitative analysis may eventually determine a cut-off level for differentiating between cancer and normal cells[38]. Although the isolation of intact RNA from bodily fluids and tissue samples is also possible, it generally requires cumbersome efforts to neutralize ubiquitous RNase enzymes[6].

Serum (peripheral blood): A meta-analysis of nine studies performed between 1998 and 2006 showed that patients with CTC positivity detected using RT-PCR of blood samples collected from the tumors' drainage veins correlated more with LN positivity (50%) than with LN negativity (21%). Furthermore, hepatic metastasis was found more often in CTC-positive patients (21%) than in CTC-negative patients (8%)[39,40]. A systematic review that evaluated CTCs after surgical resection of CRC and summarized its characteristics found that 14 reports fulfilled the inclusion criteria[7,36,41-53]. The mean CTC detection rate was 33.4%. Moreover, there were no differences among studies that obtained perioperative, early postoperative, and late postoperative samples or among studies that included patients with early-stage disease only, curative patients only, and patients with disease in all stages. The reported studies showed that perioperative CTC levels were not useful for predicting CRC recurrence[7]. The presence of CTCs in the peripheral blood at least 24 h after CRC resection is an independent prognostic marker of recurrence[7]. The sensitivity and specificity of target PCR amplification in CRC patients and control subjects were 22%-83% and 76%-100%, respectively[38,54-61].

LNs: To determine the spread of disease, many studies have attempted to detect CK mRNA in the LNs of patients with cancer using RT-PCR[2,62-66]. Isolated tumor cells or micrometastases within regional LNs that are not detected via conventional histopathological examination (hematoxylin and eosin staining) have been suspected to be markers of systemic tumor spread in these patients[11,67]. Tumor-specific changes in mRNA expression were detected in one or more LNs in 20%-54% patients with node-negative tumors that could potentially be upstaged to Dukes' grade C[2,15,62-66,68]. The detection of mRNA transcripts encoding CEA in the LNs of patients with stage Ⅱ CRC has been reported to predict outcome; the adjusted 5-year survival rate was 41% lower in the group with nodal micrometastases[62]. Micrometastatic LNs metastases identified by RT-PCR were consistently found to be prognostically significant[69].

BM: CK20 is the most commonly used marker in RT-PCR analyses of BM samples from CRC patients, with six studies published till date[46,70-74]. In three of those studies, the number of patients investigated was more than 100, with DTC detection rates of 11%-35%[73,75-77]. The CK20 detection rate in healthy controls ranged between 0% and 10%. Four groups found an association between the presence of CK20 transcripts and poor overall survival (OS)[78].

PLF: Guller *et al*[37] evaluated the clinical relevance of real-time quantitative PCR (qPCR) detection of CEA and CK20 transcripts in the PLF and blood from patients undergoing surgery for CRC and found that it was potentially related to tumor-cell dissemination. Among 39 patients with CRC, 11 had at least one sample that was positive for CEA or CK20. Six patients had evidence of disseminated CRCs before resection, whereas 10 had evidence of disseminated CRCs after resection. CEA qPCR amplification was detected in eight patients, and CK20 qPCR amplification was detected in 10 patients. Nine of the 11 PCR-positive patients developed recurrence (five distal metastases and four local metastases) after an average follow-up of 12 mo, whereas only two of the 28 qPCR-negative patients developed recurrence. In seven patients, disseminated CRCs were found in the PLF but not in the blood; five of these patients (71%) developed recurrence.

MicroRNAs

MicroRNAs (miRNAs) are small (18-24 nucleotides) RNAs that regulate the translation and stability of specific target mRNAs[79]. The deregulation of specific miRNAs contributes to a variety of diseases, most notably the development and progression of cancer, including CRC[80]. Once thought to be unstable RNA molecules, miRNAs are now known to be stably expressed in serum, plasma, urine, saliva, and other body fluids[81]. Biochemical analyses indicate that miRNAs are resistant to RNase activity as well as extreme pH and temperature[81-83]. The enormous potential of circulating miRNAs as an ideal class of cancer biomarkers is based on certain facts. First, they are remarkably stable molecules, well-preserved in harsh conditions, and resistant to RNase activity. Second, their expression profiles are specifically correlated with certain types of cancer or pathognomonic conditions. Third, they are easily accessible and can be sampled in a relatively noninvasive manner and readily detected using various techniques[81].

Serum (peripheral blood): Circulating miRNAs can be detected in the serum[79-81]. Ng *et al*[84] were the first to report that circulating miRNA levels in plasma are different between CRC patients and controls. In a population of 90 patients and 50 controls, the authors found that miR-92 was expressed at higher levels in the plasma of patients and that it could distinguish patients from healthy controls with 70% specificity and 89% sensitivity[79,84]. The expression of miR-92 decreased after surgical tumor resection, suggesting that the circulating levels of miRNAs may be a useful marker of disease recurrence[84]. A similar study found that the circulating levels

of miR-141 were elevated in metastatic CRC and that its expression was associated with poor prognosis, suggesting that this miRNA may be used in conjunction with CEA to detect CRC with distant metastasis[85].

Immunomagnetic separation

The immunomagnetic separation method using the CellSearch® System (Veridex LLC, Raritan, NJ, United States) gained approval from the United States Food and Drug Administration in 2004 for application in cases of metastatic breast cancer, and it has now been approved for application in cases of metastatic prostate cancer and CRC[85-87]. The CellSearch system detects CTCs according to the presence of the following characteristics: a round-to-oval shape by light scatter; an evident nucleus by 4',6-diamidino-2-phenylindole staining; epithelial cell adhesion molecule positivity ($EpCAM^+$); and $CK8^+$, $CK18^+$, $CK19^+$, and $CD45^-$ status by immunofluorescence. This method is more efficient in terms of sample size and processing time compared with other CTC enrichment methods. However, it is limited by its requirement of EpCAM expression; therefore, it potentiates false-negative results[39].

Plasma (peripheral blood): The CellSearch® system is the most advanced commercially available technology[9,39]. Cohen *et al*[88] published one of the largest studies of CTCs in metastatic CRC (mCRC). This study included 430 patients with mCRC who were recruited at 55 clinical centers in the United Kingdom, Netherlands, and United States, and it was performed using the CellSearch® system[88]. Patients were eligible to participate in the study if they were being administered a new first-, second-, or third-line systemic chemotherapy regimen. The peripheral blood of patients was collected before treatment initiation and at four time points after treatment initiation. For analysis, patients were categorized into favorable (< 3 CTCs/7.5 mL of blood) or unfavorable (> 3 CTCs/7.5 mL of blood) groups[39,88]. The study showed that, relative to the baseline values, the median progression-free survival (PFS) and OS rates of patients in the favorable group (PFS = 7.9 mo; OS = 18.5 mo) were approximately twice those of patients in the unfavorable group (PFS = 4.5 mo; OS = 9.4 mo)[39].

Protein

Several protein-based assays have also been developed to detect cancer cells[89,90]. Most of these are antibody-based assays, although many new approaches are being developed. Protein-based assays typically detect proteins that are overexpressed or structurally altered in cancer cells compared with those in normal cells. These approaches are generally used in research settings and are not yet applicable to larger clinical studies. The expression levels of CEA are commonly used to monitor colon cancer progression[6]. The expression of protein markers for CRC is increased in the serum of patients with other cancers and is occasionally increased in patients without disease, precluding their use as individual agents for cancer screening[6].

Serum (peripheral blood): CEA is a high molecular-weight glycoprotein that belongs to the immunoglobulin superfamily[89]. The carboxy terminal of CEA contains a hydrophobic region that is modified to provide a glycosyl phosphatidylinositol link to the cell membrane. Although its presence can be determined in biopsy samples, it is usually identified in the serum. Specifically, high CEA levels are associated with cancer progression, and the levels of this marker are expected to fall after cancer surgery[89,91]. However, in the absence of cancer, high CEA levels may also be observed in response to other conditions such as hepatitis, inflammatory bowel disease, pancreatitis, and obstructive pulmonary disease. Clinically, the potential value of the CEA test lies in its use as a prognostic marker that can be used to measure the course of cancer progression after diagnosis, with higher CEA levels being indicative of greater disease severity and poorer prognosis[89,92].

LNs: The choice of antibody used for IHC is an important factor for the accurate identification of occult disease. AE1/AE3 (DAKO, Carpinteria, CA, United States) is the most widely used antibody for IHC analysis of LNs from CRC patients[69]. This polyclonal antibody is raised against several CKs, including CK19[69]. Studies that used IHC to detect occult disease reported diverse methodologies and design. The sample size included in these studies ranged from 32 to 147 patients; therefore, none were well powered to detect smaller but potentially significant metastases[69].

BM: Most studies describing ICC for the detection of DTCs in CRC patients have either used the monoclonal antibody CK2 against CK18 or the pancytokeratin antibody A45-B/B3[93-95]. The DTC detection rate in studies that used CK2 was between 16% and 32%[96-100], whereas the detection rate was higher (24%-55%) in studies that used the A45-B/B3 antibody[101-104]. Both antibodies rarely detected CK-positive cells in the BM of noncancer controls (0%-5.5%)[78]. Flatmark *et al*[75] reported the detection of DTCs in 41 (17%) and 28 (12%) of the 235 BM samples examined by immunomagnetic selection and ICC, respectively.

PLF: A variety of monoclonal antibodies have been used for the detection of disseminated single CRCs in the peritoneal cavity, including Ber-Ep4, CA19-9, CAM5.2, CIP83, Ra96, and C54-0. Bosch *et al*[105] used a combination of three monoclonal antibodies (Ks20.8, Lu5, and Ber-Ep4) to perform ICC. The rate of detection of disseminated CRCs in the PLF ranges from 10% to 67% and from 17% to 29% using ICC alone or in combination with CYT, respectively. Bosch *et al*[105] showed that 15% and 11% washing samples taken before and after resection, respectively, tested positive by CYT, and that

Table 2 Comparison of biomarkers for detection of metastatic colorectal cancer cells

Marker	Stable	Amplifiable	Widely reported	FDA approval	False negative	False positive	Ref.
DNA methylation	•	•			•		[16]
mRNA		•	•			•	[7]
Micro RNA	•	•					[81]
CellSearch (EpCAM)	•			•	•		[39]
Genetic mutation	•	•			•		[15]

EpCAM: Epithelial cell adhesion molecule; FDA: Food and Drug Administration.

17% and 13% samples, respectively, tested positive by the combined method[105]. The best monoclonal antibody (or combination of antibodies) for the detection of disseminated CRCs has not been defined.

Cancer-associated mutations

Genetic mutation analysis is useful not only for detecting cancer in patients but also for monitoring disease spread and determining prognosis. In 1995, it was first reported that *TP53* mutations could be used to follow tumor spread into margins and the draining LNs of patients with head and neck cancer[6]. *K-RAS* and *TP53* mutations are detected in the LNs of CRC patients without histological evidence of nodal metastasis. Mutations in *K-RAS* are observed in approximately 40% patients with colon tumors. Currently, mutations in *K-RAS* are not being used for the early detection of metastatic cancer cells; however, they have significant implications in predicting the likelihood of response to antibody-based EGFR inhibitor therapy[90,106].

LNs: Studies performed on patients with CRC showed that the presence of a tumor-specific *KRAS* or *TP53* mutation in LN samples predicted poor outcome[5]. Hayashi *et al*[107,108] screened the LNs of patients without histological evidence of nodal metastasis for *KRAS* and *p53* mutations[15].

DISCUSSION

Once primary tumors are resected, metachronous metastases must arise from tumor cells that disseminate to ectopic sites before surgery. Systemic therapy primarily targets tumor cells that have detached from the primary lesion, have lodged elsewhere, are undetectable by clinical imaging, and are inaccessible to excision[5]. Therefore, metastasis-specific markers are required to accurately diagnose the existence of metastatic cancer cells. Numerous studies have demonstrated a more accurate prediction of the prognosis of patients using various immunological and PCR-based assays[109]. Each molecular method has advantages and disadvantages (Table 2). At present, it is difficult to conclude that one specific method is superior to others. Assessment of the independent prognostic impact of molecular analyses in different compartments within the same populations using comparative analyses to determine the biomarkers that provide the most accurate prognostic information remains a subject of additional investigation[11].

CTC detection may be useful for CRC patients receiving chemotherapy. Sequential peripheral blood analyses are more acceptable compared with other resources, and many research groups are currently assessing the clinical value of CTC analyses for therapy monitoring in clinical studies[10]. Monitoring of peripheral blood during and after systemic adjuvant therapy for CTCs may provide unique information for the clinical management of individual cancer patients and allow an early change in therapy, years before the appearance of overt metastasis signals that are incurable[10]. That is, if CTC levels do not drop, systemic treatment may not be effective[8].

The molecular detection of tumor cells in regional LNs is associated with an increased risk of disease recurrence and poor survival in patients with node-negative CRC[11]. These data may favor molecular or cellular biomarkers to tailor adjuvant chemotherapy in patients with node-negative disease[11].

In patients with breast cancer, the clinical significance of CTCs in the peripheral blood is less clear than that of DTCs in BM[10]. Conversely, a meta-analysis that targeted CRC patients showed that the significance of CTCs in the peripheral blood is much clearer than that of DTCs in BM[109]. The aspiration of BM is invasive, time consuming, uncomfortable for the patient, and difficult to standardize in terms of sample quality. Another major limitation is that BM aspiration is not easy to perform during control visits at outpatient centers, which hampers repeated analyses[10]. The use of DTCs in patients with CRC may provide limited information for their clinical management.

The potential of peritoneal lavage to improve outcomes is most important for the selection of patients for adjuvant chemotherapy who present with seemingly early-stage disease and who would not otherwise receive chemotherapy. Second, intraperitoneal chemotherapy may be advantageous as a prophylactic therapy for patients with positive peritoneal micrometastases[110].

The existence of cancer stem cells in CRC has been convincingly demonstrated on a functional level. In accordance with this hypothesis, it has been shown that the number of tumor cells located at the invasive front that express high amounts of nuclear beta-catenin, which is a sign of aberrant Wnt signaling activation associated with ongoing epithelial-mesenchymal transition and stem-cell formation, is strongly correlated with metastasis and poor survival in patients with rectal cancer[11,111].

CONCLUSION

The ideal biomarker is one that is found in readily available biological samples and can be noninvasively detected. More reliable molecular markers are required to accurately diagnose the existence of metastatic cancer cells. Although thousands of papers describing genetic mutations or alterations in gene expression levels associated with various types of cancers are published each year, very few of these are translated into reliable molecular markers that can be used routinely in clinical settings. At present, it is difficult to conclude that one specific molecular marker is superior to others. Comparative analyses are recommended to assess the prognostic impact of molecular analyses in the same patient and determine the biomarkers that provide the most accurate prognostic information.

REFERENCES

1 **van der Voort van Zijp J**, Hoekstra HJ, Basson MD. Evolving management of colorectal cancer. *World J Gastroenterol* 2008; **14**: 3956-3967 [PMID: 18609678 DOI: 10.3748/wjg.14.3956]
2 **Koyanagi K**, Bilchik AJ, Saha S, Turner RR, Wiese D, McCarter M, Shen P, Deacon L, Elashoff D, Hoon DS. Prognostic relevance of occult nodal micrometastases and circulating tumor cells in colorectal cancer in a prospective multicenter trial. *Clin Cancer Res* 2008; **14**: 7391-7396 [PMID: 19010855 DOI: 10.1158/1078-0432]
3 Cancer Stat Fact Sheet. Surveillance Epidemiology and End Results, 2007. Available from: URL: http://seer.cancer.gov/
4 **Bilchik AJ**, Hoon DS, Saha S, Turner RR, Wiese D, DiNome M, Koyanagi K, McCarter M, Shen P, Iddings D, Chen SL, Gonzalez M, Elashoff D, Morton DL. Prognostic impact of micrometastases in colon cancer: interim results of a prospective multicenter trial. *Ann Surg* 2007; **246**: 568-575; discussion 575-577 [PMID: 17893493 DOI: 10.1097/SLA.0b013e318155a9c7]
5 **Klein CA**. Parallel progression of primary tumours and metastases. *Nat Rev Cancer* 2009; **9**: 302-312 [PMID: 19308069 DOI: 10.1038/nrc2627]
6 **Sidransky D**. Emerging molecular markers of cancer. *Nat Rev Cancer* 2002; **2**: 210-219 [PMID: 11990857 DOI: 10.1038/nrc755]
7 **Peach G**, Kim C, Zacharakis E, Purkayastha S, Ziprin P. Prognostic significance of circulating tumour cells following surgical resection of colorectal cancers: a systematic review. *Br J Cancer* 2010; **102**: 1327-1334 [PMID: 20389297 DOI: 10.1038/sj.bjc.6605651]
8 **Groot Koerkamp B**, Rahbari NN, Büchler MW, Koch M, Weitz J. Circulating tumor cells and prognosis of patients with resectable colorectal liver metastases or widespread metastatic colorectal cancer: a meta-analysis. *Ann Surg Oncol* 2013; **20**: 2156-2165 [PMID: 23456317 DOI: 10.1245/s10434-013-2907-8]
9 **Pantel K**, Alix-Panabières C, Riethdorf S. Cancer micrometastases. *Nat Rev Clin Oncol* 2009; **6**: 339-351 [PMID: 19399023 DOI: 10.1038/nrclinonc.2009.44]
10 **Pantel K**, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. *Nat Rev Cancer* 2008; **8**: 329-340 [PMID: 18404148 DOI: 10.1038/nrc2375]
11 **Rahbari NN**, Bork U, Motschall E, Thorlund K, Büchler MW, Koch M, Weitz J. Molecular detection of tumor cells in regional lymph nodes is associated with disease recurrence and poor survival in node-negative colorectal cancer: a systematic review and meta-analysis. *J Clin Oncol* 2012; **30**: 60-70 [PMID: 22124103 DOI: 10.1200/JCO.2011.36.9504]
12 **Sadahiro S**, Suzuki T, Ishikawa K, Nakamura T, Tanaka Y, Masuda T, Mukoyama S, Yasuda S, Tajima T, Makuuchi H, Murayama C. Recurrence patterns after curative resection of colorectal cancer in patients followed for a minimum of ten years. *Hepatogastroenterology* 2003; **50**: 1362-1366 [PMID: 14571738]
13 **Koppe MJ**, Boerman OC, Oyen WJ, Bleichrodt RP. Peritoneal carcinomatosis of colorectal origin: incidence and current treatment strategies. *Ann Surg* 2006; **243**: 212-222 [PMID: 16432354 DOI: 10.1097/01.sla.0000197702.46394.16]
14 **Kobayashi H**, Kotake K, Sugihara K. Prognostic significance of peritoneal lavage cytology in patients with colorectal cancer. *Int J Clin Oncol* 2013; **18**: 411-417 [PMID: 22370903 DOI: 10.1007/s10147-012-0394-8]
15 **Jubb AM**, Quirke P, Oates AJ. DNA methylation, a biomarker for colorectal cancer: implications for screening and pathological utility. *Ann N Y Acad Sci* 2003; **983**: 251-267 [PMID: 12724230 DOI: 10.1111/j.1749-6632.2003.tb05980.x]
16 **Laird PW**. The power and the promise of DNA methylation markers. *Nat Rev Cancer* 2003; **3**: 253-266 [PMID: 12671664 DOI: 10.1038/nrc1045]
17 **Cottrell SE**. Molecular diagnostic applications of DNA methylation technology. *Clin Biochem* 2004; **37**: 595-604 [PMID: 15234241 DOI: 10.1016/j.clinbiochem.2004.05.010]
18 **Lao VV**, Grady WM. Epigenetics and colorectal cancer. *Nat Rev Gastroenterol Hepatol* 2011; **8**: 686-700 [PMID: 22009203 DOI: 10.1038/nrgastro.2011.173]
19 **Belinsky SA**. Gene-promoter hypermethylation as a biomarker in lung cancer. *Nat Rev Cancer* 2004; **4**: 707-717 [PMID: 15343277 DOI: 10.1038/nrc1432]
20 **Noda H**, Mashima R, Kamiyama H, Okada S, Kawamura YJ, Konishi F. Promoter hypermethylation of tumor-related genes in sporadic colorectal cancer in young patients. *J Exp Clin Cancer Res* 2007; **26**: 521-526 [PMID: 18365548]
21 **Noda H**, Miyaji Y, Nakanishi A, Konishi F, Miki Y. Frequent reduced expression of alpha-1B-adrenergic receptor caused by aberrant promoter methylation in gastric cancers. *Br J Cancer* 2007; **96**: 383-390 [PMID: 17242706 DOI: 10.1038/sj.bjc.6603555]
22 **Noda H**, Kato Y, Yoshikawa H, Arai M, Togashi K, Nagai H, Konishi F, Miki Y. Microsatellite instability caused by hMLH1 promoter methylation increases with tumor progression in right-sided sporadic colorectal cancer. *Oncology* 2005; **69**: 354-362 [PMID: 16293975 DOI: 10.1159/000089768]
23 **Herman JG**, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996; **93**: 9821-9826 [PMID: 8790415 DOI: 10.1073/pnas.93.18.9821]
24 **Shivapurkar N**, Gazdar AF. DNA methylation based biomarkers in non-invasive cancer screening. *Curr Mol Med* 2010; **10**: 123-132 [PMID: 20196733 DOI: 10.2174/156652410790963303]
25 **Qureshi SA**, Bashir MU, Yaqinuddin A. Utility of DNA methylation markers for diagnosing cancer. *Int J Surg* 2010; **8**: 194-198 [PMID: 20139036 DOI: 10.1016/j.ijsu.2010.02.001]
26 **Wallner M**, Herbst A, Behrens A, Crispin A, Stieber P, Göke B, Lamerz R, Kolligs FT. Methylation of serum DNA is an independent prognostic marker in colorectal cancer. *Clin Cancer Res* 2006; **12**: 7347-7352 [PMID: 17189406 DOI: 10.1158/1078-0432.CCR-06-1264]
27 **Jahr S**, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, Knippers R. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res* 2001; **61**: 1659-1665 [PMID: 11245480]
28 **Nakayama H**, Hibi K, Takase T, Yamazaki T, Kasai Y, Ito K, Akiyama S, Nakao A. Molecular detection of p16 promoter methylation in the serum of recurrent colorectal cancer patients. *Int J Cancer* 2003; **105**: 491-493 [PMID: 12712439 DOI: 10.1002/ijc.11117]

29 **Grady WM**, Rajput A, Lutterbaugh JD, Markowitz SD. Detection of aberrantly methylated hMLH1 promoter DNA in the serum of patients with microsatellite unstable colon cancer. *Cancer Res* 2001; **61**: 900-902 [PMID: 11221878]

30 **Leung WK**, To KF, Man EP, Chan MW, Bai AH, Hui AJ, Chan FK, Sung JJ. Quantitative detection of promoter hypermethylation in multiple genes in the serum of patients with colorectal cancer. *Am J Gastroenterol* 2005; **100**: 2274-2279 [PMID: 16181380 DOI: 10.1111/j.1572-0241.2005.50412.x]

31 **Nakayama H**, Hibi K, Taguchi M, Takase T, Yamazaki T, Kasai Y, Ito K, Akiyama S, Nakao A. Molecular detection of p16 promoter methylation in the serum of colorectal cancer patients. *Cancer Lett* 2002; **188**: 115-119 [PMID: 12406556 DOI: 10.1016/S0304-3835(01)00839-4]

32 **Zou HZ**, Yu BM, Wang ZW, Sun JY, Cang H, Gao F, Li DH, Zhao R, Feng GG, Yi J. Detection of aberrant p16 methylation in the serum of colorectal cancer patients. *Clin Cancer Res* 2002; **8**: 188-191 [PMID: 11801557]

33 **Hiraki M**, Kitajima Y, Sato S, Nakamura J, Hashiguchi K, Noshiro H, Miyazaki K. Aberrant gene methylation in the peritoneal fluid is a risk factor predicting peritoneal recurrence in gastric cancer. *World J Gastroenterol* 2010; **16**: 330-338 [PMID: 20082478 DOI: 10.3748/wjg.v16.i3.330]

34 **Kamiyama H**, Noda H, Takata O, Suzuki K, Kawamura Y, Konishi F. Promoter hypermethylation of tumor-related genes in peritoneal lavage and the prognosis of patients with colorectal cancer. *J Surg Oncol* 2009; **100**: 69-74 [PMID: 19384904 DOI: 10.1002/jso.21291]

35 **Hundt S**, Haug U, Brenner H. Blood markers for early detection of colorectal cancer: a systematic review. *Cancer Epidemiol Biomarkers Prev* 2007; **16**: 1935-1953 [PMID: 17932341 DOI: 10.1158/1055-9965.EPI-06-0994]

36 **Katsumata K**, Sumi T, Mori Y, Hisada M, Tsuchida A, Aoki T. Detection and evaluation of epithelial cells in the blood of colon cancer patients using RT-PCR. *Int J Clin Oncol* 2006; **11**: 385-389 [PMID: 17058136 DOI: 10.1007/s10147-006-0590-5]

37 **Guller U**, Zajac P, Schnider A, Bösch B, Vorburger S, Zuber M, Spagnoli GC, Oertli D, Maurer R, Metzger U, Harder F, Heberer M, Marti WR. Disseminated single tumor cells as detected by real-time quantitative polymerase chain reaction represent a prognostic factor in patients undergoing surgery for colorectal cancer. *Ann Surg* 2002; **236**: 768-775; discussion 775-776 [PMID: 12454515 DOI: 10.1097/00000658-200212000-00009]

38 **Giribaldi G**, Procida S, Ulliers D, Mannu F, Volpatto R, Mandili G, Fanchini L, Bertetto O, Fronda G, Simula L, Rimini E, Cherchi G, Bonello L, Maule MM, Turrini F. Specific detection of cytokeratin 20-positive cells in blood of colorectal and breast cancer patients by a high sensitivity real-time reverse transcriptase-polymerase chain reaction method. *J Mol Diagn* 2006; **8**: 105-112 [PMID: 16436641 DOI: 10.2353/jmoldx.2006.050054]

39 **Allen JE**, El-Deiry WS. Circulating Tumor Cells and Colorectal Cancer. *Curr Colorectal Cancer Rep* 2010; **6**: 212-220 [PMID: 20890370 DOI: 10.1007/s11888-010-0069-7]

40 **Katsuno H**, Zacharakis E, Aziz O, Rao C, Deeba S, Paraskeva P, Ziprin P, Athanasiou T, Darzi A. Does the presence of circulating tumor cells in the venous drainage of curative colorectal cancer resections determine prognosis? A meta-analysis. *Ann Surg Oncol* 2008; **15**: 3083-3091 [PMID: 18787906 DOI: 10.1245/s10434-008-0131-8]

41 **Taniguchi T**, Makino M, Suzuki K, Kaibara N. Prognostic significance of reverse transcriptase-polymerase chain reaction measurement of carcinoembryonic antigen mRNA levels in tumor drainage blood and peripheral blood of patients with colorectal carcinoma. *Cancer* 2000; **89**: 970-976 [PMID: 10964326]

42 **Yamaguchi K**, Takagi Y, Aoki S, Futamura M, Saji S. Significant detection of circulating cancer cells in the blood by reverse transcriptase-polymerase chain reaction during colorectal cancer resection. *Ann Surg* 2000; **232**: 58-65 [PMID: 10862196 DOI: 10.1097/00000658-200007000-00009]

43 **Ito S**, Nakanishi H, Hirai T, Kato T, Kodera Y, Feng Z, Kasai Y, Ito K, Akiyama S, Nakao A, Tatematsu M. Quantitative detection of CEA expressing free tumor cells in the peripheral blood of colorectal cancer patients during surgery with real-time RT-PCR on a LightCycler. *Cancer Lett* 2002; **183**: 195-203 [PMID: 12065095 DOI: 10.1016/S0304-3835(02)00157-X]

44 **Bessa X**, Piñol V, Castellví-Bel S, Piazuelo E, Lacy AM, Elizalde JI, Piqué JM, Castells A. Prognostic value of postoperative detection of blood circulating tumor cells in patients with colorectal cancer operated on for cure. *Ann Surg* 2003; **237**: 368-375 [PMID: 12616121 DOI: 10.1097/01.SLA.0000055223.27623.F3]

45 **Chen WS**, Chung MY, Liu JH, Liu JM, Lin JK. Impact of circulating free tumor cells in the peripheral blood of colorectal cancer patients during laparoscopic surgery. *World J Surg* 2004; **28**: 552-557 [PMID: 15366744 DOI: 10.1007/s00268-004-7276-9]

46 **Sadahiro S**, Suzuki T, Ishikawa K, Saguchi T, Maeda Y, Yasuda S, Makuuchi H, Yurimoto S, Murayama C. Detection of carcinoembryonic antigen messenger RNA-expressing cells in portal and peripheral blood during surgery does not influence relapse in colorectal cancer. *Ann Surg Oncol* 2005; **12**: 988-994 [PMID: 16244799 DOI: 10.1245/ASO.2005.03.565]

47 **Sadahiro S**, Suzuki T, Maeda Y, Yurimoto S, Yasuda S, Makuuchi H, Kamijo A, Murayama C. Detection of carcinoembryonic antigen messenger RNA-expressing cells in peripheral blood 7 days after curative surgery is a novel prognostic factor in colorectal cancer. *Ann Surg Oncol* 2007; **14**: 1092-1098 [PMID: 17200912 DOI: 10.1245/s10434-006-9289-0]

48 **Koch M**, Kienle P, Kastrati D, Antolovic D, Schmidt J, Herfarth C, von Knebel Doeberitz M, Weitz J. Prognostic impact of hematogenous tumor cell dissemination in patients with stage II colorectal cancer. *Int J Cancer* 2006; **118**: 3072-3077 [PMID: 16425256 DOI: 10.1002/ijc.21784]

49 **Lloyd JM**, McIver CM, Stephenson SA, Hewett PJ, Rieger N, Hardingham JE. Identification of early-stage colorectal cancer patients at risk of relapse post-resection by immunobead reverse transcription-PCR analysis of peritoneal lavage fluid for malignant cells. *Clin Cancer Res* 2006; **12**: 417-423 [PMID: 16428481 DOI: 10.1158/1078-0432.CCR-05-1473]

50 **Allen-Mersh TG**, McCullough TK, Patel H, Wharton RQ, Glover C, Jonas SK. Role of circulating tumour cells in predicting recurrence after excision of primary colorectal carcinoma. *Br J Surg* 2007; **94**: 96-105 [PMID: 17058316 DOI: 10.1002/bjs.5526]

51 **Uen YH**, Lin SR, Wu DC, Su YC, Wu JY, Cheng TL, Chi CW, Wang JY. Prognostic significance of multiple molecular markers for patients with stage II colorectal cancer undergoing curative resection. *Ann Surg* 2007; **246**: 1040-1046 [PMID: 18043108 DOI: 10.1097/SLA.0b013e318142d918]

52 **Uen YH**, Lu CY, Tsai HL, Yu FJ, Huang MY, Cheng TL, Lin SR, Wang JY. Persistent presence of postoperative circulating tumor cells is a poor prognostic factor for patients with stage I-III colorectal cancer after curative resection. *Ann Surg Oncol* 2008; **15**: 2120-2128 [PMID: 18481151 DOI: 10.1245/s10434-008-9961-7]

53 **Wang JY**, Lin SR, Wu DC, Lu CY, Yu FJ, Hsieh JS, Cheng TL, Koay LB, Uen YH. Multiple molecular markers as predictors of colorectal cancer in patients with normal perioperative serum carcinoembryonic antigen levels. *Clin Cancer Res* 2007; **13**: 2406-2413 [PMID: 17406027 DOI: 10.1158/1078-0432.CCR-06-2054]

54 **Castells A**, Boix L, Bessa X, Gargallo L, Piqué JM. Detection of colonic cells in peripheral blood of colorectal cancer patients by means of reverse transcriptase and polymerase chain reaction. *Br J Cancer* 1998; **78**: 1368-1372 [PMID: 9823981 DOI: 10.1038/bjc.1998.686]

55 **Guadagni F**, Kantor J, Aloe S, Carone MD, Spila A, D'Alessandro R, Abbolito MR, Cosimelli M, Graziano F, Carboni F, Carlini S, Perri P, Sciarretta F, Greiner JW, Kashmiri SV, Steinberg SM, Roselli M, Schlom J. Detection of blood-borne

cells in colorectal cancer patients by nested reverse transcription-polymerase chain reaction for carcinoembryonic antigen messenger RNA: longitudinal analyses and demonstration of its potential importance as an adjunct to multiple serum markers. *Cancer Res* 2001; **61**: 2523-2532 [PMID: 11289125]

56 **Sadahiro S**, Suzuki T, Tokunaga N, Yurimoto S, Yasuda S, Tajima T, Makuuchi H, Murayama C, Matsuda K. Detection of tumor cells in the portal and peripheral blood of patients with colorectal carcinoma using competitive reverse transcriptase-polymerase chain reaction. *Cancer* 2001; **92**: 1251-1258 [PMID: 11571740]

57 **Silva JM**, Rodriguez R, Garcia JM, Muñoz C, Silva J, Dominguez G, Provencio M, España P, Bonilla F. Detection of epithelial tumour RNA in the plasma of colon cancer patients is associated with advanced stages and circulating tumour cells. *Gut* 2002; **50**: 530-534 [PMID: 11889075 DOI: 10.1136/gut.50.4.530]

58 **Douard R**, Moutereau S, Serru V, Sales JP, Wind P, Cugnenc PH, Vaubourdolle M, Loric S. Immunobead multiplex RT-PCR detection of carcinoembryonic genes expressing cells in the blood of colorectal cancer patients. *Clin Chem Lab Med* 2005; **43**: 127-132 [PMID: 15843204 DOI: 10.1515/CCLM.2005.021]

59 **Vlems FA**, Diepstra JH, Cornelissen IM, Ruers TJ, Ligtenberg MJ, Punt CJ, van Krieken JH, Wobbes T, van Muijen GN. Limitations of cytokeratin 20 RT-PCR to detect disseminated tumour cells in blood and bone marrow of patients with colorectal cancer: expression in controls and downregulation in tumour tissue. *Mol Pathol* 2002; **55**: 156-163 [PMID: 12032226 DOI: 10.1136/mp.55.3.156]

60 **Xu D**, Li XF, Zheng S, Jiang WZ. Quantitative real-time RT-PCR detection for CEA, CK20 and CK19 mRNA in peripheral blood of colorectal cancer patients. *J Zhejiang Univ Sci B* 2006; **7**: 445-451 [PMID: 16691638 DOI: 10.1631/jzus.2006.B0445]

61 **Wang JY**, Wu CH, Lu CY, Hsieh JS, Wu DC, Huang SY, Lin SR. Molecular detection of circulating tumor cells in the peripheral blood of patients with colorectal cancer using RT-PCR: significance of the prediction of postoperative metastasis. *World J Surg* 2006; **30**: 1007-1013 [PMID: 16736329 DOI: 10.1007/s00268-005-0485-z]

62 **Liefers GJ**, Cleton-Jansen AM, van de Velde CJ, Hermans J, van Krieken JH, Cornelisse CJ, Tollenaar RA. Micrometastases and survival in stage II colorectal cancer. *N Engl J Med* 1998; **339**: 223-228 [PMID: 9673300 DOI: 10.1056/NEJM199807233390403]

63 **Cagir B**, Gelmann A, Park J, Fava T, Tankelevitch A, Bittner EW, Weaver EJ, Palazzo JP, Weinberg D, Fry RD, Waldman SA. Guanylyl cyclase C messenger RNA is a biomarker for recurrent stage II colorectal cancer. *Ann Intern Med* 1999; **131**: 805-812 [PMID: 10610624 DOI: 10.7326/0003-4819-131-11-199912070-00002]

64 **Haince JF**, Houde M, Beaudry G, L'espérance S, Garon G, Desaulniers M, Hafer LJ, Heald JI, Lyle S, Grossman SR, Têtu B, Sargent DJ, Fradet Y. Comparison of histopathology and RT-qPCR amplification of guanylyl cyclase C for detection of colon cancer metastases in lymph nodes. *J Clin Pathol* 2010; **63**: 530-537 [PMID: 20498026 DOI: 10.1136/jcp.2009.072983]

65 **Rosenberg R**, Hoos A, Mueller J, Baier P, Stricker D, Werner M, Nekarda H, Siewert JR. Prognostic significance of cytokeratin-20 reverse transcriptase polymerase chain reaction in lymph nodes of node-negative colorectal cancer patients. *J Clin Oncol* 2002; **20**: 1049-1055 [PMID: 11844829 DOI: 10.1200/JCO.20.4.1049]

66 **Noura S**, Yamamoto H, Ohnishi T, Masuda N, Matsumoto T, Takayama O, Fukunaga H, Miyake Y, Ikenaga M, Ikeda M, Sekimoto M, Matsuura N, Monden M. Comparative detection of lymph node micrometastases of stage II colorectal cancer by reverse transcriptase polymerase chain reaction and immunohistochemistry. *J Clin Oncol* 2002; **20**: 4232-4241 [PMID: 12377967 DOI: 10.1200/JCO.2002.10.023]

67 **Weitz J**, Kienle P, Magener A, Koch M, Schrödel A, Willeke F, Autschbach F, Lacroix J, Lehnert T, Herfarth C, von Knebel Doeberitz M. Detection of disseminated colorectal cancer cells in lymph nodes, blood and bone marrow. *Clin Cancer Res* 1999; **5**: 1830-1836 [PMID: 10430088]

68 **Merrie AE**, van Rij AM, Dennett ER, Phillips LV, Yun K, McCall JL. Prognostic significance of occult metastases in colon cancer. *Dis Colon Rectum* 2003; **46**: 221-231 [PMID: 12576896 DOI: 10.1007/s10350-004-6527-z]

69 **Nicastri DG**, Doucette JT, Godfrey TE, Hughes SJ. Is occult lymph node disease in colorectal cancer patients clinically significant? A review of the relevant literature. *J Mol Diagn* 2007; **9**: 563-571 [PMID: 17916603 DOI: 10.2353/jmoldx.2007.070032]

70 **Soeth E**, Röder C, Juhl H, Krüger U, Kremer B, Kalthoff H. The detection of disseminated tumor cells in bone marrow from colorectal-cancer patients by a cytokeratin-20-specific nested reverse-transcriptase-polymerase-chain reaction is related to the stage of disease. *Int J Cancer* 1996; **69**: 278-282 [PMID: 8797868]

71 **Soeth E**, Vogel I, Röder C, Juhl H, Marxsen J, Krüger U, Henne-Bruns D, Kremer B, Kalthoff H. Comparative analysis of bone marrow and venous blood isolates from gastrointestinal cancer patients for the detection of disseminated tumor cells using reverse transcription PCR. *Cancer Res* 1997; **57**: 3106-3110 [PMID: 9242433]

72 **Weitz J**, Koch M, Kienle P, Schrödel A, Willeke F, Benner A, Lehnert T, Herfarth C, von Knebel Doeberitz M. Detection of hematogenic tumor cell dissemination in patients undergoing resection of liver metastases of colorectal cancer. *Ann Surg* 2000; **232**: 66-72 [PMID: 10862197 DOI: 10.1097/00000658-200007000-00010]

73 **Conzelmann M**, Dieterle CP, Linnemann U, Berger MR. Cytokeratin 20 and guanylyl cyclase C mRNA is largely present in lymph node and liver specimens of colorectal cancer patients. *Int J Cancer* 2003; **107**: 617-628 [PMID: 14520701 DOI: 10.1002/ijc.11425]

74 **Schlimok G**, Funke I, Bock B, Schweiberer B, Witte J, Riethmüller G. Epithelial tumor cells in bone marrow of patients with colorectal cancer: immunocytochemical detection, phenotypic characterization, and prognostic significance. *J Clin Oncol* 1990; **8**: 831-837 [PMID: 1692090]

75 **Flatmark K**, Borgen E, Nesland JM, Rasmussen H, Johannessen HO, Bukholm I, Rosales R, Hårklau L, Jacobsen HJ, Sandstad B, Boye K, Fodstad Ø. Disseminated tumour cells as a prognostic biomarker in colorectal cancer. *Br J Cancer* 2011; **104**: 1434-1439 [PMID: 21448171 DOI: 10.1038/bjc.2011.97]

76 **Kienle P**, Koch M, Autschbach F, Benner A, Treiber M, Wannenmacher M, von Knebel Doeberitz M, Büchler M, Herfarth C, Weitz J. Decreased detection rate of disseminated tumor cells of rectal cancer patients after preoperative chemoradiation: a first step towards a molecular surrogate marker for neoadjuvant treatment in colorectal cancer. *Ann Surg* 2003; **238**: 324-330; discussion 330-331 [PMID: 14501498]

77 **Conzelmann M**, Linnemann U, Berger MR. K-ras codon 12 and 13 mutations are correlated with differential patterns of tumor cell dissemination in colorectal cancer patients. *Int J Oncol* 2004; **24**: 1537-1544 [PMID: 15138598]

78 **Riethdorf S**, Wikman H, Pantel K. Review: Biological relevance of disseminated tumor cells in cancer patients. *Int J Cancer* 2008; **123**: 1991-2006 [PMID: 18712708 DOI: 10.1002/ijc.23825]

79 **Schetter AJ**, Okayama H, Harris CC. The role of microRNAs in colorectal cancer. *Cancer J* 2012; **18**: 244-252 [PMID: 22647361 DOI: 10.1097/PPO.0b013e318258b78f]

80 **Cheng H**, Zhang L, Cogdell DE, Zheng H, Schetter AJ, Nykter M, Harris CC, Chen K, Hamilton SR, Zhang W. Circulating plasma MiR-141 is a novel biomarker for meta-

static colon cancer and predicts poor prognosis. *PLoS One* 2011; **6**: e17745 [PMID: 21445232 DOI: 10.1371/journal.pone.0017745]
81 **Zen K**, Zhang CY. Circulating microRNAs: a novel class of biomarkers to diagnose and monitor human cancers. *Med Res Rev* 2012; **32**: 326-348 [PMID: 22383180 DOI: 10.1002/med.20215]
82 **Chen X**, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, Li Q, Li X, Wang W, Zhang Y, Wang J, Jiang X, Xiang Y, Xu C, Zheng P, Zhang J, Li R, Zhang H, Shang X, Gong T, Ning G, Wang J, Zen K, Zhang J, Zhang CY. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* 2008; **18**: 997-1006 [PMID: 18766170 DOI: 10.1038/cr.2008.282]
83 **Mitchell PS**, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA* 2008; **105**: 10513-10518 [PMID: 18663219 DOI: 10.1073/pnas.0804549105]
84 **Ng EK**, Chong WW, Jin H, Lam EK, Shin VY, Yu J, Poon TC, Ng SS, Sung JJ. Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. *Gut* 2009; **58**: 1375-1381 [PMID: 19201770 DOI: 10.1136/gut.2008.167817]
85 **Negin BP**, Cohen SJ. Circulating tumor cells in colorectal cancer: past, present, and future challenges. *Curr Treat Options Oncol* 2010; **11**: 1-13 [PMID: 20143276 DOI: 10.1007/s11864-010-0115-3]
86 **Allard WJ**, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, Tibbe AG, Uhr JW, Terstappen LW. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin Cancer Res* 2004; **10**: 6897-6904 [PMID: 15501967 DOI: 10.1158/1078-0432.CCR-04-0378]
87 **Riethdorf S**, Fritsche H, Müller V, Rau T, Schindlbeck C, Rack B, Janni W, Coith C, Beck K, Jänicke F, Jackson S, Gornet T, Cristofanilli M, Pantel K. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system. *Clin Cancer Res* 2007; **13**: 920-928 [PMID: 17289886 DOI: 10.1158/1078-0432.CCR-06-1695]
88 **Cohen SJ**, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, Picus J, Morse M, Mitchell E, Miller MC, Doyle GV, Tissing H, Terstappen LW, Meropol NJ. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *J Clin Oncol* 2008; **26**: 3213-3221 [PMID: 18591556 DOI: 10.1200/JCO.2007.15.8923]
89 **Kim HJ**, Yu MH, Kim H, Byun J, Lee C. Noninvasive molecular biomarkers for the detection of colorectal cancer. *BMB Rep* 2008; **41**: 685-692 [PMID: 18959813 DOI: 10.5483/BMBRep.2008.41.10.685]
90 **Bolocan A**, Ion D, Ciocan DN, Paduraru DN. Prognostic and predictive factors in colorectal cancer. *Chirurgia* (Bucur) 2012; **107**: 555-563 [PMID: 23116828]
91 **Duffy MJ**. Carcinoembryonic antigen as a marker for colorectal cancer: is it clinically useful? *Clin Chem* 2001; **47**: 624-630 [PMID: 11274010]
92 **Duffy MJ**, van Dalen A, Haglund C, Hansson L, Klapdor R, Lamerz R, Nilsson O, Sturgeon C, Topolcan O. Clinical utility of biochemical markers in colorectal cancer: European Group on Tumour Markers (EGTM) guidelines. *Eur J Cancer* 2003; **39**: 718-727 [PMID: 12651195 DOI: 10.1016/S0959-8049(02)00811-0]
93 **Juhl H**, Stritzel M, Wroblewski A, Henne-Bruns D, Kremer B, Schmiegel W, Neumaier M, Wagener C, Schreiber HW, Kalthoff H. Immunocytological detection of micrometastatic cells: comparative evaluation of findings in the peritoneal cavity and the bone marrow of gastric, colorectal and pancreatic cancer patients. *Int J Cancer* 1994; **57**: 330-335 [PMID: 8168992 DOI: 10.1002/ijc.2910570307]
94 **Cohen AM**, Garin-Chesa P, Hanson M, Weyhrauch K, Kemeny N, Fong Y, Paty P, Welt S, Old L. In vitro detection of occult bone marrow metastases in patients with colorectal cancer hepatic metastases. *Dis Colon Rectum* 1998; **41**: 1112-1115 [PMID: 9749494 DOI: 10.1007/BF02239432]
95 **Schott A**, Vogel I, Krueger U, Kalthoff H, Schreiber HW, Schmiegel W, Henne-Bruns D, Kremer B, Juhl H. Isolated tumor cells are frequently detectable in the peritoneal cavity of gastric and colorectal cancer patients and serve as a new prognostic marker. *Ann Surg* 1998; **227**: 372-379 [PMID: 9527060 DOI: 10.1097/00000658-199803000-00009]
96 **Schlimok G**, Funke I, Holzmann B, Göttlinger G, Schmidt G, Häuser H, Swierkot S, Warnecke HH, Schneider B, Koprowski H, Riethmüller G. Micrometastatic cancer cells in bone marrow: in vitro detection with anti-cytokeratin and in vivo labeling with anti-17-1A monoclonal antibodies. *Proc Natl Acad Sci USA* 1987; **84**: 8672-8676 [PMID: 2446326 DOI: 10.1073/pnas.84.23.8672]
97 **Schneider BM**, Schlimok G, Riethmüller G, Witte J. [Bone marrow micrometastases in colorectal cancers]. *Fortschr Med* 1989; **107**: 59-63 [PMID: 2707688]
98 **Lindemann F**, Schlimok G, Dirschedl P, Witte J, Riethmüller G. Prognostic significance of micrometastatic tumour cells in bone marrow of colorectal cancer patients. *Lancet* 1992; **340**: 685-689 [PMID: 1381801 DOI: 10.1016/0140-6736(92)92230-D]
99 **Silly H**, Samonigg H, Stöger H, Brezinschek HP, Wilders-Truschnig M. Micrometastatic tumour cells in bone marrow in colorectal cancer. *Lancet* 1992; **340**: 1288 [PMID: 1359340 DOI: 10.1016/0140-6736(92)92987-Q]
100 **Pantel K**, Schlimok G, Angstwurm M, Weckermann D, Schmaus W, Gath H, Passlick B, Izbicki JR, Riethmüller G. Methodological analysis of immunocytochemical screening for disseminated epithelial tumor cells in bone marrow. *J Hematother* 1994; **3**: 165-173 [PMID: 7530132 DOI: 10.1089/scd.1.1994.3.165]
101 **Leinung S**, Würl P, Schönfelder A, Weiss CL, Röder I, Schönfelder M. Detection of cytokeratin-positive cells in bone marrow in breast cancer and colorectal carcinoma in comparison with other factors of prognosis. *J Hematother Stem Cell Res* 2000; **9**: 905-911 [PMID: 11177604 DOI: 10.1089/152581600750062354]
102 **Tórtola S**, Steinert R, Hantschick M, Peinado MA, Gastinger I, Stosiek P, Lippert H, Schlegel W, Reymond MA. Discordance between K-ras mutations in bone marrow micrometastases and the primary tumor in colorectal cancer. *J Clin Oncol* 2001; **19**: 2837-2843 [PMID: 11387355]
103 **Werther K**, Normark M, Brünner N, Nielsen HJ. Cytokeratin-positive cells in preoperative peripheral blood and bone marrow aspirates of patients with colorectal cancer. *Scand J Clin Lab Invest* 2002; **62**: 49-57 [PMID: 12002413 DOI: 10.1080/00365510275317208]
104 **Schoppmeyer K**, Frühauf N, Oldhafer K, Seeber S, Kasimir-Bauer S. Tumor cell dissemination in colon cancer does not predict extrahepatic recurrence in patients undergoing surgery for hepatic metastases. *Oncol Rep* 2006; **15**: 449-454 [PMID: 16391868]
105 **Bosch B**, Guller U, Schnider A, Maurer R, Harder F, Metzger U, Marti WR. Perioperative detection of disseminated tumour cells is an independent prognostic factor in patients with colorectal cancer. *Br J Surg* 2003; **90**: 882-888 [PMID: 12854118 DOI: 10.1002/bjs.4129]
106 **Migliore L**, Migheli F, Spisni R, Coppedè F. Genetics, cytogenetics, and epigenetics of colorectal cancer. *J Biomed Biotechnol* 2011; **2011**: 792362 [PMID: 21490705 DOI: 10.1155/2011/792362]
107 **Hayashi N**, Ito I, Yanagisawa A, Kato Y, Nakamori S, Imaoka

S, Watanabe H, Ogawa M, Nakamura Y. Genetic diagnosis of lymph-node metastasis in colorectal cancer. *Lancet* 1995; **345**: 1257-1259 [PMID: 7746054 DOI: 10.1016/S0140-6736(95)90922-2]

108 **Hayashi N**, Arakawa H, Nagase H, Yanagisawa A, Kato Y, Ohta H, Takano S, Ogawa M, Nakamura Y. Genetic diagnosis identifies occult lymph node metastases undetectable by the histopathological method. *Cancer Res* 1994; **54**: 3853-3856 [PMID: 8033106]

109 **Rahbari NN**, Aigner M, Thorlund K, Mollberg N, Motschall E, Jensen K, Diener MK, Büchler MW, Koch M, Weitz J. Meta-analysis shows that detection of circulating tumor cells indicates poor prognosis in patients with colorectal cancer. *Gastroenterology* 2010; **138**: 1714-1726 [PMID: 20100481 DOI: 10.1053/j.gastro.2010.01.008]

110 **Mohan HM**, O'Connor DB, O'Riordan JM, Winter DC. Prognostic significance of detection of microscopic peritoneal disease in colorectal cancer: a systematic review. *Surg Oncol* 2013; **22**: e1-e6 [PMID: 23481599 DOI: 10.1016/j.suronc.2013.01.001]

111 **Puglisi MA**, Tesori V, Lattanzi W, Gasbarrini GB, Gasbarrini A. Colon cancer stem cells: controversies and perspectives. *World J Gastroenterol* 2013; **19**: 2997-3006 [PMID: 23716979 DOI: 10.3748/wjg.v19.i20.2997]

112 **Spila A**, Ferroni P, Cosimelli M, D'Alessandro R, Abbolito MR, Mariotti S, Aloe S, Carone MD, Graziano F, Tedesco M, Martini F, Mancini R, Stigliano V, Roselli M, Guadagni F. Comparative analysis of CA 242 and CA 19-9 serum tumor markers in colorectal cancer patients. A longitudinal evaluation. *Anticancer Res* 2001; **21**: 1263-1270 [PMID: 11396197]

P- Reviewers: Di Lorenzo G, Fang BL, Shen J, Tez M, Zhang KH
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8939

World J Gastroenterol 2014 July 21; 20(27): 8939-8946
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Chemoprevention of colorectal cancer by targeting obesity-related metabolic abnormalities

Yohei Shirakami, Masahito Shimizu, Masaya Kubota, Hiroshi Araki, Takuji Tanaka, Hisataka Moriwaki, Mitsuru Seishima

Yohei Shirakami, Mitsuru Seishima, Department of Informative Clinical Medicine, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan
Masahito Shimizu, Masaya Kubota, Hiroshi Araki, Hisataka Moriwaki, Department of Gastroenterology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan
Takuji Tanaka, Department of Tumor Pathology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan
Author contributions: Shirakami Y performed the review of the literature and wrote the paper; Shimizu M and Tanaka T performed the review of the literature and edited the final draft; Kubota M and Araki H created the figures used in this review; Moriwaki H and Seishima M gave the final approval of the version to be published.
Correspondence to: Masahito Shimizu, MD, PhD, Department of Gastroenterology, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. shimim-gif@umin.ac.jp
Telephone: +81-58-2306313 Fax: +81-58-2306310
Received: September 27, 2013 Revised: December 20, 2013
Accepted: April 1, 2014
Published online: July 21, 2014

Abstract

Obesity and its related metabolic disorders, including insulin resistance and chronic inflammation, increase the risk of colorectal cancer (CRC). This observation suggests that the metabolic abnormalities associated with obesity can be effective targets for preventing the development of CRC in obese individuals. In recent years, many studies using obese and diabetic animal models have been conducted to investigate the chemoprevention of CRC using pharmaceutical or nutritional interventions. Pitavastatin, a medicine used to treat hyperlipidemia, prevents the development of obesity-related colorectal carcinogenesis by attenuating chronic inflammation. Anti-hypertensive medicines, such as captopril and telmisartan, also suppress the formation of colonic preneoplastic lesions in obese and diabetic mice. In addition, several phytochemicals, including green tea catechins, have been reported to improve metabolic disorders and prevent the development of various cancers, including CRC. Moreover, the administration of branched-chain amino acids, which improves protein malnutrition and prevents the progression of hepatic failure, is effective for suppressing obesity-related colon carcinogenesis, which is thought to be associated with improvements in insulin resistance. In the present article, we summarize the detailed relationship between metabolic abnormalities and the development of CRC. This review also outlines recent evidence, in particular drawing from basic and clinical examinations using either pharmaceutical or nutritional intervention that suggests that targeting metabolic alterations may be an effective strategy for preventing the development of CRC in obese individuals.

Key words: Colorectal cancer; Obesity; Green tea catechin; Branched-chain amino acid; Chemoprevention

Core tip: Obesity and its related metabolic disorders increase the risk of colorectal cancer (CRC). Many studies using obese animal models have been conducted to investigate the chemoprevention of CRC using pharmaceutical or nutritional interventions. Lipid-lowering and anti-hypertensive medicines suppress the development of colonic preneoplastic lesions in obese mice. Green tea catechins improve metabolic disorders and prevent the development of CRC. The administration of branched-chain amino acids may be effective for suppressing obesity-related CRC. This review summarizes recent evidence that suggests that targeting metabolic alterations may be an effective strategy for preventing the development of CRC in obese individuals.

Shirakami Y, Shimizu M, Kubota M, Araki H, Tanaka T, Moriwaki H, Seishima M. Chemoprevention of colorectal cancer by targeting obesity-related metabolic abnormalities. *World J Gastroenterol* 2014; 20(27): 8939-8946 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8939.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8939

INTRODUCTION

Obesity is recognized to be a serious health problem that is becoming more prevalent worldwide[1]. It frequently causes a number of medical problems, including type 2 diabetes mellitus, cardiovascular diseases, hypertension, and dyslipidemia[2]. In addition, recent epidemiological and experimental evidence indicates that obesity and its related metabolic abnormalities, especially diabetes mellitus, are associated with the development of certain types of epithelial malignancies, including colorectal cancer (CRC)[2-6]. Renehan *et al*[7] revealed in a large-scale meta-analysis that the magnitude of the risk of CRC is greater in obese males than in non-obese males.

Several pathophysiological mechanisms that correlate obesity with colorectal carcinogenesis have been demonstrated, including the occurrence of insulin resistance and adipocytokine imbalances, alterations in the insulin-like growth factor-1 (IGF-1)/IGF-1 receptor (IGF-1R) axis, chronic inflammation, and the induction of oxidative stress[2-6,8]. These findings also suggest that targeting obesity-associated pathophysiological disorders using nutritional or pharmaceutical interventions is a promising strategy for suppressing obesity-related colorectal carcinogenesis. For example, pitavastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, suppresses the development of colonic preneoplastic lesions by attenuating chronic inflammation in obese and diabetic mice[9]. Anti-hypertensive medicines, such as captopril and telmisartan, also prevent obesity-related colorectal carcinogenesis, and this suppressive effect appears to be associated with the reduction of oxidative stress and chronic inflammation[10].

Recently, green tea catechins (GTCs) have received significant attention due to their beneficial impact on health, as they are reported to improve metabolic abnormalities and prevent cancer[11-15]. Another phytochemical, curcumin, a component of turmeric, also demonstrates suppressive effects against colorectal carcinogenesis in obese mice[16]. Supplementation with branched-chain amino acids (BCAA: leucine, isoleucine, and valine), which can inhibit the progression of hepatic failure in patients with chronic liver disease[17-19], suppresses obesity-related colorectal carcinogenesis by improving insulin resistance in obese and diabetic mice[20].

The present review summarizes multiple mechanisms by which obesity and its related metabolic alterations influence the development of CRC, particularly focusing on the emergence of insulin resistance and the subsequent inflammatory cascade. This article also aims to review the possibility that nutritional or pharmaceutical approaches targeting pathophysiological conditions caused by obesity is effective in preventing obesity-related colorectal carcinogenesis.

POTENTIAL PATHOPHYSIOLOGICAL MECHANISMS CORRELATING OBESITY TO THE DEVELOPMENT OF CRC

Among the various obesity-related metabolic disorders, insulin resistance and hyperinsulinemia are considered to be pivotal risk factors for the development of CRC[21]. Insulin itself and the insulin-regulated signal transduction network play important roles in oncogenesis[22-24]. Insulin stimulates the growth of CRC cells[25] and promotes colorectal tumor growth in animal models[26]. In addition, IGF-1, an important endocrine and paracrine regulator of tissue growth and metabolism, is biologically activated by insulin resistance[27,28]. A number of studies have shown that the IGF-1/IGF-1R axis plays a significant role in the carcinogenesis of various cancers, including CRC[22-24]. Insulin resistance alters the IGF/IGF-1R axis, which contributes to the development of CRC[29,30]. The binding of insulin and IGF-1 to their respective receptors on cancer and/or precancerous cells activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which is responsible for cellular processes, such as growth, proliferation, and survival[22,23]. Moreover, insulin resistance and an increased fat mass create an oxidative stress environment in tissues and increase the expression of various pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which stimulate tumor growth and progression[31-35]. Increased oxidative stress promotes DNA damage and activates the PI3K/Akt signaling pathway, both of which play essential roles in cancer development[36,37]. Hence, insulin resistance and subsequent inflammatory cascades involving increased oxidative stress are thought to be significant factors in the development of obesity-related CRC.

The overproduction of fat storage causes an imbalance in adipocytokines, increasing the levels of leptin and decreasing the levels of adiponectin in the serum. This imbalance also contributes to obesity-associated carcinogenesis[38,39]. Leptin induces the production of pro-inflammatory cytokines TNF-α and IL-6[40,41], which may lead to tumor growth and progression as stated above. With respect to CRC, leptin is reported to stimulate CRC cell growth[42]. In addition, a positive association between the circulating leptin levels and the development of CRC has been indicated in an epidemiological study[43]. These findings suggest that obesity-induced abnormalities cooperatively increase the risk of cancer, including CRC, in obese individuals (Figure 1).

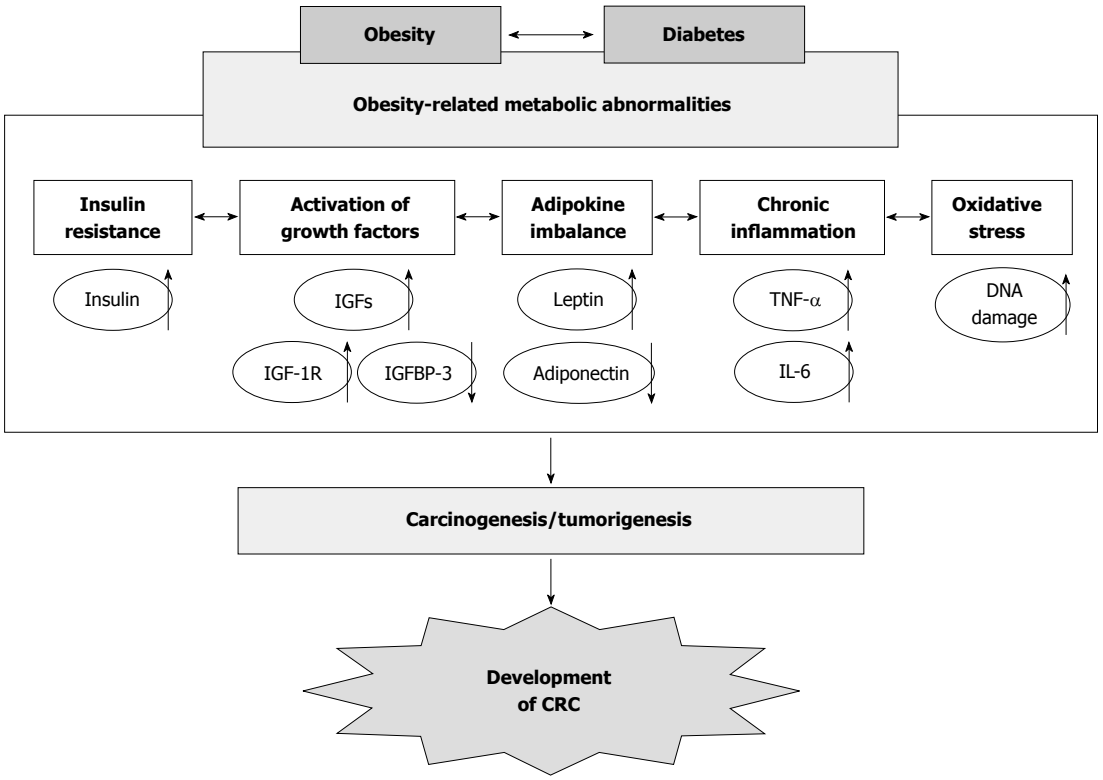


Figure 1 Proposed mechanisms linking obesity and its related metabolic abnormalities to the development of colorectal cancer. CRC: Colorectal cancer; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; IGF-1: Insulin-like growth factor-1; IGFBP-3: IGF-binding protein-3.

CHEMOPREVENTIVE EFFECTS OF GREEN TEA CATECHINS ON THE DEVELOPMENT OF METABOLIC ABNORMALITIES AND CRC

In recent years, GTCs have received considerable attention due to their salutary influence on health. Several studies have indicated that GTCs possess various useful properties, such as anti-obesity effects[11]. A recent meta-analysis of clinical trials demonstrated that GTCs appear to reduce body weight and fat[44]. Rains *et al*[45] reported several potential mechanisms whereby GTCs may affect body weight: GTCs may increase energy expenditures, promote lipid oxidation, and decrease nutrient absorption. Laboratory, epidemiological, and human interventional investigations have also shown the effects of GTCs in ameliorating metabolic syndrome[46,47]. In rodent models of obesity and diabetes, the administration of green tea or its constituents results in the significant reduction of body weight and improvements in hyperglycemia, hyperinsulinemia, hyperleptinemia, hepatic steatosis, and liver dysfunction[48-50]. Treatment with GTCs also decreases the plasma levels of insulin, TNF-α, and IL-6 in a rat model of insulin resistance[51]. These observations suggest that long-term supplementation of GTCs may be useful for preventing the progression of obesity-related metabolic abnormalities. In addition to their anti-obesity effects, GTCs are known to possess anti-cancer and cancer prevention properties[12-15]. Positive evidence for the chemopreventive effects of tea preparations against premalignant lesions has been provided in human intervention studies. Li *et al*[52] reported the preventive effects of tea on human oral precancerous mucosal lesion leukoplakia. Clinical efficacy of green tea extract in patients with human papilloma virus infected cervical lesions was demonstrated by Ahn *et al*[53]. The inhibitory effect of GTCs on the development of high-grade prostate intraepithelial neoplasia was also reported[54,55]. Moreover, a pilot study showed that the administration of GTCs successfully prevents the development of colorectal adenomas, which are considered to be precancerous lesions of CRC[56].

Several properties of GTCs are responsible for their anti-cancer and cancer prevention effects, including their antioxidant and anti-inflammatory actions[15,57]. A number of reported studies have indicated that GTCs, especially the major biologically active component epigallocatechin gallate (EGCG), inhibit proliferation and induce apoptosis in cancer cells by modulating the activities of diverse receptor tyrosine kinases (RTKs) and their downstream signaling pathways, such as the Ras/extracellular signal-regulated kinase and PI3K/Akt signaling pathways[12-14,58-61]. EGCG suppresses cell growth by inhibiting the activation of IGF-1R, a member of the RTK family, in human CRC cell lines. This inhibition is associated with a decrease in the expression of IGF-1/2 and an increase in the expression of IGF-binding protein-3 (IGFBP-3), which negatively controls the function of the IGF/IGF-1R axis[62,63]. Taken together, these

reports indicate that the IGF/IGF-1R axis, which plays a critical role in both the development of cancer and occurrence of obesity-induced pathological events[22,23], is a critical target of GTCs.

PREVENTION OF OBESITY-RELATED CRC *VIA* A NUTRACEUTICAL APPROACH

Recent investigations have shown that an increased amount of body fat and high body mass index are associated with an increased risk of colorectal malignancy[2,5-7]. While the prolonged high consumption of red and processed meat may also increase the risk of CRC[64], there is persuasive evidence that positive dietary habits, especially a high level of consumption of fruits and vegetables, can reduce the risk of this malignancy[65]. C57BLKS/J- +Lepr^{db}/+Lepr^{db} (*db*/*db*) mice, which are genetically altered and those leptin receptors are mutated, express phenotypes of obesity and having type 2 diabetes mellitus in addition to hyperlipidemia, hyperinsulinemia, and hyperleptinemia[66]. A preclinical animal model using *db*/*db* mice was established by Hirose *et al*[67] in which the intraperitoneal administration of colonic carcinogen azoxymethane (AOM) is thought to be markedly useful for determining the underlying mechanisms of how specific agents prevent the development of obesity-related CRC. Furthermore, *db*/*db* mice are susceptible to AOM, as AOM-induced colonic precancerous lesions, aberrant crypt foci (ACF) and β-catenin accumulated crypts (BCAC) develop to an obviously greater extent in these mice than in control mice[67].

Diets supplemented with certain types of flavonoids, including citrus compounds, suppress the development of premalignant lesions of CRC in *db*/*db* mice[68,69]. We also used this experimental rodent model to examine the obesity-related cancer chemopreventive effects of curcumin, a yellow pigment found in the rhizome of the spice turmeric, which is known to possess both anti-inflammatory and cancer prevention properties[70-72]. A report by Kubota *et al*[16] revealed that supplementation with curcumin effectively prevents the development of colonic preneoplastic lesions in *db*/*db* mice treated with AOM injections in association with the inhibition of the NF-κB activity and the TNF-α, IL-6, and cyclooxygenase-2 (COX-2) expression in the colonic mucosa and improvements in adipocytokine imbalances.

In the same manner, we employed a rodent model to investigate in detail the effects of EGCG and BCAA on the prevention of obesity-related colorectal carcinogenesis. The mucosa in the *db*/*db* mice colon expresses increased levels of IGF-1R, the phosphorylated form of IGF-1R (p-IGF-1R), β-catenin, and COX-2[73]. We observed that drinking water containing EGCG caused a significant reduction in the number of ACF and BCAC, which accumulate IGF-1R proteins, and this decreasing effect was associated with the inhibition of the expression of IGF-1R, p-IGF-1R, the phosphorylated form of glycogen synthase kinase-3β (GSK-3β), β-catenin, COX-2, and cyclin D1 on the colonic mucosa[73]. EGCG also decreased the serum levels of IGF-1, insulin, triglycerides, total cholesterol, and leptin, while increasing the serum level of IGFBP-3[73]. In accordance with the results of this study, another study showed that supplementation with BCAA markedly decreased the number of ACF and BCAC compared with that observed in the control diet-fed groups by inhibiting the phosphorylation of IGF-1R, GSK-3β, and Akt in the colonic mucosa[20]. In that study, the serum levels of insulin, IGF-1, IGF-2, triglycerides, total cholesterol, and leptin were also decreased in the BCAA-treated mice[20].

Taken together, these findings suggest that both EGCG and BCAA successfully suppress the development of preneoplastic lesions of obesity-related CRC *via* diverse mechanisms, including the suppression of the IGF/IGF-1R axis and improvements in hyperlipidemia, hyperinsulinemia, and hyperleptinemia. Therefore, nutraceutical approaches, for example, the administration of EGCG or BCAA, may be useful for use in the chemoprevention of colorectal tumorigenesis in obese individuals (Figure 2).

PREVENTION OF OBESITY-RELATED CRC *VIA* A PHARMACEUTICAL APPROACH

Obesity often leads to various medical problems, including hypertension and dyslipidemia[2]. Hyperactivity of the renin-angiotensin system (RAS) has been shown to be involved in the etiology of high blood pressure, obesity, and metabolic syndrome[74]. There is convincing evidence that the RAS is frequently upregulated in human malignancies due to systemic oxidative stress and hypoxia, which trigger a state of chronic inflammation[75]. We investigated the effects of an angiotensin-converting enzyme inhibitor, captopril, and an angiotensin-Ⅱ type 1 receptor blocker, telmisartan, both of which can inhibit the RAS, on the development of colonic preneoplastic lesions in an obesity-related CRC model[10]. The administration of either captopril or telmisartan significantly reduced the number of ACF and BCAC and decreased the expression of TNF-α in the colonic mucosa. Oxidative stress throughout the body is also decreased by the administration of either captopril or telmisartan[10].

Statins, HMG-CoA reductase inhibitors, are widely used for the treatment of dyslipidemia. In addition to their lipid-lowering effects, statins have been shown to possess anti-cancer properties[76,77]. Statins induce apoptosis in human CRC cells and attenuate inflammation-related colon carcinogenesis in mice[78,79]. Moreover, epidemiological studies have indicated the chemopreventive effects of statins on various types of cancer, including CRC[76,77,80]. We conducted a study using a lipophilic statin, pitavastatin, to examine the cancer prevention effects of such drugs on obesity-related colon carcinogenesis in *db*/*db* obese mice treated with AOM injection[9]. Pitavastatin treatment achieved a marked reduction in the number of BCAC by inhibiting proliferation and

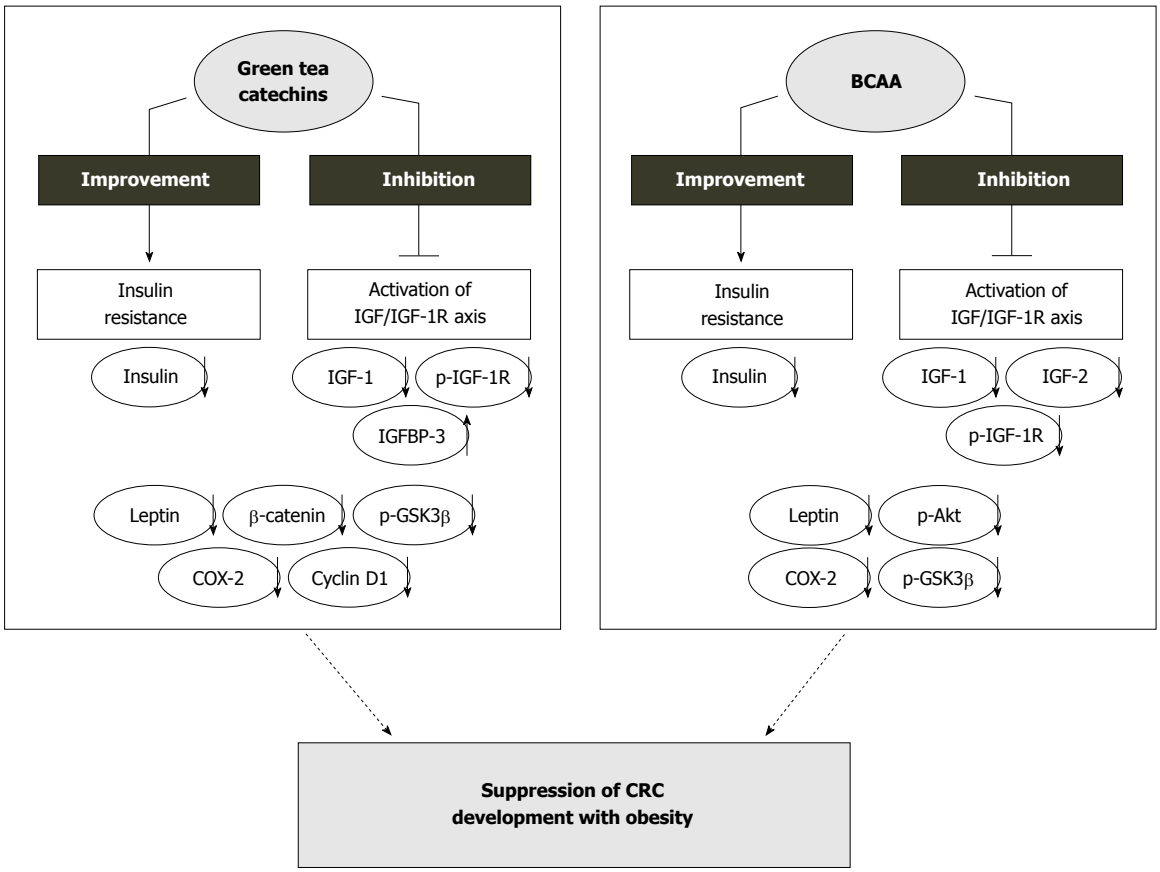


Figure 2 Mechanisms of action of green tea catechins and branched-chain amino acids in the inhibition of obesity-related colorectal carcinogenesis. IGF-1: Insulin-like growth factor-1; BCAA: Branched-chain amino acids; COX-2: Cyclooxygenase-2; IGF-1R: Insulin-like growth factors-1 receptor; GSK-3β: Glycogen synthase kinase-3β.

surrounding inflammation, in which the expression levels of TNF-α, IL-6, and COX-2 in the colonic mucosa were decreased. In addition, pitavastatin also decreased the serum levels of total cholesterol, TNF-α, IL-6, and leptin, while increasing the serum level of adiponectin[9].

These observations suggest that both anti-hypertensive and lipid-lowering agents suppress obesity-related colorectal carcinogenesis. The potential mechanisms involve improving dyslipidemia and hyperleptinemia and attenuating chronic inflammation in the colonic mucosa by decreasing the expression of pro-inflammatory cytokines. Therefore, the pharmaceutical approach described above appears to be a feasible strategy for the chemoprevention of obesity-related CRC, as these medicines exert an original pharmacological effect against the development of obesity-related metabolic disorders in addition to their cancer prevention effects.

CONCLUSION

Obesity and its related metabolic disorders, which are associated with an increased risk of several life-threatening diseases, including cancer, are critical health problems that must be addressed. Among human cancers, CRC is one of the most representative malignancies influenced by obesity. In this review, we reported the potential efficacy of nutraceutical and pharmaceutical approaches for targeting obesity-related metabolic alterations. Restoring such abnormalities to a regular state is a promising strategy for preventing the development of obesity-related CRC. Tea catechins, especially GTCs and its active constituent EGCG, can be considered feasible agents for preventing carcinogenesis, as several human interventional trials have demonstrated the efficacy of GTCs as chemopreventive agents without serious adverse effects[54,56,61]. BCAA and certain types of statins and anti-hypertensive medicines are also thought to be potential agents because they are widely used in clinical practice and their safety has been adequately proven. Moreover, a randomized controlled trial demonstrated that BCAA supplementation can prevent liver cancer in obese individuals[19,81]. Therefore, further advanced translational research should be conducted to examine whether active interventions using these agents can prevent the development and recurrence of CRC in patients with obesity.

REFERENCES

1 **Khandekar MJ**, Cohen P, Spiegelman BM. Molecular mechanisms of cancer development in obesity. *Nat Rev Cancer* 2011; **11**: 886-895 [PMID: 22113164 DOI: 10.1038/nrc3174]
2 **Calle EE**, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively

studied cohort of U.S. adults. *N Engl J Med* 2003; **348**: 1625-1638 [PMID: 12711737 DOI: 10.1056/NEJMoa021423]
3 **Aleksandrova K**, Nimptsch K, Pischon T. Obesity and colorectal cancer. *Front Biosci* (Elite Ed) 2013; **5**: 61-77 [PMID: 23276970]
4 **Anderson AS**, Caswell S. Obesity management--an opportunity for cancer prevention. *Surgeon* 2009; **7**: 282-285 [PMID: 19848061]
5 **Frezza EE**, Wachtel MS, Chiriva-Internati M. Influence of obesity on the risk of developing colon cancer. *Gut* 2006; **55**: 285-291 [PMID: 16239255]
6 **Giovannucci E**, Michaud D. The role of obesity and related metabolic disturbances in cancers of the colon, prostate, and pancreas. *Gastroenterology* 2007; **132**: 2208-2225 [PMID: 17498513]
7 **Renehan AG**, Tyson M, Egger M, Heller RF, Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. *Lancet* 2008; **371**: 569-578 [PMID: 18280327 DOI: 10.1016/S0140-6736(08)60269-X]
8 **Ramos-Nino ME**. The role of chronic inflammation in obesity-associated cancers. *ISRN Oncol* 2013; **2013**: 697521 [PMID: 23819063 DOI: 10.1155/2013/697521]
9 **Yasuda Y**, Shimizu M, Shirakami Y, Sakai H, Kubota M, Hata K, Hirose Y, Tsurumi H, Tanaka T, Moriwaki H. Pitavastatin inhibits azoxymethane-induced colonic preneoplastic lesions in C57BL/KsJ-db/db obese mice. *Cancer Sci* 2010; **101**: 1701-1707 [PMID: 20398056 DOI: 10.1111/j.1349-7006.2010.01579.x]
10 **Kubota M**, Shimizu M, Sakai H, Yasuda Y, Ohno T, Kochi T, Tsurumi H, Tanaka T, Moriwaki H. Renin-angiotensin system inhibitors suppress azoxymethane-induced colonic preneoplastic lesions in C57BL/KsJ-db/db obese mice. *Biochem Biophys Res Commun* 2011; **410**: 108-113 [PMID: 21640075 DOI: 10.1016/j.bbrc.2011.05.115]
11 **Kao YH**, Chang HH, Lee MJ, Chen CL. Tea, obesity, and diabetes. *Mol Nutr Food Res* 2006; **50**: 188-210 [PMID: 16416476 DOI: 10.1002/mnfr.200500109]
12 **Shimizu M**, Adachi S, Masuda M, Kozawa O, Moriwaki H. Cancer chemoprevention with green tea catechins by targeting receptor tyrosine kinases. *Mol Nutr Food Res* 2011; **55**: 832-843 [PMID: 21538846 DOI: 10.1002/mnfr.201000622]
13 **Shimizu M**, Shirakami Y, Moriwaki H. Targeting receptor tyrosine kinases for chemoprevention by green tea catechin, EGCG. *Int J Mol Sci* 2008; **9**: 1034-1049 [PMID: 19325845 DOI: 10.3390/ijms9061034]
14 **Shimizu M**, Weinstein IB. Modulation of signal transduction by tea catechins and related phytochemicals. *Mutat Res* 2005; **591**: 147-160 [PMID: 15992833]
15 **Yang CS**, Wang X, Lu G, Picinich SC. Cancer prevention by tea: animal studies, molecular mechanisms and human relevance. *Nat Rev Cancer* 2009; **9**: 429-439 [PMID: 19472429 DOI: 10.1038/nrc2641]
16 **Kubota M**, Shimizu M, Sakai H, Yasuda Y, Terakura D, Baba A, Ohno T, Tsurumi H, Tanaka T, Moriwaki H. Preventive effects of curcumin on the development of azoxymethane-induced colonic preneoplastic lesions in male C57BL/KsJ-db/db obese mice. *Nutr Cancer* 2012; **64**: 72-79 [PMID: 22172229 DOI: 10.1080/01635581.2012.630554]
17 **Kawaguchi T**, Nagao Y, Matsuoka H, Ide T, Sata M. Branched-chain amino acid-enriched supplementation improves insulin resistance in patients with chronic liver disease. *Int J Mol Med* 2008; **22**: 105-112 [PMID: 18575782]
18 **Marchesini G**, Bianchi G, Merli M, Amodio P, Panella C, Loguercio C, Rossi Fanelli F, Abbiati R. Nutritional supplementation with branched-chain amino acids in advanced cirrhosis: a double-blind, randomized trial. *Gastroenterology* 2003; **124**: 1792-1801 [PMID: 12806613]
19 **Muto Y**, Sato S, Watanabe A, Moriwaki H, Suzuki K, Kato A, Kato M, Nakamura T, Higuchi K, Nishiguchi S, Kumada H. Effects of oral branched-chain amino acid granules on event-free survival in patients with liver cirrhosis. *Clin Gastroenterol Hepatol* 2005; **3**: 705-713 [PMID: 16206505]
20 **Shimizu M**, Shirakami Y, Iwasa J, Shiraki M, Yasuda Y, Hata K, Hirose Y, Tsurumi H, Tanaka T, Moriwaki H. Supplementation with branched-chain amino acids inhibits azoxymethane-induced colonic preneoplastic lesions in male C57BL/KsJ-db/db mice. *Clin Cancer Res* 2009; **15**: 3068-3075 [PMID: 19366832 DOI: 10.1158/1078-0432.CCR-08-2093]
21 **Chang CK**, Ulrich CM. Hyperinsulinaemia and hyperglycaemia: possible risk factors of colorectal cancer among diabetic patients. *Diabetologia* 2003; **46**: 595-607 [PMID: 12764580 DOI: 10.1007/s00125-003-1109-5]
22 **Clayton PE**, Banerjee I, Murray PG, Renehan AG. Growth hormone, the insulin-like growth factor axis, insulin and cancer risk. *Nat Rev Endocrinol* 2011; **7**: 11-24 [PMID: 20956999 DOI: 10.1038/nrendo.2010.171]
23 **Pollak M**. Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer* 2008; **8**: 915-928 [PMID: 19029956 DOI: 10.1038/nrc2536]
24 **Giovannucci E**. Insulin, insulin-like growth factors and colon cancer: a review of the evidence. *J Nutr* 2001; **131**: 3109S-3120S [PMID: 11694656]
25 **Björk J**, Nilsson J, Hultcrantz R, Johansson C. Growth-regulatory effects of sensory neuropeptides, epidermal growth factor, insulin, and somatostatin on the non-transformed intestinal epithelial cell line IEC-6 and the colon cancer cell line HT 29. *Scand J Gastroenterol* 1993; **28**: 879-884 [PMID: 7505479]
26 **Tran TT**, Medline A, Bruce WR. Insulin promotion of colon tumors in rats. *Cancer Epidemiol Biomarkers Prev* 1996; **5**: 1013-1015 [PMID: 8959325]
27 **Alexia C**, Fallot G, Lasfer M, Schweizer-Groyer G, Groyer A. An evaluation of the role of insulin-like growth factors (IGF) and of type-I IGF receptor signalling in hepatocarcinogenesis and in the resistance of hepatocarcinoma cells against drug-induced apoptosis. *Biochem Pharmacol* 2004; **68**: 1003-1015 [PMID: 15313394 DOI: 10.1016/j.bcp.2004.05.029]
28 **Sandhu MS**, Dunger DB, Giovannucci EL. Insulin, insulin-like growth factor-I (IGF-I), IGF binding proteins, their biologic interactions, and colorectal cancer. *J Natl Cancer Inst* 2002; **94**: 972-980 [PMID: 12096082]
29 **Durai R**, Yang W, Gupta S, Seifalian AM, Winslet MC. The role of the insulin-like growth factor system in colorectal cancer: review of current knowledge. *Int J Colorectal Dis* 2005; **20**: 203-220 [PMID: 15650828 DOI: 10.1007/s00384-004-0675-4]
30 **Singh P**, Rubin N. Insulinlike growth factors and binding proteins in colon cancer. *Gastroenterology* 1993; **105**: 1218-1237 [PMID: 7691674]
31 **Esposito K**, Nappo F, Marfella R, Giugliano G, Giugliano F, Ciotola M, Quagliaro L, Ceriello A, Giugliano D. Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: role of oxidative stress. *Circulation* 2002; **106**: 2067-2072 [PMID: 12379575]
32 **Hotamisligil GS**, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 1996; **271**: 665-668 [PMID: 8571133]
33 **Kern PA**, Ranganathan S, Li C, Wood L, Ranganathan G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am J Physiol Endocrinol Metab* 2001; **280**: E745-E751 [PMID: 11287357]
34 **Szlosarek P**, Charles KA, Balkwill FR. Tumour necrosis factor-alpha as a tumour promoter. *Eur J Cancer* 2006; **42**: 745-750 [PMID: 16517151]
35 **Flores MB**, Rocha GZ, Damas-Souza DM, Osório-Costa F, Dias MM, Ropelle ER, Camargo JA, de Carvalho RB, Carvalho HF, Saad MJ, Carvalheira JB. Obesity-induced increase in tumor necrosis factor-α leads to development of colon cancer in mice. *Gastroenterology* 2012; **143**: 741-753.e1-4 [PMID: 22677195 DOI: 10.1053/j.gastro.2012.05.045]

36 **Leslie NR**. The redox regulation of PI 3-kinase-dependent signaling. *Antioxid Redox Signal* 2006; **8**: 1765-1774 [PMID: 16987030 DOI: 10.1089/ars.2006.8.1765]

37 **Valko M**, Izakovic M, Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence. *Mol Cell Biochem* 2004; **266**: 37-56 [PMID: 15646026]

38 **Barb D**, Williams CJ, Neuwirth AK, Mantzoros CS. Adiponectin in relation to malignancies: a review of existing basic research and clinical evidence. *Am J Clin Nutr* 2007; **86**: s858-s866 [PMID: 18265479]

39 **Considine RV**, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* 1996; **334**: 292-295 [PMID: 8532024 DOI: 10.1056/NEJM199602013340503]

40 **Fenton JI**, Hursting SD, Perkins SN, Hord NG. Interleukin-6 production induced by leptin treatment promotes cell proliferation in an Apc (Min/+) colon epithelial cell line. *Carcinogenesis* 2006; **27**: 1507-1515 [PMID: 16597643]

41 **Molina A**, Vendrell J, Gutiérrez C, Simón I, Masdevall C, Soler J, Gómez JM. Insulin resistance, leptin and TNF-alpha system in morbidly obese women after gastric bypass. *Obes Surg* 2003; **13**: 615-621 [PMID: 12935365 DOI: 10.1381/096089203322190844]

42 **Amemori S**, Ootani A, Aoki S, Fujise T, Shimoda R, Kakimoto T, Shiraishi R, Sakata Y, Tsunada S, Iwakiri R, Fujimoto K. Adipocytes and preadipocytes promote the proliferation of colon cancer cells in vitro. *Am J Physiol Gastrointest Liver Physiol* 2007; **292**: G923-G929 [PMID: 17170030]

43 **Stattin P**, Lukanova A, Biessy C, Söderberg S, Palmqvist R, Kaaks R, Olsson T, Jellum E. Obesity and colon cancer: does leptin provide a link? *Int J Cancer* 2004; **109**: 149-152 [PMID: 14735482 DOI: 10.1002/ijc.11668]

44 **Hursel R**, Viechtbauer W, Westerterp-Plantenga MS. The effects of green tea on weight loss and weight maintenance: a meta-analysis. *Int J Obes* (Lond) 2009; **33**: 956-961 [PMID: 19597519 DOI: 10.1038/ijo.2009.135]

45 **Rains TM**, Agarwal S, Maki KC. Antiobesity effects of green tea catechins: a mechanistic review. *J Nutr Biochem* 2011; **22**: 1-7 [PMID: 21115335 DOI: 10.1016/j.jnutbio.2010.06.006]

46 **Grove KA**, Lambert JD. Laboratory, epidemiological, and human intervention studies show that tea (Camellia sinensis) may be useful in the prevention of obesity. *J Nutr* 2010; **140**: 446-453 [PMID: 20089791 DOI: 10.3945/jn.109.115972]

47 **Thielecke F**, Boschmann M. The potential role of green tea catechins in the prevention of the metabolic syndrome - a review. *Phytochemistry* 2009; **70**: 11-24 [PMID: 19147161 DOI: 10.1016/j.phytochem.2008.11.011]

48 **Bose M**, Lambert JD, Ju J, Reuhl KR, Shapses SA, Yang CS. The major green tea polyphenol, (-)-epigallocatechin-3-gallate, inhibits obesity, metabolic syndrome, and fatty liver disease in high-fat-fed mice. *J Nutr* 2008; **138**: 1677-1683 [PMID: 18716169]

49 **Murase T**, Nagasawa A, Suzuki J, Hase T, Tokimitsu I. Beneficial effects of tea catechins on diet-induced obesity: stimulation of lipid catabolism in the liver. *Int J Obes Relat Metab Disord* 2002; **26**: 1459-1464 [PMID: 12439647 DOI: 10.1038/sj.ijo.0802141]

50 **Ramadan G**, El-Beih NM, Abd El-Ghffar EA. Modulatory effects of black v. green tea aqueous extract on hyperglycaemia, hyperlipidaemia and liver dysfunction in diabetic and obese rat models. *Br J Nutr* 2009; **102**: 1611-1619 [PMID: 19825205 DOI: 10.1017/S000711450999208X]

51 **Qin B**, Polansky MM, Harry D, Anderson RA. Green tea polyphenols improve cardiac muscle mRNA and protein levels of signal pathways related to insulin and lipid metabolism and inflammation in insulin-resistant rats. *Mol Nutr Food Res* 2010; **54** Suppl 1: S14-S23 [PMID: 20112301 DOI: 10.1002/mnfr.200900306]

52 **Li N**, Sun Z, Han C, Chen J. The chemopreventive effects of tea on human oral precancerous mucosa lesions. *Proc Soc Exp Biol Med* 1999; **220**: 218-224 [PMID: 10202392]

53 **Ahn WS**, Yoo J, Huh SW, Kim CK, Lee JM, Namkoong SE, Bae SM, Lee IP. Protective effects of green tea extracts (polyphenon E and EGCG) on human cervical lesions. *Eur J Cancer Prev* 2003; **12**: 383-390 [PMID: 14512803 DOI: 10.1097/01.cej.0000090186.08740.82]

54 **Bettuzzi S**, Brausi M, Rizzi F, Castagnetti G, Peracchia G, Corti A. Chemoprevention of human prostate cancer by oral administration of green tea catechins in volunteers with high-grade prostate intraepithelial neoplasia: a preliminary report from a one-year proof-of-principle study. *Cancer Res* 2006; **66**: 1234-1240 [PMID: 16424063]

55 **Brausi M**, Rizzi F, Bettuzzi S. Chemoprevention of human prostate cancer by green tea catechins: two years later. A follow-up update. *Eur Urol* 2008; **54**: 472-473 [PMID: 18406041 DOI: 10.1016/j.eururo.2008.03.100]

56 **Shimizu M**, Fukutomi Y, Ninomiya M, Nagura K, Kato T, Araki H, Suganuma M, Fujiki H, Moriwaki H. Green tea extracts for the prevention of metachronous colorectal adenomas: a pilot study. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 3020-3025 [PMID: 18990744 DOI: 10.1158/1055-9965.EPI-08-0528]

57 **Shirakami Y**, Shimizu M, Tsurumi H, Hara Y, Tanaka T, Moriwaki H. EGCG and Polyphenon E attenuate inflammation-related mouse colon carcinogenesis induced by AOM plus DDS. *Mol Med Rep* 2008; **1**: 355-361 [PMID: 21479417]

58 **Adachi S**, Nagao T, Ingolfsson HI, Maxfield FR, Andersen OS, Kopelovich L, Weinstein IB. The inhibitory effect of (-)-epigallocatechin gallate on activation of the epidermal growth factor receptor is associated with altered lipid order in HT29 colon cancer cells. *Cancer Res* 2007; **67**: 6493-6501 [PMID: 17616711]

59 **Adachi S**, Nagao T, To S, Joe AK, Shimizu M, Matsushima-Nishiwaki R, Kozawa O, Moriwaki H, Maxfield FR, Weinstein IB. (-)-Epigallocatechin gallate causes internalization of the epidermal growth factor receptor in human colon cancer cells. *Carcinogenesis* 2008; **29**: 1986-1993 [PMID: 18586691 DOI: 10.1093/carcin/bgn128]

60 **Shirakami Y**, Shimizu M, Adachi S, Sakai H, Nakagawa T, Yasuda Y, Tsurumi H, Hara Y, Moriwaki H. (-)-Epigallocatechin gallate suppresses the growth of human hepatocellular carcinoma cells by inhibiting activation of the vascular endothelial growth factor-vascular endothelial growth factor receptor axis. *Cancer Sci* 2009; **100**: 1957-1962 [PMID: 19558547 DOI: 10.1111/j.1349-7006.2009.01241.x]

61 **Shirakami Y**, Shimizu M, Moriwaki H. Cancer chemoprevention with green tea catechins: from bench to bed. *Curr Drug Targets* 2012; **13**: 1842-1857 [PMID: 23140294]

62 **Shimizu M**, Deguchi A, Hara Y, Moriwaki H, Weinstein IB. EGCG inhibits activation of the insulin-like growth factor-1 receptor in human colon cancer cells. *Biochem Biophys Res Commun* 2005; **334**: 947-953 [PMID: 16053920]

63 **Shimizu M**, Shirakami Y, Sakai H, Tatebe H, Nakagawa T, Hara Y, Weinstein IB, Moriwaki H. EGCG inhibits activation of the insulin-like growth factor (IGF)/IGF-1 receptor axis in human hepatocellular carcinoma cells. *Cancer Lett* 2008; **262**: 10-18 [PMID: 18164805 DOI: 10.1016/j.canlet.2007.11.026]

64 **Chao A**, Thun MJ, Connell CJ, McCullough ML, Jacobs EJ, Flanders WD, Rodriguez C, Sinha R, Calle EE. Meat consumption and risk of colorectal cancer. *JAMA* 2005; **293**: 172-182 [PMID: 15644544 DOI: 10.293/2/172]

65 **Forte A**, De Sanctis R, Leonetti G, Manfredelli S, Urbano V, Bezzi M. Dietary chemoprevention of colorectal cancer. *Ann Ital Chir* 2008; **79**: 261-267 [PMID: 19093628]

66 **Lee GH**, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM. Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 1996; **379**: 632-635 [PMID: 8628397 DOI: 10.1038/379632a0]

67 **Hirose Y**, Hata K, Kuno T, Yoshida K, Sakata K, Yamada Y, Tanaka T, Reddy BS, Mori H. Enhancement of development

of azoxymethane-induced colonic premalignant lesions in C57BL/KsJ-db/db mice. *Carcinogenesis* 2004; **25**: 821-825 [PMID: 14729596 DOI: 10.1093/carcin/bgh059]

68 **Hayashi K**, Suzuki R, Miyamoto S, Shin-Ichiroh Y, Kohno H, Sugie S, Takashima S, Tanaka T. Citrus auraptene suppresses azoxymethane-induced colonic preneoplastic lesions in C57BL/KsJ-db/db mice. *Nutr Cancer* 2007; **58**: 75-84 [PMID: 17571970 DOI: 10.1080/01635580701308216]

69 **Suzuki R**, Kohno H, Yasui Y, Hata K, Sugie S, Miyamoto S, Sugawara K, Sumida T, Hirose Y, Tanaka T. Diet supplemented with citrus unshiu segment membrane suppresses chemically induced colonic preneoplastic lesions and fatty liver in male db/db mice. *Int J Cancer* 2007; **120**: 252-258 [PMID: 17066427 DOI: 10.1002/ijc.22240]

70 **Aggarwal BB**, Kumar A, Bharti AC. Anticancer potential of curcumin: preclinical and clinical studies. *Anticancer Res* 2003; **23**: 363-398 [PMID: 12680238]

71 **Goel A**, Boland CR, Chauhan DP. Specific inhibition of cyclooxygenase-2 (COX-2) expression by dietary curcumin in HT-29 human colon cancer cells. *Cancer Lett* 2001; **172**: 111-118 [PMID: 11566484]

72 **Mukhopadhyay A**, Banerjee S, Stafford LJ, Xia C, Liu M, Aggarwal BB. Curcumin-induced suppression of cell proliferation correlates with down-regulation of cyclin D1 expression and CDK4-mediated retinoblastoma protein phosphorylation. *Oncogene* 2002; **21**: 8852-8861 [PMID: 12483537 DOI: 10.1038/sj.onc.1206048]

73 **Shimizu M**, Shirakami Y, Sakai H, Adachi S, Hata K, Hirose Y, Tsurumi H, Tanaka T, Moriwaki H. (-)-Epigallocatechin gallate suppresses azoxymethane-induced colonic premalignant lesions in male C57BL/KsJ-db/db mice. *Cancer Prev Res* (Phila) 2008; **1**: 298-304 [PMID: 19138973 DOI: 10.1158/1940-6207.CAPR-08-0045]

74 **de Kloet AD**, Krause EG, Woods SC. The renin angiotensin system and the metabolic syndrome. *Physiol Behav* 2010; **100**: 525-534 [PMID: 20381510 DOI: 10.1016/j.physbeh.2010.03.018]

75 **Smith GR**, Missailidis S. Cancer, inflammation and the AT1 and AT2 receptors. *J Inflamm* (Lond) 2004; **1**: 3 [PMID: 15813980]

76 **Demierre MF**, Higgins PD, Gruber SB, Hawk E, Lippman SM. Statins and cancer prevention. *Nat Rev Cancer* 2005; **5**: 930-942 [PMID: 16341084]

77 **Gauthaman K**, Fong CY, Bongso A. Statins, stem cells, and cancer. *J Cell Biochem* 2009; **106**: 975-983 [PMID: 19224538 DOI: 10.1002/jcb.22092]

78 **Cho SJ**, Kim JS, Kim JM, Lee JY, Jung HC, Song IS. Simvastatin induces apoptosis in human colon cancer cells and in tumor xenografts, and attenuates colitis-associated colon cancer in mice. *Int J Cancer* 2008; **123**: 951-957 [PMID: 18521906 DOI: 10.1002/ijc.23593]

79 **Yasui Y**, Suzuki R, Miyamoto S, Tsukamoto T, Sugie S, Kohno H, Tanaka T. A lipophilic statin, pitavastatin, suppresses inflammation-associated mouse colon carcinogenesis. *Int J Cancer* 2007; **121**: 2331-2339 [PMID: 17657716 DOI: 10.1002/ijc.22976]

80 **Poynter JN**, Gruber SB, Higgins PD, Almog R, Bonner JD, Rennert HS, Low M, Greenson JK, Rennert G. Statins and the risk of colorectal cancer. *N Engl J Med* 2005; **352**: 2184-2192 [PMID: 15917383 DOI: 10.352/21/2184]

81 **Muto Y**, Sato S, Watanabe A, Moriwaki H, Suzuki K, Kato A, Kato M, Nakamura T, Higuchi K, Nishiguchi S, Kumada H, Ohashi Y. Overweight and obesity increase the risk for liver cancer in patients with liver cirrhosis and long-term oral supplementation with branched-chain amino acid granules inhibits liver carcinogenesis in heavier patients with liver cirrhosis. *Hepatol Res* 2006; **35**: 204-214 [PMID: 16737844]

P- Reviewers: de Bree E, Elisaf MS
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8947

World J Gastroenterol 2014 July 21; 20(27): 8947-8956
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Fluoroquinolone-based protocols for eradication of *Helicobacter pylori*

Antonio Rispo, Pietro Capone, Fabiana Castiglione, Luigi Pasquale, Matilde Rea, Nicola Caporaso

Antonio Rispo, Pietro Capone, Fabiana Castiglione, Matilde Rea, Nicola Caporaso, Division of Gastroenterology, Department of Clinical Medicine and Surgery - University "Federico Ⅱ" of Naples, 80131 Napoli, Italy
Luigi Pasquale, Division of Gastroenterology, P.O. "Sant'Ottone Frangipane", Ariano Irpino, 80131 Napoli, Italy
Author contributions: Rispo A wrote the article; Capone P and Rea M identified the articles by Medline search; Castiglione F and Pasquale L reviewed the papers; Caporaso N contributed to critical revision of the manuscript.
Correspondence to: Antonio Rispo, MD, Division of Gastroenterology, Department of Clinical Medicine and Surgery - University "Federico Ⅱ" of Naples, Via S. Pansini 5, 80131 Napoli, Italy. antoniorispo@email.it
Telephone: +39-81-7463849 Fax: +39-81-5465649
Received: September 4, 2013 Revised: February 10, 2014
Accepted: April 1, 2014
Published online: July 21, 2014

Abstract

Helicobacter pylori (*H. pylori*) is a widespread pathogen infecting about 40% of people living in urban areas and over 90% of people living in the developing regions of the world. *H. pylori* is well-documented as the main factor in the pathogenesis of peptic ulcer disease, chronic gastritis, and gastric malignancies such as cancer and mucosa-associated lymphoid tissue-lymphoma; hence, its eradication is strongly recommended. The Maastricht Ⅳ consensus, which focused on the management of *H. pylori* infection, set important new strategies in terms of treatment approaches, particularly with regards to first- and second-line treatment protocols and led to improved knowledge and understanding of *H. pylori* resistance to antibiotics. In recent years, various fluoroquinolone-based protocols, mainly including levofloxacin, have been proposed and effectively tested at all therapeutic lines for *H. pylori* eradication. The aim of the present paper is to review the scientific literature focused on the use of fluoroquinolones in eradicating *H. pylori*.

Key words: *Helicobacter pylori*; Eradication; Fluoroquinolone; Therapy

Core tip: The Maastricht Ⅳ consensus, which focused on the management of *Helicobacter pylori* (*H. pylori*) infection, set important new strategies in terms of treatment approaches, particularly with regards to first- and second-line treatment protocols and led to improved knowledge and understanding of *H. pylori* resistance to antibiotics. In recent years, various fluoroquinolone-based protocols, mainly including levofloxacin, have been proposed and effectively tested at all therapeutic lines for *H. pylori* eradication. The aim of the present invited paper is to review the scientific literature focused on the use of fluoroquinolones in eradicating *H. pylori*.

Rispo A, Capone P, Castiglione F, Pasquale L, Rea M, Caporaso N. Fluoroquinolone-based protocols for eradication of *Helicobacter pylori*. *World J Gastroenterol* 2014; 20(27): 8947-8956 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8947.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8947

INTRODUCTION

Nalidixic acid, the precursor of all fluoroquinolones, was developed and marketed during the 1960s for the oral treatment of urinary tract infections and is still prescribed with this indication. Several fluoroquinolones were developed since; however, the role of new molecules only became significant when it was demonstrated that substitutions at the Carbon atoms in C-6 and C-7 positions

Table 1 Current recommendations for *Helicobacter pylori* eradication

Treatment	Region with low clarithromycin prevalence	Region with high clarithromycin prevalence
First line	PPI - clarithromycin - amoxicillin/metronidazole or bismuth quadruple	Bismuth quadruple[1]. If not available: non-bismuth quadruple (either sequential or concomitant)
Second line	Bismuth quadruple[1] or PPI - levofloxacin/amoxicillin	PPI - levofloxacin/amoxicillin
Third line	Based on susceptibility testing only. Besides clarithromycin and levofloxacin, rifabutin is another candidate that may be used	

[1]PPI + tetracyclines + bismuth + metronidazole. PPI: Proton pump inhibitors.

improved both antibacterial activity and pharmacological features. From that point onwards, fluoroquinolones were tested and used in the treatment of urinary, respiratory, gastrointestinal, urogenital, and intra-abdominal infections in the context of several pathological conditions[1-5].

Helicobacter pylori (*H. pylori*) is a widespread pathogen infecting about 40% of people living in urban areas and over 90% of people living in the developing regions of the world[6,7]. *H. pylori* is well-documented as the main factor in the pathogenesis of peptic ulcer disease, chronic gastritis, and gastric malignancies such as cancer and mucosa-associated lymphoid tissue-lymphoma. Hence, its eradication is strongly recommended[8-13]. The Maastricht Ⅲ consensus proposed that triple therapy protocols containing clarithromycin and metronidazole should be used as first-line treatment for *H. pylori* infection, in view of their high efficacy and safety[14]. However, more recent data show that these antibiotics have lost some efficacy because of increased primary/secondary drug resistance, so that they permit *H. pylori* eradication in only a maximum of 70% of the affected patients (a percentage significantly lower than the one that can be expected for the treatment of an infectious disease - about 90% at per-protocol analysis). Thus, antibiotics different from clarithromycin and metronidazole have been proposed for eradicating *H. pylori*[15,16]. The Maastricht Ⅳ consensus generated important new information with regard to the treatment of *H. pylori* infection. In particular, it proposed the prescription of three antibiotics together with a proton pump inhibitor (PPI; non-bismuth sequential or quadruple therapy) as first-line treatment for *H. pylori* infection in areas of high clarithromycin resistance[17-21].

In recent years, various fluoroquinolone-based protocols, mainly including levofloxacin, have been proposed and tested at all therapeutic lines for *H. pylori* eradication. The aim of this paper is to review the scientific literature focused on the use of fluoroquinolones in eradicating *H. pylori*.

Studies providing information on the use of levofloxacin-based anti-*H. pylori* protocols were identified through systematic searches in the MEDLINE and EMBASE databases. Various combinations of the terms "*H. pylori*", "fluoroquinolone", "levofloxacin", "ciprofloxacin", "eradication", "first-line", "second-line" and "rescue" were used for the searches. Additionally, references of retrieved articles were screened to identify additional relevant studies (cross-referencing). We also performed a manual search of all review articles, recently published editorials, and retrieved original studies presented at the Digestive Disease Week, United European Gastroenterology Week, and European Helicobacter Study Group conferences. In addition, reference lists from relevant identified papers were manually searched. All original research articles and abstracts published up to August 1, 2013 were included. Searches were limited to randomized controlled trials and studies comparing fluoroquinolone-based protocols to other treatment regimens. Two investigators (Dr Capone and Dr Rea) independently extracted data from the included studies by using a structured form. Only data from patients undergoing fluoroquinolone-based eradication protocols were included in the analysis. There was a > 95% agreement in data extraction between the two investigators.

CURRENT RECOMMENDATIONS FOR ERADICATING *H. PYLORI*

As mentioned above, the Maastricht Ⅳ consensus introduced important changes to the treatment of *H. pylori* infection (Table 1)[17].

The consensus led to abandoning the use of clarithromycin-containing triple therapy in regions where clarithromycin resistance rate is over 15%-20% - for example, many areas of Europe and North America - if susceptibility testing cannot be carried out[16,22]. However, in areas of known low clarithromycin resistance, clarithromycin-containing protocols are still recommended for the first-line empirical treatment of *H. pylori* infection. In these areas of low clarithromycin resistance bismuth-containing quadruple treatment protocols are considered an effective alternative, whilst in regions with high clarithromycin resistance, they are the recommended protocols for first-line empirical treatment of *H. pylori* infection[23-25]. In countries where a bismuth-based regimen is not easily available, sequential treatment or a non-bismuth quadruple treatment is recommended as first-line eradication protocol[26-29].

With regard to the role of fluoroquinolones for *H. pylori* eradication, current recommendations stress their efficacy as a second-line treatment option. In particular, either bismuth-containing quadruple therapy or levofloxacin-containing triple therapy is recommended after failure of a regimen containing PPI-clarithromycin. In areas of low fluoroquinolone resistance, a levofloxacin-containing regimen (together with a PPI and clarithromycin) can prove an effective second-line alternative in the presence of penicillin allergy[30-35]. However, when considering a

treatment approach including fluoroquinolones, clinicians should be aware of the rising rates of levofloxacin resistance, particularly in Europe and North America.

DRUG RESISTANCE IN THE TREATMENT OF *H. PYLORI* INFECTION

The success of treatment protocols for the eradication of *H. pylori* is currently being compromised by the increase in antimicrobial resistance[36,37].

Clarithromycin resistance in particular has a major negative impact on the efficacy of the recommended first-line triple therapy and a progressive increase in its prevalence may limit its use. The almost two-fold increase (from 9.8% to 17.5%) of the prevalence of clarithromycin resistance over the past 10 years (in specific areas of Southern Europe it is higher than 30%) could have been anticipated on the grounds of the genetic basis of this resistance. By contrast, metronidazole resistance, although highly prevalent (particularly in Mediterranean Africa), can be partly overcome and is therefore of secondary importance. As for amoxicillin, all the surveys performed so far have reported a resistance rate lower than 1%, indicating that resistance to this drug is not yet a clinical concern[38-42].

At present, it is well known that fluoroquinolones are the only class of antibiotics for clinical use that directly inhibit bacterial DNA synthesis. Fluoroquinolones inhibit DNA gyrase and topoisomerase Ⅳ, two bacterial enzymes which have essential and distinct roles in DNA replication. Resistance to fluoroquinolones occurs mainly by means of a mutation in the chromosomal genes for gyrase and topoisomerase Ⅳ. Miyachi *et al*[43] showed that primary levofloxacin resistance, found in approximately 15% of *H. pylori* strains, was related to point mutations in gyrA at Asn-87 or Asp-91 in 84% of cases; only 14% of the susceptible strains had gyrA mutations. The difference in occurrence of gyrA mutations between levofloxacin-resistant and -susceptible strains was significant. Other mechanisms that can determine bacterial resistance to fluoroquinolones could be microbial SOS response, auto-induction of resistance and plasmid-mediated resistance, with the latter being more frequent for other kind of urinary, pulmonary and intestinal infections. In contrast to other bacteria, resistant *H. pylori* strains show no spread of resistance through horizontal transfer of mobile genetic elements (*e.g.* plasmids)[44]. As a consequence, the low transmission rate of *H. pylori* and the lack of expansion of specific clones in the community imply that antibiotic resistance in *H. pylori* is caused by previous and direct exposure to antibiotics in infected patients. *H. pylori* infection is an example of long-lasting infection and it should be highlighted that the exposure to antibiotics for this microbe may be much longer than that for most other pathogens.

A recent paper by Mégraud *et al*[37] focused on the antibiotic resistance of *H. pylori*. The study by these authors included more than 2000 patients with *H. pylori* infection and showed resistance rates of 14.1% for levofloxacin, 17.5% for clarithromycin, and 34.9% for metronidazole, with significantly higher fluoroquinolone resistance in Western/Central and Southern Europe (> 20%) than in Northern European countries (< 10%). The results of this paper correlate well with those reported by O'Connor *et al*[45] in Ireland. These authors encountered a rate of levofloxacin resistance of 2.6 % in the under-45 age group, compared to 19.1% in patients above 45 years of age. In keeping with the suggested mechanisms of *H. pylori* resistance to fluoroquinolones, a significant association was found between fluoroquinolone use among outpatients and the proportion of levofloxacin resistance.

Carothers *et al*[46] have already shown how *H. pylori* resistance to fluoroquinolones and its impact on treatment outcomes are influenced by previous use of this class of antibiotics. In their study, resistance rates appeared to be significantly associated with any prior fluoroquinolone assumption over the previous 10 years and with the total number of courses prescribed. For patients who have previously undergone treatment with fluoroquinolones, a treatment protocol not comprising levofloxacin should be suggested.

However, previous use of fluoroquinolones for therapeutic purposes is not the only mechanism through which resistance to the drug occurs. A recent paper from Germany demonstrated that about 85% of all antibiotics used in general practice is administered in food animals; animal manure waste is spread onto agricultural land and will influence ecosystem compartments. Antibiotics such as fluoroquinolones and tetracyclines are not biodegradable; they can persist in soil for long periods and/or in high concentrations and can be detected in soil and water[47]. Antibiotic resistance pre-dates the use of antibiotics because resistance determinants have been circulating within the microbial genome for millennia[48]. Ongoing non-therapeutic use of antibiotics in food animals will increase the pool and occurrence rate of resistance genes in many bacterial species, thus having dramatic public health consequences. The Maastricht Ⅳ consensus recommendations relating to the rising rate of levofloxacin resistance are therefore extremely relevant for every-day clinical practice[17,5,49].

FLUOROQUINOLONE-BASED PROTOCOLS IN THE FIRST-LINE TREATMENT OF *H. PYLORI* INFECTION

Fluoroquinolones - levofloxacin being the main representative of this class of molecules - are antibacterial agents with a wide spectrum of activity against Gram-positive and -negative bacteria, including *in vitro* activity against *H. pylori* and atypical pathogens. Levofloxacin is widely used for the rescue treatment of resistant *H. pylori* infections. Recent meta-analyses have underlined its better efficacy and tolerability profile in the second-line treatment of *H. pylori* infection when compared with the

Table 2 Randomized controlled trials containing levofloxacin in first-line triple therapy of *Helicobacter pylori*

Ref.	Year	Nation	Patients	Therapy	Posology	Duration	Comparator	ITT
Shah *et al*[53]	2013	India	131	LTE	500	7		85.0%
Qian *et al*[54]	2012	China	345	LAE	500	7	SEQ-L	78.1%
Cuadrado-Lavín *et al*[61]	2012	Spain	250	LAO	500	10	CAO	82.8%
Pan *et al*[62]	2010	China	199	LAE	500	7	NAR	87.1%
Chen *et al*[63]	2010	Taiwan	189	LCE	500	7	CAE	78.9%
Assem *et al*[64]	2010	Egypt	450	LAE	500	7	CLE/CAE	84.7%
Erçin *et al*[65]	2010	Turkey	91	LAL	500	14	LAL (7)	72.0%
Liou *et al*[66]	2010	Taiwan	432	LAL	500	7	CAL	74.0%
Chen *et al*[63]	2010	China	300	LAL	500	7	CAL	74.0%
Molina-Infante *et al*[69]	2010	Spain	460	LAO	500	10	SEQ-L	82.6%
Castro-Fernández *et al*[59]	2009	Spain	135	LAO	500	10		71.8%
Gisbert *et al*[55]	2007	Spain	64	LARBIS	500	10		84.4%
Rispo *et al*[52]	2007	Italy	130	LAE	500	7	CAE	90.8%
Nista *et al*[51]	2006	Italy	300	CLE	500	7	CME/CAE	87.0%
Lee *et al*[56]	2006	South Korea	267	LAE	500	7	CAE	69.8%
Cammarota[50]	2004	Italy	100	CLR	500	7	CLR (250)	84.0%

LTE: Levofloxacin + tinidazole + esomeprazole; LAE: Levofloxacin + amoxicillin + esomeprazole; SEQ-L: Standard sequential therapy or levofloxacin-containing sequential therapy; LAO: Levofloxacin + amoxicillin + omeprazole; CAO: Clarithromycin + amoxicillin + omeprazole; LCE: Levofloxacin + clarithromycin + esomeprazole; CAE: Clarithromycin + amoxicillin + esomeprazole; CLE: Clarithromycin + levofloxacin + esomeprazole; LAL: Levofloxacin + amoxicillin + lansoprazole; CAL: Clarithromycin + amoxicillin + lansoprazole; LARBIS: Levofloxacin + amoxicillin + ranitidine bismuth citrate; CLR: Clarithromycin + levofloxacin + rifabutin; ITT: Intention-to-treat.

quadruple protocol comprising bismuth (which we discuss shortly).

Since 2006, several clinical trials have tested the efficacy of levofloxacin in the first-line treatment of *H. pylori*. The majority of authors utilised levofloxacin as part of a triple drug regimen including a PPI and another antibiotic agent (frequently amoxicillin). The first experience by Cammarota *et al*[50] in a trial including 100 patients with *H. pylori* infection treated with levofloxacin (500 mg/d), clarithromycin and rabeprazole showed an eradication rate of 84%. An analogous rate of *H. pylori* eradication (87%) was reported by Nista *et al*[51], who treated 300 infected patients with levofloxacin, clarithromycin and esomeprazole. Drawing upon this existing research, our team carried out a study to evaluate the efficacy of a triple therapy including levofloxacin in the first-line treatment of *H. pylori* infection when compared to the conventional protocol containing clarithromycin. We prospectively randomized 130 consecutive outpatients with histological first diagnosis of *H. pylori* infection in two treatment groups: the LAE group (65 patients) was treated with levofloxacin 250 mg *bid*, amoxicillin 1 g *bid*, esomeprazole 20 mg *bid*; and the CAE group (65 patients) with clarithromycin 500 mg *bid*, amoxicillin 1 g *bid*, and esomeprazole 20 mg *bid*. The success rate was assessed by means of ^{13}C urea breath test, which showed *H. pylori* eradication in 90.8% of patients in the LAE group, compared to 76.9% of those in the CAE group ($P < 0.01$; NNT = 7). In our experience the eradication rate was unrelated to the baseline characteristics of the patients and their underlying gastro-duodenal disease[52].

However, subsequent trials, mainly conducted in Spain and Asia, did not confirm our remarkable results. In effect, almost all recent papers coming from Spain, Northern Africa, and Asia highlighted that the rate of *H. pylori* eradication achieved by means of levofloxacin-based triple protocols is less noteworthy than expected (about 85%), probably as a result of the increased rate of *H. pylori* resistance to fluoroquinolones (Table 2). Indeed, these quite rather inadequate eradication rates are likely to be related to a higher prevalence of levofloxacin resistant *H. pylori* strains in that particular geographical areas in which the studies were carried out. Furthermore, not only has fluoroquinolone resistance readily increased over the last decade, but regional differences within the same country can be significant; this appears clearly and particularly true for Spain and China[53-66].

More recently, levofloxacin has been effectively used in first-line sequential and quadruple protocols. Romano *et al*[67] carried out a randomised trial aimed at evaluating the efficacy of a levofloxacin-containing sequential regimen compared to a clarithromycin containing sequential therapy in the eradication of *H. pylori* infection in patients from Southern Italy, a geographical area with > 15% prevalence of clarithromycin resistance. Eradication rates in these authors' intention-to-treat analyses were: 80.8% with clarithromycin sequential treatment; 96.0% with levofloxacin-250 sequential treatment; and 96.8% with levofloxacin-500 sequential treatment. The levofloxacin-250 sequential treatment appeared to be cost-saving compared to the clarithromycin sequential therapy. Two years after this study, the same authors performed a non-inferiority randomized trial to determine whether a 5-d treatment course of levofloxacin-containing quadruple concomitant regimen was as safe and effective as the 10-d course of sequential regimen in eradicating *H. pylori* in previously untreated patients. The intention-to-treat analysis showed similar eradication rates for concomitant (92.2%) and sequential regimens (93.3%). In addition, the authors showed that the concomitant regimen cost $9

less than the sequential one[67,68]. However, once again the remarkable results of using levofloxacin as the first-line eradication drug were not similarly satisfying in different geographical areas. The trials by Molina-Infante *et al*[69] (Spain) and by Qian *et al*[54] (China), using a modified levofloxacin-based quadruple sequential protocol, showed an eradication rate of 80%-85%, highlighting once again the importance of geographical differences in terms of *H. pylori* resistance to antibiotics[70].

We agree with all the experts who emphasise that susceptibility testing may help to identify the most suitable treatment protocol and to therefore use only the antibiotic agent that works well locally.

FLUOROQUINOLONE-BASED PROTOCOLS IN THE SECOND-LINE TREATMENT OF *H. PYLORI* INFECTION

With regard to the role of fluoroquinolones in *H. pylori* eradication, current recommendations underline their efficacy as a second-line option, in particular as levofloxacin containing triple therapy. Levofloxacin has been widely used for the rescue treatment of resistant infection, and a meta-analysis by Gisbert *et al*[34] has highlighted its better efficacy and tolerability profile in the second-line treatment of *H. pylori* infection compared to the quadruple protocol comprising bismuth. More specifically, this meta-analysis - which included 14 trials with a total of 977 patients - showed that the mean eradication rate with levofloxacin-based regimens was 80%, with 10-d regimens appearing to be more effective than 7-d combinations (81% *vs* 73%; $P < 0.01$). The meta-analysis also showed better results with levofloxacin than with the quadruple combination (81% *vs* 70%; OR = 1.80), and a better safety profile for levofloxacin than for the quadruple regimen, both overall (19% *vs* 44%) and in terms of severe adverse effects (0.8% *vs* 8.4%).

More recently, Di Caro *et al*[71] updated Gisbert *et al*[72], s meta-analysis by comparing the effectiveness of levofloxacin/amoxicillin-based schemes to that of quadruple regimens for the eradication of *H. pylori* in second-line treatment. In total, 10 articles and four abstracts were identified; the analysis, including 14 trials with a total of 677 patients, showed an overall eradication rate of 76.5% in the group treated with levofloxacin-amoxicillin and of 67.4% in that treated with quadruple regimen, with a cure rate of 70.6% for 7-d regimens and 88.7% for 10-d combinations. Interestingly, even though the 7-d levofloxacin-amoxicillin and quadruple protocols showed comparable efficacy, the 10-d fluoroquinolone-based regimen was significantly more effective than the quadruple regimen (OR = 0.5). No differences were reported in quadruple protocol-based eradication rates among Asian and European studies, whereas levofloxacin-amoxicillin regimens were more effective in European populations (78.3% *vs* 67.7%; $P = 0.05$). The incidence of side effects was lower in the levofloxacin-amoxicillin treatment group than in the quadruple regiment group (OR = 0.39; 95%CI: 0.18-0.85; $P = 0.02$). Consequently, the meta-analysis supported the use of 10-d levofloxacin-amoxicillin regimens as a second-line treatment for the eradication of *H. pylori* with excellent tolerability and eradication rates[71].

More recently, Gisbert *et al*[72] have re-assessed this issue in 100 consecutive patients in whom a non-bismuth quadruple regimen, administered either sequentially (PPI + amoxicillin for 5 d followed by PPI plus clarithromycin plus metronidazole for 5 more days) or concomitantly (PPI plus amoxicillin plus clarithromycin plus metronidazole for 10 d) had previously failed. At the end of the study the per-protocol and intention-to-treat *H. pylori* eradication rates were 75.5% and 74%. Intention-to-treat eradication rates achieved with levofloxacin in the "sequential" and "concomitant" failed regimen groups were 74.4% and 71.4%, respectively. A rate of *H. pylori* eradication of approximately 75% obtained using levofloxacin-based triple protocol as a second-line regimen was confirmed also by Manfredi *et al*[73] in Italy. Furthermore, the efficacy of levofloxacin-based protocols and their value over time were explored by a Spanish multicenter study. The study sample comprised 1000 consecutive patients who had not responded to previous treatment with the standard clarithromycin-based triple protocol. It showed per-protocol and intention-to-treat eradication rates of 75.1% and 73.8%, respectively. The treatment (intention-to-treat) efficacy was 76% in year 2006; 68% in year 2007; 70% in year 2008; 76% in year 2009; 74% in year 2010; and 81% in year 2011, underlying the fact that the efficacy of levofloxacin-based protocols tends to remain stable over time[74].

However, once again, different results in terms of efficacy were reported from Eastern countries. Moon *et al*[75] evaluated the efficacy and safety of triple therapy with levofloxacin, metronidazole, and lansoprazole as a second-line treatment, compared to those of quadruple therapy. According to the intention-to-treat analysis, the infection was eradicated in 38 of the 56 patients (67.9%) treated with triple therapy and in 48 of the 57 (84.2%) treated with quadruple therapy ($P = 0.042$). Per-protocol analysis showed successful eradication in 38 of 52 patients (73.1%) from the triple protocol group and 48 of 52 patients (92.3%) from the quadruple protocol group ($P = 0.01$). Even though the choice of metronidazole instead of amoxicillin could partially explain the results of this study, geographical differences in terms of *H. pylori* resistance to fluoroquinolones should not be ignored.

More recently, levofloxacin has been used in a non-bismuth quadruple second-line protocol. Calhan *et al*[76] designed a study aiming to investigate the efficacy of two levofloxacin-containing second-line treatment protocols for *H. pylori* infection. The patients were randomized consecutively to two treatment groups: 73 patients were assigned to the levofloxacin-containing sequential regimen and 75 to the levofloxacin-containing quadruple regimen group. The first group received pantoprazole 40 mg and amoxicillin 1000 mg twice daily for 5 d followed

by pantoprazole 40 mg twice daily and metronidazole 500 mg three times daily and levofloxacin 500 mg once daily for 7 d. The second group received pantoprazole 40 mg twice daily, tetracycline 500 mg four times daily, bismuth subcitrate 300 mg four times daily and levofloxacin 500 mg once daily for 10 d. The intention-to-treat analysis showed eradication rates of 82.2% and 90.6%, respectively, for the two treatment groups, with no statistically significant difference.

On the basis of these studies and findings, the role of levofloxacin (and the modalities of its use) in the second-line treatment of *H. pylori* infection is well defined by Statement 14 of the Maastricht Ⅳ Consensus. According to these recommendations, after failure of a PPI- and clarithromycin-containing treatment, either a bismuth-containing quadruple protocol or a levofloxacin-containing triple protocol is recommended, although the rising rates of levofloxacin resistance should be taken into account.

FLUOROQUINOLONE-BASED PROTOCOLS IN THE THIRD-LINE TREATMENT OF *H. PYLORI* INFECTION

The Maastricht Ⅳ consensus clearly states that after failure of second-line treatment, the therapeutic approach should be guided by antimicrobial susceptibility testing whenever possible. The work by Cammarota *et al*[77] assessed the efficacy of a third-line, culture-guided treatment approach for the eradication of *H. pylori* infection. Patterns of resistance to antibiotics were analysed in *H. pylori* isolates from 94 consecutive patients in whom the infection had persisted after two eradication protocols. Using the *E*-test, susceptibility analysis was performed for amoxicillin, clarithromycin, metronidazole, tetracycline and levofloxacin. Patients were then treated with a culture-guided, third-line regimen: 89 patients with a 1-wk quadruple regimen including omeprazole, bismuth, doxycycline and amoxicillin, and 5 patients with a 1-wk triple regimen containing omeprazole, amoxicillin and levofloxacin or clarithromycin. The study showed that 94 patients (100%) were resistant to metronidazole, 89 (95%) to clarithromycin, 29 (31%) to levofloxacin and 5 (5%) to tetracycline. No resistance to amoxicillin was found. The overall eradication rate was 90%. The quadruple regimen was effective in 91% of patients (ITT). Four patients (80%, both per protocol and intention-to-treat analyses) were *H. pylori* negative after the triple regimen.

Regrettably, antimicrobial susceptibility testing is not widely and promptly available, being performed almost exclusively at third-level centres. In view of these limitations, a number of studies have assessed the effectiveness of empirical third-line *H. pylori* eradicating protocols, which frequently included levofloxacin. Gisbert *et al*[78] reported on a prospective multicentre study which focused on this type of treatment. The authors included in their study 100 patients for whom a first treatment with omeprazole-clarithromycin-amoxicillin and a second with omeprazole-bismuth-tetracycline-metronidazole (or ranitidine bismuth citrate with these antibiotics) had failed in eradicating the *H. pylori* infection. These patients were treated with a 10-d third-line eradication protocol comprising levofloxacin (500 mg *bid*), amoxicillin (1 g *bid*), and omeprazole (20 mg *bid*). Per-protocol and intention-to-treat eradication rates were 66% and 60%, respectively. A prospective study carried out in Spain evaluated the efficacy of different "rescue" treatments empirically prescribed over the course of 10 years to 500 (consecutive) patients for whom at least one eradication regimen had failed to cure the *H. pylori* infection. The 'rescue' regimens included: quadruple therapy with omeprazole-bismuth-tetracycline-metronidazole; ranitidine bismuth citrate-tetracycline-metronidazole; omeprazole-amoxicillin-levofloxacin; and omeprazole-amoxicillin-rifabutin. Antibiotic susceptibility was unknown (rescue regimens were chosen empirically). Overall, *H. pylori* eradication rates with the second-, third- (mostly levofloxacin-based), and fourth-line rescue regimens were 70%, 74%, and 76%, respectively. Cumulative *H. pylori* eradication rate with four successive treatments was 99.5%[32].

A levofloxacin-based third-line *H. pylori* eradicating protocol was also compared to the rescue treatment based on rifabutin. Forty patients were randomised to receive a 10-d treatment course with either rifabutin (150 mg b.d.) or levofloxacin (500 mg b.d.), plus amoxicillin (1 g b.d.) and omeprazole (20 mg b.d.). At the end of the study, per-protocol eradication rates were 45% in the rifabutin group and 81% in the levofloxacin group ($P < 0.05$). Intention-to-treat eradication rates were 45% and 85%, respectively ($P < 0.01$)[79]. However, bearing in mind the efficacy of levofloxacin compared to rifabutin, regional differences in *H. pylori* resistance to antibiotics should be carefully considered. A study by Jeong *et al*[80] from South Korea compared rifabutin and levofloxacin rescue regimens in patients with first- and second-line *H. pylori* eradication failures. These patients received treatment with either rifabutin or levofloxacin, plus amoxicillin (1 g b.d) and standard dose PPI. Eradication rates were 71.4% in the rifabutin group, and 57.1% in the levofloxacin group. Although there was no significant difference in *H. pylori* eradication rates between the two groups ($P = 0.656$), the rifabutin based regimen showed a relatively higher eradication rate in that geographical region. Once again the choice of antibiotics should be based on available data on regional *H. pylori* antibiotic resistance and susceptibility.

CONCLUSION

Even the most effective regimens for the treatment of *H. pylori* infection are likely to fail to eradicate *H. pylori* in more than 20% of affected patients. At present, clinicians need to have solid up-to-date knowledge of the first-line eradication regimens - including the more recent quadruple and sequential (bismuth-including or not) protocols -

and to be prepared to face treatment failures.

The treatment strategies for the eradication of *H. pylori* have been enriched by the use and diffusion of fluoroquinolones, an effective and safe option in eradicating *H. pylori* infection. However, as highlighted in the current review and in accordance with the Maastricht Ⅳ consensus, the choice of a first or "rescue" treatment based on fluoroquinolones should be based on regional *H. pylori* antibiotic resistance. It follows that clinicians should be aware of the prevalence of *H. pylori* drug resistance in the geographical area in which they operate. As for second and third line protocols, another crucial variable for the selection of the right drugs is the accurate assessment of the treatment/s that was/were previously used.

In summary, current *H. pylori* eradication guidelines recommend the prescription of levofloxacin (a fluoroquinolone) as part of a sequential treatment or a non-bismuth quadruple treatment in first-line eradication protocols in counties where bismuth-based regimens are not easily available. With regard to second-line treatment regimens, levofloxacin-based protocols constitute an encouraging strategy, representing an alternative to quadruple therapy in patients with previous PPI-clarithromycin-amoxicillin failure and offering the advantages of efficacy, simplicity, and safety. Finally, with regards to third-line and "rescue" protocols, the antibiotic choice should be guided by antimicrobial susceptibility testing irrespective of the efficacy of levofloxacin in the empirical eradication strategies. This appears to be the most sensible and effective treatment option[81-83].

ACKNOWLEDGMENTS

Many thanks to Giovanna Affinito, our endoscopy nurse, for her precious help and contribution.

REFERENCES

1 **Appelbaum PC**, Hunter PA. The fluoroquinolone antibacterials: past, present and future perspectives. *Int J Antimicrob Agents* 2000; **16**: 5-15 [PMID: 11185413 DOI: 10.1016/S0924-8579(00)00192-8]

2 **Zhanel GG**, Ennis K, Vercaigne L, Walkty A, Gin AS, Embil J, Smith H, Hoban DJ. A critical review of the fluoroquinolones: focus on respiratory infections. *Drugs* 2002; **62**: 13-59 [PMID: 11790155]

3 **Takahashi H**, Hayakawa I, Akimoto T. [The history of the development and changes of quinolone antibacterial agents]. *Yakushigaku Zasshi* 2003; **38**: 161-179 [PMID: 15143768]

4 **Shariff V A AR**, Shenoy M S, Yadav T, M R. The antibiotic susceptibility patterns of uropathogenic Escherichia coli, with special reference to the fluoroquinolones. *J Clin Diagn Res* 2013; **7**: 1027-1030 [PMID: 23905095 DOI: 10.7860/JCDR/2013/4917.3038]

5 **Dalhoff A**. Global fluoroquinolone resistance epidemiology and implictions for clinical use. *Interdiscip Perspect Infect Dis* 2012; **2012**: 976273 [PMID: 23097666 DOI: 10.1155/2012/976273]

6 **Tonkic A**, Tonkic M, Lehours P, Mégraud F. Epidemiology and diagnosis of Helicobacter pylori infection. *Helicobacter* 2012; **17** Suppl 1: 1-8 [PMID: 22958148 DOI: 10.1111/j.1523-5378.2012.00975.x]

7 **Goh KL**, Chan WK, Shiota S, Yamaoka Y. Epidemiology of Helicobacter pylori infection and public health implications. *Helicobacter* 2011; **16** Suppl 1: 1-9 [PMID: 21896079 DOI: 10.1111/j.1523-5378.2011.00874.x]

8 **Rugge M**, Capelle LG, Cappellesso R, Nitti D, Kuipers EJ. Precancerous lesions in the stomach: from biology to clinical patient management. *Best Pract Res Clin Gastroenterol* 2013; **27**: 205-223 [PMID: 23809241 DOI: 10.1016/j.bpg.2012.12.007]

9 **Shiota S**, Mahachai V, Vilaichone RK, Ratanachu-ek T, Tshering L, Uchida T, Matsunari O, Yamaoka Y. Seroprevalence of Helicobacter pylori infection and gastric mucosal atrophy in Bhutan, a country with a high prevalence of gastric cancer. *J Med Microbiol* 2013; **62**: 1571-1578 [PMID: 23831768]

10 **Uemura N**, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. Helicobacter pylori infection and the development of gastric cancer. *N Engl J Med* 2001; **345**: 784-789 [PMID: 11556297]

11 **Zhang C**, Yamada N, Wu YL, Wen M, Matsuhisa T, Matsukura N. Helicobacter pylori infection, glandular atrophy and intestinal metaplasia in superficial gastritis, gastric erosion, erosive gastritis, gastric ulcer and early gastric cancer. *World J Gastroenterol* 2005; **11**: 791-796 [PMID: 15682469]

12 **Kapadia CR**. Gastric atrophy, metaplasia, and dysplasia: a clinical perspective. *J Clin Gastroenterol* 2003; **36**: S29-S36; discussion S61-S62 [PMID: 12702963]

13 **Roesler BM**, Costa SC, Zeitune JM. Eradication Treatment of Helicobacter pylori Infection: Its Importance and Possible Relationship in Preventing the Development of Gastric Cancer. *ISRN Gastroenterol* 2012; **2012**: 935410 [PMID: 22778979 DOI: 10.5402/2012/935410]

14 **Malfertheiner P**, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, Hunt R, Rokkas T, Vakil N, Kuipers EJ. Current concepts in the management of Helicobacter pylori infection: the Maastricht III Consensus Report. *Gut* 2007; **56**: 772-781 [PMID: 17170018]

15 **Mégraud F**. Current recommendations for Helicobacter pylori therapies in a world of evolving resistance. *Gut Microbes* 2013; **4**: 541-548 [PMID: 23929066]

16 **Megraud F**, Coenen S, Versporten A, Kist M, Lopez-Brea M, Hirschl AM, Andersen LP, Goossens H, Glupczynski Y. Helicobacter pylori resistance to antibiotics in Europe and its relationship to antibiotic consumption. *Gut* 2013; **62**: 34-42 [PMID: 22580412 DOI: 10.1136/gutjnl-2012-302254]

17 **Malfertheiner P**, Megraud F, O'Morain CA, Atherton J, Axon AT, Bazzoli F, Gensini GF, Gisbert JP, Graham DY, Rokkas T, El-Omar EM, Kuipers EJ. Management of Helicobacter pylori infection--the Maastricht IV/ Florence Consensus Report. *Gut* 2012; **61**: 646-664 [PMID: 22491499 DOI: 10.1136/gutjnl-2012-302084]

18 **Gatta L**, Vakil N, Leandro G, Di Mario F, Vaira D. Sequential therapy or triple therapy for Helicobacter pylori infection: systematic review and meta-analysis of randomized controlled trials in adults and children. *Am J Gastroenterol* 2009; **104**: 3069-3079; quiz 1080 [PMID: 19844205 DOI: 10.1038/ajg.2009.555]

19 **Luther J**, Higgins PD, Schoenfeld PS, Moayyedi P, Vakil N, Chey WD. Empiric quadruple vs. triple therapy for primary treatment of Helicobacter pylori infection: Systematic review and meta-analysis of efficacy and tolerability. *Am J Gastroenterol* 2010; **105**: 65-73 [PMID: 19755966 DOI: 10.1038/ajg.2009.508]

20 **Jafri NS**, Hornung CA, Howden CW. Meta-analysis: sequential therapy appears superior to standard therapy for Helicobacter pylori infection in patients naive to treatment. *Ann Intern Med* 2008; **148**: 923-931 [PMID: 18490667]

21 **Horvath A**, Dziechciarz P, Szajewska H. Meta-analysis: sequential therapy for Helicobacter pylori eradication in children. *Aliment Pharmacol Ther* 2012; **36**: 534-541 [PMID: 22827718 DOI: 10.1111/j.1365-2036.2012.05229.x]

22 **Oleastro M**, Cabral J, Ramalho PM, Lemos PS, Paixão E, Benoliel J, Santos A, Lopes AI. Primary antibiotic resistance of Helicobacter pylori strains isolated from Portuguese children: a prospective multicentre study over a 10 year period.

J Antimicrob Chemother 2011; **66**: 2308-2311 [PMID: 21764826 DOI: 10.1093/jac/dkr293]
23 **Laine L**, Hunt R, El-Zimaity H, Nguyen B, Osato M, Spénard J. Bismuth-based quadruple therapy using a single capsule of bismuth biskalcitrate, metronidazole, and tetracycline given with omeprazole versus omeprazole, amoxicillin, and clarithromycin for eradication of Helicobacter pylori in duodenal ulcer patients: a prospective, randomized, multicenter, North American trial. *Am J Gastroenterol* 2003; **98**: 562-567 [PMID: 12650788]
24 **O'Morain C**, Borody T, Farley A, De Boer WA, Dallaire C, Schuman R, Piotrowski J, Fallone CA, Tytgat G, Mégraud F, Spénard J. Efficacy and safety of single-triple capsules of bismuth biskalcitrate, metronidazole and tetracycline, given with omeprazole, for the eradication of Helicobacter pylori: an international multicentre study. *Aliment Pharmacol Ther* 2003; **17**: 415-420 [PMID: 12562455]
25 **Malfertheiner P**, Bazzoli F, Delchier JC, Celiński K, Giguère M, Rivière M, Mégraud F. Helicobacter pylori eradication with a capsule containing bismuth subcitrate potassium, metronidazole, and tetracycline given with omeprazole versus clarithromycin-based triple therapy: a randomised, open-label, non-inferiority, phase 3 trial. *Lancet* 2011; **377**: 905-913 [PMID: 21345487 DOI: 10.1016/S0140-6736(11)60020-2]
26 **Gisbert JP**, Calvet X, O'Connor A, Mégraud F, O'Morain CA. Sequential therapy for Helicobacter pylori eradication: a critical review. *J Clin Gastroenterol* 2010; **44**: 313-325 [PMID: 20054285 DOI: 10.1097/MCG.0b013e3181c8a1a3]
27 **Vaira D**, Zullo A, Vakil N, Gatta L, Ricci C, Perna F, Hassan C, Bernabucci V, Tampieri A, Morini S. Sequential therapy versus standard triple-drug therapy for Helicobacter pylori eradication: a randomized trial. *Ann Intern Med* 2007; **146**: 556-563 [PMID: 17438314]
28 **Essa AS**, Kramer JR, Graham DY, Treiber G. Meta-analysis: four-drug, three-antibiotic, non-bismuth-containing "concomitant therapy" versus triple therapy for Helicobacter pylori eradication. *Helicobacter* 2009; **14**: 109-118 [PMID: 19298338 DOI: 10.1111/j.1523-5378.2009.00671.x]
29 **Gisbert JP**, Calvet X. Review article: non-bismuth quadruple (concomitant) therapy for eradication of Helicobater pylori. *Aliment Pharmacol Ther* 2011; **34**: 604-617 [PMID: 21745241 DOI: 10.1111/j.1365-2036.2011.04770.x]
30 **Rokkas T**, Sechopoulos P, Robotis I, Margantinis G, Pistiolas D. Cumulative H. pylori eradication rates in clinical practice by adopting first and second-line regimens proposed by the Maastricht III consensus and a third-line empirical regimen. *Am J Gastroenterol* 2009; **104**: 21-25 [PMID: 19098844 DOI: 10.1038/ajg.2008.87]
31 **Lee JM**, Breslin NP, Hyde DK, Buckley MJ, O'Morain CA. Treatment options for Helicobacter pylori infection when proton pump inhibitor-based triple therapy fails in clinical practice. *Aliment Pharmacol Ther* 1999; **13**: 489-496 [PMID: 10215733]
32 **Gisbert JP**, Gisbert JL, Marcos S, Jimenez-Alonso I, Moreno-Otero R, Pajares JM. Empirical rescue therapy after Helicobacter pylori treatment failure: a 10-year single-centre study of 500 patients. *Aliment Pharmacol Ther* 2008; **27**: 346-354 [PMID: 17999716]
33 **Lee BH**, Kim N, Hwang TJ, Lee SH, Park YS, Hwang JH, Kim JW, Jeong SH, Lee DH, Jung HC, Song IS. Bismuth-containing quadruple therapy as second-line treatment for Helicobacter pylori infection: effect of treatment duration and antibiotic resistance on the eradication rate in Korea. *Helicobacter* 2010; **15**: 38-45 [PMID: 20302588 DOI: 10.1111/j.1523-5378.2009.00735.x]
34 **Gisbert JP**, Morena F. Systematic review and meta-analysis: levofloxacin-based rescue regimens after Helicobacter pylori treatment failure. *Aliment Pharmacol Ther* 2006; **23**: 35-44 [PMID: 16393278]
35 **Saad RJ**, Schoenfeld P, Kim HM, Chey WD. Levofloxacin-based triple therapy versus bismuth-based quadruple therapy for persistent Helicobacter pylori infection: a meta-analysis. *Am J Gastroenterol* 2006; **101**: 488-496 [PMID: 16542284]
36 **Fischbach L**, Evans EL. Meta-analysis: the effect of antibiotic resistance status on the efficacy of triple and quadruple first-line therapies for Helicobacter pylori. *Aliment Pharmacol Ther* 2007; **26**: 343-357 [PMID: 17635369]
37 **Mégraud F**. H pylori antibiotic resistance: prevalence, importance, and advances in testing. *Gut* 2004; **53**: 1374-1384 [PMID: 15306603]
38 **Debets-Ossenkopp YJ**, Herscheid AJ, Pot RG, Kuipers EJ, Kusters JG, Vandenbroucke-Grauls CM. Prevalence of Helicobacter pylori resistance to metronidazole, clarithromycin, amoxycillin, tetracycline and trovafloxacin in The Netherlands. *J Antimicrob Chemother* 1999; **43**: 511-515 [PMID: 10350380]
39 **Osato MS**, Reddy R, Reddy SG, Penland RL, Malaty HM, Graham DY. Pattern of primary resistance of Helicobacter pylori to metronidazole or clarithromycin in the United States. *Arch Intern Med* 2001; **161**: 1217-1220 [PMID: 11343444]
40 **Prazeres Magalhães P**, De Magalhães Queiroz DM, Campos Barbosa DV, Aguiar Rocha G, Nogueira Mendes E, Santos A, Valle Corrêa PR, Camargos Rocha AM, Martins Teixeira L, Affonso de Oliveira C. Helicobacter pylori primary resistance to metronidazole and clarithromycin in Brazil. *Antimicrob Agents Chemother* 2002; **46**: 2021-2023 [PMID: 12019131]
41 **Mohammadi M**, Doroud D, Massarrat S, Farahvash MJ. Clarithromycin resistance in Iranian H. pylori strains before introduction of clarithromycin. *Helicobacter* 2003; **8**: 80 [PMID: 12603622]
42 **Teo EK**, Fock KM, Ng TM, Khor CJ, Tan AL. Metronidazole-resistant Helicobacter pylori in an urban Asian population. *J Gastroenterol Hepatol* 2000; **15**: 494-497 [PMID: 10847434]
43 **Miyachi H**, Miki I, Aoyama N, Shirasaka D, Matsumoto Y, Toyoda M, Mitani T, Morita Y, Tamura T, Kinoshita S, Okano Y, Kumagai S, Kasuga M. Primary levofloxacin resistance and gyrA/B mutations among Helicobacter pylori in Japan. *Helicobacter* 2006; **11**: 243-249 [PMID: 16882327]
44 **Cattoir V**, Nectoux J, Lascols C, Deforges L, Delchier JC, Megraud F, Soussy CJ, Cambau E. Update on fluoroquinolone resistance in Helicobacter pylori: new mutations leading to resistance and first description of a gyrA polymorphism associated with hypersusceptibility. *Int J Antimicrob Agents* 2007; **29**: 389-396 [PMID: 17303392]
45 **O'Connor A**, Taneike I, Nami A, Fitzgerald N, Ryan B, Breslin N, O'Connor H, McNamara D, Murphy P, O'Morain C. Helicobacter pylori resistance rates for levofloxacin, tetracycline and rifabutin among Irish isolates at a reference centre. *Ir J Med Sci* 2013; **182**: 693-695 [PMID: 23625165]
46 **Carothers JJ**, Bruce MG, Hennessy TW, Bensler M, Morris JM, Reasonover AL, Hurlburt DA, Parkinson AJ, Coleman JM, McMahon BJ. The relationship between previous fluoroquinolone use and levofloxacin resistance in Helicobacter pylori infection. *Clin Infect Dis* 2007; **44**: e5-e8 [PMID: 17173210]
47 **Wellington EM**, Boxall AB, Cross P, Feil EJ, Gaze WH, Hawkey PM, Johnson-Rollings AS, Jones DL, Lee NM, Otten W, Thomas CM, Williams AP. The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria. *Lancet Infect Dis* 2013; **13**: 155-165 [PMID: 23347633 DOI: 10.1016/S1473-3099(12)70317-1]
48 **D'Costa VM**, King CE, Kalan L, Morar M, Sung WW, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R, Golding GB, Poinar HN, Wright GD. Antibiotic resistance is ancient. *Nature* 2011; **477**: 457-461 [PMID: 21881561 DOI: 10.1038/nature10388]
49 **Dalhoff A**. Resistance surveillance studies: a multifaceted problem--the fluoroquinolone example. *Infection* 2012; **40**: 239-262 [PMID: 22460782 DOI: 10.1007/s15010-012-0257-2]
50 **Cammarota G**, Cianci R, Cannizzaro O, Martino A, Fedeli P, Lecca PG, di Caro S, Cesaro P, Branca G, Gasbarrini G. High-dose versus low-dose clarithromycin in 1-week triple

therapy, including rabeprazole and levofloxacin, for Helicobacter pylori eradication. *J Clin Gastroenterol* 2004; **38**: 110-114 [PMID: 14745283]
51 **Nista EC**, Candelli M, Zocco MA, Cremonini F, Ojetti V, Finizio R, Spada C, Cammarota G, Gasbarrini G, Gasbarrini A. Levofloxacin-based triple therapy in first-line treatment for Helicobacter pylori eradication. *Am J Gastroenterol* 2006; **101**: 1985-1990 [PMID: 16968503]
52 **Rispo A**, Di Girolamo E, Cozzolino A, Bozzi R, Morante A, Pasquale L. Levofloxacin in first-line treatment of Helicobacter pylori infection. *Helicobacter* 2007; **12**: 364-365 [PMID: 17669111]
53 **Shah A**, Javid G, Zargar SA, Teli F, Khan BA, Yattoo GN, Gulzar GM, Sodhi JS, Khan MA, Shoukat A, Saif R. Safety and efficacy of 1-week levofloxacin-based triple therapy in first-line treatment for Helicobacter pylori-related peptic ulcer disease in Kashmir, India. *Indian J Gastroenterol* 2013; **32**: 32-36 [PMID: 23224792 DOI: 10.1007/s12664-012-0285-y]
54 **Qian J**, Ye F, Zhang J, Yang YM, Tu HM, Jiang Q, Shang L, Pan XL, Shi RH, Zhang GX. Levofloxacin-containing triple and sequential therapy or standard sequential therapy as the first line treatment for Helicobacter pylori eradication in China. *Helicobacter* 2012; **17**: 478-485 [PMID: 23067317 DOI: 10.1111/j.1523-5378.2012.00993.x]
55 **Gisbert JP**, Fernández-Bermejo M, Molina-Infante J, Pérez-Gallardo B, Prieto-Bermejo AB, Mateos-Rodríguez JM, Robledo-Andrés P, González-García G. First-line triple therapy with levofloxacin for Helicobacter pylori eradication. *Aliment Pharmacol Ther* 2007; **26**: 495-500 [PMID: 17635384]
56 **Lee JH**, Hong SP, Kwon CI, Phyun LH, Lee BS, Song HU, Ko KH, Hwang SG, Park PW, Rim KS, Kim S. [The efficacy of levofloxacin based triple therapy for Helicobacter pylori eradication]. *Korean J Gastroenterol* 2006; **48**: 19-24 [PMID: 16861877]
57 **Gisbert JP**, Bermejo MF, Infante JM, Gallardo BP, Bermejo AB, Rodríguez JM, Andrés PR, García GG. Levofloxacin, Amoxicillin, and Omeprazole as first-line triple therapy for Helicobacter pylori eradication. *J Clin Gastroenterol* 2009; **43**: 384-385 [PMID: 19020466 DOI: 10.1097/MCG.0b013e31816d921c]
58 **Zhang ZF**, Zhao G, Liu LN. [Effectiveness and safety of proton pump inhibitor and levofloxacin based first-line triple therapy in the eradication of Helicobacter pylori: a meta-analysis]. *Zhonghua Yixue Zazhi* 2008; **88**: 2722-2725 [PMID: 19080698]
59 **Castro-Fernández M**, Lamas E, Pérez-Pastor A, Pabón M, Aparcero R, Vargas-Romero J, Larraona JL, Romero-Gómez M. Efficacy of triple therapy with a proton pump inhibitor, levofloxacin, and amoxicillin as first-line treatment to eradicate Helicobacter pylori. *Rev Esp Enferm Dig* 2009; **101**: 395-398, 399-402 [PMID: 19630462]
60 **Gisbert JP**, Pérez-Aisa A, Castro-Fernández M, Barrio J, Rodrigo L, Cosme A, Gisbert JL, Marcos S, Moreno-Otero R. Helicobacter pylori first-line treatment and rescue option containing levofloxacin in patients allergic to penicillin. *Dig Liver Dis* 2010; **42**: 287-290 [PMID: 19632166 DOI: 10.1016/j.dld.2009.06.007]
61 **Cuadrado-Lavín A**, Salcines-Caviedes JR, Carrascosa MF, Dierssen-Sotos T, Cobo M, Campos MR, Ayestarán B, Fernández-Pousa A, González-Colominas E, Aresti-Zárate S, Hernández M, Pascual EL. Levofloxacin versus clarithromycin in a 10 day triple therapy regimen for first-line Helicobacter pylori eradication: a single-blind randomized clinical trial. *J Antimicrob Chemother* 2012; **67**: 2254-2259 [PMID: 22687889 DOI: 10.1093/jac/dks209]
62 **Pan X**, Li Y, Qiu Y, Tang Q, Qian B, Yao L, Shi R, Zhang G. Efficacy and tolerability of first-line triple therapy with levofloxacin and amoxicillin plus esomeprazole or rabeprazole for the eradication of Helicobacter pylori infection and the effect of CYP2C19 genotype: a 1-week, randomized, open-label study in Chinese adults. *Clin Ther* 2010; **32**: 2003-2011 [PMID: 21118735 DOI: 10.1016/j.clinthera.2010.11.005]
63 **Chen LW**, Chien RN, Chang JJ, Fang KM, Chang LC. Comparison of the once-daily levofloxacin-containing triple therapy with the twice-daily standard triple therapy for first-line Helicobacter pylori eradication: a prospective randomised study. *Int J Clin Pract* 2010; **64**: 1530-1534 [PMID: 20846200 DOI: 10.1111/j.1742-1241.2010.02482.x]
64 **Assem M**, El Azab G, Rasheed MA, Abdelfatah M, Shastery M. Efficacy and safety of Levofloxacin, Clarithromycin and Esomeprazol as first line triple therapy for Helicobacter pylori eradication in Middle East. Prospective, randomized, blind, comparative, multicenter study. *Eur J Intern Med* 2010; **21**: 310-314 [PMID: 20603042 DOI: 10.1016/j.ejim.2010.05.011]
65 **Erçin CN**, Uygun A, Toros AB, Kantarcioğlu M, Kilciler G, Polat Z, Bağci S. Comparison of 7- and 14-day first-line therapies including levofloxacin in patients with Helicobacter pylori positive non-ulcer dyspepsia. *Turk J Gastroenterol* 2010; **21**: 12-16 [PMID: 20533106]
66 **Liou JM**, Lin JT, Chang CY, Chen MJ, Cheng TY, Lee YC, Chen CC, Sheng WH, Wang HP, Wu MS. Levofloxacin-based and clarithromycin-based triple therapies as first-line and second-line treatments for Helicobacter pylori infection: a randomised comparative trial with crossover design. *Gut* 2010; **59**: 572-578 [PMID: 20427390 DOI: 10.1136/gut.2009.198309]
67 **Romano M**, Cuomo A, Gravina AG, Miranda A, Iovene MR, Tiso A, Sica M, Rocco A, Salerno R, Marmo R, Federico A, Nardone G. Empirical levofloxacin-containing versus clarithromycin-containing sequential therapy for Helicobacter pylori eradication: a randomised trial. *Gut* 2010; **59**: 1465-1470 [PMID: 20947881 DOI: 10.1136/gut.2010.215350]
68 **Federico A**, Nardone G, Gravina AG, Iovene MR, Miranda A, Compare D, Pilloni PA, Rocco A, Ricciardiello L, Marmo R, Loguercio C, Romano M. Efficacy of 5-day levofloxacin-containing concomitant therapy in eradication of Helicobacter pylori infection. *Gastroenterology* 2012; **143**: 55-61.e1; quize e13-14 [PMID: 22484118 DOI: 10.1053/j.gastro.2012.03.043]
69 **Molina-Infante J**, Perez-Gallardo B, Fernandez-Bermejo M, Hernandez-Alonso M, Vinagre G, Dueñas C, Mateos-Rodriguez JM, Gonzalez-Garcia G, Abadia EG, Gisbert JP. Clinical trial: clarithromycin vs. levofloxacin in first-line triple and sequential regimens for Helicobacter pylori eradication. *Aliment Pharmacol Ther* 2010; **31**: 1077-1084 [PMID: 20180787 DOI: 10.1111/j.1365-2036.2010.04274.x]
70 **Molina-Infante J**, Gisbert JP. Levofloxacin in first-line eradication regimens for Helicobacter pylori: better test antibiotic susceptibility before treating. *Gut* 2011; **60**: 1605; author reply 1605-1606 [PMID: 21193443 DOI: 10.1136/gut.2010.233015]
71 **Di Caro S**, Fini L, Daoud Y, Grizzi F, Gasbarrini A, De Lorenzo A, Di Renzo L, McCartney S, Bloom S. Levofloxacin/amoxicillin-based schemes vs quadruple therapy for Helicobacter pylori eradication in second-line. *World J Gastroenterol* 2012; **18**: 5669-5678 [PMID: 23155306 DOI: 10.3748/wjg.v18.i40.5669]
72 **Gisbert JP**, Molina-Infante J, Marin AC, Vinagre G, Barrio J, McNicholl AG. Second-line rescue triple therapy with levofloxacin after failure of non-bismuth quadruple "sequential" or "concomitant" treatment to eradicate H. pylori infection. *Scand J Gastroenterol* 2013; **48**: 652-656 [PMID: 23556551 DOI: 10.3109/00365521.2013.786132]
73 **Manfredi M**, Bizzarri B, de'Angelis GL. Helicobacter pylori infection: sequential therapy followed by levofloxacin-containing triple therapy provides a good cumulative eradication rate. *Helicobacter* 2012; **17**: 246-253 [PMID: 22759323 DOI: 10.1111/j.1523-5378.2012.00945.x]
74 **Gisbert JP**, Pérez-Aisa A, Bermejo F, Castro-Fernández M, Almela P, Barrio J, Cosme A, Modolell I, Bory F, Fernández-Bermejo M, Rodrigo L, Ortuño J, Sánchez-Pobre P, Khorrami S, Franco A, Tomas A, Guerra I, Lamas E, Ponce J, Calvet X. Second-line therapy with levofloxacin after failure of treatment to eradicate helicobacter pylori infection: time trends

in a Spanish Multicenter Study of 1000 patients. *J Clin Gastroenterol* 2013; **47**: 130-135 [PMID: 22647827 DOI: 10.1097/MCG.0b013e318254ebdd]

75 **Moon JY**, Kim GH, You HS, Lee BE, Ryu DY, Cheong JH, Jung JI, Jeong JH, Song CS, Song GA. Levofloxacin, Metronidazole, and Lansoprazole Triple Therapy Compared to Quadruple Therapy as a Second-Line Treatment of Helicobacter pylori Infection in Korea. *Gut Liver* 2013; **7**: 406-410 [PMID: 23898379 DOI: 10.5009/gnl.2013.7.4.406]

76 **Calhan T**, Kahraman R, Sahin A, Senates E, Doganay HL, Kanat E, Ozdil K, Sokmen HM. Efficacy of two levofloxacin-containing second-line therapies for Helicobacter pylori: a pilot study. *Helicobacter* 2013; **18**: 378-383 [PMID: 23601026 DOI: 10.1111/hel.12056]

77 **Cammarota G**, Martino A, Pirozzi G, Cianci R, Branca G, Nista EC, Cazzato A, Cannizzaro O, Miele L, Grieco A, Gasbarrini A, Gasbarrini G. High efficacy of 1-week doxycycline- and amoxicillin-based quadruple regimen in a culture-guided, third-line treatment approach for Helicobacter pylori infection. *Aliment Pharmacol Ther* 2004; **19**: 789-795 [PMID: 15043520]

78 **Gisbert JP**, Castro-Fernández M, Bermejo F, Pérez-Aisa A, Ducons J, Fernández-Bermejo M, Bory F, Cosme A, Benito LM, López-Rivas L, Lamas E, Pabón M, Olivares D. Third-line rescue therapy with levofloxacin after two H. pylori treatment failures. *Am J Gastroenterol* 2006; **101**: 243-247 [PMID: 16454825]

79 **Gisbert JP**, Gisbert JL, Marcos S, Moreno-Otero R, Pajares JM. Third-line rescue therapy with levofloxacin is more effective than rifabutin rescue regimen after two Helicobacter pylori treatment failures. *Aliment Pharmacol Ther* 2006; **24**: 1469-1474 [PMID: 17032282]

80 **Jeong MH**, Chung JW, Lee SJ, Ha M, Jeong SH, Na S, Na BS, Park SK, Kim YJ, Kwon KA, Ko KI, Jo Y, Hahm KB, Jung HY. [Comparison of rifabutin- and levofloxacin-based third-line rescue therapies for Helicobacter pylori]. *Korean J Gastroenterol* 2012; **59**: 401-406 [PMID: 22735872]

81 **Gisbert JP**. Letter: third-line rescue therapy with levofloxacin after failure of two treatments to eradicate Helicobacter pylori infection. *Aliment Pharmacol Ther* 2012; **35**: 1484-1485; author reply 1486 [PMID: 22582841 DOI: 10.1111/j.1365-2036.2012.05117.x]

82 **Gisbert JP**. Rescue Therapy for Helicobacter pylori Infection 2012. *Gastroenterol Res Pract* 2012; **2012**: 974594 [PMID: 22536225 DOI: 10.1155/2012/974594]

83 **Gisbert JP**. "Rescue" regimens after Helicobacter pylori treatment failure. *World J Gastroenterol* 2008; **14**: 5385-5402 [PMID: 18803350]

P- Reviewers: Aghakhani A, Buzas GM, Leitman M, Martin-Villa JM, Misra SP, Paulssen EJ, Said ZNA, Zullo A
S- Editor: Ma YJ **L- Editor**: Wang TQ **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8957

World J Gastroenterol 2014 July 21; 20(27): 8957-8963
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Helicobacter pylori and functional dyspepsia: An unsolved issue?

Angelo Zullo, Cesare Hassan, Vincenzo De Francesco, Alessandro Repici, Raffaele Manta, Silverio Tomao, Bruno Annibale, Dino Vaira

Angelo Zullo, Cesare Hassan, Gastroenterology and Digestive Endoscopy, "Nuovo Regina Margherita" Hospital, 00153 Rome, Italy
Vincenzo De Francesco, Gastroenterology Unit, "Riuniti" Hospital, 24128 Foggia, Italy
Alessandro Repici, Digestive Endoscopy Unit, "IRCCS Istituto Clinico Humanitas", 20089 Milan, Italy
Raffaele Manta, Gastroenterology and Endoscopy Unit, "Nuovo S Agostino" Hospital, 41100 Modena, Italy
Silverio Tomao, Oncology Unit, "SM Goretti" Hospital, Latina, "Sapienza" University, 00185 Rome, Italy
Bruno Annibale, Department of Digestive and Liver Diseases, "Sant'Andrea" Hospital, "Sapienza" University, 00185 Rome, Italy
Dino Vaira, Department of Internal Medicine and Gastroenterology, University of Bologna, 40126 Bologna, Italy
Author contributions: Zullo A and Hassan C designed research; De Francesco V, Repici A and Manta R performed research; Tomao S, Annibale B and Vaira D contributed with constructive criticisms; Zullo A and Hassan C analyzed data and wrote the paper.
Correspondence to: Angelo Zullo, MD, Gastroenterology and Digestive Endoscopy, "Nuovo Regina Margherita" Hospital, Via E Morosini 30, 00153 Roma, Italy. zullo66@yahoo.it
Telephone: +39-6-58446608 Fax: +39-6-58446533
Received: November 16, 2013 Revised: December 30, 2013
Accepted: April 2, 2014
Published online: July 21, 2014

Abstract

Patients with *Helicobacter pylori* (*H. pylori*) infection may complain of dyspeptic symptoms without presence of macroscopic lesions on gastroduodenal mucosa. Such a condition is usually recognized as functional dyspepsia, and different pathogenetic mechanisms are involved. The role of *H. pylori* in these patients is controversial. Several trials assessed the potential role of *H. pylori* eradication in improving dyspeptic symptoms, and data of some meta-analyses demonstrated that cure of infection is associated with a small (10%), but significant therapeutic gain as compared to placebo. The reason for which dyspeptic symptoms regress in some patients following bacterial eradication, but persist in others remains unclear. Regrettably, trials included in the meta-analyses are somewhat different for study design, definition of symptoms, assessment of symptoms changes, and some may be flawed by potential pitfalls. Consequently, the information could be not consistent. We critically reviewed the main available trials, attempting to address future research in this field

Key words: *Helicobacter pylori*; Dyspepsia; Symptoms; Therapy; Pathogenesis

Core tip: The role of *Helicobacter pylori* infection in functional dyspesia is still controversial. Some meta-analyses indicated the infection eradication is associated with a significant therapeutic gain as compared to placebo. However, the considered trials differ for study design, definition of symptoms, assessment of symptoms changes, and some may be flawed by potential pitfalls. Therefore, further studies are needed.

Zullo A, Hassan C, De Francesco V, Repici A, Manta R, Tomao S, Annibale B, Vaira D. *Helicobacter pylori* and functional dyspepsia: An unsolved issue? *World J Gastroenterol* 2014; 20(27): 8957-8963 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8957.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8957

INTRODUCTION

Functional dyspepsia (FD) is classically defined as continuous or frequently recurring epigastric pain or discomfort centred in upper abdomen for which no organic

cause can be determined[1]. It includes a heterogeneous and broad range of chronic upper abdominal symptoms which are widely shared with different gastrointestinal disorders, including reflux disease, irritable bowel disease, gallbladder and pancreatic dysfunction, and celiac disease. Therefore, FD is generally diagnosed when other frequent gastrointestinal diseases are excluded, and upper endoscopy ruled out macroscopic lesions - *i.e.*, investigated dyspepsia. Such a condition is frequently encountered in clinical practice, its prevalence being close to 20%-30% in the general population. Of note, dyspeptic symptoms persist over the long-term in the majority of patients, despite periods of remission may occur, so that several FD patients recurrently require medical consultations and therapies[2]. Consequently, the management of FD patients has a relevant economic impact, and it involves both general practitioners and gastroenterologists. Regrettably, the aetiology of FD remains largely unknown - different factors being presumably involved - and no definite and effective treatment is currently available for all these patients.

The discovery of *Helicobacter pylori* (*H. pylori*) in the '80[thies] triggered the expectation that dyspeptic symptoms could be caused by such a persistent infection in the stomach, which invariably causes chronic active gastritis. Starting at the end of '90[thies], several studies have been performed to assess the potential role of *H. pylori* eradication on FD, and a Cochrane meta-analysis including data of 12 trials involving 2541 patients was published on 2003[3]. It was calculated that *H. pylori* eradication had a small (37% *vs* 29%; 8%), but statistically significant effect (RR = 0.91; 95%CI: 0.86-0.95) in reducing dyspepsia symptoms as compared to placebo at 12 mo follow-up, with a number need to treat (NNT) of 15. An update of data including 17 trials with 3,566 patients showed that there was a 10% (95%CI: 6-14) relative risk reduction of dyspepsia following *H. pylori* eradication as compared to placebo, with a NNT of 14 (95%CI: 10-25)[4]. The last Consensus of an International panel of clinical investigators on gastroduodenal functional disorders (Rome Ⅲ) recommended *H. pylori* eradication in all infected patients with non-ulcer dyspepsia diagnosed at upper endoscopy, also suggesting non-invasive testing followed by *H. pylori* eradication ("test and treat") in those patients with no alarm features, although many infected patients with FD may not gain symptomatic benefit[2].

Can we therefore conclude that everything is clear on this issue? Unfortunately, results of different studies are conflicting, so that no definitive information emerges from the available data. This could depend on different factors, such as the study design, definition of symptoms, assessment of symptoms changes, and potential pitfalls. The knowledge of these limitations could add "a further light at the end on the tunnel" of FD.

H. PYLORI AND FD PATHOGENESIS

The pathogenesis of FD still remains unrevealed, and several alterations have been invoked as putative mechanisms responsible of dyspeptic symptoms. These include altered gastric emptying (delayed or accelerated), impaired accommodation of proximal stomach, and sensory abnormalities (gastroduodenal hypersensitivity)[2]. However, the underlying causes of these alterations are unknown. To date, no conclusive data have been reported on the role of *H. pylori* infection on these dysfunctions. Indeed, disturbances of either motor or sensory function in the gastroduodenal tract of patients with *H. pylori* are not consistent[5,6]. Some studies found that *H. pylori* infection delays gastric emptying, it is associated with a loss of gastric phase Ⅲ of the migrating motor complex, and its eradication significantly improves gastric emptying. However, other studies failed to confirm these observations[7].

Nevertheless, at least in theory, *H. pylori* infection may cause dyspeptic symptoms through other mechanisms such as: (1) alterations of gastric acid secretion; (2) persistent and active inflammation of gastric mucosa; and (3) post-infective changes in gastroduodenal mucosa.

It has been found that gastrin releasing peptide-stimulated maximal acid output (MAO) is 3-fold increased in dyspeptic patients with *H. pylori* infection as compared to uninfected controls[8], and approximately 50% of the non-ulcer dyspepsia infected patients had a similarly stimulated MAO when compared with duodenal ulcer patients[9]. Indeed, *H. pylori* alters production of both gastrin (increased) and somatostatin (decreased) in gastric mucosa, as well as of ghrelin which is involved in acid secretion, hunger sensations, and gastrointestinal motility[10]. Noteworthy, these alterations of gastric acid secretion normalize 6-12 mo following a successful *H. pylori* eradication[10]. Therefore, it is expected that at least some dyspeptic symptoms probably linked to acid hypersecretion, such as epigastric pain, could regress 1 year after the cure of infection. Although the level of stimulated gastric acid secretion at entry was not a predictive factor of FD improvement following *H. pylori* therapy[11], no study assessed the relationship between gastric acid output levels and dyspeptic symptoms 1 year following a successful *H. pylori* eradication.

As far as gastritis is concerned, no consistent data demonstrated that such an inflammatory status of gastric mucosa may be associated with dyspeptic symptoms. A certain genetic predisposition to develop dyspeptic symptoms in patients with *H. pylori*-associated gastritis has been suggested[12]. Indeed, more than half of the patients with *H. pylori* infection are asymptomatic, despite the infection invariably causes a chronic active gastritis. However, it has been widely reported that the active component of gastritis - *i.e.*, polymorphonuclear infiltration - quickly and completely recovers following bacterial eradication, whilst the presence of lymphocytic infiltrate in gastric mucosa may persist several months or years[13]. It has been speculated that these cells may cause alterations of gastric mucosa function by producing different cytokines, similarly to what it is accepted for irritable bowel disease (IBS)[14,15]. Therefore, to correlate dyspeptic symptoms modification and gastritis grade (*i.e.*, lymphocytic infil-

trate) 1 year following a successful bacterial eradication would be an ideal clinical situation to unravel such an issue. Indeed, at least in theory, a total recovery of gastric function should be expected only in those patients in whom both the infection is cured and the inflammation disappeared - *i.e.*, grade 0 gastritis according to the update Sydney System classification[13]. Of note, a trial found that FD symptoms disappeared after curing *H. pylori* infection more frequently in patients with gastritis grade score 0-1 than in those with grade 2-3 (32% *vs* 17%, $P = 0.008$)[16].

Similarly to IBS in which the role for an acute gastrointestinal infection is recognized as a trigger of symptoms in a subset of patients, some studies suggest that dyspeptic symptoms may develop following an infection[17]. A study found that the risk of dyspepsia is 5.2-fold (95%CI: 2.7-9.8) increased 1 year following an acute *Salmonella*-associated gastroenteritis[18]. Similarly, another study found that the risk of hunger pain was 5.77-fold (95%CI: 1.3-25.7) increased 1 year after a water-borne viral gastroenteritis with Norovirus[19]. As for IBS, an increased infiltration of both enterochromaffin cells (EC) and mast cells in gastroduodenal mucosa in post-infectious FD patients has been recently reported[20]. In addition, a role for eosinophils, inflammatory cells, and neuroendocrine cells (*i.e.*, serotonin) infiltration in duodenal mucosa in post-infectious FD patients has been suggested[21]. Since it is widely recognized that *H. pylori* initiates as an acute infection with dyspeptic symptoms, it would appear worthy to assess whether dyspeptic symptoms persist following *H. pylori* eradication in a subset of patients due to a persistence of these cells in gastroduodenal mucosa.

CONCERNS ON *H. PYLORI* ERADICATION TRIALS IN FD PATIENTS

Several trials on the role of *H. pylori* eradication in FD patients are now available, and data of the majority of these studies have been pooled in some meta-analyses[3,4,22]. However, it is remarkable to note that the evaluation of symptoms at entry as well as the assessment of their modifications following bacterial eradication is not the same among different studies. In detail, type and number of symptoms evaluated at entry are not the same, and the definition of symptoms regression or improvement are somewhat different.

By taking into account only double-blind, placebo-controlled trials with at least 50 patients for arms followed for 6-12 mo, in which the final evaluation of *H. pylori* status was performed by means of either upper endoscopy with biopsies or a ^{13}C-urea breath test, we identified 12 studies (Table 1)[11,16,23-32]. Noteworthy, the number and type of symptoms evaluated at entry, the score used to evaluate symptoms modifications, and the period considered for final symptoms assessment are very different among studies. In addition, the use of non-steroid anti-inflammatory drugs (NSAIDs), and the presence of gastric erosions at endoscopy were considered as exclusion criteria in some, but not in other studies. Symptoms modification was evaluated by assessing the score of symptoms present in the last 1-7 d preceding the end of follow-up in some studies and in the last 1-6 mo in others. Furthermore, some trials considered symptomatic improvement following the eradication therapy, irrespectively of whether the patients were actually eradicated or not. Unfortunately, only few studies correlated the symptoms modifications and gastritis score at the end of follow-up, in order to assess whether a complete gastritis regression was also associated with symptom resolution[16]. Similarly, no study tested the potential correlation between gastric acid secretion normalization and symptom disappearance following a successful bacterial eradication. Finally, in different multicenter trials, several participating centres enrolled less than 5-10 patients - with only 1 patient included in a centre[16] - so that a β-type error in patients enrolment cannot be ruled out. Based on all these observations, it remains unclear whether it is appropriate to pool data of these heterogeneous trials, and how robust the final data interpretation may actually be.

Another aspect deserving consideration is that *H. pylori* eradication observed in the placebo group would appear unexpectedly high in some of these trials, with cure rates approaching 6.3%, 6.6%, 7.4%, 8%, and 12%[23,26,28,29,31], whilst the cure rate was reasonably low (0%-2%) in others studies[24,27,32]. *H. pylori* infection virtually persists long-life whether not opportunely treated, and spontaneous disappearance is considered as an infrequent event[33].

Unexpectedly, in a trial[11], comparison of symptom modification was performed between patients receiving eradication therapy and those receiving placebo, rather than between patients cured from *H. pylori* and those with persistent infection. To establish whether *H. pylori* infection really impacts on FD symptoms, the comparison should be between patients definitely cured and those not cured. Indeed, the eradication rate following the triple therapy used is distinctly lower than 100% and, most likely, as many as 15%-20% of patients computed in the eradication therapy group probably remained infected. Both patients and physicians would know whether FD symptoms disappear by really eliminating *H. pylori* infection, rather than following a potentially eradicating therapy.

In the majority of studies (Table 1), assessment of symptom modification was based on the overall score, instead of evaluation of each independent symptom. This would consider FD as a well-definite disease, rather than a combination of symptoms, most likely depending on different pathogenetic mechanisms[34,35]. Intuitively, it would appear at least implausible to expect that *H. pylori* eradication would improve some symptoms - such as abdominal pain, flatulence, diarrhoea, constipation, bloating - which are generally attributed to large bowel[36].

It is widely recognized that NSAIDs - including low-dose aspirin - may cause dyspeptic symptoms, irrespective of onset of either gastroduodenal ulcers or erosions[37].

Table 1 Double-blind, placebo-controlled trials on *Helicobacter pylori* eradication in functional dyspepsia patients

Patients (follow-up)	Low-dose aspirin	Gastric erosions	Symptoms evaluated	Score used (period)	Primary end-point	Comment	Ref.
Therapy = 160 Placebo = 158 (12 mo)	No	NA	Pain or discomfort; heartburn; nausea; postprandial fullness	GDSS: 0-20 (the last 6 mo)	Therapy better than placebo	Evaluation according to the final *H. pylori* status was lacking	[11]
Therapy = 170 Placebo = 167 (12 mo)	Yes	< 5	Ulcer-like; motility-like; reflux-like	GSRS: 0-6 (the last 7 d)	No difference	Rate of family history of ulcers (34% *vs* 23%) and caffeine use (82% *vs* 73%) were significantly higher in therapy group than placebo. Distribution of low-dose aspirin use was lacking. The rate of "indeterminate" UBT result was unexpectedly high (16%)	[16]
Therapy = 129 Placebo = 124 (12 mo)	No	No	Epigastric pain; nausea; abdominal distension; eructation; vomiting; early satiety; regurgitation; retrosternal burning	Likert score: 0-5 (the last 7 d)	Therapy better than placebo	Difference remained significant when symptoms resolution was assessed according to final *H. pylori* status	[23]
Therapy = 81 Placebo = 80 (12 mo)	No	NA	Epigastric pain; burning; postprandial fullness; nausea; vomiting	Score: 0-3 (the last 1 d)	No difference	No difference was observed when symptoms resolution was assessed according to final *H. pylori* status	[24]
Therapy = 135 Placebo = 143 (12 mo)	NA	< 5	Ulcer-like; motility-like; reflux-like	GSRS: 1-7 (the last 7 d)	No difference	No difference according to final *H. pylori* status. A significant difference was observed between patients with persistent gastritis and those with gastritis healing	[25]
Therapy = 135 Placebo = 143 (6 mo)	No	< 10	Epigastric pain or burning; epigastric fullness; heartburn, regurgitation; nausea vomiting, abdominal pain; flatulence; diarrhoea; constipation	Likert score: 0-3 (the last 7 d)	No difference	No difference was observed when symptoms resolution was assessed according to final *H. pylori* status	[26]
Therapy = 71 Placebo = 65 (12 mo)	Yes	No	Epigastric pain; nausea; bloating; heartburn early satiety; vomiting; regurgitation; hunger pain	Score: 0-16 (the last 1 d)	No difference	No difference was observed according to final *H. pylori* status. Both patient groups received omeprazole therapy during the first 3 mo	[27]
Therapy = 201 Placebo = 203 (12 mo)	Yes	Yes	Rome Ⅲ criteria	PADYQ: 0-44 (the last 30 d)	Therapy better than placebo	Therapy success occurred for "postprandial distress syndrome" but not for "epigastric pain syndrome"	[28]
Therapy = 75 Placebo =82 (12 mo)	Yes	< 5	Epigastric pain; belching; heartburn, bloating; flatulence; sour taste; nausea; halitosis	DSS likert score: 1-5	No difference	No difference was observed when symptoms resolution was assessed according to final *H. pylori* status	[29]
Therapy = 50 Placebo = 50 (12 mo)	No	No	Ulcer-like; motility-like; reflux-like; unspecified	Score for severity: 0-2 Score for frequency: 0-3	Therapy better than placebo	Difference was based on the reduction on mean score. Difference remained significant according to final *H. pylori* status. The rate of asymptomatic patients was lacking	[30]
Therapy = 50 Placebo = 50 (12 mo)	NA	NA	Abdominal fullness; early satiety; bloating; nausea (Rome Ⅱ criteria)	Likert score: 0-6	No difference	No difference was observed when symptoms resolution was assessed according to final *H. pylori* status	[31]
Therapy = 164 Placebo = 164 (12 mo)	NA	< 5	Indigestion; diarrhoea constipation; reflux; abdominal pain	GSRS: 1-7 (the last 7 d)	No difference	No difference was observed when symptoms resolution was assessed according to final *H. pylori* status	[32]

GSRS: Gastrointestinal symptoms rating score; GDSS: Glasgow dyspepsia severity score; PADYQ: Porto Alegre dyspeptic symptoms questionnaire; DSS: Dyspepsia summary score; NA: Not available.

Therefore, inclusion of patients taking these drugs in trials aimed to assess an independent role of *H. pylori* in FD should be regarded as confounding. Regrettably, in a trial[16], the rate of low-dose aspirin users in either therapy and placebo arm was lacking, so that a different distribution between the two groups cannot be ruled out.

The potential role of gastroduodenal erosions in dyspeptic patients remains largely unclear. Indeed, gastroduodenal erosions may be encountered in asymptomatic patients. However, at least in theory, the presence of breaks on gastric mucosa - irrespective of diameter - may favour a H^+ back diffusion and, consequently, stimulation of visceral nerve terminations with epigastric pain. In the considered trials (Table 1), patients with gastroduodenal erosions were excluded in some studies, whilst enrolled in others when erosions were either < 5 or < 10. However, no data on erosions resolution at the end follow-up were provided, and their potential association with symptom modification was not assessed. Some data would suggest that gastric erosions did not regress following *H. pylori* eradication[38].

CONCLUSION

The most definite information currently available on the role of *H. pylori* eradication in FD patients arises from two meta-analyses where trials with a follow-up of 6-12 mo were included[3,4,22]. Basically, it has been calculated that *H. pylori* eradication is associated with a 10% (95%CI: 6-14) therapeutic gain as compared to placebo, with a NNT of 14 (95%CI: 10-25), and that symptoms improvement ultimately occurs in nearly 40% of eradicated patients[4]. Another meta-analysis, including data more recent studies (overall 14 trials with 2993 patients), confirmed that improvement of dyspepsia symptoms occurs more frequently after *H. pylori* therapy than placebo (OR = 1.38, 95%CI: 1.18-1.62, $P < 0.0001$), without differences among Europe, United States and Asia[22]. However, as we discussed, criteria adopted regarding type of symptoms at entry, evaluation of symptoms modification at follow-up, and clinical characteristics of enrolled patients (gastroduodenal erosions, use of NSAIDs, *etc.*) frequently varied among these studies. In addition, the role of dietary and lifestyle factors was not considered[39]. These factors could affect symptoms evaluation, particularly when only 1 d - or few days - recording is used, as occurred in several trials. On the other hand, some alterations induced by *H. pylori* infection - *i.e.*, low-grade chronic inflammation, gastric acid out-put perturbation, EC and mast cells infiltration, *etc.* - with a putative role in FD symptoms were not opportunely assessed following a successful cure of infection. Unfortunately, despite several trials with some thousands of patients, no clear predictive factors of FD regression after bacterial eradiation have been identified. During the last decades, progressive attempts aimed to unify different dyspeptic symptoms in few, well-structured categories were performed. The latter functional disorders classification (Rome Ⅲ) proposed two main type of FD, there is postprandial distress syndrome (postprandial fullness and early satiation) and epigastric pain syndrome (EPS: pain or burning localized to the epigastrium, not generalized or localized to other abdominal or chest regions, not relieved by defecation or passage of flatus, not fulfilling criteria for gallbladder and sphincter of oddi disorders). For both conditions, criteria should fulfilled for the last 3 mo with symptom onset at least 6 mo before diagnosis. This could reduce the confusion in FD definition in future studies, allowing a more reliable comparison among therapeutic trials. Regrettably, a recent Korean trial showed that application of these Rome Ⅲ criteria to FD is very difficult, and only 4 patients fulfilling these criteria were identified by gastroenterologists at 11 tertiary referral hospitals during 1 year[40]. In addition, the observation that EPS frequently overlaps with non-erosive reflux disease - which is not associated with *H. pylori* infection[41] - further puzzles data interpretation[42]. Therefore, the saga continues.

While waiting to understand what subset of FD patients may actually benefit of *H. pylori* eradication, it should be considered that bacterial eradication in dyspeptic patients significantly reduces the number of upper endoscopies[43], medical visits[44], and the use of drugs[45], so that it is cost-effective at long-term follow-up[46]. Prevention of peptic ulcer onset, and reduction of both cancer and lymphoma development in the stomach are other remarkable gains favouring *H. pylori* eradication in symptomatic patients[47].

REFERENCES

1 **Talley NJ**, Stanghellini V, Heading RC, Koch KL, Malagelada JR, Tytgat GN. Functional gastroduodenal disorders. *Gut* 1999; **45** Suppl 2: II37-II42 [PMID: 10457043 DOI: 10.1136/gut.45.2008.ii37]

2 **Tack J**, Talley NJ, Camilleri M, Holtmann G, Hu P, Malagelada JR, Stanghellini V. Functional gastroduodenal disorders. *Gastroenterology* 2006; **130**: 1466-1479 [PMID: 16678560 DOI: 10.1053/j.gastro.2005.11.059]

3 **Moayyedi P**, Deeks J, Talley NJ, Delaney B, Forman D. An update of the Cochrane systematic review of Helicobacter pylori eradication therapy in nonulcer dyspepsia: resolving the discrepancy between systematic reviews. *Am J Gastroenterol* 2003; **98**: 2621-2626 [PMID: 14687807 DOI: 10.1111/j.1572-0241.2003.08724.x]

4 **Moayyedi P**, Soo S, Deeks J, Delaney B, Harris A, Innes M, Oakes R, Wilson S, Roalfe A, Bennett C, Forman D. Eradication of Helicobacter pylori for non-ulcer dyspepsia. *Cochrane Database Syst Rev* 2005; **2**: CD002096 [PMID: 15674892 DOI: 10.1002/14651858.CD002096.pub5]

5 **Tack J**, Piessevaux H, Coulie B, Caenepeel P, Janssens J. Role of impaired gastric accommodation to a meal in functional dyspepsia. *Gastroenterology* 1998; **115**: 1346-1352 [PMID: 9834261 DOI: 10.1016/S0016-5085(98)70012-5]

6 **Sarnelli G**, Cuomo R, Janssens J, Tack J. Symptom patterns and pathophysiological mechanisms in dyspeptic patients with and without Helicobacter pylori. *Dig Dis Sci* 2003; **48**: 2229-2236 [PMID: 14714606 DOI: 10.1023/B:DDAS.0000007856.71462.6c]

7 **Talley NJ**, Xia HH. Helicobacter pylori infection and non-ulcer dyspepsia. *Br Med Bull* 1998; **54**: 63-69 [PMID: 9604431 DOI: 10.1093/oxfordjournals.bmb.a011680]

8 **el-Omar EM**, Penman ID, Ardill JE, Chittajallu RS, Howie

C, McColl KE. Helicobacter pylori infection and abnormalities of acid secretion in patients with duodenal ulcer disease. *Gastroenterology* 1995; **109**: 681-691 [PMID: 7657096 DOI: 10.1016/0016-5085(95)90374-7]
9 **el-Omar E**, Penman I, Ardill JE, McColl KE. A substantial proportion of non-ulcer dyspepsia patients have the same abnormality of acid secretion as duodenal ulcer patients. *Gut* 1995; **36**: 534-538 [PMID: 7737559 DOI: 10.1136/gut.36.4.534]
10 **Suzuki H**, Moayyedi P. Helicobacter pylori infection in functional dyspepsia. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 168-174 [PMID: 23358394 DOI: 10.1038/nrgastro.2013.9]
11 **McColl K**, Murray L, El-Omar E, Dickson A, El-Nujumi A, Wirz A, Kelman A, Penny C, Knill-Jones R, Hilditch T. Symptomatic benefit from eradicating Helicobacter pylori infection in patients with nonulcer dyspepsia. *N Engl J Med* 1998; **339**: 1869-1874 [PMID: 9862941 DOI: 10.1056/NEJM199812243392601]
12 **Tahara T**, Shibata T, Yamashita H, Hirata I, Arisawa T. The Role of RANTES Promoter Polymorphism in Functional Dyspepsia. *J Clin Biochem Nutr* 2009; **45**: 235-240 [PMID: 19794934 DOI: 10.3164/jcbn.09-31]
13 **Dixon MF**, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 1996; **20**: 1161-1181 [PMID: 8827022 DOI: 10.1097/00000478-199610000-00001]
14 **Fock KM**. Functional dyspepsia, H. pylori and post infectious FD. *J Gastroenterol Hepatol* 2011; **26** Suppl 3: 39-41 [PMID: 21443707 DOI: 10.1111/j.1440-1746.2011.06649.x]
15 **Li XB**, Chen HM, Lu H, Zheng Q, Chen XY, Peng YS, Ge ZZ, Liu WZ. Role of Helicobacter pylori infection on neuronal expression in the stomach and spinal cord of a murine model. *J Dig Dis* 2009; **10**: 286-292 [PMID: 19906107 DOI: 10.1111/j.1751-2980.2009.00397.x]
16 **Talley NJ**, Vakil N, Ballard ED, Fennerty MB. Absence of benefit of eradicating Helicobacter pylori in patients with nonulcer dyspepsia. *N Engl J Med* 1999; **341**: 1106-1111 [PMID: 10511608 DOI: 10.1056/NEJM199910073411502]
17 **Tack J**, Demedts I, Dehondt G, Caenepeel P, Fischler B, Zandecki M, Janssens J. Clinical and pathophysiological characteristics of acute-onset functional dyspepsia. *Gastroenterology* 2002; **122**: 1738-1747 [PMID: 12055579 DOI: 10.1053/gast.2002.33663]
18 **Mearin F**, Pérez-Oliveras M, Perelló A, Vinyet J, Ibañez A, Coderch J, Perona M. Dyspepsia and irritable bowel syndrome after a Salmonella gastroenteritis outbreak: one-year follow-up cohort study. *Gastroenterology* 2005; **129**: 98-104 [PMID: 16012939 DOI: 10.1053/j.gastro.2005.04.012]
19 **Zanini B**, Ricci C, Bandera F, Caselani F, Magni A, Laronga AM, Lanzini A. Incidence of post-infectious irritable bowel syndrome and functional intestinal disorders following a water-borne viral gastroenteritis outbreak. *Am J Gastroenterol* 2012; **107**: 891-899 [PMID: 22525306 DOI: 10.1038/ajg.2012.102]
20 **Li X**, Chen H, Lu H, Li W, Chen X, Peng Y, Ge Z. The study on the role of inflammatory cells and mediators in post-infectious functional dyspepsia. *Scand J Gastroenterol* 2010; **45**: 573-581 [PMID: 20163288 DOI: 10.3109/00365521003632576]
21 **Futagami S**, Shindo T, Kawagoe T, Horie A, Shimpuku M, Gudis K, Iwakiri K, Itoh T, Sakamoto C. Migration of eosinophils and CCR2-/CD68-double positive cells into the duodenal mucosa of patients with postinfectious functional dyspepsia. *Am J Gastroenterol* 2010; **105**: 1835-1842 [PMID: 20461070 DOI: 10.1038/ajg.2010.151]
22 **Zhao B**, Zhao J, Cheng WF, Shi WJ, Liu W, Pan XL, Zhang GX. Efficacy of Helicobacter pylori eradication therapy on functional dyspepsia: a meta-analysis of randomized controlled studies with 12-month follow-up. *J Clin Gastroenterol* 2014; **48**: 241-247 [PMID: 24002127 DOI: 10.1097/MCG.0b013e31829f2e25]
23 **Bruley Des Varannes S**, Fléjou JF, Colin R, Zaïm M, Meunier A, Bidaut-Mazel C. There are some benefits for eradicating Helicobacter pylori in patients with non-ulcer dyspepsia. *Aliment Pharmacol Ther* 2001; **15**: 1177-1185 [PMID: 11472320 DOI: 10.1046/j.1365-2036.2001.01014.x]
24 **Hsu PI**, Lai KH, Tseng HH, Lo GH, Lo CC, Lin CK, Cheng JS, Chan HH, Ku MK, Peng NJ, Chien EJ, Chen W, Hsu PN. Eradication of Helicobacter pylori prevents ulcer development in patients with ulcer-like functional dyspepsia. *Aliment Pharmacol Ther* 2001; **15**: 195-201 [PMID: 11148437 DOI: 10.1046/j.1365-2036.2001.00903.x]
25 **Talley NJ**, Janssens J, Lauritsen K, Rácz I, Bolling-Sternevald E. Eradication of Helicobacter pylori in functional dyspepsia: randomised double blind placebo controlled trial with 12 months' follow up. The Optimal Regimen Cures Helicobacter Induced Dyspepsia (ORCHID) Study Group. *BMJ* 1999; **318**: 833-837 [PMID: 10092259 DOI: 10.1136/bmj.318.7187.833]
26 **Koelz HR**, Arnold R, Stolte M, Fischer M, Blum AL. Treatment of Helicobacter pylori in functional dyspepsia resistant to conventional management: a double blind randomised trial with a six month follow up. *Gut* 2003; **52**: 40-46 [PMID: 12477757 DOI: 10.1136/gut.52.1.40]
27 **Koskenpato J**, Farkkilä M, Sipponen P. Helicobacter pylori eradication and standardized 3-month omeprazole therapy in functional dyspepsia. *Am J Gastroenterol* 2001; **96**: 2866-2872 [PMID: 11693319 DOI: 10.1111/j.1572-0241.2001.04240.x]
28 **Mazzoleni LE**, Sander GB, Francesconi CF, Mazzoleni F, Uchoa DM, De Bona LR, Milbradt TC, Von Reisswitz PS, Berwanger O, Bressel M, Edelweiss MI, Marini SS, Molina CG, Folador L, Lunkes RP, Heck R, Birkhan OA, Spindler BM, Katz N, Colombo Bda S, Guerrieri PP, Renck LB, Grando E, Hocevar de Moura B, Dahmer FD, Rauber J, Prolla JC. Helicobacter pylori eradication in functional dyspepsia: HEROES trial. *Arch Intern Med* 2011; **171**: 1929-1936 [PMID: 22123802 DOI: 10.1001/archinternmed.2011.533]
29 **Veldhuyzen van Zanten S**, Fedorak RN, Lambert J, Cohen L, Vanjaka A. Absence of symptomatic benefit of lansoprazole, clarithromycin, and amoxicillin triple therapy in eradication of Helicobacter pylori positive, functional (nonulcer) dyspepsia. *Am J Gastroenterol* 2003; **98**: 1963-1969 [PMID: 14499772 DOI: 10.1016/S0002-9270(03)00432-5]
30 **Gilvarry J**, Buckley MJ, Beattie S, Hamilton H, O'Morain CA. Eradication of Helicobacter pylori affects symptoms in non-ulcer dyspepsia. *Scand J Gastroenterol* 1997; **32**: 535-540 [PMID: 9200283]
31 **Sodhi JS**, Javid G, Zargar SA, Tufail S, Shah A, Khan BA, Yattoo GN, Gulzar GM, Khan MA, Lone MI, Saif Ru, Parveen S, Shoukat A. Prevalence of Helicobacter pylori infection and the effect of its eradication on symptoms of functional dyspepsia in Kashmir, India. *J Gastroenterol Hepatol* 2013; **28**: 808-813 [PMID: 23432600 DOI: 10.1111/jgh.12178]
32 **Blum AL**, Talley NJ, O'Moráin C, van Zanten SV, Labenz J, Stolte M, Louw JA, Stubberöd A, Theodórs A, Sundin M, Bolling-Sternevald E, Junghard O. Lack of effect of treating Helicobacter pylori infection in patients with nonulcer dyspepsia. Omeprazole plus Clarithromycin and Amoxicillin Effect One Year after Treatment (OCAY) Study Group. *N Engl J Med* 1998; **339**: 1875-1881 [PMID: 9862942 DOI: 10.1056/NEJM199812243392602]
33 **Cover TL**, Blaser MJ. Helicobacter pylori in health and disease. *Gastroenterology* 2009; **136**: 1863-1873 [PMID: 19457415 DOI: 10.1053/j.gastro.2009.01.073]
34 **Camilleri M**, Stanghellini V. Current management strategies and emerging treatments for functional dyspepsia. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 187-194 [PMID: 23381190 DOI: 10.1038/nrgastro.2013.11]
35 **Keohane J**, Quigley EM. Functional dyspepsia: the role of visceral hypersensitivity in its pathogenesis. *World J Gastroenterol* 2006; **12**: 2672-2676 [PMID: 16718751 DOI: 10.3748/wjg.v12.i17.2672]

36 **Brandt LJ**, Chey WD, Foxx-Orenstein AE, Schiller LR, Schoenfeld PS, Spiegel BM, Talley NJ, Quigley EM. An evidence-based position statement on the management of irritable bowel syndrome. *Am J Gastroenterol* 2009; **104** Suppl 1: S1-35 [PMID: 19521341 DOI: 10.1038/ajg.2008.122]

37 **Laine L**. Review article: gastrointestinal bleeding with low-dose aspirin - what's the risk? *Aliment Pharmacol Ther* 2006; **24**: 897-908 [PMID: 16948802 DOI: 10.1111/j.1365-2036.2006.03077.x]

38 **Sanchez-Mete L**, Zullo A, Hassan C, Rinaldi V, Magno MS, Festuccia F, Morini S, Attili AF. Helicobacter pylori diagnosis in patients with liver cirrhosis. *Dig Liver Dis* 2003; **35**: 566-570 [PMID: 14567461 DOI: 10.1016/S1590-8658(03)00029-X]

39 **Hong SJ**, Sung IK, Kim JG, Lee SW, Choi SC, Yang CH, Lee SI, Lee DH, Kim GH, Jeon SW, Park MI, Park SK, Park BJ, Seol SY. Failure of a Randomized, Double-Blind, Placebo-Controlled Study to Evaluate the Efficacy of H. pylori Eradication in H. pylori-Infected Patients with Functional Dyspepsia. *Gut Liver* 2011; **5**: 468-471 [PMID: 22195245 DOI: 10.5009/gnl.2011.5.4.468]

40 **Feinle-Bisset C**, Azpiroz F. Dietary and lifestyle factors in functional dyspepsia. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 150-157 [PMID: 23296252 DOI: 10.1038/nrgastro.2012.246]

41 **Zullo A**, Hassan C, Repici A, Bruzzese V. Helicobacter pylori eradication and reflux disease onset: did gastric acid get "crazy"? *World J Gastroenterol* 2013; **19**: 786-789 [PMID: 23429673 DOI: 10.3748/wjg.v19.i6.786]

42 **Noh YW**, Jung HK, Kim SE, Jung SA. Overlap of Erosive and Non-erosive Reflux Diseases With Functional Gastrointestinal Disorders According to Rome III Criteria. *J Neurogastroenterol Motil* 2010; **16**: 148-156 [PMID: 20535345 DOI: 10.5056/jnm.2010.16.2.148]

43 **Manes G**, Menchise A, de Nucci C, Balzano A. Empirical prescribing for dyspepsia: randomised controlled trial of test and treat versus omeprazole treatment. *BMJ* 2003; **326**: 1118 [PMID: 12763982 DOI: 10.1136/bmj.326.7399.1118]

44 **Harvey RF**, Lane JA, Nair P, Egger M, Harvey I, Donovan J, Murray L. Clinical trial: prolonged beneficial effect of Helicobacter pylori eradication on dyspepsia consultations - the Bristol Helicobacter Project. *Aliment Pharmacol Ther* 2010; **32**: 394-400 [PMID: 20491744 DOI: 10.1111/j.1365-2036.2010.04363.x]

45 **Maconi G**, Sainaghi M, Molteni M, Bosani M, Gallus S, Ricci G, Alvisi V, Porro GB. Predictors of long-term outcome of functional dyspepsia and duodenal ulcer after successful Helicobacter pylori eradication--a 7-year follow-up study. *Eur J Gastroenterol Hepatol* 2009; **21**: 387-393 [PMID: 19182682 DOI: 10.1097/MEG.0b013e3283069db0]

46 **Lassen AT**, Hallas J, Schaffalitzky de Muckadell OB. Helicobacter pylori test and eradicate versus prompt endoscopy for management of dyspeptic patients: 6.7 year follow up of a randomised trial. *Gut* 2004; **53**: 1758-1763 [PMID: 15542510 DOI: 10.1136/gut.2004.043570]

47 **Wong BC**, Lam SK, Wong WM, Chen JS, Zheng TT, Feng RE, Lai KC, Hu WH, Yuen ST, Leung SY, Fong DY, Ho J, Ching CK, Chen JS. Helicobacter pylori eradication to prevent gastric cancer in a high-risk region of China: a randomized controlled trial. *JAMA* 2004; **291**: 187-194 [PMID: 14722144]

P- Reviewers: Shimatani T, Tovey FI **S- Editor**: Gou SX
L- Editor: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8964

World J Gastroenterol 2014 July 21; 20(27): 8964-8970
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Laryngopharyngeal reflux and *Helicobacter pylori*

Taner Yılmaz, Münir Demir Bajin, Rıza Önder Günaydın, Serdar Özer, Tevfik Sözen

Taner Yılmaz, Münir Demir Bajin, Rıza Önder Günaydın, Serdar Özer, Tevfik Sözen, Department of Otolaryngology Head and Neck Surgery, Hacettepe University Hospital, 06100 Ankara, Turkey

Author contributions: Yılmaz T and Bajin MD designed the research; Günaydın RÖ and Özer S contributed new analytic tools; Sözen T analyzed the data; Yılmaz T and Bajin MD wrote the paper.

Correspondence to: Dr. Münir Demir Bajin, Department of Otolaryngology Head and Neck Surgery, Hacettepe University Hospital, Hacettepe Mh, 06100 Ankara, Turkey. dbajin@hacettepe.edu.tr
Telephone: +90-505-7723547 Fax: +90-312-3113500
Received: September 28, 2013 Revised: January 17, 2014
Accepted: April 1, 2014
Published online: July 21, 2014

Abstract

Laryngopharyngeal reflux (LPR) occurs when gastric contents pass the upper esophageal sphincter, causing symptoms such as hoarseness, sore throat, coughing, excess throat mucus, and globus. The pattern of reflux is different in LPR and gastroesophageal reflux. LPR usually occurs during the daytime in the upright position whereas gastroesophageal reflux disease more often occurs in the supine position at night-time or during sleep. Ambulatory 24-h double pH-probe monitoring is the gold standard diagnostic tool for LPR. Acid suppression with proton pump inhibitor on a long-term basis is the mainstay of treatment. *Helicobacter pylori* (*H. pylori*) is found in many sites including laryngeal mucosa and interarytenoid region. In this paper, we aim to present the relationship between LPR and *H. pylori* and review the current literature.

Key words: Laryngophrayngeal reflux; *Helicobacter pylori*; Gastroesophageal reflux disease; Proton pump inhibitors

Core tip: This paper reviews the literature regarding the relationship between laryngophrayngeal reflux (LPR) and *Helicobacter pylori*. The otolaryngology perspective of LPR and the importance of endoscopic examination are emphasized.

Yılmaz T, Bajin MD, Günaydın RÖ, Özer S, Sözen T. Laryngopharyngeal reflux and *Helicobacter pylori*. *World J Gastroenterol* 2014; 20(27): 8964-8970 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8964.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8964

INTRODUCTION

Gastroesophageal reflux (GER) can be a normal physiological phenomenon that occurs in most people, especially after meals. GER disease (GERD) develops when the reflux causes symptoms like heartburn and acid regurgitation. Laryngopharyngeal reflux (LPR) happens when gastric contents pass the upper esophageal sphincter, causing symptoms such as hoarseness, sore throat, coughing, excess throat mucus, and globus. The pattern of reflux is different in LPR and GER. LPR usually occurs during the daytime in the upright position, whereas GERD takes place more often in the supine position at night-time or during sleep[1]. Interestingly, the patients are different in terms of body type as well. There are reports suggesting a relationship between GERD and obesity[2,3]. In contrast, in a group of patients with laryngeal and pharyngeal symptoms, those with abnormal pharyngeal reflux events did not have a higher mean body mass index (BMI) than those with normal BMI[4]. A significantly higher percentage of esophageal reflux events was seen in obese versus non-obese participants. The authors concluded that abnormal esophageal reflux (GERD) is associated with increasing BMI and obesity, although this was not true for patients with pharyngeal

Table 1 Reflux Symptom Index

Within the past month, how did the following problems affect you?	0 = no problem					5 = severe problem
Hoarseness or a problem with your voice	0	1	2	3	4	5
Clearing your throat	0	1	2	3	4	5
Excess throat mucus or postnasal drip	0	1	2	3	4	5
Difficulty swallowing food, liquids, or pills	0	1	2	3	4	5
Coughing after you ate or after lying down	0	1	2	3	4	5
Breathing difficulties or choking episodes	0	1	2	3	4	5
Troublesome or annoying cough	0	1	2	3	4	5
Sensations of something sticking in your throat or a lump in your throat	0	1	2	3	4	5
Heartburn, chest pain, indigestion, or stomach acid coming up	0	1	2	3	4	5

A total score of 13 is thought to be clinically significant.

reflux.

Helicobacter pylori (*H. pylori*) was originally identified by Marshall and Warren[5]. It was called a *Campylobacter*-like organism at first. It is a Gram-negative bacterium with a spiral shape and four to six flagella. It is obligate microaerophilic, and urease, catalase and oxidase positive. Although it is susceptible to acid, it is protected from the harmful effects of acid by both its motility and its ability to convert urea to ammonium by urease and form a basic milieu around itself. Although it occurs less commonly in developed countries and in children, and is more common in developing countries and adults, its prevalence varies between different regions and socioeconomic strata of the same country. Probable routes of contamination are fecal-oral, oral-oral, gastro-oral (reflux and vomiting), and iatrogenic (*e.g.*, insufficiently disinfected endoscopes)[6]. The relationship of *H. pylori* with gastritis, peptic ulcer, gastric carcinoma, and mucosa-associated lymphoid tissue lymphoma has been demonstrated in many studies[7]. Different noninvasive [urea breath test (UBT), serological tests, and stool tests] and invasive [histological and microbiological examination of biopsy materials, rapid urease test, and polymerase chain reaction (PCR)] tests with varying specificity and sensitivity may be performed for the diagnosis for *H. pylori* in tissue[8]. *H. pylori* is localized primarily in the gastric mucosa. It is reported that the microorganism may exist in paranasal sinuses, tonsils, adenoids, and even middle ear mucosa[9-12]. It may also exist in atypical locations like dental plaque and saliva[13,14]. In many other studies *H. pylori* could not be found in tonsils, adenoids, dental plaque, saliva, or the oral cavity, which may mean that these tissues are only temporary colonization sites[15-18].

It has been estimated that half of otolaryngology patients with laryngeal and voice disorders have LPR[19]. LPR is considered one of the most important and common factors causing inflammation in the upper airways. The tissue damage is demonstrated in both animals and humans. It may be caused by direct exposure to acid, pepsin and bile, and by vagally mediated reflexes[20,21]. Besides acid and pepsin, the presence of *H. pylori* may be related to the symptoms and findings of LPR.

The variance between esophageal symptoms and upper aerodigestive tract disease may reflect the relative susceptibility of the epithelium of the larynx and pharynx to reflux-related injury. LPR may also occur in healthy individuals without symptoms or laryngeal pathology. LPR-related laryngeal disease and findings tend to resolve over a longer time and more often need higher levels of medication and therapy. LPR has an impact on various laryngeal pathologies including stenosis, malignancy, benign lesions, dysphagia, and functional disorders.

DIAGNOSIS

Laryngeal and voice disorders may present with diverse clinical manifestations. Many voice clinicians recommend that LPR be routinely assessed in patients with laryngeal and voice disorders; however, even among otolaryngologists who have a relatively high index of suspicion for LPR, it appears that this disorder is still often underdiagnosed and undertreated[22]. The symptoms, manifestations, patterns, and mechanisms of LPR and GERD are different. Patients with LPR usually deny symptoms of heartburn and/or regurgitation[21]. Less than half of otorhinolaryngology patients with LPR documented by pH monitoring complain of heartburn or regurgitation[23].

As with the majority of diseases, the diagnosis of LPR begins with history taking. It is then confirmed by laryngoscopy and subsequently validated by response to a trial of proton pump inhibitor (PPI) therapy. Although some institutions do perform routine pH testing, for the majority of cases this testing is reserved for refractory or complicated cases. The most common symptoms associated with LPR are cough, throat clearing, sore throat, globus, excess throat mucus, choking, and asthma. However, these entities have a multifactorial etiology and may be caused by recent sinusitis or other respiratory infections, smoking, voice abuse, and allergy, or these symptoms may be lacking. Therefore, accurate diagnosis based on history is a challenge. Belafsky *et al*[24] developed the Reflux Symptom Index (RSI), a self-administered nine-item questionnaire to help categorize the severity of LPR (Table 1). An RSI > 13 is considered abnormal. Symptoms of GERD, which include heartburn, chest pain, indigestion or acid regurgitation, are important, but it should be noted that more than half of the patients with LPR do not have these classic GERD symptoms[25]. Laryngeal

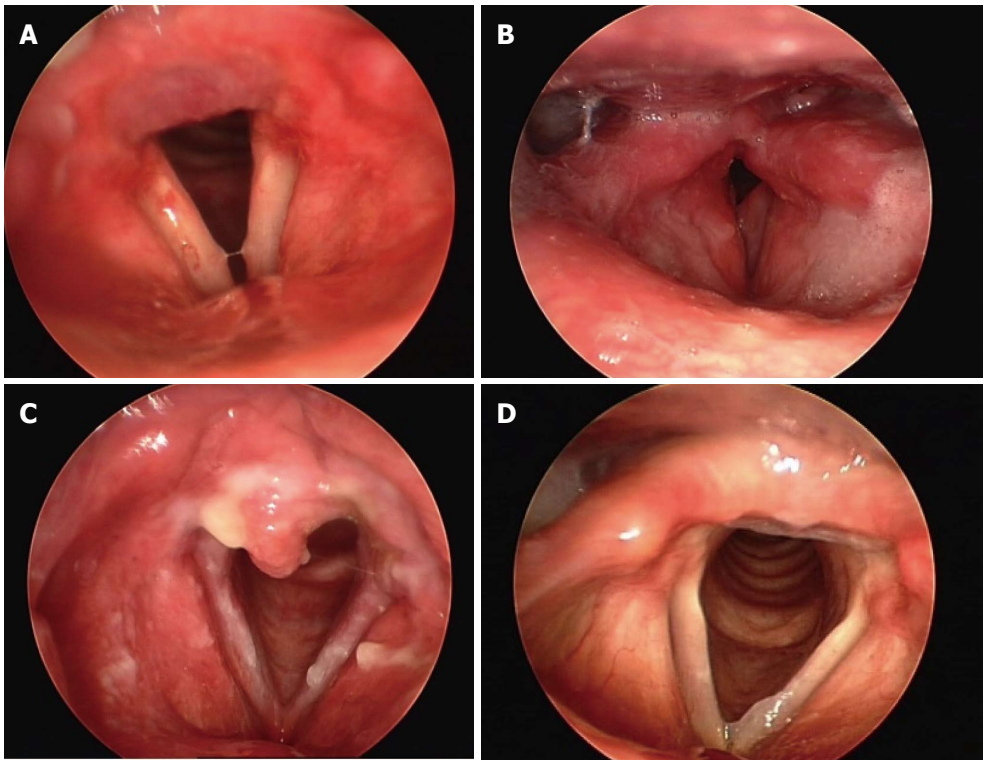


Figure 1 Diagnosis. A: Posterior laryngitis and erythema of the arytenoids; B: Laryngeal edema secondary to laryngopharyngeal reflux (LPR); C: Excessive posterior laryngitis. Note the thick mucus over the interarytenoid area and vocal cords; D: Normal larynx with only a mild posterior laryngitis treated by proton pump inhibitors with documented LPR by ambulatory double probe pH measurement.

Table 2 Reflux finding score

Subglottic edema	2 = present
0 = absent	
Ventricular obliteration	2 = partial
4 = complete	
Erythema/hyperemia	2 = arytenoids only
4 = diffuse	
Vocal cord edema	1 = mild
2 = moderate	
3 = severe	
4 = polypoid	
Diffuse laryngeal edema	1 = mild
2 = moderate	
3 = severe	
4 = obstructing	
Posterior commissure hypertrophy	1 = mild
2 = moderate	
3 = severe	
4 = obstructing	
Granuloma/granulation	0 = absent
2 = present	
Thick endolaryngeal mucus/other	0 = absent
2 = present	
Total	

A total score of 7 is thought to be clinically significant.

examination is the second step in the diagnostic evaluation. The findings of LPR on laryngeal examination vary considerably. According to Belafsky *et al*[25-27], laryngeal edema is the hallmark finding of LPR. However, most otolaryngologists rely solely on the findings of erythema or posterior laryngitis (PL) (Figure 1A). Unfortunately, those findings are not present in many LPR patients. In our experience, edema (Figure 1B) is the principal, and most common, finding of LPR along with PL. PL is characterized by edema or hypertrophy, and sometimes erythema and hyperemia on the posterior wall of the glottis. Inflammation may reach the medial surface of the arytenoid cartilages and aryepiglottic folds. Furthermore, diffuse vocal fold edema and infraglottic edema reaching from the anterior commissure to the posterior wall may create an illusion of sulcus vocalis[27]. The nature of endolaryngeal mucus if it is thick and tenacious also points to PL (Figure 1C). LPR patients may present with one or all of these findings[21]. The difficulty in making an LPR diagnosis is that the findings are sometimes quite subtle; signs of inflammation and irritation are absent, and patients may have a normal-looking larynx (Figure 1D). Therefore, a high index of suspicion is needed. Reflux finding score (RFS) may be useful in categorizing the severity of the mucosal injury on laryngoscopy[26] (Table 2). Laryngoscopy should be done by both flexible and rigid endoscopes. There are also controversial studies on this matter. Branski *et al*[28] have reported on a series of patients in whom reflux findings were scrutinized by five observers reviewing videotaped examinations. The conclusions of the paper were that significant variability exists in describing reflux-related findings. In another important study by Hicks *et al*[29], 100 normal subjects underwent laryngoscopy; their examinations were then reviewed by otolaryngologists and speech-language pathologists to estimate the presence of reflux-related lesions in these

healthy volunteers. The key finding in this study was that nearly 80% of the normal volunteers had an interarytenoid bar or posterior commissure hypertrophy. Unfortunately, this finding has been taken by many to be an indication that laryngoscopy is not useful in the clinical evaluation of reflux disease. However, it is important to realize that the physician's own perception during a laryngoscopy strongly affects the diagnosis. That is, mild laryngeal edema might be the sole finding of LPR.

Traditional diagnostic tests for GERD lack both sensitivity and specificity for LPR. As mentioned before, these two patient groups differ in terms of symptoms and diagnosis. Barium esophagography, radionuclide scanning, the Bernstein acid-perfusion test, and esophagoscopy with biopsy are all often negative in LPR patients[21]. This is probably because most LPR patients do not develop esophagitis, because esophageal mucosa is more resistant to acid- and pepsin-related injury than the laryngeal and pharyngeal mucosa is[21,23]. Therefore, evaluating a patient depending on GERD protocols may lead the otolaryngologist to misdiagnosis. At the present time, ambulatory 24-h double pH probe (simultaneous esophageal and pharyngeal) monitoring has become the diagnostic gold standard for LPR[30-32]. The upper probe must be placed in a consistent zone at or above (2 cm) the functional upper esophageal sphincter. This allows the lower probe to be placed about 5 cm above the lower esophageal sphincter. However, it is expensive and is not widely available. Nevertheless, pH monitoring effectively documents LPR with a high degree of specificity and sensitivity. Esophageal manometry is also important for accurate placement of the pH electrodes and particularly useful in patients with chronic cough.

Endoscopic examination of the esophagus (transnasal esophagoscopy; TNE) is performed in the clinical setting with or without sedation. It is generally used to check GERD-related complications and exclude other diseases. TNE allows otolaryngologists to screen the esophagus. In a large series, Postma *et al*[33] reported that 50% of the patients had positive findings on TNE, including 17% esophagitis, 8% hiatal hernia, 5% Barrett's metaplasia, 5% *Candida* esophagitis, and 4% stricture. Esophagoscopy alone does not diagnose LPR, and only a small percentage of LPR patients have abnormal esophagoscopy.

H. pylori infects the stomach, usually during childhood. The most common cause of peptic ulcers, *H. pylori* infection, is present in about half the world's population. There are several different methods to test for *H. pylori* infection.

Breath test (carbon isotope-urea breath test or UBT)

Up to 2 wk before the test, the patient must stop taking any antibiotics, bismuth-containing medications such as Pepto-Bismol, and PPIs. The patient swallows a special substance containing urea (a waste product the body produces as it breaks down protein) that has been made harmlessly radioactive. If *H. pylori* is present, the bacteria convert the urea into CO_2, which is detected and recorded in the exhaled breath after 10 min. This test can identify almost all people who have *H. pylori* and confirm that the infection has been fully treated.

Blood tests

Blood tests are used to measure *H. pylori* IgG, and *H. pylori* CagA IgG antibodies. This test is not as accurate as the other tests. These blood tests can be used to diagnose whether an *H. pylori* infection is present. However, the test cannot determine whether you have an infection at the time of the test or how long you have had it because the test remains positive for several years, even if the infection is cured. As a result, it cannot be used to see if the infection has been eradicated.

Stool test

A test to detect the genetic traces of *H. pylori* in the feces appears to be as accurate as the breath test for initially detecting the bacteria, and for detecting recurrences after antibiotic therapy. This test can also be used to diagnose *H. pylori* infection and confirm that it has been eradicated.

Biopsy

The most accurate way to identify the presence of *H. pylori* is by taking a tissue biopsy. *H. pylori* DNA was screened using a nested PCR amplification method for a portion of the 23S rRNA gene. Tissue samples can also be cultured on homogenized brain-heart infusion agar. Suspected colonies are tested and catalase-, oxidase- and urease-positive, curved Gram-negative rods are defined as *H. pylori*.

Another important diagnostic tool is an empiric trial of PPI therapy over a prolonged period, which has been proposed as a valid diagnostic test for LPR. The typical regime is twice daily PPI therapy for 1-6 mo. This recommendation is based on the fact that we have not identified the specific symptom combination, or combination of symptoms and laryngeal signs, pathognomonic to LPR. Besides, ambulatory 24-h double-probe pH measurement is not available in all clinics. The principal disadvantage of PPI therapy is its high cost, patient unwillingness, and placebo effect. Nevertheless it is a useful diagnostic tool in many cases.

TREATMENT

Treatment for LPR includes lifestyle modifications, acid-suppressive medication, and surgical therapy. Lifestyle modifications include elevation of the head of the bed, decreased intake of fat, citrus, tomato, chocolate, caffeine, and alcohol, cessation of smoking, and avoiding recumbency and further eating 3 h before bedtime. These measures are helpful if there is associated abnormal esophageal acid exposure[1]. If only LPR is present, these measures may be less meaningful because pharyngeal reflux occurs most often in the upright position during the daytime. Although Hanson *et al*[34] have described a 50% response rate to these measures alone in patients with chronic laryngitis, there are minimal supportive data on the efficacy of these measures in LPR. Medical acid

suppression is the most important and common method of treatment. The treatment of LPR has dramatically changed since the introduction of PPIs, which are the most widely used drugs for the treatment of reflux. They maintain a potent and consistent effect on gastric acid secretion with few adverse effects. Comparisons between the five available compounds (omeprazole, rabeprazole, lansoprazole, esomeprazole, and pantoprazole) shows that they have a similar antisecretory potency on a milligram basis. Treatment recommendation at present is twice daily dosing of PPIs for at least 3-4 mo. Most authors suggest a longer duration of at least 6 mo up to 1 year[22,25]. Symptoms frequently improve before the laryngoscopic findings resolve[25]. Although PPIs effectively reduce the acid secretion, reflux still continues, meaning that the larynx and pharynx are still exposed to pepsin and bile.

Surgical therapy or antireflux surgery has been shown to be effective for patients with aggressive or life-threatening LPR[35]. The main procedure for antireflux surgery is Nissen fundoplication. The fundus of the stomach is wrapped around the lower esophageal sphincter to provide an antireflux barrier. Patients who have good control of GER and LPR symptoms with acid suppression may not need surgical intervention. However, in patients who do not respond to medical therapy, the symptoms can be attributed to pepsin and bile reflux. This patient group are considered to the best candidates for Nissen fundoplication[36]. However, it is not a widely accepted treatment choice.

No single drug cures *H. pylori* infection. Treatment involves taking several medications for 14 d. The recommended first-line therapy is PPI-clarithromycin-amoxicillin or metronidazole. The consensus is that 14 rather than 7 d treatment has a slight advantage in terms of treatment success. With regard to second-line therapies, bismuth-based quadruple therapies remain the best option. If unavailable, PPI-amoxicillin or tetracycline and metronidazole are recommended. There are increasing numbers of patients with *H. pylori* infection that is resistant to antibiotics, so it is important to take all the medications prescribed and to have a test that confirms that the infection has been cleared. Antimicrobial susceptibility testing is required in the resistant cases or treatment failures[37].

DISCUSSION

Regarding the relationship between LPR and *H. pylori*, the literature is limited. Rouev *et al*[38] compared 46 patients with GERD and LPR symptoms and found that there was an increasing tendency in GERD patients that develop LPR symptoms. They found 11 patients with *H. pylori* infection but the treatment did not affect the overall outcome. In one of the first studies investigating the relationship between *H. pylori* positivity and LPR, Oridate *et al*[39] compared *H. pylori* antibody positivity, laryngopharyngeal reflux symptoms, objective laryngopharyngeal findings, and rate of response to acid-suppressive therapy in 42 patients who were diagnosed with GERD. They found that the laryngopharyngeal, but not esophageal, symptom relief induced by acid suppression was significantly lower among *H. pylori* antibody-negative than antibody-positive cases. This was a surprising finding. Kountouras *et al*[40] have suggested that the increasing incidence of GERD complications after *H. pylori* eradication may be explained not just by the diminishing prevalence of *H. pylori* infection, but rather by healing of *H. pylori*-associated peptic ulcer disease, which coexists with GERD. The appearance of GERD depends on the esophageal acid exposure, and its symptomatology is related to acid hypersecretion; a condition that predisposes to peptic ulcer disease. Given that the vast majority of peptic ulcer cases are caused by *H. pylori* infection, the bacterium could therefore also promote GERD development by inducing esophageal acidity, but this does not necessarily promote LPR. Cekin *et al*[41] found no association between *H. pylori* and LPR status. In addition, they analyzed two subgroups based on whether their lesions were benign or malignant/premalignant and found a significant relationship between LPR positivity and the presence of malignant/premalignant laryngeal lesions. Again, they found no association between *H. pylori* status and either of the two subgroup categories.

LPR in the pediatric population is believed to contribute to failure to thrive, laryngomalacia, recurrent respiratory papillomatosis, chronic cough, hoarseness, esophagitis, and aspiration, among other pathologies. Thus, LPR should be considered as a chronic disease with a variety of presentations. High clinical suspicion along with consultation with an otolaryngologist, who can evaluate for laryngeal findings, is necessary to diagnose LPR accurately[42]. The majority of infected persons acquire the bacteria during early childhood and one of the risk factors may be immunological. These factors are possibly the cause of divergent manifestations of *H. pylori* infection in children compared with adults.

Tezer *et al*[43] have concluded that the expression of *H. pylori* positivity and degree of GERD correlated with LPR in 45 patients. *H. pylori* positivity and degree of GERD were more adverse in patients with an RFS of $\geqslant 7$. However, their findings relied only on RFS; ambulatory 24-h double pH probe monitoring was not used. Toros *et al*[44] have investigated 45 patients. Although the percentage of *H. pylori* positivity was high, there was no significant relationship between the symptoms and *H. pylori* positivity. All patients underwent medical therapy mostly for gastroenterological indications rather than laryngopharyngeal symptoms. In a recent article by Youssef and Ahmed[45], *H. pylori* treatment and LPR symptom resolution was investigated. *H. pylori* stool antigen (HPSA) test was positive in 57% of the study group. Patients with negative HPSA were treated with esomeprazole as a single modality with a reported improvement score of 96.6%. Patients with positive HPSA test results were divided into two groups: one received only esomeprazole, with reported improvement in 40%, whereas the second group was treated with esomeprazole, plus amoxicillin sodium and

clarithromycin (triple therapy) and reported a 90% incidence of symptom improvement. The incidence of *H. pylori* infection in patients with LPR was 57%. They concluded that *H. pylori* infection should be considered when treatment is prescribed to patients with LPR because the standard therapy for GERD might be insufficient. Also, the use of triple therapy for LPR with *H. pylori* infection might result in a higher cure rate. However, in a study by Ercan *et al*[46], 32 LPR patients were investigated regarding the presence of *H. pylori* and sex, age, degree of gastritis and esophagitis, and also the number of reflux episodes, fractional acid exposure times regarding proximal probe readings. They found that there was no relationship between the presence of *H. pylori* and LPR. Islam *et al*[47] took biopsies from the vocal fold and interarytenoid region of 50 patients. *H. pylori* was not found in the histological specimens of the vocal fold and interarytenoid region. The presence of *H. pylori* in the gastric mucosa, determined by UBT and *H. pylori* antibodies, does not have an effect on the RFS and RSI. In their prospective study, Siupsinskiene *et al*[48] found *H. pylori* in the biopsy material from the larynx in more than one-third of the patients, equally suffering from benign laryngeal disease and laryngeal cancer, but significantly more often than in the control group. Patients with chronic laryngitis and laryngeal cancer showed the highest rate of *H. pylori* infection in the larynx. However, the relationship was not clearly identified and they concluded that further studies are needed to confirm the importance of *H. pylori* infection for the development of different laryngeal diseases, as well as the effect of *H. pylori* eradication on the course of laryngeal diseases.

CONCLUSION

Detailed history taking and laryngoscopic examination constitute the basis for diagnosis of LPR. Most LPR patients have only mild symptoms. Unlike GERD patients, they seldom have heartburn or regurgitation. Laryngoscopic examination most commonly demonstrates findings in the posterior glottis and vocal folds. Laryngeal edema is an important indicator for LPR that is most often neglected. Ambulatory 24-h double pH-probe monitoring is the gold standard diagnostic tool for LPR. Acid suppression with PPI on a long-term basis is the mainstay of treatment; a trial of PPIs may also be useful as a diagnostic maneuver but it should be at least 4 mo. Laryngeal acid and pepsin sensitivity is greater in oropharyngeal mucosa than esophageal mucosa and this constitutes the main difference of LPR and GERD pathophysiology. *H. pylori* is found in many sites, including laryngeal mucosa and interarytenoid region; however, the importance of this colonization and its effects on disease progress and treatment outcome is yet to be identified with prospective clinical studies.

REFERENCES

1 **Ylitalo R**. Reflux and Its Impact on Laryngology. Textbook of Laryngology. In: Merati AL, Bielamowicz SA, editors. Hong Kong: Plural Publishing, 2006: 203-303

2 **Smith KJ**, O'Brien SM, Smithers BM, Gotley DC, Webb PM, Green AC, Whiteman DC. Interactions among smoking, obesity, and symptoms of acid reflux in Barrett's esophagus. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 2481-2486 [PMID: 16284367 DOI: 10.1158/1055-9965.EPI-05-0370]

3 **Stein DJ**, El-Serag HB, Kuczynski J, Kramer JR, Sampliner RE. The association of body mass index with Barrett's oesophagus. *Aliment Pharmacol Ther* 2005; **22**: 1005-1010 [PMID: 16268976 DOI: 10.1111/j.1365-2036.2005.02674.x]

4 **Halum SL**, Postma GN, Johnston C, Belafsky PC, Koufman JA. Patients with isolated laryngopharyngeal reflux are not obese. *Laryngoscope* 2005; **115**: 1042-1045 [PMID: 15933517 DOI: 10.1097/01.MLG.0000162656.05715.57]

5 **Marshall BJ**, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **1**: 1311-1315 [PMID: 6145023 DOI: 10.1016/S0140-6736(84)91816-6]

6 **Mitchell H**, Mégraud F. Epidemiology and diagnosis of Helicobacter pylori infection. *Helicobacter* 2002; **7** Suppl 1: 8-16 [PMID: 12197904 DOI: 10.1046/j.1523-5378.7.s1.10.x]

7 **Eslick GD**. Helicobacter pylori infection causes gastric cancer? A review of the epidemiological, meta-analytic, and experimental evidence. *World J Gastroenterol* 2006; **12**: 2991-2999 [PMID: 16718777]

8 **Mégraud F**, Lehours P. Helicobacter pylori detection and antimicrobial susceptibility testing. *Clin Microbiol Rev* 2007; **20**: 280-322 [PMID: 17428887 DOI: 10.1128/CMR.00033-06]

9 **Morinaka S**, Ichimiya M, Nakamura H. Detection of Helicobacter pylori in nasal and maxillary sinus specimens from patients with chronic sinusitis. *Laryngoscope* 2003; **113**: 1557-1563 [PMID: 12972933 DOI: 10.1097/00005537-200309000-00027]

10 **Cirak MY**, Ozdek A, Yilmaz D, Bayiz U, Samim E, Turet S. Detection of Helicobacter pylori and its CagA gene in tonsil and adenoid tissues by PCR. *Arch Otolaryngol Head Neck Surg* 2003; **129**: 1225-1229 [PMID: 14623755 DOI: 10.1001/archotol.129.11.1225]

11 **Yilmaz T**, Ceylan M, Akyön Y, Ozçakýr O, Gürsel B. Helicobacter pylori: a possible association with otitis media with effusion. *Otolaryngol Head Neck Surg* 2006; **134**: 772-777 [PMID: 16647533 DOI: 10.1016/j.otohns.2006.02.002]

12 **Ozmen S**, Yücel OT, Sinici I, Ozmen OA, Süslü AE, Oğretmenoğlu O, Onerci M. Nasal pepsin assay and pH monitoring in chronic rhinosinusitis. *Laryngoscope* 2008; **118**: 890-894 [PMID: 18520187 DOI: 10.1097/MLG.0b013e318165e324]

13 **Cammarota G**, Tursi A, Montalto M, Papa A, Veneto G, Bernardi S, Boari A, Colizzi V, Fedeli G, Gasbarrini G. Role of dental plaque in the transmission of Helicobacter pylori infection. *J Clin Gastroenterol* 1996; **22**: 174-177 [PMID: 8724252 DOI: 10.1097/00004836-199604000-00004]

14 **Li C**, Musich PR, Ha T, Ferguson DA, Patel NR, Chi DS, Thomas E. High prevalence of Helicobacter pylori in saliva demonstrated by a novel PCR assay. *J Clin Pathol* 1995; **48**: 662-666 [PMID: 7560176 DOI: 10.1136/jcp.48.7.662]

15 **Bernander S**, Dalén J, Gästrin B, Hedenborg L, Lamke LO, Ohrn R. Absence of Helicobacter pylori in dental plaques in Helicobacter pylori positive dyspeptic patients. *Eur J Clin Microbiol Infect Dis* 1993; **12**: 282-285 [PMID: 8513816 DOI: 10.1007/BF01967259]

16 **Di Bonaventura G**, Catamo G, Neri M, Neri G, Piccolomini R. Absence of Helicobacter pylori in tonsillar swabs from dyspeptic patients. *New Microbiol* 2000; **23**: 445-448 [PMID: 11061634]

17 **Skinner LJ**, Winter DC, Curran AJ, Barnes C, Kennedy S, Maguire AJ, Charles DA, Timon CI, Burns HP. Helicobacter pylori and tonsillectomy. *Clin Otolaryngol Allied Sci* 2001; **26**: 505-509 [PMID: 11843933 DOI: 10.1046/j.1365-2273.2001.00513.x]

18 **Bitar MA**, Soweid A, Mahfouz R, Zaatari G, Fuleihan N. Is Helicobacter pylori really present in the adenoids of children? *Eur Arch Otorhinolaryngol* 2005; **262**: 987-992 [PMID:

15924276 DOI: 10.1007/s00405-005-0926-1]
19 **Koufman JA**, Amin MR, Panetti M. Prevalence of reflux in 113 consecutive patients with laryngeal and voice disorders. *Otolaryngol Head Neck Surg* 2000; **123**: 385-388 [PMID: 11020172 DOI: 10.1067/mhn.2000.109935]
20 **Shaker R**, Dodds WJ, Ren J, Hogan WJ, Arndorfer RC. Esophagoglottal closure reflex: a mechanism of airway protection. *Gastroenterology* 1992; **102**: 857-861 [PMID: 1537523]
21 **Koufman JA**. The otolaryngologic manifestations of gastroesophageal reflux disease (GERD): a clinical investigation of 225 patients using ambulatory 24-hour pH monitoring and an experimental investigation of the role of acid and pepsin in the development of laryngeal injury. *Laryngoscope* 1991; **101**: 1-78 [PMID: 1895864]
22 **Koufman JA**, Aviv JE, Casiano RR, Shaw GY. Laryngopharyngeal reflux: position statement of the committee on speech, voice, and swallowing disorders of the American Academy of Otolaryngology-Head and Neck Surgery. *Otolaryngol Head Neck Surg* 2002; **127**: 32-35 [PMID: 12161727 DOI: 10.1067/mhn.2002.125760]
23 **Koufman JA**, Belafsky PC, Bach KK, Daniel E, Postma GN. Prevalence of esophagitis in patients with pH-documented laryngopharyngeal reflux. *Laryngoscope* 2002; **112**: 1606-1609 [PMID: 12352672 DOI: 10.1097/00005537-200209000-00014]
24 **Belafsky PC**, Postma GN, Koufman JA. Validity and reliability of the reflux symptom index (RSI). *J Voice* 2002; **16**: 274-277 [PMID: 12150380 DOI: 10.1016/S0892-1997(02)00097-8]
25 **Belafsky PC**, Postma GN, Koufman JA. Laryngopharyngeal reflux symptoms improve before changes in physical findings. *Laryngoscope* 2001; **111**: 979-981 [PMID: 11404607 DOI: 10.1097/00005537-200106000-00009]
26 **Belafsky PC**, Postma GN, Koufman JA. The validity and reliability of the reflux finding score (RFS). *Laryngoscope* 2001; **111**: 1313-1317 [PMID: 11568561 DOI: 10.1097/00005537-200108000-00001]
27 **Belafsky PC**, Postma GN, Koufman JA. The association between laryngeal pseudosulcus and laryngopharyngeal reflux. *Otolaryngol Head Neck Surg* 2002; **126**: 649-652 [PMID: 12087332 DOI: 10.1067/mhn.2002.125603]
28 **Branski RC**, Bhattacharyya N, Shapiro J. The reliability of the assessment of endoscopic laryngeal findings associated with laryngopharyngeal reflux disease. *Laryngoscope* 2002; **112**: 1019-1024 [PMID: 12160267 DOI: 10.1097/00005537-200206000-00016]
29 **Hicks DM**, Ours TM, Abelson TI, Vaezi MF, Richter JE. The prevalence of hypopharynx findings associated with gastroesophageal reflux in normal volunteers. *J Voice* 2002; **16**: 564-579 [PMID: 12512644 DOI: 10.1016/S0892-1997(02)00132-7]
30 **Grøntved AM**, West F. pH monitoring in patients with benign voice disorders. *Acta Otolaryngol Suppl* 2000; **543**: 229-231 [PMID: 10909027 DOI: 10.1080/000164800454468]
31 **Postma GN**. Ambulatory pH monitoring methodology. *Ann Otol Rhinol Laryngol Suppl* 2000; **184**: 10-14 [PMID: 11051424]
32 **Wiener GJ**, Koufman JA, Wu WC, Cooper JB, Richter JE, Castell DO. Chronic hoarseness secondary to gastroesophageal reflux disease: documentation with 24-h ambulatory pH monitoring. *Am J Gastroenterol* 1989; **84**: 1503-1508 [PMID: 2596451]
33 **Postma GN**, Cohen JT, Belafsky PC, Halum SL, Gupta SK, Bach KK, Koufman JA. Transnasal esophagoscopy: revisited (over 700 consecutive cases). *Laryngoscope* 2005; **115**: 321-323 [PMID: 15689759 DOI: 10.1097/01.mlg.0000154741.25443.fe]
34 **Hanson DG**, Kamel PL, Kahrilas PJ. Outcomes of antireflux therapy for the treatment of chronic laryngitis. *Ann Otol Rhinol Laryngol* 1995; **104**: 550-555 [PMID: 7598368]
35 **Westcott CJ**, Hopkins MB, Bach K, Postma GN, Belafsky PC, Koufman JA. Fundoplication for laryngopharyngeal reflux disease. *J Am Coll Surg* 2004; **199**: 23-30 [PMID: 15217625 DOI: 10.1016/j.jamcollsurg.2004.03.022]
36 **Lindstrom DR**, Wallace J, Loehrl TA, Merati AL, Toohill RJ. Nissen fundoplication surgery for extraesophageal manifestations of gastroesophageal reflux (EER). *Laryngoscope* 2002; **112**: 1762-1765 [PMID: 12368611 DOI: 10.1097/00005537-200210000-00010]
37 **Selgrad M**, Malfertheiner P. Treatment of Helicobacter pylori. *Curr Opin Gastroenterol* 2011; **27**: 565-570 [PMID: 21946029 DOI: 10.1097/MOG.0b013e32834bb818]
38 **Rouev P**, Chakarski I, Doskov D, Dimov G, Staykova E. Laryngopharyngeal symptoms and gastroesophageal reflux disease. *J Voice* 2005; **19**: 476-480 [PMID: 15936924 DOI: 10.1016/j.jvoice.2004.07.008]
39 **Oridate N**, Takeda H, Yamamoto J, Asaka M, Mesuda Y, Nishizawa N, Mori M, Furuta Y, Fukuda S. Helicobacter pylori seropositivity predicts outcomes of acid suppression therapy for laryngopharyngeal reflux symptoms. *Laryngoscope* 2006; **116**: 547-553 [PMID: 16585857 DOI: 10.1097/01.MLG.0000201907.24514.6A]
40 **Kountouras J**, Zavos C, Chatzopoulos D. The role of gastric Helicobacter pylori infection in laryngopharyngeal reflux disease. *Otolaryngol Head Neck Surg* 2007; **136**: 334; author reply 334-335 [PMID: 17275574 DOI: 10.1016/j.otohns.2006.10.027]
41 **Cekin E**, Ozyurt M, Erkul E, Ergunay K, Cincik H, Kapucu B, Gungor A. The association between Helicobacter pylori and laryngopharyngeal reflux in laryngeal pathologies. *Ear Nose Throat J* 2012; **91**: E6-E9 [PMID: 22430349]
42 **Venkatesan NN**, Pine HS, Underbrink M. Laryngopharyngeal reflux disease in children. *Pediatr Clin North Am* 2013; **60**: 865-878 [PMID: 23905824 DOI: 10.1016/j.pcl.2013.04.011]
43 **Tezer MS**, Kockar MC, Koçkar O, Celik A. Laryngopharyngeal reflux finding scores correlate with gastroesophageal reflux disease and Helicobacter pylori expression. *Acta Otolaryngol* 2006; **126**: 958-961 [PMID: 16864494 DOI: 10.1080/00016480500529314]
44 **Toros SZ**, Toros AB, Yüksel OD, Ozel L, Akkaynak C, Naiboglu B. Association of laryngopharyngeal manifestations and gastroesophageal reflux. *Eur Arch Otorhinolaryngol* 2009; **266**: 403-409 [PMID: 18648836 DOI: 10.1007/s00405-008-0761-2]
45 **Youssef TF**, Ahmed MR. Treatment of clinically diagnosed laryngopharyngeal reflux disease. *Arch Otolaryngol Head Neck Surg* 2010; **136**: 1089-1092 [PMID: 20855671 DOI: 10.1001/archoto.2010.165]
46 **Ercan I**, Cakir BO, Uzel TS, Sakiz D, Karaca C, Turgut S. The role of gastric Helicobacter pylori infection in laryngopharyngeal reflux disease. *Otolaryngol Head Neck Surg* 2006; **135**: 52-55 [PMID: 16815182 DOI: 10.1016/j.otohns.2006.03.020]
47 **Islam A**, Oguz H, Yucel M, Koca G, Gonultas MA, Arslan N, Demirci M. Does Helicobacter pylori exist in vocal fold pathologies and in the interarytenoid region? *Dysphagia* 2013; **28**: 382-387 [PMID: 23315289 DOI: 10.1007/s00455-012-9444-7]
48 **Siupsinskiene N**, Jurgutaviciute V, Katutiene I, Janciauskas D, Vaitkus S, Adamonis K. Helicobacter pylori infection in laryngeal diseases. *Eur Arch Otorhinolaryngol* 2013; **270**: 2283-2288 [PMID: 23572292 DOI: 10.1007/s00405-013-2475-3]

P- Reviewers: Murakami K, Naito Y **S- Editor**: Gou SX
L- Editor: Kerr C **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8971

World J Gastroenterol 2014 July 21; 20(27): 8971-8978
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Natural products and food components with anti-*Helicobacter pylori* activities

Hiroaki Takeuchi, Vu Thu Trang, Norihito Morimoto, Yoshie Nishida, Yoshihisa Matsumura, Tetsuro Sugiura

Hiroaki Takeuchi, Norihito Morimoto, Yoshie Nishida, Yoshihisa Matsumura, Tetsuro Sugiura, Department of Clinical Laboratory Medicine, Kochi Medical School, Kochi 783-8505, Japan
Vu Thu Trang, School of Biotechnology and Food Technology, Hanoi University of Science and Technology, Hanoi 12771, Vietnam
Author contributions: Takeuchi H designed and performed research, and wrote the paper; Trang VT performed research and contributed analytic tools; Morimoto N and Nishida Y contributed analytic tools; Matsumura Y and Sugiura T analyzed data.
Supported by JSPS KAKENHI Grant, No. 24590697 and No. 24590698; and the Vietnam National Foundation for Science and Technology Development (NAFOSTED, 106.99-2011.22)
Correspondence to: Hiroaki Takeuchi, PhD, Department of Clinical Laboratory Medicine, Kochi Medical School, Kohasu, Oko-cho, Nankoku-city, Kochi 783-8505, Japan. htake@kochi-u.ac.jp
Telephone: +81-88-8802427 Fax: +81-88-8802428
Received: September 27, 2013 Revised: January 20, 2014
Accepted: April 1, 2014
Published online: July 21, 2014

Abstract

The bacterial pathogen *Helicobacter pylori* (*H. pylori*) colonizes in over half of the world's population. *H. pylori* that establishes life-long infection in the stomach is definitely associated with gastro-duodenal diseases and a wide variety of non-gastrointestinal tract conditions such as immune thrombocytopenia. Triple therapy which consists of a proton pump inhibitor and combinations of two antibiotics (amoxicillin, clarithromycin or amoxicillin, metronidazol) is commonly used for *H. pylori* eradication. Recently, the occurrence of drug-resistant *H. pylori* and the adverse effect of antibiotics have severely weakened eradication therapy. Generally antibiotics induce the disturbance of human gastrointestinal microflora. Furthermore, there are inappropriate cases of triple therapy such as allergy to antibiotics, severe complications (liver and/or kidney dysfunction), the aged and people who reject the triple therapy. These prompt us to seek alterative agents instead of antibiotics and to develop more effective and safe therapy with these agents. The combination of these agents actually may result in lower a dose of antibiotics. There are many reports world-wide that non-antibiotic substances from natural products potentially have an anti-*H. pylori* agent. We briefly review the constituents derived from nature that fight against *H. pylori* in the literature with our studies.

Key words: Anti-*Helicobacter pylori* effect; Natural product; Food component; *In vitro* and *in vivo* effects; Human health; *Helicobacter pylori* treatment; Combined effect

Core tip: The present review summarized the natural products and food components with anti-*Helicobacter pylori* (*H. pylori*) activities in the literatures and showed the possibility for its application on human health. There are many promising *in vitro* effects on *H. pylori* and other infections (infectious diseases). Next, further *in vivo* evidence is required. There are many guidelines for *H. pylori* treatment which are not always the same among countries. Thus, we should address the evaluation of *in vivo* effects using such components in clinical investigation to make an adequate guideline useful for all countries for the application on *H. pylori* treatment.

Takeuchi H, Trang VT, Morimoto N, Nishida Y, Matsumura Y, Sugiura T. Natural products and food components with anti-*Helicobacter pylori* activities. *World J Gastroenterol* 2014; 20(27): 8971-8978 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8971.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8971

INTRODUCTION

The bacterial pathogen *Helicobacter pylori* (*H. pylori*) colonizes in over half of the world's population[1]. *H. pylori* that establishes life-long infection in the stomach is definitely associated with gastro-duodenal diseases and a wide variety of non-gastrointestinal tract conditions such as immune thrombocytopenia[2,3]. Foods and the components possessing anti-*H. pylori* activity are summarized in Table 1. Anti-*H. pylori* effects and combined effects with agents in clinical trial are summarized in Table 2.

LACTOFERRIN

Lactoferrin is a multifunctional iron-binding glycoprotein found in milk (human and bovine), neutrophils, saliva and lacrimal fluid. The inhibitory activity of bovine lactoferrin (bLF) against *H. pylori* is known *in vitro* and animal experiments using BALB/c mouse[4]. Clinical trials were performed to evaluate whether oral administration of bLF suppressed *H. pylori* colonized in the stomach with bLF alone or with a combination of bLF and antibiotics[5-7]. The clinical study with a combination of bLF and antibiotics in 150 consecutive *H. pylori*-positive patients showed a 100% eradication rate[5], which was significantly higher than those without prescription. Similarly, Di Mario *et al*[6] indicated that the eradication rate of a combination of triple therapy and bLF was 93%, significantly higher than the other two groups; triple therapy without bLF or administrating before triple therapy. On the other hand, a randomized, double-blind, placebo-controlled study with 59 *H. pylori*-positive patients indicated that administration of bLF alone effectively suppressed the colonization of *H. pylori* in the stomach[7]. Anti-*H. pylori* activity of human lactoferrin was reported *in vitro*[8], but not in clinical trials[9,10]. These results showed that bLF could be a new effective agent against *H. pylori* and could enhance the eradication rate when combined with antibiotics. The possible mechanism of bLF is that the cationic lactoferrin binds to the anionic cell wall materials and allows a greater penetration of the antibiotics.

GREEN TEA (CATECHIN COMPOUNDS)

Among the catechin compounds, epigallocatechin-3-gallate (EGCg) showed the lowest MIC against *H. pylori*. The anti-*H. pylori* activity of EGCg obviously exhibited itself even in the antibiotic-resistant [amoxicillin (AMPC), metronidazole (MNZ) and clarithromycin (CAM)] isolates and showed additive effects in regard to antibiotics[11]. In Mongolian gerbils, the eradication rate of EGCg was 36.4% due probably to the inhibition of *H. pylori* urease activity[12]. However, green tea catechins (GTCs) failed to show any clear-cut activities against *H. pylori* *in vivo*. The most likely reason for the *in vivo* inefficacy was the short gastric transit time of GTCs. Solutions of GTCs adsorbed to sucralfate (GTC-scf) were used in animal experiments to prolong the gastric transit time of GTCs. As a result, colony forming unit of *H. pylori* in the stomach significantly decreased using GTC-scf compared to solutions of GTCs[13]. The administration of green tea polyphenol in a drinking water dose-dependently suppressed *H. pylori* infection in Mongolian gerbils[14]. One of the postulated mechanisms of suppression by green tea polyphenols against *H. pylori* infection was the inhibition of urease activity *via* disturbance of cell membrane, leading to the prevention or even eradication of *H. pylori* infection[14]. Another proposed mechanism, the blockage of toll-like receptor 4 activation by EGCg was reported[15]. Anti-*H. pylori* activity of epicatechin gallate was second next to EGCg, and hence pyrogallol and gallate substituent groups of catechin compounds are an important element of antimicrobial activity.

POLYPHENOL COMPOUNDS

Ginger (*Zingiber officinale*) belonging to the family Zingiberaceae is cultivated world-wide. Dietary plant phenolic compounds have been shown to exert varieties of biological actions including anti-*H. pylori* activity. The effective compounds possessing anti-*H. pylori* activity were identified as 6-gingerol, 8-gingerol, 10-gingerol, 6-shogaol and phenolic acids and their derivatives. The aqueous and ethanol extracts of ginger inhibited the growth of antibiotic-resistant *H. pylori in vitro*[16]. In addition, the combined use of ginger extract and CAM strengthened growth inhibition of *H. pylori* with synergic and additive effects *in vitro*[17]. The methanol extract of ginger containing 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol also effectively inhibited the growth of CagA-positive *H. pylori*[18]. Siddaraju *et al*[19] reported that ginger-free phenolic (GRFP) and ginger hydrolysed phenolic (GRHP) fractions of ginger inhibited *H. pylori* growth *in vitro*. GRHP with higher content of cinnamic and coumaric acid showed better inhibition than GRFP, indicating that phenolic acids have anti-*H. pylori* activity. Similar effectiveness was reported with phenolic fractions of *Curcuma amada*, known as mango ginger[20]. Mango ginger free phenolics including caffeic, gentisic and ferulic acids, and mango ginger bound phenolics including ferulic, cinnamic and p-coumaric acids inhibited *H. pylori* growth *in vitro*. Turmeric (*Curcuma longa*) possesses curcumin, the major polyphenolic constituent. Both the methanol extract of the dried powdered turmeric rhizome and curcumin inhibited the growth of all *H. pylori* strains examined *in vitro*[21].

Propolis, a resinous hive product collected by honeybees, is composed of resins (flavonoids and a various kinds of polyphenols), wax, essential oils and organic compounds. Propolis exhibits antimicrobial activity with inhibitions of bacterial motility and enzyme activity most likely due to the damage of cytoplasmic membrane[22]. Anti-*H. pylori* activities of Brazilian propolis and Bulgarian propolis were found by *in vitro* studies[23,24]. The labdan-type diterpenes and some of the prenylated phenolic compounds in Brazilian propolis were putative antimicrobial constituents derived from propolis[23]. Furthermore, the combined use of propolis extract and CAM increased

Table 1 Foods and products posessing anti-*Helicobacter pylori* potential

Food	Putative active component	Stage of experiment	Ref.
Bovine milk	Lactoferrin	*In vitro*, *in vivo* (animal) *in vivo* (human)	[4-10]
Green tea	Catechin compounds	*In vitro*, *in vivo* (animal)	[11-15]
Ginger (*Zingiber officinale*)	6-gingerol, 8-gingerol, 10-gingerol, 6-shogaol, phenolic acids (cinnamic, caffeic, ferulic, syringic, p-coumaric, protocatechuic, gentisic, gallic)	*In vitro*	[16-19]
Curcuma amada	Phenolic acids (cinnamic, caffeic, ferulic, syringic, p-coumaric, protocatechuic, gentisic, gallic)	*In vitro*	[20]
Turmeric (*Curcuma longa*)	Curcumin	*In vitro*	[21]
Propolis	Phenolic compounds	*In vitro*	[17,22-24]
Acacia nilotica	Unknown (phenolics, alkaloids, terpenes, flavonoids, tannins)	*In vitro*	[25,26,28]
Calotropis procera	Unknown	*In vitro*	[27,28]
Muscadine grape skin	Polyphenols (quercetin, resveratrol)	*In vitro*, *in vivo* (animal)	[29,30]
Apple peel	Quercetin glycosides	*In vitro*	[31,32]
Virgin oil	Phenolics	*In vitro*, *in vivo* (human)	[33,34]
Cranberry (Vaccinium macrocarpon) Cranbery juice	Polyphenol compound	*In vitro*, *in vivo* (human)	[35-37]
Plants	Tannins (tellimagrandin- Ⅰ, - Ⅱ)	*In vitro*	[38]
Broccoli sprout (*Brassica oleracea*)	Sulforaphane	*In vitro*, *in vivo* (animal), *in vivo* (human)	[39,40]
Paeonia lactiflora	Paeonol, benzoic acid, unknown	*In vitro*	[41-43]
Decalepis hamiltonii	2-hydroxy-4-methoxy benzaldehyde (HMBA) Unknown	*In vitro*	[44,45]
(Maillard reaction products)	Melanoidin	*In vitro*, *in vivo* (animal), *in vivo* (human)	[46]
(Maillard reaction products)	Aminoreductone	*In vitro*	[47]
Milk (Maillard reaction products)	Casein polymer (FP-10),	*In vitro*, *in vivo* (animal); *in vivo* (human)	[48,49]
Okinawamozuku (*Cladosiphon okamuranus*)	Fucoidan	*In vitro*, *in vivo* (animal)	[50,51]
Garlic (*Allium sativum*)	Allicin, diallyl sulfur components	*In vitro*	[52-55]
Chinese chive (*Allium tuberosum*)	Unknown	*In vitro*	[56]
Deep seawater	Unknown	*In vitro*, *in vivo* (animal); *in vivo* (human)	[63]
Essential oils	Unknown (geranial in lemongrass)	*In vitro*, *in vivo* (animal)	[64-66]

the anti-*H. pylori* activity with synergic and additive effects *in vitro*[17].

The plant *Acacia nilotica* (*A. nilotica*) contains phenolics, alkaloids, terpenes, flavonoids and tannins[25] as secondary metabolites, which exhibits beneficial function for human health[26]. *Calotropis procera* (*C. procera*), a wild-growing plant, has multifarious medicinal and biological properties[27]. Amin *et al*[28] demonstrated that methanol and acetone extracts of *A. nilotica* and *C. procera* exhibited stronger anti-*H. pylori* activity than MNZ, but not AMPC and CAM. The anti-*H. pylori* activity was due to the suppression of *H. pylori* urease activity.

Muscadine grapes (*Vitis rotundifolia*), common in the south-eastern United States, have unique anthocyanin profiles and high flavonoid concentrations. Brown *et al*[29] previously reported that muscadine grapes exhibited anti-*H. pylori* potential *in vitro* through their major phenolic compounds acting alone or in synergy. Anti-*H. pylori* effects of quercetin and resveratrol, active polyphenols identified in muscadine grape skin (MGS) extracts, were confirmed *in vitro* experiment irrespective of the pH condition[30]. In the case of *in vivo* tests on mice, MGS and quercetin did not significantly reduce *H. pylori* growth but regulated the inflammatory response to *H. pylori* infection[29]. The concentration of polyphenols in apple peel could be up to three times higher than that found in the pulp. Apple peel polyphenols derived from a standardized apple peel extract (APPE, 60% of total polyphenols; 58% of flavonoids; 30% of flavan-3-ols and procyanidins) was investigated for anti-*H. pylori* activity on a few strains *in vitro*[31,32]. APPE (mainly quercetin glycosides) showed growth inhibition of *H. pylori via* suppression of urease activity and inhibited the respiratory burst of neutrophils induced by *H. pylori* leading to the protection of gastric mucosa.

Virgin olive oil, one of the few edible vegetable oils that are consumed unrefined, contains a significant amount of phenolic compounds. Extracts of virgin olive oil and a very low concentration of the pure dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol (TyEDA) effectively killed the *H. pylori in vitro*[33]. A successful eradication with administration of virgin olive oil was confirmed in two clinical trials consisting of 60 *H. pylori*-infected adults (30 subjects per trial). These data revealed[34] a moderate effectiveness of virgin oil in eradication of *H. pylori*. Further studies are necessary to confirm these findings including administration conditions, types of olive oils and combination with common antibiotics.

Native Americans have conveniently used cranberry

Table 2 Anti-*Helicobacter pylori* effects and combination effects in clinical studies

Food	Putative anti-*H. pylori* effect	Effect comminbed with agents in clinical trial			Ref.
		Agents	Eradication rate	Study design	
Bovine milk	Penetration of the antibiotics to *H. pylori*	bLF + triple therapy	100%	Open, randomized, single-center	[5]
	(damage of cell membrane)	(rabeprazole, CAM, tinidazole)	93%	Open, randomized, multi-center	[6]
Green tea	Inhibition of urease activity *via* disrupted cell membrane				
Ginger (*Zingiber officinale*)	Blockage of Toll-like receptor 4 (TLR4) activation				
Curcuma amada	-				
Turmeric (*Curcuma longa*)	-				
Propolis	Damage of cytoplasmic membrane				
Acacia nilotica	Suppression of urease activity				
Calotropis procera	Suppression of urease activity				
Muscadine grape skin	-				
Apple peel	Inhibition of urease activity				
Virgin oil	-				
Cranberry (Vaccinium macrocarpon)	Inhibition of *H. pylori* adhesion to gastric mucosa	Cranbery juice + *Lactobacillus* (La1)	22.90%	Multicentric, randomized, controlled, double-blind	[37]
Cranbery juice					
Plants	Damage of lipid bilayer membrane				
Broccoli sprout (*Brassica oleracea*)	-				
Paeonia lactiflora	Inhibition of urease activity				
Decalepis hamiltonii	Bacterial lysis (cell death) (interference of DNA/protein involved in DNA protection and bioavailability)				
(Maillard reaction products)	Inhibition of *H. pylori* urease binding to gastric mucin				
(Maillard reaction products)	-				
Milk (Maillard reaction products)	Blockage of interaction between *H. pylori* and gastric mucin				
Okinawamozuku (*Cladosiphon okamuranus*)	Inhibition of *H. pylori* binding to gastric cell				
Garlic (*Allium sativum*)	-				
Chinese chive (*Allium tuberosum*)	Interference of the cell division process				
Deep seawater	-				
Essential oils	-				

H. pylori: *Helicobacter pylori*; CAM: Clarithromycin.

(*Vaccinium macrocarpon*) originated in North America for infectious diseases. Burger *et al*[35] reported that certain high molecular constituents of cranberry juice inhibited *H. pylori* adhesion to human gastric mucus *in vitro*. Direct *in vitro* study using cranberry, polyphenol-rich fruit, documented that the extracts effectively suppressed *H. pylori* proliferation compared to other polyphenol-poor fruits (oranges, pineapples, apples, and white grapes). The polyphenol-rich fraction obtained by ion-exchange column chromatography showed a higher growth inhibition of *H. pylori* than that of the sugar/organic acid-rich fraction. Thus, the effective antimicrobial component in cranberry is thought to be polyphenol compounds[36]. Interestingly, a clinical trial with a combination of cranberry juice and probiotic *Lactobacillus johnsonii* La1 (La1) in 271 *H. pylori*-infected children assigned into 4 groups was performed[37]. The eradication rates of 4 groups were 1.5% (placebo juice/heat-killed La1), 14.9% (placebo juice/La1), 16.9% (cranberry juice/heat-killed La1) and 22.9% (cranberry juice/La1), respectively ($P < 0.01$). The highest rate was found in the group who had been administrated cranberry juice/La1 but showed no statistical significance between placebo juice/La1 and cranberry juice/heat-killed La1 groups. These suggested that regular intake of cranberry juice or La1 may be useful in the management of asymptomatic children colonized by *H. pylori*. However, no synergistic inhibitory effects on *H. pylori* colonization were observed when both foodstuffs were simultaneously consumed.

Tannins are naturally occurring plant polyphenols and well known to be present in various materials such as fruits, tea, chocolate, coffee, legume forages, legumes, trees and grasses, *etc*. *In vitro* study with 36 polyphenols and 4 terpenoids from medicinal plants, monomeric hydrolyzable tannins such as tellimagrandin I and II revealed especially strong bactericidal activity with the damage of lipid bilayer membranes[38].

SULFORAPHANE

The sulforaphane, abundant in broccoli (*Brassica oleracea*) sprout in the form of its glucosinolate precursor, exhib-

ited bactericidal activity against *H. pylori* including antibiotic-resistant strains *in vitro* assay[39]. *In vivo* study (animal and human) with administration of glucoraphanin (precursor of sulforaphane)-rich broccoli sprouts was reported[40]. The bacterial colonization of *H. pylori*-infected C57BL/6 female mice treated with broccoli sprout was significantly reduced and the broccoli sprout attenuated gastric inflammation (gastritis) in *H. pylori*-infected mice. Furthermore, in a clinical trial with 48 *H. pylori*-positive patients, 70 g/d of glucoraphanin-rich broccoli sprouts was consumed for 8 wk. As a result, the levels of clinical laboratory examinations (urea breath test and *H. pylori* antigen in the stool) were significantly lower after consumption for 8 wk but reverted to the baseline at 8 wk after the end of the trial. They suggested that the dual actions of sulforaphane were the anti-*H. pylori* activity and the blocking gastric tumor formation due to induction of antioxidant enzymes[40].

PAEONIA LACTIFLORA PALLAS

Paeonia lactiflora (*P. lactiflora*) Pallas (Paeoniaceae) is composed of monoterpene glycosides (albiflorin, benzoylpaeoniflorin, oxypaeoniflorin, and paeoniflorin), monoterpenes (lactoflorin, paeoniflorigenone, and paeonilactones), benzoic acid and its esters, and gallotannins[41]. *P. lactiflora* root was also shown to inhibit the growth of any bacteria[42] except of *H. pylori*. Ngan *et al*[43] reported that paeonol and benzoic acid identified in *P. lactiflora* root possessed a strong *in vitro* bactericidal effect even in the antibiotic-resistant *H. pylori* strains. 1,2,3,4,6-penta-*O*-galloyl-β-*D*-glucopyranose showed a relatively higher inhibition of *H. pylori* urease activity compared to acetohydroxamic acid, suggesting that *P. lactiflora* root globally affected growth inhibition of *H. pylori*.

DECALEPIS HAMILTONII

Pectic polysaccharide from *Decalepis hamiltonii* (*D. hamiltonii*) (Swallow root) containing a sulfonamide group and phenolics was investigated *in vitro* assay. Carbohydrate and pectic polysaccharide of swallow root at a 200 μg/mL concentration exhibited anti-*H. pylori* activity as equivalent to that of AMPC (10 g/mL). Anti-*H. pylori* activity resulted from bacterial lysis observed by the scanning electron microscopy analysis[44]. Later, 2-hydroxy-4-methoxy benzaldehyde (HMBA), identified from the roots of *D. hamiltonii* by the hydrodistillation and cold crystallization method, was shown to inhibit the growth of *H. pylori in vitro*. Increased binding ability of HMBA to DNA and protein involved in DNA protection and bioavailability, leads to cell death of *H. pylori*[45] .

MAILLARD REACTION PRODUCTS

The maillard reaction between amino and carbonyl groups in the food is ubiquitously caused by a thermal process. Melanoidin, the final product of the Maillard reaction, is a high-molecular-weight compound. The *in vivo* effects of melanoidin, prepared by the Maillard reaction between casein and lactose, on *H. pylori* colonized in the stomach of euthymic hairless mice and humans were investigated. Melanoidin I inhibited the binding of urease to gastric mucin and suppressed *H. pylori* colonization in mice as well as in human subjects[46]. These results are critically interesting because melanoidin are common ingredients in a variety of heat-treated foods. Furthermore, the anti-*H. pylori* activity of other Maillard reaction products, aminoreductone (AR), was discovered *in vitro* assay[47]. AR effectively exhibited growth inhibition with bactericidal effects on all 24 *H. pylori* strains including antibiotic-resistant strains. The killing activity of AR was significantly higher than that of its derived melanoidin and was observed even in acidic condition (pH = 3). These results indicated that foods containing AR, such as milk or dairy products are valuable sources for preventing colonization of *H. pylori* in the stomach and its associated tissue damages. Casein polymer (FP-10), made from the casein of milk with maillard reaction, blocked the interaction between *H. pylori* and gastric mucin in the stomach. Therefore, the intake of FP-10 decreased the density of *H. pylori* colonized in the human stomach without serious side effects[48,49].

FUCOIDAN

Similar to melanoidin, polysaccharides are also well known as a high-molecular- weight compound. Among the polysaccharides, fucoidan, one of the sulfated polysaccharides, extracted from Okinawamozuku (*Cladosiphon okamuranus*) was reported to effectively inhibit the binding of *H. pylori* to gastric cell *in vitro*[50]. *In vivo* experiments with Mongolian gerbils, fucoidan reduced the prevalence of *H. pylori*-infected animals and also the onset of *H. pylori*-induced gastritis in a dose-dependent manner[51].

GARLIC (*ALLIUM SATIVUM*) AND CHINESE CHIVE (*ALLIUM TUBEROSUM*)

Garlic, like all allium vegetables, contains a wide range of thiosulphinates such as allicin (allyl 2-propene thiosulfinate) which is thought to be responsible for the antibacterial activity. The allicin in garlic was also reported to show anti-*H. pylori* activity and synergic effect with omeprazole, PPI, *in vitro*[52]. On the other hand, a clinical trial with fresh garlic (10 sliced cloves) or capsaicin-containing peppers (six sliced fresh jalapeños) demonstrated that neither garlic nor capsaicin had any *in vivo* effects on *H. pylori*[53]. Later, *in vitro* effectiveness of the anti-*H. pylori* activity of pure garlic oil and garlic powder and their diallyl sulfur components in a variety of garlic substances were described[54]. Interestingly, the anti-*H. pylori* activity of garlic oil was noticeably affected by food materials and mucin by *in vitro* assay. These data suggested that under suitable fasting or fed conditions in the stomach, admin-

istration of garlic oil might be effective for prevention and treatment of *H. pylori* infections[55]. Furthermore, Chinese chive (*Allium tuberosum*)[56], one of the *Allium* vegetables, definitely inhibited the growth of *H. pylori* strains including antibiotic-resistant isolates *in vitro*. The inhibitory activity of water extracts in Chinese chive was stable under severe stress conditions such as heat and low pH. The water extract did not disturb the antibiotics' activity by combination assay with antibiotics frequently used in clinical practice.

DEEP SEAWATER

Deep seawater is collected at Muroto promontory in Japan. Refined deep seawater (RDSW) produced from deep seawater, a mineral-rich healthy drinking water for humans, is widely consumed. Beyond satisfying the general need for water to support life, RDSW has additional merits for the human body such as hemorheology, allergy and immunology as previously described[57-63]. It should be noted that all types of RDSW have no side effects in long-time heavy consumers or adverse effects in persons with medical problems. Our *in vitro* and *in vivo* studies including animals (Mongolian gerbils) and clinical trial with *H. pylori*-positive patients indicated that RDSW actually exhibited anti-*H. pylori* activity *in vitro* and intake of RDSW significantly decreased the level of urea breath test value in *H. pylori*-infected patients[63]. In addition, amelioration of the intestinal flora condition was observed in RDSW-drinking group. These implicate that the application of RDSW promotes human health and provides eurhythmic body.

ESSENTIAL OILS

Essential oils, which are extracted from plants (*e.g.*, leaves, peels), showed the growth inhibition of *H. pylori in vitro*[64-66] and *in vivo* study with mice[66]. Among 13 essential oils used *in vitro* study, lemongrass oil was utilized *in vivo* study because of the highest MIC *in vitro* experiment. The density of *H. pylori* colonized in the stomach of mice treated with lemongrass oil was significantly reduced compared with untreated mice[66].

CONCLUSION

The great benefits obtained from nature such as milk, plant, vegetable, fruits, water, *etc* are adequate to ameliorate human health. Many foodstuffs have exhibited inhibitory activity against the growth of *H. pylori in vitro* and *in vivo* as reviewed. Furthermore, probiotics and vitamins also possess anti-*H. pylori* potentials and may be readily considered as effective alternative and adjuvant therapy for *H. pylori* treatment. Basically, natural products consumed daily are safe and beneficial for humans. If effective components identified *in vitro* actually show less anti-*H. pylori* activities *in vivo*, intake of these foodstuffs have no serious problem for human health. However, it is better that the effectiveness (merit and demerit) is confirmed *in vivo* experiments, particularly in the clinical trials, at the point of translational medicine. There are many guidelines for *H. pylori* treatment which are not always the same among all countries. We need to evaluate *in vivo* effects using such components in clinical investigation to make an adequate guideline useful for all countries for the application on *H. pylori* treatment. Furthermore, caution must be used when attempting to extrapolate data from *in vitro* studies to the *in vivo* condition. Much effort has been focused on plant preparations and their constituents as potential antibacterial products for prevention or eradication of *H. pylori* and other bacteria. We hope that natural products and food components may be useful for the prevention and/or treatment of *H. pylori* infection as well as in other disorders. Therefore, novel, diet-based therapeutics for use when conventional antibiotic therapies have failed and/or are unavailable, have received considerable attention.

ACKNOWLEDGMENTS

We appreciate that Mr. Ricky Barrow, English teacher, kindly checked the manuscript.

REFERENCES

1 **Dunn BE**, Cohen H, Blaser MJ. Helicobacter pylori. *Clin Microbiol Rev* 1997; **10**: 720-741 [PMID: 9336670]
2 **Gasbarrini A**, Franceschi F. Autoimmune diseases and Helicobacter pylori infection. *Biomed Pharmacother* 1999; **53**: 223-226 [PMID: 10424243 DOI: 10.1016/S0753-3322(99)80092-4]
3 **Morimoto N**, Takeuchi H, Takahashi T, Ueta T, Tanizawa Y, Kumon Y, Kobayashi M, Sugiura T. Helicobacter pylori-associated chronic idiopathic thrombocytopenic purpura and low molecular weight H. pylori proteins. *Scand J Infect Dis* 2007; **39**: 409-416 [PMID: 17464863 DOI: 10.1080/00365540601105723]
4 **Wada T**, Aiba Y, Shimizu K, Takagi A, Miwa T, Koga Y. The therapeutic effect of bovine lactoferrin in the host infected with Helicobacter pylori. *Scand J Gastroenterol* 1999; **34**: 238-243 [PMID: 10232866 DOI: 10.1080/00365529950173627]
5 **Di Mario F**, Aragona G, Bò ND, Ingegnoli A, Cavestro GM, Moussa AM, Iori V, Leandro G, Pilotto A, Franzè A. Use of lactoferrin for Helicobacter pylori eradication. Preliminary results. *J Clin Gastroenterol* 2003; **36**: 396-398 [PMID: 12702979 DOI: 10.1097/00004836-200305000-00006]
6 **Di Mario F**, Aragona G, Dal Bó N, Cavallaro L, Marcon V, Olivieri P, Benedetti E, Orzès N, Marin R, Tafner G, Chilovi F, De Bastiani R, Fedrizzi F, Franceschi M, Salvat MH, Monica F, Piazzi L, Valiante F, Vecchiati U, Cavestro GM, Comparato G, Iori V, Maino M, Leandro G, Pilotto A, Rugge M, Franzè A. Bovine lactoferrin for Helicobacter pylori eradication: an open, randomized, multicentre study. *Aliment Pharmacol Ther* 2006; **23**: 1235-1240 [PMID: 16611285 DOI: 10.1111/j.1365-2036.2006.02851.x]
7 **Okuda M**, Nakazawa T, Yamauchi K, Miyashiro E, Koizumi R, Booka M, Teraguchi S, Tamura Y, Yoshikawa N, Adachi Y, Imoto I. Bovine lactoferrin is effective to suppress Helicobacter pylori colonization in the human stomach: a randomized, double-blind, placebo-controlled study. *J Infect Chemother* 2005; **11**: 265-269 [PMID: 16369731 DOI: 10.1007/s10156-005-0407-x]
8 **Yamazaki N**, Yamauchi K, Kawase K, Hayasawa H, Nakao K, Imoto I. Antibacterial effects of lactoferrin and its N-terminal

peptide against Helicobacter pylori on vitro. *J Infect Chemother* 1997; **3**: 85-89 [DOI: 10.1007/BF02490180]
9 **Opekun AR**, El-Zaimaity HM, Osato MS, Gilger MA, Malaty HM, Terry M, Headon DR, Graham DY. Novel therapies for Helicobacter pylori infection. *Aliment Pharmacol Ther* 1999; **13**: 35-42 [PMID: 9892877 DOI: 10.1046/j.1365-2036.1999.00435.x]
10 **Guttner Y**, Windsor HM, Viiala CH, Marshall BJ. Human recombinant lactoferrin is ineffective in the treatment of human Helicobacter pylori infection. *Aliment Pharmacol Ther* 2003; **17**: 125-129 [PMID: 12492741 DOI: 10.1046/j.1365-2036.2003.01395.x]
11 **Yanagawa Y**, Yamamoto Y, Hara Y, Shimamura T. A combination effect of epigallocatechin gallate, a major compound of green tea catechins, with antibiotics on Helicobacter pylori growth in vitro. *Curr Microbiol* 2003; **47**: 244-249 [PMID: 14570277 DOI: 10.1007/s00284-002-3956-6]
12 **Mabe K**, Yamada M, Oguni I, Takahashi T. In vitro and in vivo activities of tea catechins against Helicobacter pylori. *Antimicrob Agents Chemother* 1999; **43**: 1788-1791 [PMID: 10390246]
13 **Takabayashi F**, Harada N, Yamada M, Murohisa B, Oguni I. Inhibitory effect of green tea catechins in combination with sucralfate on Helicobacter pylori infection in Mongolian gerbils. *J Gastroenterol* 2004; **39**: 61-63 [PMID: 14767736 DOI: 10.1007/s00535-003-1246-0]
14 **Matsubara S**, Shibata H, Ishikawa F, Yokokura T, Takahashi M, Sugimura T, Wakabayashi K. Suppression of Helicobacter pylori-induced gastritis by green tea extract in Mongolian gerbils. *Biochem Biophys Res Commun* 2003; **310**: 715-719 [PMID: 14550260 DOI: 10.1016/j.bbrc.2003.09.066]
15 **Lee KM**, Yeo M, Choue JS, Jin JH, Park SJ, Cheong JY, Lee KJ, Kim JH, Hahm KB. Protective mechanism of epigallocatechin-3-gallate against Helicobacter pylori-induced gastric epithelial cytotoxicity via the blockage of TLR-4 signaling. *Helicobacter* 2004; **9**: 632-642 [PMID: 15610077 DOI: 10.1111/j.1083-4389.2004.00281.x]
16 **Nostro A**, Cellini L, Di Bartolomeo S, Di Campli E, Grande R, Cannatelli MA, Marzio L, Alonzo V. Antibacterial effect of plant extracts against Helicobacter pylori. *Phytother Res* 2005; **19**: 198-202 [PMID: 15934015 DOI: 10.1002/ptr.1640]
17 **Nostro A**, Cellini L, Di Bartolomeo S, Cannatelli MA, Di Campli E, Procopio F, Grande R, Marzio L, Alonzo V. Effects of combining extracts (from propolis or Zingiber officinale) with clarithromycin on Helicobacter pylori. *Phytother Res* 2006; **20**: 187-190 [PMID: 16521108 DOI: 10.1002/ptr.1830]
18 **Mahady GB**, Pendland SL, Yun GS, Lu ZZ, Stoia A. Ginger (Zingiber officinale Roscoe) and the gingerols inhibit the growth of Cag A+ strains of Helicobacter pylori. *Anticancer Res* 2003; **23**: 3699-3702 [PMID: 14666666]
19 **Siddaraju MN**, Dharmesh SM. Inhibition of gastric H+, K+-ATPase and Helicobacter pylori growth by phenolic antioxidants of Zingiber officinale. *Mol Nutr Food Res* 2007; **51**: 324-332 [PMID: 17295419 DOI: 10.1002/mnfr.200600202]
20 **Siddaraju MN**, Dharmesh SM. Inhibition of gastric H(+),K(+)-ATPase and Helicobacter pylori growth by phenolic antioxidants of Curcuma amada. *J Agric Food Chem* 2007; **55**: 7377-7386 [PMID: 17725316 DOI: 10.1021/jf070719r]
21 **Mahady GB**, Pendland SL, Yun G, Lu ZZ. Turmeric (Curcuma longa) and curcumin inhibit the growth of Helicobacter pylori, a group 1 carcinogen. *Anticancer Res* 2002; **22**: 4179-4181 [PMID: 12553052]
22 **Mirzoeva OK**, Grishanin RN, Calder PC. Antimicrobial action of propolis and some of its components: the effects on growth, membrane potential and motility of bacteria. *Microbiol Res* 1997; **152**: 239-246 [PMID: 9352659 DOI: 10.1016/S0944-5013(97)80034-1]
23 **Banskota AH**, Tezuka Y, Adnyana IK, Ishii E, Midorikawa K, Matsushige K, Kadota S. Hepatoprotective and anti-Helicobacter pylori activities of constituents from Brazilian propolis. *Phytomedicine* 2001; **8**: 16-23 [PMID: 11292234 DOI: 10.1078/0944-7113-00004]
24 **Boyanova L**, Gergova G, Nikolov R, Derejian S, Lazarova E, Katsarov N, Mitov I, Krastev Z. Activity of Bulgarian propolis against 94 Helicobacter pylori strains in vitro by agar-well diffusion, agar dilution and disc diffusion methods. *J Med Microbiol* 2005; **54**: 481-483 [PMID: 15824428 DOI: 10.1099/jmm.0.45880-0]
25 **Sultana B**, Anwar F, Przybylski R. Antioxidant activity of phenolic components present in barks of Azadirachta indica, Terminalia arjuna, Acacia nilotica, and Eugenia jambolana Lam. trees. *Food Chem* 2007; **104**: 1106–1114 [DOI: 10.1016/j.foodchem.2007.01.019]
26 **Mahesh B**, Satesh S. Antimicrobial activity of some medicinal plants against plant and human pathogen. *World J Agric Sci* 2008; **4**: 839-843
27 **Yesmin MN**, Din SN, Mubassara S, Akond MA. Antioxidant and Antibacterial Activities of Calotropis procera Linn. *Am Eurasian J Agric Environ Sci* 2008; **4**: 550-553
28 **Amin M**, Anwar F, Naz F, Mehmood T, Saari N. Anti-Helicobacter pylori and urease inhibition activities of some traditional medicinal plants. *Molecules* 2013; **18**: 2135-2149 [PMID: 23434867 DOI: 10.3390/molecules18022135]
29 **Brown JC**, Wang J, Kasman L, Jiang X, Haley-Zitlin V. Activities of muscadine grape skin and quercetin against Helicobacter pylori infection in mice. *J Appl Microbiol* 2011; **110**: 139-146 [PMID: 20955190 DOI: 10.1111/j.1365-2672.2010.04870.x]
30 **Brown JC**, Jiang X. Activities of muscadine grape skin and polyphenolic constituents against Helicobacter pylori. *J Appl Microbiol* 2013; **114**: 982-991 [PMID: 23294280 DOI: 10.1111/jam.12129]
31 **Pastene E**, Troncoso M, Figueroa G, Alarcón J, Speisky H. Association between polymerization degree of apple peel polyphenols and inhibition of Helicobacter pylori urease. *J Agric Food Chem* 2009; **57**: 416-424 [PMID: 19128009 DOI: 10.1021/jf8025698]
32 **Pastene E**, Speisky H, Troncoso M, Alarcón J, Figueroa G. In vitro inhibitory effect of apple peel extract on the growth of Helicobacter pylori and respiratory burst induced on human neutrophils. *J Agric Food Chem* 2009; **57**: 7743-7749 [PMID: 19691323 DOI: 10.1021/jf9006592]
33 **Romero C**, Medina E, Vargas J, Brenes M, De Castro A. In vitro activity of olive oil polyphenols against Helicobacter pylori. *J Agric Food Chem* 2007; **55**: 680-686 [PMID: 17263460 DOI: 10.1021/jf0630217]
34 **Castro M**, Romero C, de Castro A, Vargas J, Medina E, Millán R, Brenes M. Assessment of Helicobacter pylori eradication by virgin olive oil. *Helicobacter* 2012; **17**: 305-311 [PMID: 22759331 DOI: 10.1111/j.1523-5378.2012.00949.x]
35 **Burger O**, Ofek I, Tabak M, Weiss EI, Sharon N, Neeman I. A high molecular mass constituent of cranberry juice inhibits helicobacter pylori adhesion to human gastric mucus. *FEMS Immunol Med Microbiol* 2000; **29**: 295-301 [PMID: 11118911 DOI: 10.1111/j.1574-695X.2000.tb01537.x]
36 **Matsushima M**, Suzuki T, Masui A, Kasai K, Kouchi T, Takagi A, Shirai T, Mine T. Growth inhibitory action of cranberry on Helicobacter pylori. *J Gastroenterol Hepatol* 2008; **23** Suppl 2: S175-S180 [PMID: 19120894 DOI: 10.1111/j.1440-1746.2008.05409.x]
37 **Gotteland M**, Andrews M, Toledo M, Muñoz L, Caceres P, Anziani A, Wittig E, Speisky H, Salazar G. Modulation of Helicobacter pylori colonization with cranberry juice and Lactobacillus johnsonii La1 in children. *Nutrition* 2008; **24**: 421-426 [PMID: 18343637 DOI: 10.1016/j.nut.2008.01.007]
38 **Funatogawa K**, Hayashi S, Shimomura H, Yoshida T, Hatano T, Ito H, Hirai Y. Antibacterial activity of hydrolyzable tannins derived from medicinal plants against Helicobacter pylori. *Microbiol Immunol* 2004; **48**: 251-261 [PMID: 15107535 DOI: 10.1111/j.1348-0421.2004.tb03521.x]
39 **Fahey JW**, Haristoy X, Dolan PM, Kensler TW, Scholtus

I, Stephenson KK, Talalay P, Lozniewski A. Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo[a]pyrene-induced stomach tumors. *Proc Natl Acad Sci USA* 2002; **99**: 7610-7615 [PMID: 12032331 DOI: 10.1073/pnas.112203099]

40 **Yanaka A**, Fahey JW, Fukumoto A, Nakayama M, Inoue S, Zhang S, Tauchi M, Suzuki H, Hyodo I, Yamamoto M. Dietary sulforaphane-rich broccoli sprouts reduce colonization and attenuate gastritis in Helicobacter pylori-infected mice and humans. *Cancer Prev Res* (Phila) 2009; **2**: 353-360 [PMID: 19349290 DOI: 10.1158/1940-6207.CAPR-08-0192]

41 **He CN**, Peng Y, Zhang YC, Xu LJ, Gu J, Xiao PG. Phytochemical and biological studies of paeoniaceae. *Chem Biodivers* 2010; **7**: 805-838 [PMID: 20397219 DOI: 10.1002/cbdv.200800341]

42 **Ngan LT**, Moon JK, Kim JH, Shibamoto T, Ahn YJ. Growth-inhibiting effects of Paeonia lactiflora root steam distillate constituents and structurally related compounds on human intestinal bacteria. *World J Microbiol Biotechnol* 2012; **28**: 1575-1583 [PMID: 22805939 DOI: 10.1007/s11274-011-0961-6]

43 **Ngan LT**, Moon JK, Shibamoto T, Ahn YJ. Growth-inhibiting, bactericidal, and urease inhibitory effects of Paeonia lactiflora root constituents and related compounds on antibiotic-susceptible and -resistant strains of Helicobacter pylori. *J Agric Food Chem* 2012; **60**: 9062-9073 [PMID: 22891951 DOI: 10.1021/jf3035034]

44 **Srikanta B**, Siddaraju M, Dharmesh S. A novel phenol-bound pectic polysaccharide from Decalepis hamiltonii with multi-step ulcer preventive activity. *World J Gastroenterol* 2007; **13**: 5196-5207 [PMID: 17876890]

45 **Srikanta BM**, Nayaka MA, Dharmesh SM. Inhibition of Helicobacter pylori growth and its cytotoxicity by 2-hydroxy 4-methoxy benzaldehyde of Decalepis hamiltonii (Wight & amp; Arn); a new functional attribute. *Biochimie* 2011; **93**: 678-688 [PMID: 21185348 DOI: 10.1016/j.biochi.2010.12.009]

46 **Hiramoto S**, Itoh K, Shizuuchi S, Kawachi Y, Morishita Y, Nagase M, Suzuki Y, Nobuta Y, Sudou Y, Nakamura O, Kagaya I, Goshima H, Kodama Y, Icatro FC, Koizumi W, Saigenji K, Miura S, Sugiyama T, Kimura N. Melanoidin, a food protein-derived advanced maillard reaction product, suppresses Helicobacter pylori in vitro and in vivo. *Helicobacter* 2004; **9**: 429-435 [PMID: 15361082 DOI: 10.1111/j.1083-4389.2004.00263.x]

47 **Trang VT**, Takeuchi H, Kudo H, Aoki A, Katsuno S, Shimamura T, Sugiura T, Ukeda H. Antimicrobial activity of aminoreductone against Helicobacter pylori. *J Agric Food Chem* 2009; **57**: 11343-11348 [PMID: 19891477 DOI: 10.1021/jf9026876]

48 **Inoue T**, Koji S, Beppu H, Kawai K, Shimpo K, Sonoda S, Nakano H. Clinical effects of FP-10 against H. pylori infection. *J Anal Bio-Sci* 2005; **28**: 428-432

49 **Okudaira K**, Kato S, Ito K, Miura S. Helicobacter pylori adhesion inhibitory effect of casein polymer FP-10. *Helicobacter Research* (Japanese) 2006; **42**: 330-335

50 **Shibata H**, KimuraTakagi I, Nagaoka M, Hashimoto S, Sawada H, Ueyama S, Yokokura T. Inhibitory effect of Cladosiphon fucoidan on the adhesion of Helicobacter pylori to human gastric cells. *J Nutr Sci Vitaminol* (Tokyo) 1999; **45**: 325-336 [PMID: 10524351 DOI: 10.3177/jnsv.45.325]

51 **Shibata H**, Iimuro M, Uchiya N, Kawamori T, Nagaoka M, Ueyama S, Hashimoto S, Yokokura T, Sugimura T, Wakabayashi K. Preventive effects of Cladosiphon fucoidan against Helicobacter pylori infection in Mongolian gerbils. *Helicobacter* 2003; **8**: 59-65 [PMID: 12603617 DOI: 10.1046/j.1523-5378.2003.00124.x]

52 **Jonkers D**, van den Broek E, van Dooren I, Thijs C, Dorant E, Hageman G, Stobberingh E. Antibacterial effect of garlic and omeprazole on Helicobacter pylori. *J Antimicrob Chemother* 1999; **43**: 837-839 [PMID: 10404325 DOI: 10.1093/jac/43.6.837]

53 **Graham DY**, Anderson SY, Lang T. Garlic or jalapeño peppers for treatment of Helicobacter pylori infection. *Am J Gastroenterol* 1999; **94**: 1200-1202 [PMID: 10235193 DOI: 10.1111/j.1572-0241.1999.01066.x]

54 **O'Gara EA**, Hill DJ, Maslin DJ. Activities of garlic oil, garlic powder, and their diallyl constituents against Helicobacter pylori. *Appl Environ Microbiol* 2000; **66**: 2269-2273 [PMID: 10788416 DOI: 10.1128/AEM.66.5.2269-2273.2000]

55 **O'Gara EA**, Maslin DJ, Nevill AM, Hill DJ. The effect of simulated gastric environments on the anti-Helicobacter activity of garlic oil. *J Appl Microbiol* 2008; **104**: 1324-1331 [PMID: 18028365 DOI: 10.1111/j.1365-2672.2007.03637.x]

56 **Kudo H**, Takeuchi H, Shimamura T, Kadota Y, Sugiura T, Ukeda H. In vitro anti-Helicobacter pylori activity of Chinese chive (Allium tuberosum). *Food Sci Technol Res* 2011; **17**: 505-513

57 **Tai H**, Watanabe Y, Nakagawa K, Nozaki Y, Kikuchi Y. Effect of Deep Sea Water on Blood Rheological Properties. *Hemorheology and Related Research* (Japanese) 1999; **2**: 43-47

58 **Tai H**, Nakagawa K, Watanabe Y, Yokoyama Y, Nakajima H. Ikegami Y. Nozaki Y. Kikuchi Y. Effect of long-term drinking of high mineral water prepared from deep sea water on hemorheology and blood pressure. *Hemorheology and Related Research* (Japanese) 2001; **4**: 21-25

59 **Kimata H**, Tai H, Nkajima H. Reduction of Allergic Skin Responses and Serum Allergen-Specific IgE and IgE-Inducing Cytokines by Drinking Deep-Sea Water in Patients with Allergic Rhinitis. *Otorhinolaryngol Nova* 2001; **11**: 302-303 [DOI: 10.1159/000068306]

60 **Kimata H**, Tai H, Nakagawa K, Yokoyama Y, Nakajima H, Ikegami Y. Improvement of skin symptoms and mineral imbalance by drinking deep sea water in patients with atopic eczema/dermatitis syndrome (AEDS). *Acta Medica* (Hradec Kralove) 2002; **45**: 83-84 [PMID: 12325458]

61 **Hataguchi Y**, Tai H, Nakajima H, Kimata H. Drinking deep-sea water restores mineral imbalance in atopic eczema/dermatitis syndrome. *Eur J Clin Nutr* 2005; **59**: 1093-1096 [PMID: 16015263 DOI: 10.1038/sj.ejcn.1602218]

62 **Hataguchi Y**, Fukuda T, Uozumi Y, Watanabe Y, Asano M, Moro I. Effect of magnesium chloride on the S-IgA in saliva. *Digestive Organ and Mucosal Immunology* (Japanese) 2005; **42**: 48-51

63 **Kawada M**, Takeuchi H, Con SA, Yamamoto E, Yasukawa T, Nakagawa K, Ikegami Y, Sugiura T. Antibacterial activities of refined deep seawater on Helicobacter pylori. *J Med Microbiol Diagn* 2012; S1-S7 [DOI: 10.4172/2161-0703]

64 **Imai H**, Osawa K, Yasuda H, Hamashima H, Arai T, Sasatsu M. Inhibition by the essential oils of peppermint and spearmint of the growth of pathogenic bacteria. *Microbios* 2001; **106** Suppl 1: 31-39 [PMID: 11549238]

65 **Kalpoutzakis E**, Aligiannis N, Mentis A, Mitaku S, Charvala C. Composition of the essential oil of two Nepeta species and in vitro evaluation of their activity against Helicobacter pylori. *Planta Med* 2001; **67**: 880-883 [PMID: 11745033 DOI: 10.1055/s-2001-18851]

66 **Ohno T**, Kita M, Yamaoka Y, Imamura S, Yamamoto T, Mitsufuji S, Kodama T, Kashima K, Imanishi J. Antimicrobial activity of essential oils against Helicobacter pylori. *Helicobacter* 2003; **8**: 207-215 [PMID: 12752733 DOI: 10.1046/j.1523-5378.2003.00146.x]

P- Reviewers: Balbinotti R, Slomiany BL, Sugimoto M
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8979

World J Gastroenterol 2014 July 21; 20(27): 8979-8985
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Helicobacter pylori: Friend or foe?

Stephen David Howard Malnick, Ehud Melzer, Malka Attali, Gabriel Duek, Jacob Yahav

Stephen David Howard Malnick, Department of Internal Medicine C, Kaplan Medical Center, 76100 Rehovot, Israel
Ehud Melzer, Gabriel Duek, Gastroenterology Institute, Kaplan Medical Center, 76100 Rehovot, Israel
Malka Attali, Department of Internal Medicine B, Kaplan Medical Center, 76100 Rehovot, Israel
Jacob Yahav, Kaplan Medical Center, Affiliated to the Hebrew University, Jerusalem, 76100 Rehovot, Israel
Author contributions: Malnick SDH developed the concept and wrote the manuscript; Melzer E, Attali M and Duek G developed the theme and researched the literature; Yahav J contributed to the writing and revision of the manuscript, in addition to developing the concept.
Correspondence to: Stephen David Howard Malnick, Director, Department of Internal Medicine C, Kaplan Medical Center, PO Box 1, 76100 Rehovot, Israel. stephen@malnick.net
Telephone: +97-289-441371 Fax: +97-289-441852
Received: September 24, 2013 Revised: January 23, 2014
Accepted: April 21, 2014
Published online: July 21, 2014

Abstract

Helicobacter pylori (*H. pylori*) is a Gram-negative spiral bacterium that is present in nearly half the world's population. It is the major cause of peptic ulcer disease and a recognized cause of gastric carcinoma. In addition, it is linked to non-ulcer dyspepsia, vitamin B12 deficiency, iron-deficient anemia and immune thrombocytopenic purpura. These conditions are indications for testing and treatment according to current guidelines. An additional indication according to the guidelines is "anyone with a fear of gastric cancer" which results in nearly every infected person being eligible for eradication treatment. There may be beneficial effects of *H. pylori* in humans, including protection from gastroesophageal reflux disease and esophageal adenocarcinoma. In addition, universal treatment will be extremely expensive (more than $32 billion in the United States), may expose the patients to adverse effects such as anaphylaxis and *Clostridium difficile* infection, as well as contributing to antibiotic resistance. There may also be an as yet uncertain effect on the fecal microbiome. There is a need for robust clinical data to assist in decision-making regarding treatment of *H. pylori* infection.

Key words: *Helicobacter pylori*; Treatment; Cost; Benefit; Cancer

Core tip: *Helicobacter pylori* (*H. pylori*) is found in more than half the world's population. It is a major cause of peptic ulcer disease and gastric carcinoma. The overwhelming majority of those infected will not suffer any consequences during their lifetime. Furthermore, there may be a beneficial effect of *H. pylori* infection on allergy and asthma in young children and a protection against gastroesophageal reflux disease and its feared complication of esophageal carcinoma. Universal eradication will be prohibitively expensive, have adverse effects and needs to be evaluated on the basis of robust clinical data that is not yet available.

Malnick SDH, Melzer E, Attali M, Duek G, Yahav J. *Helicobacter pylori*: Friend or foe? *World J Gastroenterol* 2014; 20(27): 8979-8985 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8979.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8979

INTRODUCTION

Helicobacter pylori (H. pylori) is a Gram-negative bacterium found on the luminal surface of the gastric epithelium[1]. It induces chronic inflammation of the underlying mucosa. The infection is usually contracted in the first years of life and persists indefinitely unless treated[2]. The prevalence varies with age and socioeconomic status in childhood and therefore varies between countries[3].

Approximately 50% of the world's population is infected with *H. pylori*[2]. *H. pylori* infection has been linked

Table 1 Recommendations for testing and treating *Helicobacter pylori* infection[5]

Recommendations
Do not test if not prepared to treat
Peptic ulcers
Unexplained iron deficiency anemia
Idiopathic thrombocytopenic purpura
Vitamin B12 deficiency
Long-term proton pump inhibitor therapy
Functional dyspepsia?
Family history of gastric cancer

to gastric and duodenal ulcers (in 1%-10% of infected patients), gastric carcinoma (0.1%-3%) and gastric mucosa-associated lymphoid tissue lymphoma (less than 0.01%)[4]. However, the vast majority of the infected population will never develop symptoms related to *H. pylori* infection.

Consensus guidelines have been developed and updated[5] (Table 1). The recommendations for treatment include peptic ulcer, mucosa-associated lymphoid tissue (MALT)-lymphoma, gastric cancer, first-degree relatives of patents with gastric cancer, unexplained iron-deficiency anemia and immune thrombocytopenia. In addition, the Maastricht Guidelines state that "*H. pylori*-positive patients with fear of gastric cancer should receive eradication treatment"[5]. This last recommendation makes it likely that anyone found to be *H. pylori*-positive will receive eradication treatment.

EPIDEMIOLOGY

The prevalence of *H. pylori* infection varies from 20% to 50% in industrialized countries to over 80% in developing countries[4,6]. In developing countries, the majority of children are infected before the age of 10 and the prevalence peaks at more than 80% before the age of 50. In developed countries, infection in children is unusual but becomes more common in adulthood. Serology is negative in the vast majority under the age of 10, increases to 10% in those between 18 and 30 years and to 50% in those older than 60[7].

The route for infection by *H. pylori* is unclear[8]. It seems most likely to be by the oral-fecal or oral-oral route[9].

The risk of acquiring *H. pylori* is related to socio-economic status and early life living conditions[10]. In some countries there is a link between a decline in *H. pylori* prevalence and economic development. In Japan, 70%-80% of adults born before 1950, 45% of those born between 1950 and 1960 and 25% of those born between 1960 and 1970 are infected[11].

Reinfection with *H. pylori* following successful bacterial eradication is unusual. In adults the rate is less than 2% per year[12] which is similar to the primary adult rate of infection[13].

DISEASES ASSOCIATED WITH *H. PYLORI*

H. pylori is present in the majority of the patients with uncomplicated duodenal ulcers, especially in those with no history of recent non-steroidal anti-inflammatory drug consumption[14]. *H. pylori* is not found in up to 27% of patients with endoscopically proven duodenal ulcers[15] and thus needs to be tested for. In addition *H. pylori* is found in the majority of uncomplicated gastric ulcers[16]. *H. pylori* infection has also been linked with unexplained iron deficiency anemia[17], vitamin B12 deficiency[18] and immune thrombocytopenic purpura[19]. It is clear that *H. pylori* should be tested and treated to eradication in the above cases.

H. pylori is also linked to gastric cancer. There is a 6-fold increase in the risk of gastric cancer in *H. pylori*-positive populations compared with uninfected populations[20]. In a nested case control study of Japanese Americans living in Hawaii, *H. pylori* seropositvity was present in 94% of those with gastric cancer compared with 76% of matched controls (OR = 6.0)[21].

There is also an association between *H. pylori* infection and MALT lymphoma[22]. In addition MALT lymphoma regresses following successful treatment for *H. pylori*.

In summary, *H. pylori* infection is clearly linked to peptic ulcer disease, gastric cancer and MALT lymphoma, immune thrombocytopenia and some cases of vitamin B12 and iron deficiency. In such situations, it is reasonable to proceed to eradication.

DIAGNOSIS OF *H. PYLORI* INFECTION

There are several methods for diagnosing *H. pylori* infection, both noninvasive and invasive. The invasive tests are performed on specimens obtained at endoscopy These include biopsy urease testing, histology and less commonly bacterial culture and sensitivity. The sensitivity of a biopsy urease test is between 90%-95% and the specificity is 95%-100%[15].

In addition, there are non-invasive tests, including urea breath testing, stool antigen testing, and serology. The urea breath test is based on the hydrolysis of urea by *H. pylori* to produce carbon dioxide and ammonia[23]. A labeled carbon isotope is given by mouth and *H. pylori* liberates tagged carbon dioxide that can be detected in the exhaled air.

Laboratory-based enzyme-linked immunosorbent assay testing to detect immunoglobulin G is inexpensive and non-invasive. There is a high sensitivity (90%-100%), but variable specificity (76%-96%). In low prevalence areas, a positive serology result has a low predictive value for active infection. In such areas, stool antigen or breath testing is recommended. Conversion of positive serology to negative 1 year after treatment suggests bacterial eradication[24].

The presence of *H. pylori* in the stool of infected patients has enabled the development of fecal assays[15] which have high sensitivity, specificity and diagnostic accuracy[25].

In summary, there are a range of tests, both invasive and non-invasive, that are available for the diagnosis of *H. pylori* infection.

Table 2 Randomized controlled trials of *Helicobacter pylori* eradication and risk of gastric cancer

Ref.	Province, country	Gastric cancer per 10^5	No. of patients treatment/control	Follow up (yr)	No. of patients with gastric cancer	*P* value
Wong *et al*[30]	Fujian, China	$99/10^5$	817/813	7.5	7 (0.9)/11 (1.4)	0.330
Fukase *et al*[31]	Japan	$62/10^5$	272/272	3	9 (3.3)/24 (8.8)	0.009

INDICATIONS FOR *H. PYLORI* ERADICATION

Peptic ulcer disease

H. pylori is found in the majority of duodenal ulcers[14], especially if there is no history of consumption of non-steroidal antiinflammatory drugs (NSAIDS). In those patients with a duodenal ulcer who do not have *H. pylori* infection, there seems to be a worse prognosis with a higher incidence of ulcer relapse, non-healed ulcer, and relapse of severe dyspeptic symptoms[26].

H. pylori seems to be associated with the majority of gastric ulcers[16] but again there is an increasing proportion of patients with gastric ulcers in whom *H. pylori* is not detected. Some of these cases may be related to surreptitious use of NSAIDS.

Thus, in cases of peptic ulcer disease, routine testing and treating of *H. pylori* is recommended and justified. The recommendations for testing and treatment of *H. pylori* are shown in Table 1.

Carcinoma of the stomach

H. pylori is linked to the development of chronic active gastritis and atrophic gastritis which are early stages in the carcinogenesis sequence. There is a clear association between *H. pylori* infection and gastric adenocarcinoma. *H. pylori* has been recognized as a grade 1 carcinogen by the International Agency for Research on Cancer[27]. It is thought that long-term chronic inflammation caused by *H. pylori* is the main mechanism for the development of gastric carcinoma[28].

In spite of the definite connection between gastric carcinoma and *H. pylori* infection, it has not been convincingly shown that *H. pylori* eradication decreases the incidence of gastric carcinoma. This is due to the fact in order to perform trials with cancer as the endpoint, more than 18000 patients will need to be recruited and will need to be followed up for 10-20 years[29]. In addition, it may be unethical to include an untreated arm since *H. pylori* has been classified as a type 1 carcinogen. There are only two randomized controlled interventional trials with gastric cancer development as the primary outcome[30,31] (Table 2). Both of these studies were performed in high risk areas of the Far East. In the study of Wong *et al*[30], 1630 *H. pylori*-positive patients were followed up for 7 years. In the eradication group 7/817 (0.9%) of the patients developed gastric carcinoma compared with 11/813 (1.3%) in the placebo group ($P = 0.33$). It is of interest to note that none of the patients without precancerous lesions at baseline histology developed cancer. The authors suggest that the chemopreventive effect of *H. pylori* eradication is only effective before preneoplastic lesions have developed.

The majority of intestinal-type gastric carcinoma arises from atrophic gastric mucosa. Although eradication of *H. pylori* results in a decrease in inflammation, it is not clear that mucosal atrophy is improved by *H. pylori* eradication[32-35]. One study with follow-up of 13.7 years after *H. pylori* eradication found no significant inflammatory cell infiltration at the time of cancer diagnosis. This suggests that the decrease in mucosal inflammation resulting from *H. pylori* eradication is insufficient to prevent gastric carcinoma once severe mucosal atrophy has developed[25].

Fukase *et al*[31] enrolled 544 patients in a multicenter study with a 3 year follow-up. The odds ratio for developing gastric cancer was 0.353 in the eradication group ($P = 0.009$). A meta-analysis of published trials found gastric cancer in 33/3112 (1%) of eradication patients *vs* 50/3031 (1.6%) of untreated patients[36]. This had a relative risk of 0.65 ($P = 0.05$).

The influence of *H. pylori* eradication may decrease with time. In a study of 268 *H. pylori*-positive patients who had undergone endoscopic resection of early gastric cancer, there were 177 patients who had undergone successful *H. pylori* eradication and 91 who had persistent *H. pylori* infection[37]. Although the incidence of metachronous gastric carcinoma was lower in the eradicated group at 5 years of follow-up ($P = 0.007$), this difference was no longer significant in the follow-up period extending to 11.1 years ($P = 0.262$). Interestingly, in this study too, multivariate analysis showed severe mucosal atrophy, but not *H. pylori* status, as an independent risk factor for metachronous gastric cancer.

There may be a precancerous state, with moderate to severe gastric mucosal atrophy or intestinal metaplasia representing a point of no return in terms of developing gastric cancer, from which *H. pylori* eradication can no longer prevent gastric cancer. It thus may be preferable to try to identify those patients at risk of developing atrophic gastritis and then treat for *H. pylori* eradication. It has been suggested that *H. pylori* eradication will be most beneficial in terms of preventing cancer in patients who have chronic atrophic gastritis and negative serum pepsinogen[38].

Functional dyspepsia

Dyspepsia is a common symptom with an extensive differential diagnosis. It is thought to be present in about 25% of the population in any year, although the majority of affected people do not seek medical care. About 25%

Table 3 Inverse association of *Helicobacter pylori* with asthma and allergy[55]

H. pylori status (*H. pylori*/cagA)	< 15 yr OR (95%CI)	> 15 yr OR (95%CI)
-	1	1
+/-	0.97 (0.65-1.45)	0.95 (0.68-1.33)
+/+	0.63 (0.43-0.93)	0.97 (0.72-1.32)

H. pylori: *Helicobacter pylori*.

of those suffering from dyspepsia have an underlying organic cause, but the remainder have nonulcer dyspepsia in which there is no clear organic cause after diagnostic evaluation. Functional dyspepsia (FD) is classified into postprandial distress syndrome and epigastric pain syndrome[39].

H. pylori eradication has been associated with significant benefits in a subset of patients suffering from FD[40]. Four hundred and four patients with FD who were infected with *H. pylori* were randomized to receive placebo or eradication treatment of *H. pylori*. At 12-mo follow-up, patients in whom *H. pylori* was eradicated were more likely to have symptomatic improvement compared with the control group (49% *vs* 36%, $P = 0.01$). In addition, a systematic review of 17 randomized controlled trials, including 3566 patients with FD, found that eradication of *H. pylori* was associated with a small but significant benefit; 14 patients needed to be treated in order to cure one case of FD[41].

However, it is possible that alterations in the upper gastrointestinal tract microbiome may result in the development of dyspepsia. Dyspepsia is more likely to occur after an episode of gastroenteritis[42,43]. It has been suggested that the effect of *H. pylori* therapy in improving the symptoms of FD is due to its impact on the gut microbiome rather than the eradication of *H. pylori* alone[44]. The clinical management of *H. pylori* infection has recently been reviewed[45].

BENEFICIAL EFFECTS OF *H. PYLORI* INFECTION

H. pylori has been colonizing the human stomach for more than 58000 years[46] and has been found in Egyptian mummies. This long-standing relationship suggests that there may be some adverse effects in altering the colonization of the human microbiome.

There does appear to be an inverse relationship between *H. pylori* infection and Barrett's esophagus[47]. Sonnenberg *et al*[47] reported a study of more than 78000 patients in the United States who underwent upper gastrointestinal endoscopy and histopathological analysis of gastric biopsies. They found that there was a strong correlation between the presence of *H. pylori*, chronic gastritis and intestinal metaplasia. In addition, there was an inverse association with Barrett's esophagus. Barrett's esophagus is thought to be an intermediate lesion along the pathway between reflux esophagitis and esophageal adenocarcinoma. In recent years, there has been an increase in the incidence of esophageal adenocarcinoma in the developed world, together with an increase in the incidence of Barrett's esophagus and esophageal reflux (Table 3)[48-53].

H. pylori infection is usually acquired in childhood and generally persists for life[54]. Thus *H. pylori* has infected the majority of the world's population for the majority of their lifetime[54] and in most cases causes no symptoms. In recent years, there has been a decrease in the prevalence of *H. pylori* infection in developed countries. In the United States less than 6% of children are infected by *H. pylori*[55]. A similar trend is becoming apparent in other parts of the developed world[56,57].

There have been reports of an inverse association between childhood-onset asthma and *H. pylori* infection[55,58,59] and protection from other infections[60,61] (Table 3). Recently, it has become clear that the gut microbiota has an important effect on many disease processes[62] and that disturbing the balance of the bacteria by antibiotics can produce a state of dysbiosis, with an effect on pathogen evolution[63]. *Clostridium difficile* infection linked to antibiotic use is one example of a deleterious effect related to antibiotic consumption and its effect on the microbiome.

Many organisms that are considered as commensals such as *Kelbsiella*, *Strep viridans* and *Candida* can become opportunistic pathogens, especially in the aged population. There is no coordinated attempt to eradicate these organisms from the human population and we suggest that there should not be a similar effort to eradicate *H. pylori*. There is a complex biological relationship between humans and commensal bacteria that is only now beginning to be understood. The "test and treat" approach to *H. pylori* does not address this issue at all.

COST OF ERADICATION OF *H. PYLORI*

The current recommendations for treating *H. pylori* make a strong case for universal eradication. The assertion that "*H. pylori*-positive patients with a fear of gastric cancer should receive eradication treatment"[5] makes it likely that the majority of the world's infected population will receive treatment. The economic implications are enormous.

In the United States the population in 2012 was approximately 300 million. A urease breath test costs $15 and thus the cost of testing would be approximately $4.5 billion. Assuming a 30% positivity rate, then retesting to confirm eradication would need to be performed on 90 million people with an additional cost of $1.5 billion.

First-line therapy consisting of amoxycillin 1 g *bid*, omeprazole 20 mg *bid*, and clarithromycin 500 mg *bid* for 10 d costs $203 (based on www.goodrx.com). This would cost $18.27 billion for 90 million people who are *H. pylori*-positive. This treatment is about 80% effective and thus 18 million people would still be infected with *H. pylori*. Second-line therapy with omeprazole, bismuth sub-

salicylate, tetracycline and metronidazole costs $2.68 billion and is expected to be about 70% successful. Repeat testing of these 18 million people would cost $270 million and there would still be 5.4 million people infected with *H. pylori*.

Further treatment would require gastroscopy, biopsy, bacterial culture and sensitivity testing. The cost of gastroscopy to medicare in an ambulatory surgery clinic is $341 for the center and $351 for the physician and thus the total cost for 5.4 million people is $3.74 billion. The cost of a *Helicobacter* culture is $159 with a further $222 for susceptibility testing for 4 drugs (Ellie Goldstein, personal communication).This would result in a total cost of $2 billion for 5.4 million people. Thus the total cost for eliminating *H. pylori* from the population of the United States is in the region of $33 billion dollars!

IS ERADICATION OF *H. PYLORI* COST-EFFECTIVE ?

The question of whether eradication of *H. pylori* is cost-effective is complex. There is a difference between treating anyone found to be positive, or those with non-ulcer dyspepsia, or people with a high risk for gastric cancer. In addition there is still not a complete understanding of the beneficial effects of commensal *H. pylori* infection as well as the risks associated with universal treatment.

There have been studies estimating the financial implications of screening for *H. pylori* in a subpopulation of dyspeptic patients, or related to one *H. pylori*-associated disease such as peptic ulcer or gastric cancers[64,65]. In these studies, screening for and treating *H. pylori* was found to be cost-effective in patients with peptic ulcer or for preventing gastric cancer[65]. Furthermore it has been estimated that screening and treatment for *H. pylori* is likely to be cost-effective taking into account both gastric cancer and peptic ulcer disease[66,67]. A meta-analysis of trials of eradication therapy in *H. pylori*-positive peptic ulcer disease found a reduction in the recurrence of peptic ulcer disease and concluded that it was cost effective[68]. A comparison of a strategy of screening and treating everyone found to be positive *vs* testing and treatment only if symptoms arise found an incremental cost per case of $26 in the screened cohort[69].

A comprehensive cost-benefit analysis is difficult to perform since not all of the variables are known. The effect on the fecal microbiome of widespread eradication is not known. In addition, the decrease in prevalence of *H. pylori* will cause a corresponding decrease in the incidence of new infection in the next generation. To the best of our knowledge, a cost-benefit analysis incorporating these variables has not been performed.

In summary, *H. pylori* is a common infection of the human stomach. It is a major cause of peptic ulcer disease, a recognized carcinogen, and is linked to both iron and vitamin B12 deficiency. It may have some beneficial effects protecting from gastroesophageal reflux disease and associated esophageal carcinoma, as well as protecting young children from asthma and allergic diseases.

Near universal eradication, consistent with current guidelines, will be prohibitively expensive. Furthermore, it is likely there will be some fatalities from previously unknown allergic reactions to antibiotics employed, drug adverse effects, an increase in bacterial antibiotic resistance in treated populations, an increase in *Clostridium difficile* infection and unknown effects on the fecal microbiome.

There is an urgent need for robust clinical data to enable and support decisions regarding treatment of *H. pylori* infection before committing to a huge expenditure of limited health-care resources for which the overall impact is uncertain.

REFERENCES

1 **Marshall BJ**, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **1**: 1311-1315 [PMID: 6145023]
2 **Everhart JE**. Recent developments in the epidemiology of Helicobacter pylori. *Gastroenterol Clin North Am* 2000; **29**: 559-578 [PMID: 11030073]
3 **Woodward M**, Morrison C, McColl K. An investigation into factors associated with Helicobacter pylori infection. *J Clin Epidemiol* 2000; **53**: 175-181 [PMID: 10729690]
4 **McColl KE**. Clinical practice. Helicobacter pylori infection. *N Engl J Med* 2010; **362**: 1597-1604 [PMID: 20427808 DOI: 10.1056/NEJMcp1001110]
5 **Malfertheiner P**, Megraud F, O'Morain CA, Atherton J, Axon AT, Bazzoli F, Gensini GF, Gisbert JP, Graham DY, Rokkas T, El-Omar EM, Kuipers EJ. Management of Helicobacter pylori infection--the Maastricht IV/ Florence Consensus Report. *Gut* 2012; **61**: 646-664 [PMID: 22491499 DOI: 10.1136/gutjnl-2012-302084]
6 **Suerbaum S**, Michetti P. Helicobacter pylori infection. *N Engl J Med* 2002; **347**: 1175-1186 [PMID: 12374879 DOI: 10.1056/NEJMra020542]
7 **Pounder RE**, Ng D. The prevalence of Helicobacter pylori infection in different countries. *Aliment Pharmacol Ther* 1995; **9** Suppl 2: 33-39 [PMID: 8547526]
8 **Cave DR**. Transmission and epidemiology of Helicobacter pylori. *Am J Med* 1996; **100**: 12S-17S; discussion 17S-18S [PMID: 8644777]
9 **Mégraud F**. Transmission of Helicobacter pylori: faecal-oral versus oral-oral route. *Aliment Pharmacol Ther* 1995; **9** Suppl 2: 85-91 [PMID: 8547533]
10 **Webb PM**, Knight T, Greaves S, Wilson A, Newell DG, Elder J, Forman D. Relation between infection with Helicobacter pylori and living conditions in childhood: evidence for person to person transmission in early life. *BMJ* 1994; **308**: 750-753 [PMID: 8142828]
11 **Asaka M**, Kimura T, Kudo M, Takeda H, Mitani S, Miyazaki T, Miki K, Graham DY. Relationship of Helicobacter pylori to serum pepsinogens in an asymptomatic Japanese population. *Gastroenterology* 1992; **102**: 760-766 [PMID: 1537513]
12 **Archimandritis A**, Balatsos V, Delis V, Manika Z, Skandalis N. "Reappearance" of Helicobacter pylori after eradication: implications on duodenal ulcer recurrence: a prospective 6 year study. *J Clin Gastroenterol* 1999; **28**: 345-347 [PMID: 10372933]
13 **Parsonnet J**. The incidence of Helicobacter pylori infection. *Aliment Pharmacol Ther* 1995; **9** Suppl 2: 45-51 [PMID: 8547528]
14 NIH Consensus Conference. Helicobacter pylori in peptic ulcer disease. NIH Consensus Development Panel on Helicobacter pylori in Peptic Ulcer Disease. *JAMA* 1994; **272**: 65-69

[PMID: 8007082]
15 **Chey WD**, Wong BC; Practice Parameters Committee of the American College of Gastroenterology. American College of Gastroenterology guideline on the management of Helicobacter pylori infection. *Am J Gastroenterol* 2007; **102**: 1808-1825 [PMID: 17608775 DOI: 10.1111/j.1572-0241.2007.01393.x]
16 **Laine L**. Helicobacter pylori, gastric ulcer, and agents noxious to the gastric mucosa. *Gastroenterol Clin North Am* 1993; **22**: 117-125 [PMID: 8449561]
17 **DuBois S**, Kearney DJ. Iron-deficiency anemia and Helicobacter pylori infection: a review of the evidence. *Am J Gastroenterol* 2005; **100**: 453-459 [PMID: 15667507 DOI: 10.1111/j.1572-0241.2005.30252.x]
18 **Kaptan K**, Beyan C, Ural AU, Cetin T, Avcu F, Gülşen M, Finci R, Yalçín A. Helicobacter pylori--is it a novel causative agent in Vitamin B12 deficiency? *Arch Intern Med* 2000; **160**: 1349-1353 [PMID: 10809040]
19 **Papagiannakis P**, Michalopoulos C, Papalexi F, Dalampoura D, Diamantidis MD. The role of Helicobacter pylori infection in hematological disorders. *Eur J Intern Med* 2013; **24**: 685-690 [PMID: 23523153 DOI: 10.1016/j.ejim.2013.02.011]
20 An international association between Helicobacter pylori infection and gastric cancer. The EUROGAST Study Group. *Lancet* 1993; **341**: 1359-1362 [PMID: 8098787]
21 **Nomura A**, Stemmermann GN, Chyou PH, Kato I, Perez-Perez GI, Blaser MJ. Helicobacter pylori infection and gastric carcinoma among Japanese Americans in Hawaii. *N Engl J Med* 1991; **325**: 1132-1136 [PMID: 1891021 DOI: 10.1056/NEJM199110173251604]
22 **Wotherspoon AC**, Ortiz-Hidalgo C, Falzon MR, Isaacson PG. Helicobacter pylori-associated gastritis and primary B-cell gastric lymphoma. *Lancet* 1991; **338**: 1175-1176 [PMID: 1682595]
23 **Howden CW**, Hunt RH. Guidelines for the management of Helicobacter pylori infection. Ad Hoc Committee on Practice Parameters of the American College of Gastroenterology. *Am J Gastroenterol* 1998; **93**: 2330-2338 [PMID: 9860388 DOI: 10.1111/j.1572-0241.1998.00684.x]
24 **Feldman M**, Cryer B, Lee E, Peterson WL. Role of seroconversion in confirming cure of Helicobacter pylori infection. *JAMA* 1998; **280**: 363-365 [PMID: 9686554]
25 **Trevisani L**, Sartori S, Ruina M, Caselli M, Rossi MR, Costa F, Bellini M, Iaquinto G, Gardullo N, Todisco A. Helicobacter pylori stool antigen test: clinical evaluation and cost analysis of a new enzyme immunoassay. *Dig Dis Sci* 1999; **44**: 2303-2306 [PMID: 10573378]
26 **Bytzer P**, Teglbjaerg PS; Danish Ulcer Study Group. Helicobacter pylori-negative duodenal ulcers: prevalence, clinical characteristics, and prognosis--results from a randomized trial with 2-year follow-up. *Am J Gastroenterol* 2001; **96**: 1409-1416 [PMID: 11374675 DOI: 10.1111/j.1572-0241.2001.03774.x]
27 **Helicobacter and Cancer Collaborative Group**. Gastric cancer and Helicobacter pylori: a combined analysis of 12 case control studies nested within prospective cohorts. *Gut* 2001; **49**: 347-353 [PMID: 11511555]
28 **Sotoudeh M**, Derakhshan MH, Abedi-Ardakani B, Nouraie M, Yazdanbod A, Tavangar SM, Mikaeli J, Merat S, Malekzadeh R. Critical role of Helicobacter pylori in the pattern of gastritis and carditis in residents of an area with high prevalence of gastric cardia cancer. *Dig Dis Sci* 2008; **53**: 27-33 [PMID: 17492381 DOI: 10.1007/s10620-007-9817-1]
29 **Graham DY**, Shiotani A. The time to eradicate gastric cancer is now. *Gut* 2005; **54**: 735-738 [PMID: 15888771 DOI: 10.1136/gut.2004.056549]
30 **Wong BC**, Lam SK, Wong WM, Chen JS, Zheng TT, Feng RE, Lai KC, Hu WH, Yuen ST, Leung SY, Fong DY, Ho J, Ching CK, Chen JS. Helicobacter pylori eradication to prevent gastric cancer in a high-risk region of China: a randomized controlled trial. *JAMA* 2004; **291**: 187-194 [PMID: 14722144 DOI: 10.1001/jama.291.2.187]
31 **Fukase K**, Kato M, Kikuchi S, Inoue K, Uemura N, Okamoto S, Terao S, Amagai K, Hayashi S, Asaka M. Effect of eradication of Helicobacter pylori on incidence of metachronous gastric carcinoma after endoscopic resection of early gastric cancer: an open-label, randomised controlled trial. *Lancet* 2008; **372**: 392-397 [PMID: 18675689 DOI: 10.1016/S0140-6736(08)61159-9]
32 **Zhou LY**, Lin SR, Ding SG, Huang XB, Zhang L, Meng LM, Cui RL, Zhu J. The changing trends of the incidence of gastric cancer after Helicobacter pylori eradication in Shandong area. *Chin J Dig Dis* 2005; **6**: 114-115 [PMID: 16045599 DOI: 10.1111/j.1443-9573.2005.00204.x]
33 **Mera R**, Fontham ET, Bravo LE, Bravo JC, Piazuelo MB, Camargo MC, Correa P. Long term follow up of patients treated for Helicobacter pylori infection. *Gut* 2005; **54**: 1536-1540 [PMID: 15985559 DOI: 10.1136/gut.2005.072009]
34 **Ito M**, Haruma K, Kamada T, Mihara M, Kim S, Kitadai Y, Sumii M, Tanaka S, Yoshihara M, Chayama K. Helicobacter pylori eradication therapy improves atrophic gastritis and intestinal metaplasia: a 5-year prospective study of patients with atrophic gastritis. *Aliment Pharmacol Ther* 2002; **16**: 1449-1456 [PMID: 12182744]
35 **Sung JJ**, Lin SR, Ching JY, Zhou LY, To KF, Wang RT, Leung WK, Ng EK, Lau JY, Lee YT, Yeung CK, Chao W, Chung SC. Atrophy and intestinal metaplasia one year after cure of H. pylori infection: a prospective, randomized study. *Gastroenterology* 2000; **119**: 7-14 [PMID: 10889149]
36 **Fuccio L**, Zagari RM, Eusebi LH, Laterza L, Cennamo V, Ceroni L, Grilli D, Bazzoli F. Meta-analysis: can Helicobacter pylori eradication treatment reduce the risk for gastric cancer? *Ann Intern Med* 2009; **151**: 121-128 [PMID: 19620164]
37 **Maehata Y**, Nakamura S, Fujisawa K, Esaki M, Moriyama T, Asano K, Fuyuno Y, Yamaguchi K, Egashira I, Kim H, Kanda M, Hirahashi M, Matsumoto T. Long-term effect of Helicobacter pylori eradication on the development of metachronous gastric cancer after endoscopic resection of early gastric cancer. *Gastrointest Endosc* 2012; **75**: 39-46 [PMID: 22018552 DOI: 10.1016/j.gie.2011.08.030]
38 **Yanaoka K**, Oka M, Ohata H, Yoshimura N, Deguchi H, Mukoubayashi C, Enomoto S, Inoue I, Iguchi M, Maekita T, Ueda K, Utsunomiya H, Tamai H, Fujishiro M, Iwane M, Takeshita T, Mohara O, Ichinose M. Eradication of Helicobacter pylori prevents cancer development in subjects with mild gastric atrophy identified by serum pepsinogen levels. *Int J Cancer* 2009; **125**: 2697-2703 [PMID: 19610064 DOI: 10.1002/ijc.24591]
39 **Talley NJ**; American Gastroenterological Association. American Gastroenterological Association medical position statement: evaluation of dyspepsia. *Gastroenterology* 2005; **129**: 1753-1755 [PMID: 16285970 DOI: 10.1053/j.gastro.2005.09.019]
40 **Mazzoleni LE**, Sander GB, Francesconi CF, Mazzoleni F, Uchoa DM, De Bona LR, Milbradt TC, Von Reisswitz PS, Berwanger O, Bressel M, Edelweiss MI, Marini SS, Molina CG, Folador L, Lunkes RP, Heck R, Birkhan OA, Spindler BM, Katz N, Colombo Bda S, Guerrieri PP, Renck LB, Grando E, Hocevar de Moura B, Dahmer FD, Rauber J, Prolla JC. Helicobacter pylori eradication in functional dyspepsia: HEROES trial. *Arch Intern Med* 2011; **171**: 1929-1936 [PMID: 22123802 DOI: 10.1001/archinternmed.2011.533]
41 **Moayyedi P**, Soo S, Deeks J, Delaney B, Harris A, Innes M, Oakes R, Wilson S, Roalfe A, Bennett C, Forman D. Eradication of Helicobacter pylori for non-ulcer dyspepsia. *Cochrane Database Syst Rev* 2005; **(1)**: CD002096 [PMID: 15674892 DOI: 10.1002/14651858.CD002096.pub2]
42 **Mearin F**, Pérez-Oliveras M, Perelló A, Vinyet J, Ibañez A, Coderch J, Perona M. Dyspepsia and irritable bowel syndrome after a Salmonella gastroenteritis outbreak: one-year follow-up cohort study. *Gastroenterology* 2005; **129**: 98-104 [PMID: 16012939]
43 **Ford AC**, Thabane M, Collins SM, Moayyedi P, Garg AX,

Clark WF, Marshall JK. Prevalence of uninvestigated dyspepsia 8 years after a large waterborne outbreak of bacterial dysentery: a cohort study. *Gastroenterology* 2010; **138**: 1727-1736; quiz e12 [PMID: 20117111 DOI: 10.1053/j.gastro.2010.01.043]
44 **Moayyedi P**. Helicobacter pylori eradication for functional dyspepsia: what are we treating?: comment on "Helicobacter pylori eradication in functional dyspepsia". *Arch Intern Med* 2011; **171**: 1936-1937 [PMID: 22123803 DOI: 10.1001/archinternmed.2011.541]
45 **Malfertheiner P**, Venerito M, Selgrad M. Helicobacter pylori infection: selected aspects in clinical management. *Curr Opin Gastroenterol* 2013; **29**: 669-675 [PMID: 24100726 DOI: 10.1097/MOG.Ob012e328365d443]
46 **Linz B**, Balloux F, Moodley Y, Manica A, Liu H, Roumagnac P, Falush D, Stamer C, Prugnolle F, van der Merwe SW, Yamaoka Y, Graham DY, Perez-Trallero E, Wadstrom T, Suerbaum S, Achtman M. An African origin for the intimate association between humans and Helicobacter pylori. *Nature* 2007; **445**: 915-918 [PMID: 17287725 DOI: 10.1038/nature05562]
47 **Sonnenberg A**, Lash RH, Genta RM. A national study of Helicobactor pylori infection in gastric biopsy specimens. *Gastroenterology* 2010; **139**: 1894-1901.e2; quiz e12 [PMID: 20727889 DOI: 10.1053/j.gastro.2010.08.018]
48 **Shaheen N**, Ransohoff DF. Gastroesophageal reflux, barrett esophagus, and esophageal cancer: scientific review. *JAMA* 2002; **287**: 1972-1981 [PMID: 11960540]
49 **Lagergren J**, Bergström R, Lindgren A, Nyrén O. Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. *N Engl J Med* 1999; **340**: 825-831 [PMID: 10080844 DOI: 10.1056/NEJM199903183401101]
50 **Devesa SS**, Blot WJ, Fraumeni JF. Changing patterns in the incidence of esophageal and gastric carcinoma in the United States. *Cancer* 1998; **83**: 2049-2053 [PMID: 9827707]
51 **Pohl H**, Welch HG. The role of overdiagnosis and reclassification in the marked increase of esophageal adenocarcinoma incidence. *J Natl Cancer Inst* 2005; **97**: 142-146 [PMID: 15657344 DOI: 10.1093/jnci/dji024]
52 **Bollschweiler E**, Wolfgarten E, Gutschow C, Hölscher AH. Demographic variations in the rising incidence of esophageal adenocarcinoma in white males. *Cancer* 2001; **92**: 549-555 [PMID: 11505399]
53 **Bresalier RS**. Barrett's Esophagus and esophageal adenocarcinoma. *Annu Rev Med* 2009; **60**: 221-231 [PMID: 18783330 DOI: 10.1146/annurev.med.59.061206.112706]
54 **Atherton JC**, Blaser MJ. Coadaptation of Helicobacter pylori and humans: ancient history, modern implications. *J Clin Invest* 2009; **119**: 2475-2487 [PMID: 19729845 DOI: 10.1172/JCI38605]
55 **Chen Y**, Blaser MJ. Helicobacter pylori colonization is inversely associated with childhood asthma. *J Infect Dis* 2008; **198**: 553-560 [PMID: 18598192 DOI: 10.1086/590158]
56 **Segal I**, Otley A, Issenman R, Armstrong D, Espinosa V, Cawdron R, Morshed MG, Jacobson K. Low prevalence of Helicobacter pylori infection in Canadian children: a cross-sectional analysis. *Can J Gastroenterol* 2008; **22**: 485-489 [PMID: 18478134]
57 **Rothenbacher D**, Bode G, Berg G, Gommel R, Gonser T, Adler G, Brenner H. Prevalence and determinants of Helicobacter pylori infection in preschool children: a population-based study from Germany. *Int J Epidemiol* 1998; **27**: 135-141 [PMID: 9563707]
58 **Chen Y**, Blaser MJ. Inverse associations of Helicobacter pylori with asthma and allergy. *Arch Intern Med* 2007; **167**: 821-827 [PMID: 17452546 DOI: 10.1001/archinte.167.8.821]
59 **Janson C**, Asbjornsdottir H, Birgisdottir A, Sigurjonsdottir RB, Gunnbjörnsdottir M, Gislason D, Olafsson I, Cook E, Jögi R, Gislason T, Thjodleifsson B. The effect of infectious burden on the prevalence of atopy and respiratory allergies in Iceland, Estonia, and Sweden. *J Allergy Clin Immunol* 2007; **120**: 673-679 [PMID: 17586034 DOI: 10.1016/j.jaci.2007.05.003]
60 **Rothenbacher D**, Blaser MJ, Bode G, Brenner H. Inverse relationship between gastric colonization of Helicobacter pylori and diarrheal illnesses in children: results of a population-based cross-sectional study. *J Infect Dis* 2000; **182**: 1446-1449 [PMID: 11015236 DOI: 10.1086/315887]
61 **Chang AH**, Haggerty TD, de Martel C, Leung CW, Parsonnet J. Effect of Helicobacter pylori infection on symptoms of gastroenteritis due to enteropathogenic Escherichia coli in adults. *Dig Dis Sci* 2011; **56**: 457-464 [PMID: 20635147 DOI: 10.1007/s10620-010-1309-z]
62 **Pflughoeft KJ**, Versalovic J. Human microbiome in health and disease. *Annu Rev Pathol* 2012; **7**: 99-122 [PMID: 21910623 DOI: 10.1146/annurev-pathol-011811-132421]
63 **Stecher B**, Maier L, Hardt WD. 'Blooming' in the gut: how dysbiosis might contribute to pathogen evolution. *Nat Rev Microbiol* 2013; **11**: 277-284 [PMID: 23474681 DOI: 10.1038/nrmicro2989]
64 **Briggs AH**, Sculpher MJ, Logan RP, Aldous J, Ramsay ME, Baron JH. Cost effectiveness of screening for and eradication of Helicobacter pylori in management of dyspeptic patients under 45 years of age. *BMJ* 1996; **312**: 1321-1325 [PMID: 8646042]
65 **Parsonnet J**, Harris RA, Hack HM, Owens DK. Modelling cost-effectiveness of Helicobacter pylori screening to prevent gastric cancer: a mandate for clinical trials. *Lancet* 1996; **348**: 150-154 [PMID: 8684154]
66 **Mason J**, Axon AT, Forman D, Duffett S, Drummond M, Crocombe W, Feltbower R, Mason S, Brown J, Moayyedi P. The cost-effectiveness of population Helicobacter pylori screening and treatment: a Markov model using economic data from a randomized controlled trial. *Aliment Pharmacol Ther* 2002; **16**: 559-568 [PMID: 11876711]
67 **Roderick P**, Davies R, Raftery J, Crabbe D, Pearce R, Bhandari P, Patel P. The cost-effectiveness of screening for Helicobacter pylori to reduce mortality and morbidity from gastric cancer and peptic ulcer disease: a discrete-event simulation model. *Health Technol Assess* 2003; **7**: 1-86 [PMID: 12709294]
68 **Ford AC**, Delaney BC, Forman D, Moayyedi P. Eradication therapy in Helicobacter pylori positive peptic ulcer disease: systematic review and economic analysis. *Am J Gastroenterol* 2004; **99**: 1833-1855 [PMID: 15330927 DOI: 10.1111/j.1572-0241.2004.40014.x]
69 **Leivo T**, Salomaa A, Kosunen TU, Tuominen R, Färkkilä M, Linna M, Sintonen H. Cost-benefit analysis of Helicobacter pylori screening. *Health Policy* 2004; **70**: 85-96 [PMID: 15312711 DOI: 10.1016/j.healthpol.2004.02.004]

P- Reviewers: Ihara E, Kim YJ, Suk KT **S- Editor**: Gou SX
L- Editor: Cant MR **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8986

World J Gastroenterol 2014 July 21; 20(27): 8986-8992
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Novel findings about management of gastric cancer: A summary from 10th IGCC

Danila Penon, Letizia Cito, Antonio Giordano

Danila Penon, Department of Biochemistry and Medical Biotechnology, University of Naples Federico II, 80131 Naples, Italy
Letizia Cito, Antonio Giordano, Istituto Nazionale per lo Studio e la Cura dei Tumori "Fondazione Giovanni Pascale", IRCCS, 80131 Naples, Italy
Antonio Giordano, Sbarro Institute for Cancer Research and Molecular Medicine, Philadelphia, PA 19122, United States
Antonio Giordano, Center of Biotechnology, College of Science and Technology, Temple University, Philadelphia, PA 19122, United States
Antonio Giordano, Department of Medicine, Surgery and Neuroscience, University of Siena, 53100 Siena, Italy
Author contributions: Penon D and Cito L attended the meeting and prepared the manuscript; Cito L revised the language; Giordano A analyzed the manuscript and gave his final approval.
Correspondence to: Antonio Giordano, MD PhD, Director of Sbarro Institute for Cancer Research and Molecular Medicine, 1801 N Broad St, Philadelphia, PA 19122, United States. giordano@temple.edu
Telephone: +1-215-2049520 Fax: +1-215-2049522
Received: October 31, 2013 Revised: March 17, 2014
Accepted: April 5, 2014
Published online: July 21, 2014

Abstract

The Tenth International Gastric Cancer Congress (IGCC) was held in Verona, Italy, from June 19 to 22, 2013. The meeting enclosed various aspects of stomach tumor management, including both tightly clinical approaches, and topics more related to basic research. Moreover, an overview on gastrointestinal stromal tumors was provided too, although here not discussed. Here we will discuss some topics related to molecular biology of gastric cancer (GC), inherent to prognostic, diagnostic and therapeutic tools shown at the conference. Results about well known subjects, such as E-cadherin loss of expression/function, were presented. They revealed that other mutations of the gene were identified, showing a continuous research to improve diagnosis and prognosis of stomach tumor. Simultaneously, new possible molecular markers with an established role for other neoplasms, were discussed, such as mesothelin, stomatin-like protein 2 and Notch-1. Hence, a wide overview including both old and new diagnostic/prognostic tools was offered. Great attention was also dedicated to possible drugs to be used against GC. They included monoclonal antibodies, such as MS57-2.1, drugs used in other pathologies, such as maraviroc, and natural extracts from plants such as biflorin. We would like to contribute to summarize the most impressive studies presented at the IGCC, concerning novel findings about molecular biology of gastric cancer. Although further investigations will be necessary, it can be inferred that more and more tools were developed, so as to better face stomach neoplasms.

Key words: Gastric cancer; Prognostic tools; Markers; Therapy

Core tip: Gastric cancer (GC) is one of the most common tumors in the world, although scientists' knowledge about this neoplasm grew in the last years. In June, an international meeting (10th International Gastric Cancer Congress), focused on GC management, was held in Verona (Italy). It gave an overview about the state-of-the-art stomach tumor treatments, including chemotherapy, surgical therapies and nutritional support. Moreover, several new possible prognostic markers were shown. Here we report a summary of novel findings taken from some molecular biology sessions, focused on prognosis and treatment of GC.

Penon D, Cito L, Giordano A. Novel findings about management of gastric cancer: A summary from 10th IGCC. *World J Gastroenterol* 2014; 20(27): 8986-8992 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8986.htm DOI: http://

dx.doi.org/10.3748/wjg.v20.i27.8986

INTRODUCTION

Gastric cancer (GC) represents one of the most frequent cause of cancer death[1,2], although most of the mechanisms leading to its development have been clarified. *Helicobacter pylori* (*H. pylori*) infection, salted/smoked food consumption and E-cadherin mutations[2-4] are the main causes of stomach tumor, according to multifactoriality characterizing almost all neoplasms. Improving early diagnosis is one of the most yearned target, because of possible misunderstanding of first GC symptom. Therapies are based above all on surgery and, however, the use of drugs was recently supported by GC gene expression analysis, which led to performing target specific treatments[5,6]. Reflecting the need of a multidisciplinary approach, the (10th IGCC) predisposed several sessions in which the authors were allowed to present their results in a well targeted context. Discussed topics ranged from surgical techniques, to patient nutrition, to diagnosis and chemotherapy. We focused our attention on subjects related to possible diagnostic/prognostic factors and to molecular targeted therapies. As we report, together with novel findings about well characterized molecules, a role in GC development of markers involved in other neoplasms growth was also found. Moreover, discussed therapies provided interesting starting points, as the results obtained with natural extracts of *Capraria biflora* on a GC cell line and the treatment of a mouse model of peritoneal metastases with maraviroc, an Food and Drug Administration (FDA) approved drug used for human immunodeficiency virus (HIV) patients. Hence we can assert that the 10th IGCC gave an all-round view about GC, showing the most important trend in this neoplasm management.

NOVEL FINDINGS ABOUT E-CADHERIN

Many studies about E-cadherin (also known as CDH1) have been performed over the past several years. Its role in GC development is ascertained by now[7-9] and several germline mutations were effectively well characterized[7]. Yet, Sugimoto *et al*[10] reported the first case of a *de novo* large genomic deletion of *CDH1* associated with early-onset diffuse GC. The patient, with a deletion of the exon 11, was a 41-year-old man with no familial history of GC. His son was a carrier of the same deletion, hence according to authors' conclusions, CDH1 mutational status should be considered also in the absence of familial history of GC. Again, the investigation of CDH1 mutational status led also to decision of profilactic gastrectomy, as shown by Biffi *et al*[11]. Authors presented a case of 41-year-old female patient positive for germline CDH1 mutation, who had previously undergone surgical resection of a lobular breast cancer. The case reported by authors was the first Italian prophylactic surgical intervention, whereas in the United States this kind of radical management in carriers is usually performed[12-15].

MARKERS KNOWN IN OTHER CANCERS: POSSIBLE ROLE IN GC DIAGNOSIS AND PROGNOSIS

Nearby novel discoveries related to well-known markers, such as CDH1, novel potential diagnostic and prognostic tools were described.

Santos-Sousa *et al*[16] presented an emerging role for mesothelin, a glycosylphosphatidylinositol-anchored cell surface protein overexpressed both in mesothelioma[17-19] and in ovarian cancer[18-20]. They found that mesothelin expression in GC tissue specimens was correlated with tumor location, macroscopic appearance, Lauren histological classification and stage. Moreover, its cytoplasmic expression was correlated with lymphatic invasion and associated with poorer survival. In 2012, Baba *et al*[21] discussed the role of mesothelin in GC development and its possible usefulness as a prognostic factor. They found that patients positive for mesothelin expression in gastric tissues showed broader nodal involvement and deeper tumor invasion. Yet, when the analysis was limited to only advanced GC cases, a higher survival rate was found in mesothelin positive patients. Considering the papers of Santos-Sousa *et al*[16] and of Baba *et al*[21], it can be inferred that mesothelin is an independent prognostic factor of GC, as stressed from authors themselves. But the first authors showed its cytoplasmic placement as a key element in exerting prognostic role, whereas the second ones referred its expected cell membrane localization. Hence further studies are necessary to better answer questions about mesothelin expression and localization so as to improve our knowledge on its role in GC development.

Few contributions were presented about the role of cell cycle regulators in stomach tumor development. Very interesting were the results presented by Kim *et al*[22], which focused their attention on p16 protein, whose expression was found lost in other neoplasms[23-26]. The same result was obtained in intestinal histotype of GC from the analysis performed by the authors, who showed that loss of p16 expression was related to a higher rate of cancer recurrence and poorer 5-year disease-free survival. This finding led the authors to hypothesize a role of loss of p16 expression in GC development, which is similar to data observed in other cancers.

Stomatin-like protein 2 (SLP-2) is a protein belonging to the stomatin superfamily, which has been found overexpressed in several kind of tumors[27]. Its overexpression is generally associated with poor prognosis in esophageal squamous cell carcinoma, human gallbladder cancer and HER2 negative breast cancer[27-29]. Liu *et al*[30] confirmed SLP-2 as a prognostic tool to manage GC. High level of SLP-2 expression was significantly associated with the depth of invasion, lymph node and distant metastases, and tumor node metastasis (TNM) stage in

GC. Notch1 is another possible marker overexpressed in GC, as shown in two independent cohort studies performed by Chu *et al*[31]. Both of them showed that higher Notch1 expression was correlated with a shorter survival time, while lower Notch-1 expression was correlated with a better survival of GC patients. These results suggest that Notch1, whose prognostic role was found in other tumors[32,33], has a predictive role in clinical outcomes of GC patients too. Moreover, the authors highlighted the dependence of Notch1 prognostic value on p65 status, hypothesizing a role as a promising novel target for GC therapy.

An unexpected result was reported by Chen *et al*[34]. They found that CD44 positive expression in surgical specimens of primary GC was not correlated with clinicopathological features and survival outcomes. These data may be considered surprising because CD44 is a well-recognized tumor marker[35-37]. It may be possible that GC development is independent from CD44 expression levels, although it has to be mentioned that in 2013, a paper of Hirata *et al*[38] was published, in which the authors found a correlation between expression of a CD44 variant and GC recurrence.

Epigenetic control of DNA expression was often found pivotal in etiogenesis of various cancers, since it leads to gene silencing and therefore to loss of expression of oncosuppressors too[39-41]. Calcagno *et al*[42] investigated the expression levels of enzymes with methyltransferase activity, showing that they may exert an important role in GC development. They found high levels of DNMT1, DNMT3A and DNMT3B (DNA-methyl-transferase 1, 3A and 3B) expression in gastric adenocarcinoma tissues, when compared to normal specimens. However, they found no correlation between DNMT1, DNMT3A and DNMT3B overexpression and clinicopathological features, drawing the conclusion that the increased expression may be placed in the early stage GC development. Besides, the same authors investigated the effects of hypermethylation in GC cell lines[43]. They treated two gastric adenocarcinoma cell lines, ACP02 (diffuse-type) and ACP03 (intestinal-type), with a demethylating agent and evaluated gene expression compared to untreated cells. The genes neuritin 1 (NRN1) and tumor necrosis factor alpha-induced protein (TNFAIP) were found upregulated in both GC cell lines compared to controls, while metastasis associated lung adenocarcinoma transcript 1 (MALAT1) and small nucleolar RNA D (SNORD) were overexpressed only in intestinal-type GC cell line. They obtained these data as before by microarray assay and confirmed them by real-time PCR, finding new genes epigenetically altered in GC. Also, the overexpression of nonmuscle myosin IIA (NMIIA) may be associated with progression and poor prognosis of GC, as revealed by Liu *et al*[44], because high expression of this protein is significantly correlated with the depth of wall invasion, lymph node metastasis, distant metastasis and TNM stage. Another very interesting prognostic tool was presented by Choi[45], who found that in GC patients, the number of loss of eterozygosity (LOH) may be a determinant poor prognostic factor. The author analyzed LOH of 5 chromosomes having tumor suppressor genes such as p16, PTEN, Rb, E-cadherin and p53 in 100 surgically resected tumors. Patients with 2 or more LOHs displayed a poorer 5-year survival rate than those who had less than 2 LOHs. In particular, LOH in 17p13 (p53 locus) contributed to a lower survival rate. Therefore, the number and the type of LOH in GC may be useful prognostic indicators. A very innovative diagnostic tool was presented by Linē *et al*[46], who identified a tumor-associated autoantibody signature that can be used for the early detection of GC among high-risk individuals. The autoantibody production, which does not correlate with histotype, already occurs in early GC and it could be associated with shorter overall survival. *H. pylori* status, grade, localization and size of the primary tumor were not related to autoantibody signature. Diagnosis of GC at advanced stages is considered a major reason for lower five-year overall survival rate in developing countries[47]. Hence, early diagnosis of GC is fundamental for patient survival.

VASCULAR ENDOTHELIAL GROWTH FACTOR AS POSSIBLE GC MARKERS

VEGF (vascular endothelial growth factor) has been largely investigated because of its active role in angiogenesis. It was found that its overexpression is a poorer prognostic marker in various neoplasms such as osteosarcoma[48], non-small cell lung carcinoma[49] and melanoma[50].

Some authors analyzed the expression levels of VEGFs either alone, or together with other possible prognostic factors. Kruszyna *et al*[51] showed that VEGF, hypoxia inducible factor-1 (HIF-1) and CXC chemokine receptor 4 (CXCR4) were up-regulated in tumoral, but not in normal specimens. Von Hippel-Lindau tumor suppressor (VHL) and HIF-prolyl hydroxylase 2 (PHD2) were, instead, expressed at very low levels in tumor tissues. All these results were found related with malignant tumor progression and lymph node metastasis, drawing attention to the possibility of considering VEGF, CXCR4, VHL and PHD2 as prognostic markers of GC. Noteworthy were also the results obtained by Partika *et al*[52]. Although in few patients, they observed the absence of VEGF within the GC tissue despite its high plasma concentration. The authors hypothesized that VEGF in somehow was quickly eliminated into the blood stream. Hence, further investigations may be useful to cast light on the possibility to use plasma levels of VEGF as a biomarker. Finally, Yingwei *et al*[53] investigated the expression levels of VEGF, EGF and their receptors in GC cell lines of different biological properties and the outcomes of their targeted inhibition. They found that EGF, EGFR, VEGF and VEGFR mRNA expression increased sequentially in SGC7901, BGC823, HGC27 and MGC803 cell lines, allowing them to increase their proliferation, motility and adhesion. Therefore, spe-

cific inhibition of VEGF and EGF may impair cellular properties related to tumoral phenotype, representing a possible therapeutic strategy for GC. On the other hand, Donizy *et al*[54] have not found any clinical significance of VEGF-C, VEGF-D, VEGFR-3 expression in GC patients. The only statistically significant parameter which they found related to poor prognosis and shorter long-term survival was the lower level of matrix metalloproteinase-2 (MMP-2). Hence it can be deduced that, although there are some exceptions, VEGF pathways may be considered as possible prognostic tools and/or therapeutic targets.

MICROSATELLITES INSTABILITY

Interesting studies about prognostic significance of microsatellites instability (MSI) were also presented. Pascale *et al*[55] analyzed the differences in MSI between two groups of patients living in higher and lower Italian risk areas. The authors found that GC patients living in higher risk areas showed a higher rate of MSI than those in low-risk areas. These results stress the relationship among environment, genome and cancer, topic of investigations for a long time. Although not always demonstrated, it is undeniable that many authors contributed to reinforce the aforementioned relationship. The analysis performed by Pascale *et al*[55] is hence particularly interesting because it highlighted the clinical implications derived from possible impact of environment on human genome. Kim *et al*[56] presented similarly interesting results, related to role of MSI in GC medical evaluation. They studied the link between the MSI-high (MSI-H) and GC prognosis in patients who underwent gastrectomy. In few patients MSI-H was detected and there was no relationship with lymph node involvement. Yet, MSI-H correlated with a poorer prognosis than MSI-low (MSI-L) and microsatellite stable (MSS) context. It has to be noticed that in literature different results were reported too. Some authors showed that MSI-H was related to a better prognosis[57-59], whereas others reported no significant correlation between MSI and GC prognosis[60,61]. And in more recent reviews, the role exerted by MSI in GC development is discussed but not definitely clarified[62]. Hence it can be deduced that far from being well understood, the role of MSI in GC, although challenging cues were provided during the 10th IGCC, deserves further investigations in order to better clarify its role in GC development.

TREATMENT AND THERAPY OF GC

One of the most challenging topics of the 10th IGCC, representing also one of the most innovative section of 10th IGCC, was referred to biomolecular analysis of therapeutic management of GC. Various authors obtained promising results, identifying novel potential therapeutic tools that could have a future clinical application. Liu *et al*[63] identified a novel immunological method that can not only detect GC cells and but also inhibit migration and invasion. In particular, a functional monoclonal antibody (mAb) MS57-2.1 against novel antigenic markers on the gastric cancer cell surface, MS57A and MS57B, was generated. Both antigens are membrane bound glycosylated enzymes and belong to the alkaline phosphatase family[64]. MS57-2.1 mAb was produced by hybridoma method and it was able to bind specifically to GC cell membrane with high affinity. Through this binding, a cellular signal inhibiting tumor cell migration and invasion was found activated *in vitro* and tumor metastasis impairment was detected *in vivo*. Hence, MS57-2.1 mAb could represent an effective novel tool in GC therapy because it may help to impair progression of tumoral phenotype. Other authors tested the effect of drugs both *in vitro* and *in vivo* experiments. *In vitro*, Calcagno *et al*[65] evaluated the cytotoxic and genotoxic potential of E-2-Benzo[D]thiazol in normal and gastric tumor cells (ACP02 - diffuse-type gastric adenocarcinoma cell line). Their results showed DNA damage and apoptosis in tumor cells, without significant damage to lymphocytes. These findings suggest E-2-Benzo[D]thiazol as a potential drug to improve GC management. Protein kinase D (PKD) inhibitor CID755673 may be another anti-neoplastic treatment for GC, as shown by Tsuboi *et al*[66]. PKD regulates multiple normal and abnormal biological processes, including angiogenesis[67,68]. VEGF pathway seems to be an important driver of tumorigenesis in GC, as previously reported[69,70], also in a paragraph above. Analysis of mechanism of action of CID755673, performed in MKN45 cell line, showed inhibition of PKD phosphorylation, induced by phorbol myristate acetate, and of VEGF secretion levels in a dose-dependent manner. Hence, PKD inhibitors may contribute to angiogenesis regression in GC. In an *in vivo* model, Graziosi *et al*[71] studied the effect of maraviroc, a chemokine CCR5-receptor antagonist, in GC treatment. Maraviroc is the first member of a new class of antiretroviral drugs, whose mechanism of action is pivoted on blocking R5-tropic HIV entry into CD4 cells[72,73]. It was approved by United States FDA to be used, in combination with other antiretroviral agents, for treatment of patients carrying both drug-sensitive and -resistant HIV strains[74]. In cancer, metastasis prevention induced by maraviroc was observed in hepatocellular carcinoma[75] and basal breast cancer[76,77]. In their study, Graziosi *et al*[71] analyzed a mouse model of peritoneal carcinomatosis in which maraviroc reduced both GC cell dissemination and tumor growth. These findings provide evidences for an important role of CCR5 in cancer cell invasiveness, suggesting also a possible use of maraviroc as a further therapy to reduce the risk of metastasis in GC patients. Finally, among the various possible chemotherapeutic strategies, it has to be mentioned the potential efficacy of biflorin, a prenyl-ortonaftoquinone obtained from the roots of Capraria biflora L., in ACP02 cell line. Calcagno *et al*[78] reported that biflorin exerts anticancer activity; it inhibits both tumor cell line growth in culture and tumor development in mice[79,80]. In fact, biflorin showed a powerful cytotoxic effect *in vitro*, inhibiting cell

proliferation, migration and invasion. Moreover, after treatment, morphological analysis spotlighted cell death by necrosis. Results obtained by authors seemed to be focused on a possible reduction of MYC copy number in ACP02 and in the length of telomeres, to give a possible explanation for biflorin effects.

CONCLUSION

The 10th IGCC gave a complete overview about the state-of-the-art of stomach tumor management. Both in the basic research and in clinical activity, there has been a great knowledge improvement. In our opinion, original suggestions were particularly found in therapy and treatment sections. Treatment of GC with Maraviroc, generally used in HIV patients, may be a turning point, so as the promising possible use of biflorin. Yet, due to complexity of GC etiogenesis, further studies will be necessary and will have to be performed for a long period of time to reach the target of a gold standard therapy.

REFERENCES

1 **Hong L**, Han Y, Liu J, Brain L. Fibroblast growth factor receptor 2: a therapeutic target in gastric cancer. *Expert Rev Gastroenterol Hepatol* 2013; **7**: 759-765 [PMID: 24134151 DOI: 10.1586/17474124.2013.837804]

2 **Giordano A**, Cito L. Advances in gastric cancer prevention. *World J Clin Oncol* 2012; **3**: 128-136 [PMID: 23061031 DOI: 10.5306/wjco.v3.i9.128]

3 **Jakszyn P**, Gonzalez CA. Nitrosamine and related food intake and gastric and oesophageal cancer risk: a systematic review of the epidemiological evidence. *World J Gastroenterol* 2006; **12**: 4296-4303 [PMID: 16865769]

4 **Zagari RM**, Bazzoli F. Gastric cancer: who is at risk? *Dig Dis* 2004; **22**: 302-305 [PMID: 15812151 DOI: 10.1159/000083590]

5 **Zhang L**, Yang J, Cai J, Song X, Deng J, Huang X, Chen D, Yang M, Wery JP, Li S, Wu A, Li Z, Li Z, Liu Y, Chen Y, Li Q, Ji J. A subset of gastric cancers with EGFR amplification and overexpression respond to cetuximab therapy. *Sci Rep* 2013; **3**: 2992 [PMID: 24141978 DOI: 10.1038/srep02992]

6 **Teng L**, Lu J. cMET as a potential therapeutic target in gastric cancer (Review). *Int J Mol Med* 2013; **32**: 1247-1254 [PMID: 24141315 DOI: 10.3892/ijmm.2013.1531]

7 **Guilford P**, Hopkins J, Harraway J, McLeod M, McLeod N, Harawira P, Taite H, Scoular R, Miller A, Reeve AE. E-cadherin germline mutations in familial gastric cancer. *Nature* 1998; **392**: 402-405 [PMID: 9537325]

8 **Gabbert HE**, Mueller W, Schneiders A, Meier S, Moll R, Birchmeier W, Hommel G. Prognostic value of E-cadherin expression in 413 gastric carcinomas. *Int J Cancer* 1996; **69**: 184-189 [PMID: 8682585]

9 **Wei D**, Gong W, Oh SC, Li Q, Kim WD, Wang L, Le X, Yao J, Wu TT, Huang S, Xie K. Loss of RUNX3 expression significantly affects the clinical outcome of gastric cancer patients and its restoration causes drastic suppression of tumor growth and metastasis. *Cancer Res* 2005; **65**: 4809-4816 [PMID: 15930301 DOI: 10.1158/0008-5472.CAN-04-3741]

10 **Sugimoto S**, Yamada H, Takahashi M, Morohoshi Y, Yamaguchi N, Tsunoda Y, Hayashi H, Sugimura H, Komatsu H. Early-onset diffuse gastric cancer associated with a de novo large genomic deletion of CDH1 gene. *Gastric Cancer* 2013; Epub ahead of print [PMID: 23812922 DOI: 10.1007/s10120-013-0278-2]

11 **Biffi R**, Pozzi S, Feroce I, Serrano D, Trovato C, Sonzogni A, Sabbatini A R, Didier F, Galimberti V, Corso G, Cossu M L, Bonanni B. Total prophylactic gastrectomy for HDGC (Hereditary Diffuse Gastric Cancer) syndrome caused by a mutation in the E-cadherin (CDH1) gene: the first Italian case. Available from: URL: http://www.10igcc.com

12 **Li J**, McBean E, Li X, Berho M, Szomstein S, Rosenthal RJ. Laparoscopic prophylactic total gastrectomy with linear stapler side-to-side esophagojejunal anastomosis for hereditary diffuse gastric cancer syndrome in 2 siblings. *Surg Laparosc Endosc Percutan Tech* 2013; **23**: e124-e126 [PMID: 23752020 DOI: 10.1097/SLE.0b013e3182773e38]

13 **Hebbard PC**, Macmillan A, Huntsman D, Kaurah P, Carneiro F, Wen X, Kwan A, Boone D, Bursey F, Green J, Fernandez B, Fontaine D, Wirtzfeld DA. Prophylactic total gastrectomy (PTG) for hereditary diffuse gastric cancer (HDGC): the Newfoundland experience with 23 patients. *Ann Surg Oncol* 2009; **16**: 1890-1895 [PMID: 19408054 DOI: 10.1245/s10434-009-0471-z]

14 **Pandalai PK**, Lauwers GY, Chung DC, Patel D, Yoon SS. Prophylactic total gastrectomy for individuals with germline CDH1 mutation. *Surgery* 2011; **149**: 347-355 [PMID: 20719348 DOI: 10.1016/j.surg.2010.07.005]

15 **Newman EA**, Mulholland MW. Prophylactic gastrectomy for hereditary diffuse gastric cancer syndrome. *J Am Coll Surg* 2006; **202**: 612-617 [PMID: 16571431 DOI: 10.1016/j.jamcollsurg.2005.12.017]

16 **Santos-Sousa H**, Marcos-Silva L, Costa-Maia J, Pinto-De-Sousa J, David L. Mesothelin expression and its clinicopathological and prognostic significance in gastric and gastroesophageal junction cancer. Available from: URL: http://www.10igcc.com

17 **Luo L**, Shi HZ, Liang QL, Jiang J, Qin SM, Deng JM. Diagnostic value of soluble mesothelin-related peptides for malignant mesothelioma: a meta-analysis. *Respir Med* 2010; **104**: 149-156 [PMID: 19945835 DOI: 10.1016/j.rmed.2009.05.017]

18 **Ho M**, Hassan R, Zhang J, Wang QC, Onda M, Bera T, Pastan I. Humoral immune response to mesothelin in mesothelioma and ovarian cancer patients. *Clin Cancer Res* 2005; **11**: 3814-3820 [PMID: 15897581 DOI: 10.1158/1078-0432.CCR-04-2304]

19 **Hassan R**, Bera T, Pastan I. Mesothelin: a new target for immunotherapy. *Clin Cancer Res* 2004; **10**: 3937-3942 [PMID: 15217923 DOI: 10.1158/1078-0432.CCR-03-0801]

20 **Chang MC**, Chen CA, Chen PJ, Chiang YC, Chen YL, Mao TL, Lin HW, Lin Chiang WH, Cheng WF. Mesothelin enhances invasion of ovarian cancer by inducing MMP-7 through MAPK/ERK and JNK pathways. *Biochem J* 2012; **442**: 293-302 [PMID: 21999204 DOI: 10.1042/BJ20110282]

21 **Baba K**, Ishigami S, Arigami T, Uenosono Y, Okumura H, Matsumoto M, Kurahara H, Uchikado Y, Kita Y, Kijima Y, Kitazono M, Shinchi H, Ueno S, Natsugoe S. Mesothelin expression correlates with prolonged patient survival in gastric cancer. *J Surg Oncol* 2012; **105**: 195-199 [PMID: 21780126 DOI: 10.1002/jso.22024]

22 **Kim J**, Cho S, Jang Y, Park S, Park S, Kim S, Mok Y, Kim C. The prognostic significance of p16 gene protein loss in intestinal type gastric cancer. Available from: URL: http://www.10igcc.com

23 **Tam KW**, Zhang W, Soh J, Stastny V, Chen M, Sun H, Thu K, Rios JJ, Yang C, Marconett CN, Selamat SA, Laird-Offringa IA, Taguchi A, Hanash S, Shames D, Ma X, Zhang MQ, Lam WL, Gazdar A. CDKN2A/p16 inactivation mechanisms and their relationship to smoke exposure and molecular features in non-small-cell lung cancer. *J Thorac Oncol* 2013; **8**: 1378-1388 [PMID: 24077454 DOI: 10.1097/JTO.0b013e3182a46c0c]

24 **Matsuda Y**, Ichida T, Matsuzawa J, Sugimura K, Asakura H. p16(INK4) is inactivated by extensive CpG methylation in human hepatocellular carcinoma. *Gastroenterology* 1999; **116**: 394-400 [PMID: 9922321]

25 **Emig R**, Magener A, Ehemann V, Meyer A, Stilgenbauer F,

Volkmann M, Wallwiener D, Sinn HP. Aberrant cytoplasmic expression of the p16 protein in breast cancer is associated with accelerated tumour proliferation. *Br J Cancer* 1998; **78**: 1661-1668 [PMID: 9862580]
26 **Caldas C**, Hahn SA, da Costa LT, Redston MS, Schutte M, Seymour AB, Weinstein CL, Hruban RH, Yeo CJ, Kern SE. Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. *Nat Genet* 1994; **8**: 27-32 [PMID: 7726912 DOI: 10.1038/ng0994-27]
27 **Cao W**, Zhang B, Ding F, Zhang W, Sun B, Liu Z. Expression of SLP-2 was associated with invasion of esophageal squamous cell carcinoma. *PLoS One* 2013; **8**: e63890 [PMID: 23667687 DOI: 10.1371/journal.pone.0063890]
28 **Wang WX**, Lin QF, Shen D, Liu SP, Mao WD, Ma G, Qi WD. Clinicopathological significance of SLP-2 overexpression in human gallbladder cancer. *Tumour Biol* 2014; **35**: 419-423 [PMID: 23918306 DOI: 10.1007/s13277-013-1058-7]
29 **Cao W**, Zhang B, Li J, Liu Y, Liu Z, Sun B. SLP-2 overexpression could serve as a prognostic factor in node positive and HER2 negative breast cancer. *Pathology* 2011; **43**: 713-718 [PMID: 22081131 DOI: 10.1097/PAT.0b013e32834c34ed]
30 **Liu D**, Zhang L, Shen Z, Tan F, Hu Y, Yu J, Li G. Increased levels of SLP-2 correlate with poor prognosis in gastric cancer. *Gastric Cancer* 2013; **16**: 498-504 [PMID: 23371255 DOI: 10.1007/s10120-013-0232-3]
31 **Chu D**, Chu D, Wang W. Notch1 expression, which is related to p65 status, is an independent predictor of prognosis in gastric cancer. Available from: URL: http://www.10igcc.com
32 **Ahn S**, Hyeon J, Park CK. Notch1 and Notch4 are markers for poor prognosis of hepatocellular carcinoma. *Hepatobiliary Pancreat Dis Int* 2013; **12**: 286-294 [PMID: 23742774 DOI: 10.1016/S1499-3872(13)60046-6]
33 **Willander K**, Dutta RK, Ungerbäck J, Gunnarsson R, Juliusson G, Fredrikson M, Linderholm M, Söderkvist P. NOTCH1 mutations influence survival in chronic lymphocytic leukemia patients. *BMC Cancer* 2013; **13**: 274 [PMID: 23734977 DOI: 10.1186/1471-2407-13-274]
34 **Chen X**, Wen L, Wang F, Yang K, Zhang B, Chen Z, Chen J, Mo X, Zhou Z, Hu J. Protein, mRNA expressions and prognostic significance of CD44 in human gastric adenocarcinoma. Available from: URL: http://www.10igcc.com
35 **Sheridan C**, Kishimoto H, Fuchs RK, Mehrotra S, Bhat-Nakshatri P, Turner CH, Goulet R, Badve S, Nakshatri H. CD44+/CD24- breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. *Breast Cancer Res* 2006; **8**: R59 [PMID: 17062128 DOI: 10.1186/bcr1610]
36 **Misra S**, Hascall VC, Berger FG, Markwald RR, Ghatak S. Hyaluronan, CD44, and cyclooxygenase-2 in colon cancer. *Connect Tissue Res* 2008; **49**: 219-224 [PMID: 18661347 DOI: 10.1080/03008200802143356]
37 **Mărgăritescu C**, Pirici D, Simionescu C, Stepan A. The utility of CD44, CD117 and CD133 in identification of cancer stem cells (CSC) in oral squamous cell carcinomas (OSCC). *Rom J Morphol Embryol* 2011; **52**: 985-993 [PMID: 22119814]
38 **Hirata K**, Suzuki H, Imaeda H, Matsuzaki J, Tsugawa H, Nagano O, Asakura K, Saya H, Hibi T. CD44 variant 9 expression in primary early gastric cancer as a predictive marker for recurrence. *Br J Cancer* 2013; **109**: 379-386 [PMID: 23778530 DOI: 10.1038/bjc.2013.314]
39 **Bosviel R**, Garcia S, Lavediaux G, Michard E, Dravers M, Kwiatkowski F, Bignon YJ, Bernard-Gallon DJ. BRCA1 promoter methylation in peripheral blood DNA was identified in sporadic breast cancer and controls. *Cancer Epidemiol* 2012; **36**: e177-e182 [PMID: 22402307 DOI: 10.1016/j.canep.2012.02.001]
40 **Tommasi S**, Pinto R, Petriella D, Pilato B, Lacalamita R, Santini D, Zito F, Colucci G, Paradiso A, Silvestris N. Onco-suppressor methylation: a possible key role in colon metastatic progression. *J Cell Physiol* 2011; **226**: 1934-1939 [PMID: 21506124 DOI: 10.1002/jcp.22524]
41 **Li L**, Shen Y, Wang M, Tang D, Luo Y, Jiao W, Wang Z, Yang R, Tian K. Identification of the methylation of p14ARF promoter as a novel non-invasive biomarker for early detection of lung cancer. *Clin Transl Oncol* 2014; **16**: 581-589 [PMID: 24154929 DOI: 10.1007/s12094-013-1122-1]
42 **Calcagno D**, Calcagno D, Wisnieski F, Chen ES, Gigek CO, Santos L, Leal MF, Pontes TB, Demachki S, Assumpção PP, Lourenço LG, Burbano R, Smith MDAC. Clinical significance of the DNA methyltranferase mRNA expression in gastric adenocarcinoma. Available from: URL: http://www.10igcc.com
43 **Calcagno D**, Calcagno DQ, Wisnieski F, Chen ES, Gigek CO, Leal MF, Pontes TB, Demachki S, Ricardo A, Assumpção PP, Lourenço LG, Burbano RR, Smith MDAC. Identification of genes epigenetically silenced in human gastric adenicarcinoma cells. Available from: URL: http://www.10igcc.com
44 **Liu D**, Zhang L, Shen Z, Tan F, Hu Y, Yu J, Li G. Clinicopathological significance of NMIIA Overexpression in Human Gastric Cancer. *Int J Mol Sci* 2012; **13**: 15291-15304 [PMID: 23203126 DOI: 10.3390/ijms131115291]
45 **Choi W**. The significance of the number of LOH in major tumor suppressor genes as a prognostic factor. Available from: URL: http://www.10igcc.com
46 **Linē A**, Zayakin P, Ancāns G, Siliņa K, Meistere I, Kalniņa Z, Andrejeva D, Endzeliņš E, Ivanova L, Pismennaja A, Ruskule A, Doniņa S, Wex T, Malfertheiner P, Leja M. Identification of tumor-associated autoantibody signature for the early detection of gastric cancer. Available from: URL: http://www.10igcc.com
47 **Ali Z**, Deng Y, Ma C. Progress of research in gastric cancer. *J Nanosci Nanotechnol* 2012; **12**: 8241-8248 [PMID: 23421202]
48 **Chen D**, Zhang YJ, Zhu KW, Wang WC. A systematic review of vascular endothelial growth factor expression as a biomarker of prognosis in patients with osteosarcoma. *Tumour Biol* 2013; **34**: 1895-1899 [PMID: 23589053 DOI: 10.1007/s13277-013-0733-z]
49 **Fontanini G**, Vignati S, Boldrini L, Chinè S, Silvestri V, Lucchi M, Mussi A, Angeletti CA, Bevilacqua G. Vascular endothelial growth factor is associated with neovascularization and influences progression of non-small cell lung carcinoma. *Clin Cancer Res* 1997; **3**: 861-865 [PMID: 9815760]
50 **Claffey KP**, Brown LF, del Aguila LF, Tognazzi K, Yeo KT, Manseau EJ, Dvorak HF. Expression of vascular permeability factor/vascular endothelial growth factor by melanoma cells increases tumor growth, angiogenesis, and experimental metastasis. *Cancer Res* 1996; **56**: 172-181 [PMID: 8548760]
51 **Kruszyna L**, Murawa D, Murawa P, Jacodzinski P, Oszkinis G. Expression and clinical significance of genes responsible for angiogenesis and progression in gastric cancer. Available from: URL: http://www.10igcc.com
52 **Partika R**, Donocik J, Kokocinska D, Jalowiecki P. Evaluation of concentration of VEGF and EGF on biological properties of gastric cancer cells. Available from: URL: http://www.10igcc.com
53 **Yingwei P**, Chunfeng L. Experimental study on the effects of VEGF and EGF on biological properties of gastric cancer cells. Available from: URL: http://www.10igcc.com
54 **Donizy P**, Donizy P, Matkowski R, Rudno-Rudzinska J, Frejlinch E, Kabarowski J, Kelan W, Halon A. Clinical significance of gastric cancer immunoprofile related to angiogenesis (VEGF-C, VEGF-D, VEGFR3) and enzymatic degradation of extracellular matrix (MMP-2,9/TIMP-1,2). Available from: URL: http://www.10igcc.com
55 **Pascale V**, Marrelli D, Garosi L, Corso G, Flauti G, Caputo E, Roviello F. Different frequency of microsatellite instability (MSI) in patients coming from high-risk and low-risk areas of Italy: clinico-pathological implications. Available from:

URL: http://www.10igcc.com
56 **Kim SY**, Choi YY, An JY, Kwon IG, Cho I, Kim H, Cheong J, Hyung WJ, Noh SH. MSI-H related to worse prognosis in T4N0 gastric cancer patients. Available from: URL: http://www.10igcc.com
57 **Lee HS**, Choi SI, Lee HK, Kim HS, Yang HK, Kang GH, Kim YI, Lee BL, Kim WH. Distinct clinical features and outcomes of gastric cancers with microsatellite instability. *Mod Pathol* 2002; **15**: 632-640 [PMID: 12065777]
58 **Beghelli S**, de Manzoni G, Barbi S, Tomezzoli A, Roviello F, Di Gregorio C, Vindigni C, Bortesi L, Parisi A, Saragoni L, Scarpa A, Moore PS. Microsatellite instability in gastric cancer is associated with better prognosis in only stage II cancers. *Surgery* 2006; **139**: 347-356 [PMID: 16546499 DOI: 10.1016/j.surg.2005.08.021]
59 **Falchetti M**, Saieva C, Lupi R, Masala G, Rizzolo P, Zanna I, Ceccarelli K, Sera F, Mariani-Costantini R, Nesi G, Palli D, Ottini L. Gastric cancer with high-level microsatellite instability: target gene mutations, clinicopathologic features, and long-term survival. *Hum Pathol* 2008; **39**: 925-932 [PMID: 18440592 DOI: 10.1016/j.humpath.2007.10.024]
60 **Oki E**, Kakeji Y, Zhao Y, Yoshida R, Ando K, Masuda T, Ohgaki K, Morita M, Maehara Y. Chemosensitivity and survival in gastric cancer patients with microsatellite instability. *Ann Surg Oncol* 2009; **16**: 2510-2515 [PMID: 19565284 DOI: 10.1245/s10434-009-0580-8]
61 **An JY**, Kim H, Cheong JH, Hyung WJ, Kim H, Noh SH. Microsatellite instability in sporadic gastric cancer: its prognostic role and guidance for 5-FU based chemotherapy after R0 resection. *Int J Cancer* 2012; **131**: 505-511 [PMID: 21898388 DOI: 10.1002/ijc.26399]
62 **Hudler P**. Genetic aspects of gastric cancer instability. *ScientificWorldJournal* 2012; **2012**: 761909 [PMID: 22606061 DOI: 10.1100/2012/761909]
63 **Liu B**, Gao J, Chen X, Li J, Lu M, Qu Y, Zhu Z. Inhibition gastric cancer cell migration and invasion by monoclonal antibody MS57-2.1. Available from: URL: http://www.10igcc.com
64 **Gao J**, Qu Y, Li M, Zhou Y, Wang L, Lu M, Tao S, Liu B, Zhu Z. Cell membrane protein MS57A and MS57B are novel markers on gastric cancer cell lines and potential therapeutic targets of gastric cancer: Expressed on the gastric cancer cell surface defined by MS57-2.1 mAb. *Transl Gastrointest Cancer* 2012; **1** (Suppl 1)
65 **Calcagno D**, Soares BM, Barreto LDH, Ramos JAB, De Almeida SB, Pinto LC, Ferreira VF, Nogueira AF, Azevedo EC, Calcagno DQ, Burbano RR, Vasconcelos T, De Vasconcellos MC, Montenegro RC. Cytotoxic and genotoxic potential of E-2-Benzo [D] Thiazol in normal and gstric tumor cells. Available from: URL: http://www.10igcc.com
66 **Tsuboi K**, Matsuo Y, Shamoto T, Shibata T, Wakasugi T, Funahashi H, Kimura M, Takeyama H. PKD regulates VEGF secretion in gastric cancer. Available from: URL: http://www.10igcc.com
67 **Ellwanger K**, Hausser A. Physiological functions of protein kinase D in vivo. *IUBMB Life* 2013; **65**: 98-107 [PMID: 23288632 DOI: 10.1002/iub.1116]
68 **Rozengurt E**. Protein kinase D signaling: multiple biological functions in health and disease. *Physiology* (Bethesda) 2011; **26**: 23-33 [PMID: 21357900 DOI: 10.1152/physiol.00037.2010]
69 **Ilson DH**. Angiogenesis in gastric cancer: hitting the target? *Lancet* 2014; **383**: 4-6 [PMID: 24094766 DOI: 10.1016/S0140-6736(13)61892-9]
70 **Scartozzi M**, Giampieri R, Loretelli C, Bittoni A, Mandolesi A, Faloppi L, Bianconi M, Del Prete M, Andrikou K, Bearzi I, Cascinu S. Tumor angiogenesis genotyping and efficacy of first-line chemotherapy in metastatic gastric cancer patients. *Pharmacogenomics* 2013; **14**: 1991-1998 [PMID: 24090479 DOI: 10.2217/pgs.13.185]
71 **Graziosi L**, Marino E, Renga B, Santorelli C, Cavazzoni E, Fiorucci S, Donini A. Effect of the CCR5 antagonist maraviroc in reducing gastric cancer dissemination in rodent models of peritoneal metastasis. Available from: URL: http://www.10igcc.com
72 **Lieberman-Blum SS**, Fung HB, Bandres JC. Maraviroc: a CCR5-receptor antagonist for the treatment of HIV-1 infection. *Clin Ther* 2008; **30**: 1228-1250 [PMID: 18691983 DOI: 10.1016/S0149-2918(08)80048-3]
73 **Sayana S**, Khanlou H. Maraviroc: a new CCR5 antagonist. *Expert Rev Anti Infect Ther* 2009; **7**: 9-19 [PMID: 19622053 DOI: 10.1586/14787210.7.1.9]
74 **Brown KC**, Paul S, Kashuba AD. Drug interactions with new and investigational antiretrovirals. *Clin Pharmacokinet* 2009; **48**: 211-241 [PMID: 19492868 DOI: 10.2165/00003088-200948040-00001]
75 **Ochoa-Callejero L**, Pérez-Martínez L, Rubio-Mediavilla S, Oteo JA, Martínez A, Blanco JR. Maraviroc, a CCR5 antagonist, prevents development of hepatocellular carcinoma in a mouse model. *PLoS One* 2013; **8**: e53992 [PMID: 23326556 DOI: 10.1371/journal.pone.0053992]
76 **Velasco-Velázquez M**, Jiao X, De La Fuente M, Pestell TG, Ertel A, Lisanti MP, Pestell RG. CCR5 antagonist blocks metastasis of basal breast cancer cells. *Cancer Res* 2012; **72**: 3839-3850 [PMID: 22637726 DOI: 10.1158/0008-5472.CAN-11-3917]
77 **Velasco-Velázquez M**, Pestell RG. The CCL5/CCR5 axis promotes metastasis in basal breast cancer. *Oncoimmunology* 2013; **2**: e23660 [PMID: 23734321 DOI: 10.4161/onci.23660]
78 **Calcagno D**, Barbosa GS, Ralph ACL, Gregòrio L, Lemos TLG, Burbano RR, Calcagno DQ, Smith MDAC, Montenegro RC, Vasconcellos MC. Anticancer activity of biflorin on gastric cancer cell line. Available from: URL: http://www.10igcc.com
79 **Vasconcellos MC**, Bezerra DP, Fonseca AM, Araújo AJ, Pessoa C, Lemos TL, Costa-Lotufo LV, de Moraes MO, Montenegro RC. The in-vitro and in-vivo inhibitory activity of biflorin in melanoma. *Melanoma Res* 2011; **21**: 106-114 [PMID: 21346641 DOI: 10.1097/CMR.0b013e328343ecc4]
80 **Vasconcellos MC**, Bezerra DP, Fonseca AM, Pereira MR, Lemos TL, Pessoa OD, Pessoa C, Moraes MO, Alves AP, Costa-Lotufo LV. Antitumor activity of biflorin, an o-naphthoquinone isolated from Capraria biflora. *Biol Pharm Bull* 2007; **30**: 1416-1421 [PMID: 17666796 DOI: 10.1248/bpb.30.1416]

P- Reviewers: Chen JX, Fassan M, Fujiwara T, Lin ZH, Shimi SM, Wang XL, Zheng XF
S- Editor: Wen LL **L- Editor**: Wang TQ **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8993

World J Gastroenterol 2014 July 21; 20(27): 8993-8997
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

De-escalating therapy in gastric aggressive lymphoma

Rosanna Cuccurullo, Silvia Govi, Andrés JM Ferreri

Rosanna Cuccurullo, Silvia Govi, Andrés JM Ferreri, Unit of Lymphoid Malignancies, Division of Onco-Hematological Medicine, Department of Onco-Hematology, San Raffaele Scientific Institute, Milan, Italy

Author contributions: Cuccurullo R, Govi S and Ferreri AJM performed literature revision, critically analyzed reported studies and wrote the manuscript.

Correspondence to: Andrés JM Ferreri, MD, Unit of Lymphoid Malignancies, Division of OncoHematological Medicine, Department of OncoHematology, San Raffaele Scientific Institute, Via Olgettina 60, 20132 Milan, Italy. ferreri.andres@hsr.it
Telephone: +39-2-26437649 Fax: +39-2-26437625
Received: November 29, 2014 Revised: January 14, 2014
Accepted: March 12, 2014
Published online: July 21, 2014

Abstract

The treatment of primary gastric diffuse large B-cell lymphoma (DLBCL) has changed radically over the last 10–15 years, with the abandonment of routine gastrectomy in favor of more conservative therapies. Low-level evidence suggests that consolidation radiotherapy could be avoided in patients with limited-stage DLBCL of the stomach who achieve complete remission after rituximab-CHOP combination. Small, recent prospective trials suggest that selected patients with limited-stage *Helicobacter pylori* (*H. pylori*)-positive DLBCL of the stomach and favorable prognostic factors can be managed with antibiotics alone, with excellent disease control and cure rates, keeping chemo-radiotherapy for unresponsive patients. This recommendation should equally regard patients with mucosa-associated lymphoid tissue-related or *de novo* DLBCL. Future studies should be focused on the establishment of reliable variables able to distinguish the best candidates for exclusive treatment with *H. pylori* eradication from those who need for conventional chemo-immunotherapy.

Key words: Diffuse large B-cell lymphoma; Gastric lymphoma; *Helicobacter pylori*; Antibiotic therapy; Rituximab

Core tip: Therapeutic intensity has been progressively reduced in patients with limited-stage diffuse large B-cell lymphoma of the stomach, with a consequent improvement in tolerability and quality of life, and with unimpaired survival figures. In particular, patients with *Helicobacter pylori* (*H. pylori*)-positive lymphoma and favourable prognostic factors can be managed with antibiotics alone, with excellent disease control and cure rates, keeping chemo-radiotherapy for unresponsive patients. Future studies should be focused on the establishment of reliable variables able to distinguish the best candidates for exclusive treatment with *H. pylori* eradication from those who need for conventional therapy.

Cuccurullo R, Govi S, Ferreri AJM. De-escalating therapy in gastric aggressive lymphoma. *World J Gastroenterol* 2014; 20(27): 8993-8997 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8993.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8993

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoma category arising in the stomach, representing 5% of all gastric malignancies. DLBCL usually arises as a primary form, which is a tumor limited to the gastric wall, with or without involvement of perigastric lymph nodes (stage IE-IIE). The treatment of primary gastric DLBCL (PG-DLBCL) has greatly evolved in the last decades, mostly due to the development of new chemoimmunotherapy combinations, progress in radiation technology, improvement of sensitivity of procedures used in staging and response assessment and expansion of etiopathogenic knowledge. In particular, gastrectomy-based strategies have been abandoned, the role of radiation therapy has been downsized, whereas indications of

chemoimmunotherapy and antibiotic therapy have been extended, resulting in important organ-salvage benefits and iatrogenic toxicity reduction without survival impairment. However, the level of evidence supporting therapeutic choices is still low since available literature is mostly constituted by retrospective analyses of small and heterogeneous series, whereas only a few prospective trials with completed accrual are available. This review travels through the changes in the therapeutic management of patients with PG-DLBCL introduced in the last decade, defines the impact of each treatment component and critically analyzes the supporting evidence.

FROM GASTRECTOMY TO CONSERVATIVE CHEMO-RADIOTHERAPY

For several years, surgery played a central role in diagnosis, staging and treatment of PG-DLBCL. Its goal in these tumors has gradually changed from curative to staging and palliation, with less concern for radicality. Gastrectomy, once considered essential to diagnose gastric lymphomas, today has been replaced by modern endoscopy procedures through which multiple biopsies of gastric mucosa allow an accurate histopathological diagnosis. Also computer tomography-scan and ^{18}Fluorodeoxyglucose positron emission tomography (^{18}FDG-PET) are important to establish the disease's anatomical extension as this is a crucial prognostic factor, reducing the role of surgery as staging procedure.

Large retrospective studies published more than 15 years ago have suggested that patients with PG-DLBCL could be managed with an organ-sparing strategy based on a combination of anthracycline-based polychemotherapy and consolidative irradiation of the stomach and perigastric lymph-nodes[1]. These studies have suggested that the extent of surgery (excision or biopsy) has no impact on outcome of PG-DLBCL and that patients' quality of life after conservative nonsurgical treatment is remarkably better than after gastrectomy, with a reduced risk of severe malabsorption syndrome, vitamin deficits, anemia, dumping syndrome, secondary nutritional depletion and infections among others.

These preliminary observations have been confirmed by a large controlled clinical trial, where 589 patients with newly diagnosed PG-DLBCL have been randomly allocated among gastrectomy alone, gastrectomy plus radiotherapy, gastrectomy plus chemotherapy, and chemotherapy alone, with a 10-year overall survival (OS) of 54%, 53%, 91%, and 96% ($P < 0.001$), respectively[2]. Late toxicity has been more frequent and severe in patients who undergoing gastrectomy, with more cases of lethal complications. Lymphoma progression has been significantly less common among patients treated with chemotherapy, with rare cases of perforation, obstruction and hemorrhage in patients managed with chemotherapy alone. Accordingly, this trial has demonstrated that patients with PG-DLBCL must be managed with anthracycline-based chemotherapy, and that the addition of primary gastrectomy results in impaired survival figures due to significantly higher complications rates[2].

These results are in line with a retrospective comparison of two small prospective trials performed between 1988 and 1996[3]. The first one has been a Groupe d' étudé des Lymphomes Digestifs (GELD) trial addressing primary gastrectomy followed by chemotherapy in 48 patients with PG-DLBCL; the second one has been a Groupe d'étudé des Lymphomes de l'Adulte trial addressing anthracycline-based chemotherapy as exclusive treatment. Patient characteristics distribution has been similar between two series, with the exception of higher rates of increased serum lactate dehydrogenase levels and large tumors in GELD series. Comparison has been limited to patients with International Prognostic Index of 0-1. After a median follow-up of 59 mo (range 3-128), the 5-year OS has been 91% for both subgroups, suggesting that gastrectomy is superfluous in patients with low-risk PG-DLBCL[3]. Thereafter, the role of gastrectomy as part of first-line treatment of PG-DLBCL has progressively declined. Presently, surgical approach remains confined to resolution of chemotherapy-related complications, like bleeding or perforation, which affect < 1% of PG-DLBCL patients managed with upfront chemotherapy[2].

FROM CONSERVATIVE CHEMORADIOTHERAPY TO CHEMO (IMMUNO) THERAPY ALONE

Rituximab is a chimeric anti-CD20 IgG1 monoclonal antibody that has drastically changed the natural history and therapeutic approaches to DLBCL patients[4]. In the pre-rituximab era, CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) chemotherapy followed by involved-field radiotherapy (IF-RT) was the standard of care for limited-stage DLBCL and, consequently, for patients with PG-DLBCL[5]. However, large randomized trials failed to demonstrate a survival benefit with the addition of IF-RT after anthracycline-based polychemotherapy in DLBCL patients (reviewed in[4]), and the major concerns of secondary radio-induced malignancies and quality-of-life impairment in patients with a high cure rate led physicians to avoid consolidative radiotherapy in these patients, even in PG-DLBCL. In a small, pre-rituximab comparative trial[6], patients with PG-DLBCL who achieved a complete remission after four courses of anthracycline-based chemotherapy were randomly allocated between other two chemotherapy courses or IF-RT 30 Gy. The addition of IF-RT did not increase iatrogenic toxicity, but did not modify survival figures, with a 5-year OS of 82% for both arms. However, induction chemotherapy was heterogeneous and the trial was undersized for comparison since it was prematurely closed, with a consequent small number of patient accrued and scarce events.

In the rituximab era, the role of consolidation radio-

therapy as part of first-line treatment in patients with limited-stage DLBCL is still matter of debate. A worldwide use of rituximab is associated with a better quality of response, and a concomitant development of metabolic tools, like ^{18}FDG-PET, allowed a better definition of extension of disease and therapeutic response. Randomized trials assessing the role on consolidation IF-RT in DLBCL patients do not exist in the rituximab era, but large retrospective studies seem to suggest that IF-RT is unnecessary in DLBCL patients in complete remission after rituximab-CHOP (R-CHOP chemoimmunotherapy)[4]. In a retrospective Japanese study focused on PG-DLBCL[7], 23 patients have been treated with six courses of R-CHOP and 35 have been managed with 3-4 courses of R-CHOP plus radiotherapy, with a 3-year OS of 91% and 95% (P = 0.27), respectively. With all the limitations of a retrospective study, these results support the use of six cycles of R-CHOP without IF-RT as first-choice treatment option for PG-DLBCL patients[7]. Presently, the effect of radiation therapy on both carcinogenesis and quality-of-life impairment remains matter of debate. In fact, second cancers seem to be related to underlying susceptibility rather than radiation consequence[8], and the use of techniques of highly conformal irradiation is associated with improved tolerability. Nevertheless, available literature seems to suggest that consolidative radiotherapy is unnecessary in patients with newly diagnosed PG-DLBCL in complete remission after R-CHOP therapy.

FROM CHEMOIMMUNOTHERAPY TO *HELICOBACTER PYLORI*-ERADICATING ANTIBIOTIC THERAPY

Half of PG-DLBCL is associated with concomitant areas of mucosa-associated lymphoid tissue (MALT) lymphoma[9-11]. MALT lymphomas constitute an heterogeneous group of indolent malignancies usually arising from sites of infection, chronic irritation and inflammation, where tumor microenvironment, mostly orchestrated by inflammatory cells, is an unavoidable player in the neoplastic process[12]. In the stomach, *Helicobacter pylori* (*H. pylori*), a member of the superfamily VI of Gram-negative bacilli, now called Epsilonproteobacteria, classified as type I carcinogen by the International Agency of Research against Cancer, plays a central role in chronic inflammation, immune system inhibition and related lymphomagenesis[13]. In collaboration with host factors, some *H. pylori* colonization/virulence factors contribute to carcinogenesis, which is favoured by the concomitance of particular genotypes of both pathogen and host. *H. pylori*-related gastric MALT lymphomagenesis is a multi-step process initiated by infection and followed by chronic gastritis, MALT acquisition and, eventually, lymphoma development[14]. Under the antigenic stimulation of *H. pylori*, and thanks to the influence of products from some genetic abnormalities, autoreactive B lymphocytes evolve to an antigen-dependent MALT lymphoma. Some other genetic abnormalities like t(1:14), bcl-10 mutation and trisomies 3, 12 and 18 as well as the effect of DNA-damaging reactive oxygen species produced by neutrophils favour the loss of antigenic dependence[12]. Eventually, other putative karyotype damages related to t(1:14), p15, *Rb, myc*, as well as to p53 inativation, and p16 deletion result in the development of a DLBCL[13], which is usually considered an *H. pylori*-independent growing aggressive tumour.

The most important hints linking gastric MALT lymphoma to *H. pylori* were provided by the observation that *Hp*-associated gastritis reproduces features of acquired MALT, the high prevalence of *Hp* in gastric lymphoma patients (92%), mostly in endemic regions, and the high lymphoma regression rate observed in patients treated with *H. pylori*-eradicating antibiotic therapy (reviewed in[14]). In fact, *H. pylori* eradication with ample spectrum antibiotics is the standard first-line treatment for patients with limited-stage gastric MALT lymphoma associated with this microorganism[9]. This strategy is associated with a complete remission rate of 60%-70% and a 5-year OS of 93%[10,15]. Based on the frequent association among PG-DLBCL, gastric MALT lymphoma and *H. pylori* infection[11], and following the example of gastric MALT lymphomas, some investigators have treated selected patients with PG-DLBCL with antibiotic therapy alone, reporting sporadic cases of lymphoma regression[16,17] and complete remission rates of 27%-87% in a few, small retrospective case-series[18-20], with a relevant risk of reporting bias. More recently, two prospective trials demonstrated that *H. pylori* eradication is feasible and effective as exclusive treatment in patients with PG-DLBCL[21,22]. The first trial has included 16 Taiwanese patients with stage IE "high-grade transformed MALT lymphomas", obtaining a 62% remission rate and no cases of recurrence among responders at a median follow-up > 5 years[21,23,24]. The second trial, named HG-L1, has been a multicentre phase Ⅱ study addressing feasibility, activity and efficacy of *H. pylori* eradication with clarithromycin, tinidazole or metronidazole and omeprazole, as exclusive treatment for Western patients with newly diagnosed PG-DLBCL without aggressiveness indicators (bleeding ulcers, systemic symptoms, increased serum lactate dehydrogenase levels)[22]. The HG-L1 trial has demonstrated that two-thirds of these patients can be efficiently managed with antibiotics alone, thus, avoiding the use of chemotherapy and radiotherapy, which is of importance considering that these patients are often older than 70 years. In fact, this strategy has been associated with a complete remission rate of 63%, a 5-year OS of 94%, and no deaths due to lymphoma. Importantly, *H. pylori* eradication has been associated with long-term remission both in patients with MALT-related and *de novo* DLBCL[18,20], suggesting that near half of *de novo* DLBCL are actually dependent on antigenic stimulation determined by *H. pylori* infection. Conversely to previous reports suggesting that involvement of perigastric

lymph nodes is a negative predictor of response to antibiotics[18,20], half of patients with small (size < 1.5 cm) perigastric lymph nodes enrolled in the HG-L1 trial has achieved lymphoma regression[22]. In this trial, patients who did not respond to upfront antibiotics have been referred to salvage treatment with R-CHOP combination, achieving long-lasting complete remission in all cases, with a median progression-free survival of 55+ months, and antibiotic refractoriness has not been associated with lower survival rates.

Reliable parameters able to distinguish the best candidates for exclusive treatment with *H. pylori* eradication from those who need for conventional chemo-immunotherapy remain to be defined. A few studies have been performed in this context. The prognostic value of ontogenic classification of PG-DLBCL in germinal-centre B-cell like and non-germinal-centre B-cell like DLBCL has been investigated in the HG-L1 trial, reporting that lymphoma regression after *H. pylori* eradication can be observed in both DLBCL subgroups[22]. Other small studies show that nuclear expression of BCL10 predicts *H. pylori* independence of MALT-related DLBCL of the stomach[25], and that autocrine B cell-activating factor of tumor necrosis factor family (*BAFF*) signal transduction pathways may contribute to *H. pylori*-independent growth of this lymphoma[26]. In fact, *BAFF* overexpression seems to be associated with pAKT expression and nuclear expression of *BCL3*, *BCL10* and NF-kappaB, and is more common among MALT-related DLBCL of the stomach unresponsive to *H. pylori* eradication[26]. The predictive value of these and other molecules on large, prospective series remains matter of investigation.

CONCLUSION

On this background, patients with *H. pylori*-related PG-DLBCL and favourable features are eligible for bacteria eradication as exclusive treatment, keeping conventional chemo-immunotherapy for unresponsive patients. This strategy should be equally proposed to patients with *de novo* and MALT-associated DLBCL, and with germinal-centre B-cell like and non-germinal-centre B-cell like DLBCL. Small perigastric lymphadenopathies are not a major limitation to use this conservative approach, but close and accurate disease monitoring is strongly suggested in these patients. Clinical and molecular studies aimed to identify the best candidates for *H. pylori* eradication as exclusive treatment are strongly encouraged. The establishment of different molecular pathways potentially associated with antigenic independence and tumour aggressiveness as well as the analysis of their prognostic role on large series remain important steps forward a rational conservative treatment of gastric DLBCL.

REFERENCES

1 **Ferreri AJ**, Cordio S, Ponzoni M, Villa E. Non-surgical treatment with primary chemotherapy, with or without radiation therapy, of stage I-II high-grade gastric lymphoma. *Leuk Lymphoma* 1999; **33**: 531-541 [PMID: 10342580]

2 **Avilés A**, Nambo MJ, Neri N, Huerta-Guzmán J, Cuadra I, Alvarado I, Castañeda C, Fernández R, González M. The role of surgery in primary gastric lymphoma: results of a controlled clinical trial. *Ann Surg* 2004; **240**: 44-50 [PMID: 15213617 DOI: 10.1097/01.sla.0000129354.31318.f1]

3 **Binn M**, Ruskoné-Fourmestraux A, Lepage E, Haioun C, Delmer A, Aegerter P, Lavergne A, Guettier C, Delchier JC. Surgical resection plus chemotherapy versus chemotherapy alone: comparison of two strategies to treat diffuse large B-cell gastric lymphoma. *Ann Oncol* 2003; **14**: 1751-1757 [PMID: 14630680 DOI: 10.1093/annonc/mdg495]

4 **Martelli M**, Ferreri AJ, Agostinelli C, Di Rocco A, Pfreundschuh M, Pileri SA. Diffuse large B-cell lymphoma. *Crit Rev Oncol Hematol* 2013; **87**: 146-171 [PMID: 23375551 DOI: 10.1016/j.critrevonc.2012.12.009]

5 **Ferreri AJ**, Montalbán C. Primary diffuse large B-cell lymphoma of the stomach. *Crit Rev Oncol Hematol* 2007; **63**: 65-71 [PMID: 17339119 DOI: 10.1016/j.critrevonc.2007.01.003]

6 **Martinelli G**, Gigli F, Calabrese L, Ferrucci PF, Zucca E, Crosta C, Pruneri G, Preda L, Piperno G, Gospodarowicz M, Cavalli F, Moreno Gomez H. Early stage gastric diffuse large B-cell lymphomas: results of a randomized trial comparing chemotherapy alone versus chemotherapy + involved field radiotherapy. (IELSG 4). [corrected]. *Leuk Lymphoma* 2009; **50**: 925-931 [PMID: 19479614 DOI: 10.1080/10428190902912478]

7 **Tanaka T**, Shimada K, Yamamoto K, Hirooka Y, Niwa Y, Sugiura I, Kitamura K, Kosugi H, Kinoshita T, Goto H, Nakamura S. Retrospective analysis of primary gastric diffuse large B cell lymphoma in the rituximab era: a multicenter study of 95 patients in Japan. *Ann Hematol* 2012; **91**: 383-390 [PMID: 21822617 DOI: 10.1007/s00277-011-1306-0]

8 **Shenkier TN**, Voss N, Fairey R, Gascoyne RD, Hoskins P, Klasa R, Klimo P, O'Reilly SE, Sutcliffe S, Connors JM. Brief chemotherapy and involved-region irradiation for limited-stage diffuse large-cell lymphoma: an 18-year experience from the British Columbia Cancer Agency. *J Clin Oncol* 2002; **20**: 197-204 [PMID: 11773170 DOI: 10.1200/JCO.20.1.197]

9 **Ruskoné-Fourmestraux A**, Fischbach W, Aleman BM, Boot H, Du MQ, Megraud F, Montalban C, Raderer M, Savio A, Wotherspoon A. EGILS consensus report. Gastric extranodal marginal zone B-cell lymphoma of MALT. *Gut* 2011; **60**: 747-758 [PMID: 21317175 DOI: 10.1136/gut.2010.224949]

10 **Hancock BW**, Qian W, Linch D, Delchier JC, Smith P, Jakupovic I, Burton C, Souhami R, Wotherspoon A, Copie-Bergman C, Capella C, Traulle C, Levy M, Cortelazzo S, Ferreri AJ, Ambrosetti A, Pinotti G, Martinelli G, Vitolo U, Cavalli F, Gisselbrecht C, Zucca E. Chlorambucil versus observation after anti-Helicobacter therapy in gastric MALT lymphomas: results of the international randomised LY03 trial. *Br J Haematol* 2009; **144**: 367-375 [PMID: 19036078 DOI: 10.1111/j.1365-2141.2008.07486.x]

11 **Ferreri AJ**, Freschi M, Dell'Oro S, Viale E, Villa E, Ponzoni M. Prognostic significance of the histopathologic recognition of low- and high-grade components in stage I-II B-cell gastric lymphomas. *Am J Surg Pathol* 2001; **25**: 95-102 [PMID: 11145257 DOI: 10.1097/00000478-200101000-00011]

12 **Thieblemont C**, Bertoni F, Copie-Bergman C, Ferreri AJ, Ponzoni M. Chronic inflammation and extra-nodal marginal-zone lymphomas of MALT-type. *Semin Cancer Biol* 2014; **24**: 33-42 [PMID: 24333758 DOI: 10.1016/j.semcancer.2013.11.005.]

13 **Ferreri AJ**, Govi S, Ponzoni M. Marginal zone lymphomas and infectious agents. *Semin Cancer Biol* 2013; **23**: 431-440 [PMID: 24090976 DOI: 10.1016/j.semcancer.2013.09.004]

14 **Ferreri AJ**, Ernberg I, Copie-Bergman C. Infectious agents and lymphoma development: molecular and clinical aspects. *J Intern Med* 2009; **265**: 421-438 [PMID: 19298458 DOI: 10.1111/j.1365-2796.2009.02083.x]

15 **Ferreri AJ**, Govi S, Ponzoni M. The role of Helicobacter pylori eradication in the treatment of diffuse large B-cell and marginal zone lymphomas of the stomach. *Curr Opin Oncol* 2013; **25**: 470-479 [PMID: 23942292 DOI: 10.1097/01.cco.0000432523.24358.15]
16 **Ng WW**, Lam CP, Chau WK, Fen-Yau Li A, Huang CC, Chang FY, Lee SD. Regression of high-grade gastric mucosa-associated lymphoid tissue lymphoma with Helicobacter pylori after triple antibiotic therapy. *Gastrointest Endosc* 2000; **51**: 93-96 [PMID: 10625811 DOI: 10.1016/S0016-51070070399-3]
17 **Alsolaiman MM**, Bakis G, Nazeer T, MacDermott RP, Balint JA. Five years of complete remission of gastric diffuse large B cell lymphoma after eradication of Helicobacter pylori infection. *Gut* 2003; **52**: 507-509 [PMID: 12631659 DOI: 10.1136/gut.52.4.507]
18 **Tari A**, Asaoku H, Kashiwado K, Yoshino T, Kitadai Y, Tanaka S, Fujihara M. Predictive value of endoscopy and endoscopic ultrasonography for regression of gastric diffuse large B-cell lymphomas after Helicobacter pylori eradication. *Dig Endosc* 2009; **21**: 219-227 [PMID: 19961519 DOI: 10.1111/j.1443-1661.2009.00896.x]
19 **Morgner A**, Miehlke S, Fischbach W, Schmitt W, Müller-Hermelink H, Greiner A, Thiede C, Schetelig J, Neubauer A, Stolte M, Ehninger G, Bayerdörffer E. Complete remission of primary high-grade B-cell gastric lymphoma after cure of Helicobacter pylori infection. *J Clin Oncol* 2001; **19**: 2041-2048 [PMID: 11283137]
20 **Cavanna L**, Pagani R, Seghini P, Zangrandi A, Paties C. High grade B-cell gastric lymphoma with complete pathologic remission after eradication of Helicobacter pylori infection: report of a case and review of the literature. *World J Surg Oncol* 2008; **6**: 35 [PMID: 18353178 DOI: 10.1186/1477-7819-6-35]
21 **Chen LT**, Lin JT, Shyu RY, Jan CM, Chen CL, Chiang IP, Liu SM, Su IJ, Cheng AL. Prospective study of Helicobacter pylori eradication therapy in stage I(E) high-grade mucosa-associated lymphoid tissue lymphoma of the stomach. *J Clin Oncol* 2001; **19**: 4245-4251 [PMID: 11709568]
22 **Ferreri AJ**, Govi S, Raderer M, Mulè A, Andriani A, Caracciolo D, Devizzi L, Ilariucci F, Luminari S, Viale E, Müllauer L, Dell'Oro S, Arcidiacono PG, Ponzoni M, Patti C. Helicobacter pylori eradication as exclusive treatment for limited-stage gastric diffuse large B-cell lymphoma: results of a multicenter phase 2 trial. *Blood* 2012; **120**: 3858-3860 [PMID: 23118214 DOI: 10.1182/blood-2012-06-438424]
23 **Chen LT**, Lin JT, Tai JJ, Chen GH, Yeh HZ, Yang SS, Wang HP, Kuo SH, Sheu BS, Jan CM, Wang WM, Wang TE, Wu CW, Chen CL, Su IJ, Whang-Peng J, Cheng AL. Long-term results of anti-Helicobacter pylori therapy in early-stage gastric high-grade transformed MALT lymphoma. *J Natl Cancer Inst* 2005; **97**: 1345-1353 [PMID: 16174856 DOI: 10.1093/jnci/dji277]
24 **Kuo SH**, Yeh KH, Wu MS, Lin CW, Hsu PN, Wang HP, Chen LT, Cheng AL. Helicobacter pylori eradication therapy is effective in the treatment of early-stage H pylori-positive gastric diffuse large B-cell lymphomas. *Blood* 2012; **119**: 4838-4844; quiz 5057 [PMID: 22403257 DOI: 10.1182/blood-2012-01-404194]
25 **Kuo SH**, Chen LT, Yeh KH, Wu MS, Hsu HC, Yeh PY, Mao TL, Chen CL, Doong SL, Lin JT, Cheng AL. Nuclear expression of BCL10 or nuclear factor kappa B predicts Helicobacter pylori-independent status of early-stage, high-grade gastric mucosa-associated lymphoid tissue lymphomas. *J Clin Oncol* 2004; **22**: 3491-3497 [PMID: 15337797 DOI: 10.1200/JCO.2004.10.087]
26 **Kuo SH**, Yeh PY, Chen LT, Wu MS, Lin CW, Yeh KH, Tzeng YS, Chen JY, Hsu PN, Lin JT, Cheng AL. Overexpression of B cell-activating factor of TNF family (BAFF) is associated with Helicobacter pylori-independent growth of gastric diffuse large B-cell lymphoma with histologic evidence of MALT lymphoma. *Blood* 2008; **112**: 2927-2934 [PMID: 18628489 DOI: 10.1182/blood-2008-02-137513]

P- Reviewers: Li QQ, Zheng XF
S- Editor: Wen LL **L- Editor**: A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.8998

World J Gastroenterol 2014 July 21; 20(27): 8998-9016
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Hepatitis B virus: Where do we stand and what is the next step for eradication?

Haruki Komatsu

Haruki Komatsu, Department of Pediatrics, Toho University Sakura Medical Center, Chiba 285-8741, Japan
Author contributions: Komatsu H wrote this article.
Correspondence to: Haruki Komatsu, MD, PhD, Department of Pediatrics, Toho University Sakura Medical Center, 564-1 Shimoshizu Sakura, Chiba 285-8741, Japan. haruki-komatsu@chive.ocn.ne.jp
Telephone: +81-43-4628811 Fax: +81-43-4630801
Received: September 20, 2013 Revised: December 9, 2013
Accepted: April 15, 2014
Published online: July 21, 2014

Abstract

Hepatitis B (HB) virus (HBV) infection, which causes liver cirrhosis and hepatocellular carcinoma, is endemic worldwide. Hepatitis B vaccines became commercially available in the 1980s. The World Health Organization recommended the integration of the HB vaccine into the national immunisation programs in all countries. HBV prevention strategies are classified into three groups: (1) universal vaccination alone; (2) universal vaccination with screening of pregnant women plus HB immune globulin (HBIG) at birth; and (3) selective vaccination with screening of pregnant women plus HBIG at birth. Most low-income countries have adopted universal vaccine programs without screening of pregnant women. However, HB vaccines are not widely used in low-income countries. The Global Alliance for Vaccine and Immunization was launched in 2000, and by 2012, the global coverage of a three-dose HB vaccine had increased to 79%. The next challenges are to further increase the coverage rate, close the gap between recommendations and routine practices, approach high-risk individuals, screen and treat chronically infected individuals, and prevent breakthrough infections. To eradicate HBV infections, strenuous efforts are required to overcome socioeconomic barriers to the HB vaccine; this task is expected to take several decades to complete.

Key words: Cancer; Global Alliance for Vaccine and Immunization; Hepatitis B immune globulin; Hepatitis B virus; Hepatocellular carcinoma; Selective vaccination; Universal vaccination; World Health Organization

Core tip: Hepatitis B (HB) vaccines, which are the first vaccines that have been proven to prevent cancer, have played a crucial role in preventing HB virus (HBV) infection worldwide since their development in the 1980s. In particular, the HB vaccines have been rapidly integrated into the national immunisation programs of low-income countries since the Global Alliance for Vaccine and Immunization was launched in 2000. However, we have still not eradicated HBV. More than 240 million people worldwide are carriers of HBV. The vaccine strategies, current status of HBV infection, and unresolved issues related to controlling HBV infection are discussed in this review.

Komatsu H. Hepatitis B virus: Where do we stand and what is the next step for eradication? *World J Gastroenterol* 2014; 20(27): 8998-9016 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/8998.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.8998

INTRODUCTION

According to the World Health Organization (WHO), two billion people (one-third of the global population) have been infected with the hepatitis B (HB) virus (HBV) worldwide, and more than 240 million are chronic carriers (4%-6% of the world population)[1]. Chronically infected individuals have a 25% risk of dying from the

sequelae of chronic HBV infection, such as cirrhosis and hepatocellular carcinoma (HCC)[2]. Approximately 600000 people die every year due to the consequences of HBV infection[1]. Globally, chronic HBV infection accounts for 54.4% of the cases of liver cancer[3]. HBV infection is one of the vaccine-preventable infectious diseases. In 1991, the WHO recommended the integration of the HB vaccine into the national immunisation programs in countries with an HBV carrier prevalence of 8% or higher by 1995 and in all other counties by 1997[4].

In this review, the vaccine strategies for the control of HBV infection and the prospect of HBV eradication are summarised and discussed.

HB VACCINE

Discovery of the HBV

In 1963, Blumberg *et al*[5-8] unexpectedly identified a protein in the blood of Australian aborigines, which was later named the Australia antigen. The investigators were examining serum from multi-transfused patients with conditions such as leukaemia or thalassemia compared to serum from a variety of healthy individuals from different parts of the world to identify genetic polymorphisms of serum proteins[9]. The Australian antigen was initially thought to be associated with leukaemia and Down's syndrome[5,10], but further observations revealed that the Australia antigen is a component of the infectious agent for HBV. In fact, two patients with Down's syndrome and a technician in Blumberg's laboratory became positive for the antigen after developing hepatitis[6,8,10-12]. The Australia antigen was eventually confirmed to be correlated with viral hepatitis[13-16]. In 1970, Dane *et al*[17] discovered hepatitis B virions -double-coated particles approximately 42 nm in diameter- in the serum of patients with Australia antigen-associated hepatitis.

Development of the HB vaccine

After the discovery of the Australian antigen, it took over 10 years to make the HB vaccine commercially available. The virion of HBV (*i.e.*, the Dane particle) consists of an inner core and an outer membranous envelope, which contains the Australian antigen[8,18]. Electronic microscopy analyses revealed that enormous numbers of spherical and tubular particles of 22 nm in diameter, which are clearly distinct from the virions, coexist in the serum of HBV-infected patients[11,17,19]. These particles are empty viral envelopes, containing only the Australian antigen and non-infectious agents[6,8,18]. The first available vaccines consisted of purified and formalin-inactivated small empty HBV envelopes containing the hepatitis B surface antigen (HBsAg), which were harvested from the plasma of chronic HBV carriers[20-23]. These plasma-derived HB vaccines first became commercially available in the United States in 1981 and in France in 1982[24,25]. Since 1981, plasma-derived HB vaccines have been manufactured and used in many countries. However, there were concerns about whether the supply of plasma was adequate to meet the demand for the vaccine and whether the safety of a vaccine derived from human blood could be verified. A recombinant expression system was developed to address these problems. The recombinant expression of HBsAg was achieved in HBV-transfected yeast[26,27]. Electron microscopy revealed that the expressed HBsAg polypeptides showed the same appearance as the particles isolated from human plasma. In 1986, the recombinant HB vaccine (the second-generation vaccine) was approved by the United States Food and Drug Administration[8,24]. Although new recombinant HB vaccines using HBV-transfected mammalian cells containing pre-S2+S envelope proteins (*i.e.*, third generation vaccines) were commercialised in the 1990s[25,28,29], the majority of vaccine manufacturers are now adopting a yeast-system for recombinant expression[30]. The complete vaccine series induces protective levels of anti-HBs antibodies in more than 95% of infants, children, and young adults[1]. Although the duration of protection provided by the HB vaccine is controversial, the protection afforded by three or four doses of a monovalent HB vaccine persists for at least 20 years[1,31].

Universal vaccination vs selective vaccination

Two vaccine programs are being conducted worldwide to control and eradicate HBV infection. The first program is universal vaccination, which integrates a three- or four-dose series HB vaccine into routine vaccination programs. The other is selective vaccination, which targets high-risk individuals identified by assessments of chronic diseases, lifestyle, and occupation. The WHO strongly recommends universal vaccination in all countries, and nearly all of the countries throughout the world are adopting such a program[4]. In addition, high-income countries that can afford to perform screening of pregnant women give hepatitis B immune globulin (HBIG) to newborn babies born to HBsAg-positive mothers at birth. Because screening of pregnant women is costly and not feasible in low-income countries, the WHO does not recommend it for all countries. As shown in Table 1, the current HBV prevention strategies are classified into three groups: (1) universal vaccination without screening of pregnant women; (2) universal vaccination with screening of pregnant women plus HBIG; and (3) selective vaccination with screening of pregnant women plus HBIG.

In intermediate and highly endemic countries, universal vaccination without screening of pregnant women is clearly cost-effective[32]. However, the protective efficacy rate of a three- or four-dose HB vaccine series alone is 70%-80% in perinatal transmission[33-35]. In contrast, HB vaccine plus HBIG increases the protective efficacy rate to 95% in perinatal transmission[35]. Although aspects of the healthcare infrastructure, such as medical personnel, hospitals, and careful follow-up programs, are indispensable for the screening of pregnant women, the adminis-

Table 1 Classification of hepatitis B vaccine prevention strategies

	Universal vaccination alone	Universal vac. + pregnant-women screening + HBIG	Selective vaccination + pregnant-women screening + HBIG
Low- and intermediate-income countries[1]	√		
High-and intermediate-income countries[1] (*e.g.*, European countries, United States)		√	
High-income countries (*e.g.*, Scandinavian countries, United Kingdom, Japan)			√

[1]Intermediate-income countries are using a variety of vaccination strategies; HBIG: Hepatitis B immune globulin.

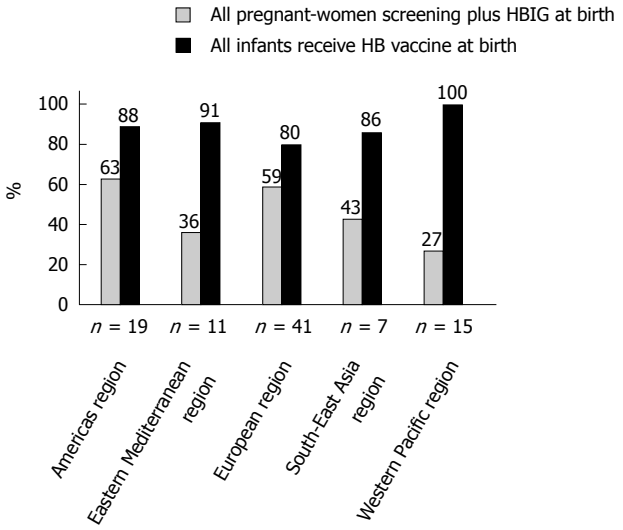


Figure 1 World Health Organization global and regional implementation of universal hepatitis B vaccination at birth and screening of pregnant women plus hepatitis B immune globulin administration at birth. The data were drawn from the WHO "Global policy report on the prevention and control of viral hepatitis"[36]. HBIG: Hepatitis B immune globulin; HB: Hepatitis B.

tration of HBIG can definitely improve the prevention rate of perinatal transmission.

Universal vaccination with screening of pregnant women plus HBIG is the most effective strategy to reduce and eradicate HBV, if and when the financial condition of a country allows for the implementation of such a program. In fact, almost all high-income countries are conducting universal vaccination with screening of pregnant women plus HBIG. The number of countries providing selective vaccination with screening of pregnant women plus HBIG is small and has gradually declined. Because the prevalence of HBV carriers is low in these counties, universal vaccination is considered not to be cost-effective; instead, the government's target high-risk individuals for vaccination.

GLOBAL POLICY REPORT ON THE PREVENTION AND CONTROL OF VIRAL HEPATITIS

In 2012, a global survey of the efforts to prevent and control viral hepatitis was conducted on the behalf of the WHO by the World Hepatitis Alliance, a non-government organisation that represents approximately 280 hepatitis B and hepatitis C patient groups around the world[36,37]. Of the 194 WHO member states, 126 (64.9%) completed the surveys, which is not a sufficiently high response rate. The rates of response to WHO regional surveys were 26.1% (12/46) in the African Region, 77.1% (27/35) in the Americas Region, 77.3% (17/22) in the Eastern Mediterranean Region, 83.0% (44/53) in the European Region, 36.4% (4/11) in the South-East Asia Region, and 55.6% (15/27) in the Western Pacific Region.

The WHO member states are classified into five groups: "high income", "upper-middle income", "lower-middle income", "low income", and "other". The rates of response to the WHO/World Hepatitis Alliance survey in these groups were 80% (40/50: high income), 64.2% (34/53: upper-middle income), 68.0% (34/50: lower-middle income), 47.4% (18/38: low income), and 0% (0/3: other). The proportion of high- and upper-middle-income countries was 2% (1/46) in the African Region, 60% (21/35) in the Americas Region, 32% (7/22) in the Eastern Mediterranean Region, 68% (36/53) in the European Region, 18% (2/11) in the South-East Asia Region, and 26% (7/27) in the Western Pacific Region.

The rates of implementation of screening for all pregnant women plus HBIG at birth and programs administering the HB vaccine to all infants at birth in each WHO region (except for the African Region) are shown in Figure 1. According to this report, the rates of screening of pregnant women plus HBIG administration were 63% (Americas), 36% (Eastern Mediterranean), 59% (Europe), 43% (South-East), and 27% (Western Pacific), with more than half of the countries in the Americas and European Regions adopting HBIG administration after birth. Because high- and upper-middle-income countries are the majority in both the Americas and European regions, these regions can afford to provide screening of all pregnant women plus HBIG administration. In contrast, the Western Pacific Region shows the lowest rate (27%) of screening of pregnant women plus HBIG administration and the second-lowest ratio of high- and upper-middle-income countries.

In light of the low ratio of high- and upper-middle-income countries in the Western Pacific Region, the WHO Western Pacific Office strongly recommends the

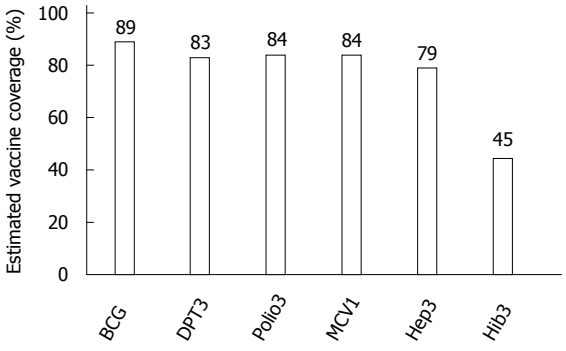


Figure 2 World Health Organization-estimated global coverage rates for the BCG, DPT3, polio, MCV1, Hep3, and Hib3 vaccines in 2012. The data are from[47]. BCG: Bacille Calmette-Guérin; DPT3: Three-dose DTP; MCV1: Measles-containing vaccine; Hep3: Three-dose hepatitis B; Hib3: Three-dose Hib vaccine

universal administration of a dose of HB vaccine to all newborn infants rather than a targeted approach for infants born to HBsAg-positive mothers[38]. The coverage of HB vaccine birth dose administration (within 24 h after birth) varies from 80% to 100% among the six WHO regions. The Western Pacific Region achieved 100% birth dose coverage, following sustained efforts by the Western Pacific Office over many years to improve the birth dose coverage in low-income counties[38]. Conversely, the birth dose coverage rate is not high in the regions where high-income counties are implementing screening of all pregnant women, such as the European Region (80%).

GLOBAL ALLIANCE FOR VACCINE AND IMMUNISATION

Vaccine shortages began to emerge in the late 1990s[39]. Vaccine manufacturers had begun phasing out the production of the traditional, less-expensive vaccines, such as the diphtheria-pertussis-tetanus (DPT) combination used in low-income countries. Between 1998 and 2001, 10 of 14 manufacturers partly or totally stopped their production of traditional vaccines. In 2001, the availability of the traditional DPT combination tuberculosis and measles vaccine dropped to the lowest level in 10 years. Moreover, the prices of these vaccines were increased.

Compared to traditional vaccines (*e.g.*, DPT, oral polio, measles), which cost (USD) $0.06-0.10 per dose, the HB vaccine was expensive in the 1980s and 1990s[40,41]. In 1981, the price of a plasma-derived HB vaccine at introduction was $30 per dose. Although recombinant HB vaccines provide a safe and stable supply of HB vaccine, the price of the recombinant HB vaccine varied from USD $30 to $40, or nearly $100 for the complete series of three shots. Therefore, the benefits of the development of HB vaccines were not experienced by low-income counties, where the vaccines were greatly needed to prevent HBV infection.

The Global Alliance for Vaccine and Immunization (the GAVI Alliance) is a public-private partnership whose partners include United Nations agencies, the WHO, the World Bank, public health institutions, donor and recipient countries, the Bill and Melinda Gates Foundation, pharmaceutical manufacturers, and other members of the philanthropic and financial communities. The GAVI Alliance was launched in 2000 to establish vaccination programs in low-income countries. By bringing together low-income countries, donor governments, research and technical institutes, civil society organisations, vaccine providers, and private philanthropists, the dynamics of the global vaccine market have been changed by the establishment of sustainable supplies of vaccines, research, competition, and price reduction. During the first phase (2000-2005), hepatitis B became one of the three under-utilised vaccines [hepatitis B, Haemophilus influenzae type b (Hib), and yellow fever] immediately available for routine infant immunisation programs through the new and under-utilised vaccine flagship program[42-44]. The HB vaccine was considered to have the greatest potential in the implementation of under-utilised vaccines. The widespread use of new and under-utilised vaccines has the potential to contribute to the United Nations Millennium Development Goal 4 of reducing global childhood mortality by two-thirds by 2015[42,45,46].

GLOBAL COVERAGE OF TRADITIONAL VACCINES AND UNDER-UTILISED VACCINES

The global coverage in 2012 of traditional vaccines was as follows: Bacille Calmette-Guérin (BCG), 89%; the three-dose DTP (DPT3) vaccine, 83%; the three-dose polio vaccine (Polio3), 84%; and the first-dose measles-containing vaccine (MCV1), 84%[47]. In contrast, as shown in Figure 2, the 2012 coverage of the three-dose HB (Hep3) vaccine and the three-dose Hib vaccine (Hib3) was 79% and 45%, respectively[47]. A slight difference in the coverage between traditional vaccines and under-utilised vaccines remains. In the 2000s, the GAVI Alliance began introducing the combination pentavalent vaccine (DPT-hepB-Hib)[48]. The GAVI Alliance increased the number of manufacturers and reduced the price of the pentavalent vaccine. Most GAVI-eligible countries thus switched to the pentavalent vaccine. By 2015, the GAVI Alliance aims to support the immunisation of an additional 230 million children with the combination pentavalent vaccine[49]. Therefore, the immunisation status of HBV and Hib will soon become the same as that of DPT.

STATUS OF HB VACCINATION

Universal vaccination without screening of pregnant women

Almost all of the countries classified by the WHO as low-income use universal vaccination without screening of pregnant women. The WHO/United Nations Children's Fund and the GAVI Alliance have important roles in the prevention of HBV infection in these countries.

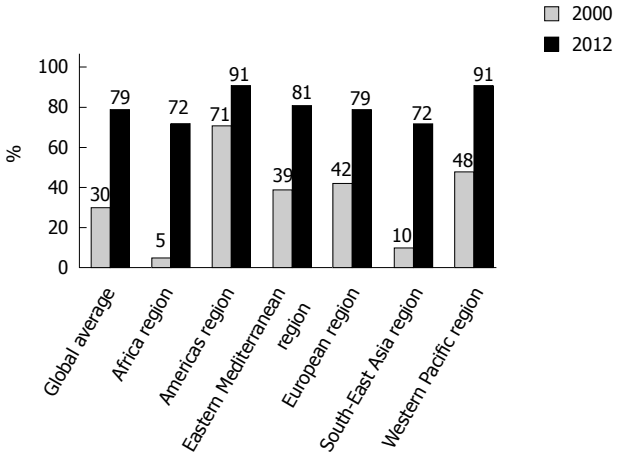


Figure 3 World Health Organization-estimated global coverage and coverage in each WHO region for the universal 3-dose hepatitis B vaccine in 2000 and 2012.

With the GAVI Alliance's support, many low-income countries were able to introduce an HB vaccine into their routine vaccine programs. As shown in Figure 3, the global average of the universal three-dose HB vaccine immunisation rate was 30% of the WHO member states in 2000[47]. Very low introduction rates were revealed in the Africa Region (5%) and the South-East Asia Region (10%)[50]. Only the Americas Region achieved above 50% in the universal three-dose HB vaccine immunisation rate in 2000[50]. However, by 2012 the global average of the universal three-dose HB vaccine immunisation rates had more than doubled to 79%[47]. In that year, the routine three-dose HB vaccination coverage was approximately 90% in the Americas Region and Western Pacific Region, and the coverage rates in the African Region and South-East Asia had increased by 14- and 7-fold, respectively.

African region: All of the African Region WHO member states except for Algeria are in Sub-Saharan Africa, which has two-thirds of all of the worldwide cases of human immunodeficiency virus (HIV)[36]. The prevalence of HBV is estimated at 8% in West Africa and 5%-7% in Central, Eastern, and Southern Africa[51,52]. Due to the lower prevalence of the serum hepatitis B e antigen (HBeAg) in Africa compared to that in Asia, HBV infection in Africa is thought to be acquired almost always in early childhood by horizontal transmission rather than by vertical transmission[53-56]. In Sub-Saharan countries, a birth dose is not used; instead, a 6-, 10-, and 14-wk ("6-10-14") after birth vaccination schedule is common[57,58]. Although universal infant HB vaccination was introduced in only 5% of the African Region member states in 2000, the introduction rate had increased to 72% by 2012[50]. Although a birth dose might be beneficial for African infants[58], no information about the birth dose or regional goals in Africa is available.

Americas region: In the WHO Americas Region, the prevalence of HBV infection varies from low to intermediate[59]. The prevalence of HBV infection is less than 2% in the central and tropical Latin America region, and it has remained between 2% and 4% in the Caribbean, Andean, and south Latin American regions[36,52]. In 2000, the Pan American Health Organization (PAHO) recommended that universal infant HB vaccination should be the primary strategy to prevent HBV transmission[59,60]. The decision to introduce a birth dose of HB vaccine depended on the prevalence of HBV carriers in each country. The PAHO recommended that a birth dose should be added to the vaccine program in the countries and territories where the prevalence of HBsAg exceed 8%[59,61]; the majority of the countries had no birth dose in their routine vaccine schedules. Without a birth dose, a 2-4-6 mo after birth schedule is predominant[59]. In 2012, however, the PAHO advised all of the region's countries to introduce a birth dose (within 24 h of birth) for universal infant HB vaccination[62]. As shown in Figure 3, the universal three-dose HB vaccine immunisation rate of the Americans Region was 91% in 2012, the highest among the WHO regions.

Eastern Mediterranean region: The epidemiology of HBV infection in this region is complex. Before the introduction of the HB vaccine into the region's routine immunisation programs, the prevalence of HBV carriers ranged from 2% to 3% in several member states; however, the prevalence was more than 10% in the region members of Somalia and Sudan[63]. Similar to other regions, the universal infant HB immunisation rate increased to 81% by 2012, which is double the figure in 2000[50]. In 2009, universal infant HB vaccination, including a birth dose (within 24 h of birth), was recommended by the Eastern Mediterranean Regional Office, and a reduction in the prevalence of chronic HBV infection to less than 1% among children over 5 years of age by 2015 was set as a time-bound regional goal[63].

European region: Although high- and upper-middle-income countries are the majority in the European Region, the rate of universal HB vaccination was only 79% in 2012, giving the region the rank of fourth highest among the six WHO regions[50]. Although Italy was one of the first countries to begin universal HB vaccination for infants, northern European countries are still using selective vaccination programs. Three countries give the first shot of HB vaccine to school-aged children[64]. It was reported that 13.3 million (1.8%) adults in the European Region had HBsAg; furthermore, two-thirds of the region's residents who are infected with HBV live in the countries that are not part of the European Union/Free Trade Treaty Association[65], indicating that in the European Region one in 50 adults is an HBV carrier. As long as northern European countries continue selective vaccination, the universal immunisation rate will not reach 100%.

South-East Asia region: The universal HB immuni-

sation rate in the South-East Asia Region in 2000 was 9%, the second lowest figure among the WHO regions. Although universal immunisation reached 72% in 2012, this figure was the lowest among the WHO regions[50]. Mirroring the universal HB vaccination rate, the 2012 DPT coverage rate of the South-East Asia Region was the second lowest (75%) among the WHO regions[66]. These findings suggest that the implementation of the entire panoply of immunisation programs is insufficient in this region. The WHO Regional Office encourages the South-East Asia member states to intensify their routine vaccine immunisation programs[67].

Western Pacific region: Although the Western Pacific Region comprises only 28% of the global population, the region bears a disproportionate burden of HB-related mortality and morbidity, accounting for almost half of all chronic HBV infections worldwide. An estimated 160 million people with chronic HBV infection live in this region, and the regional HBV-related mortality rate is comparable to that of tuberculosis[68,69]. The control and prevention of hepatitis B infection is thus the top priority in this region. In 2005, the Western Pacific region became the first region to set a time-bound goal of reducing the chronic HBV infection rate to less than 2% among five-year-old children by 2012[69]. This milestone influenced the individual countries' national policies[70] and resulted in the highest coverage rate (91%) for universal HB vaccine immunisation among the WHO regions in 2012[50].

Universal vaccination with screening of all pregnant women plus HBIG

The universal vaccination with screening of all pregnant women plus HBIG strategy is expensive and complicated, but it is the most powerful strategy to prevent and control HBV infection. High-income countries, such as the United States and Italy, are implementing universal vaccination, screening all pregnant women, and administering HBIG to babies born to HBsAg-positive mothers at birth.

In the United States in 1982, the Advisory Committee on Immunization Practices (ACIP) recommended that persons with a substantial risk of HBV infection should be vaccinated[71]. The initial strategies for preventing HBV infection focused on the immunisation of high-risk groups: healthcare workers, men who have sex with men (MSM), drug users, recipients of certain blood products, and close contacts. However, many people who had no identifiable source for infection were infected with HBV[72]. In 1991, the ACIP proposed, for the first time, that hepatitis B vaccination was recommended for all infants regardless of the HBsAg status of the mother[73]. In Italy, an HB vaccine program for the high-risk groups began in 1983[74]. Despite the decreasing circulation of HBV in the late 1980s, a compulsory universal vaccination against HBV was introduced for all newborns and for 12-year-old children (a double cohort policy of mandatory immunisation) in Italy in 1991[75-77]. In some counties, a birth dose (administration within 24 h after birth) is not given if the screened mother is negative for HBsAg[78,79]. In the United States, a birth dose of the monovalent HB vaccine is given to babies born to HBsAg-negative mothers before discharge from the hospital[80]. In Europe, a monovalent or polyvalent vaccine is given to babies born to HBsAg-negative mothers at 2 or 3 mo after birth as the first dose[78,79]. As a birth dose, a monovalent vaccine should be used. In Germany, four doses are recommended for the hexavalent vaccine, and three doses are used for the monovalent vaccine[77,78]. Serum HBsAg is usually used for the screening of pregnant women. However, serum HBeAg is also used for screening in a few countries[81]. In Taiwan, free HBIG is administered to newborn babies born to HBs-positive and HBe-positive mothers[81-83]. Because the risk of mother-to-child transmission is considered low in babies born to HBsAg-positive and HBe-negative mothers, the administration of self-paid HBIG is optional for the families of babies born to HBeAg-negative mothers[82,83].

Selective vaccination with screening of pregnant women plus HBIG

Denmark, Finland, Iceland, Japan, Norway, Sweden, and the United Kingdom, which are low-endemic countries (prevalence of HBsAg < 1%)[65], use selective vaccination[77,78]. Of these seven countries, in 2012, five were among the 10 countries with the lowest mortality rates for children under 5 years of age, and all seven are among the 24 richest countries in 2013[84,85]. In these countries, selective vaccination is considered more cost-effective than universal vaccination. In 2008, however, Ireland gave up selective vaccination and introduced a universal childhood vaccination program with a hexavalent vaccine[86,87]; the Netherlands decided to implement universal vaccination in 2011[88-90]. Selective vaccination targets individuals at high risk of HBV infection, but the definition of high risk varies from country to country. In 70% or more of the countries with selective immunisation programs, high-risk individuals include the following: injection drug users; non-injection users who are living with current injectors; sexual partners of injection users; children of injectors; MSM; close family contacts; healthcare workers; laboratory staff; police, fire, and rescue services; babies born to mothers who are chronically infected with HBV or to mothers who have had acute hepatitis B during pregnancy; people traveling to or going to reside in an area of high or intermediate prevalence; individuals receiving regular blood or blood products; and individuals in residential accommodations for those with learning difficulties[77]. In these countries, HBIG is given to babies born to HBsAg-positive mothers. In England, the indications for HBIG are as follows: infants of mothers with acute hepatitis, mothers who are HBsAg-positive and HBeAg-positive, mothers who are HBsAg-positive and HBeAg/anti-HBe-negative, mothers whose HBeAg/anti-HBe status is unknown,

Table 2 Hepatitis B surface antigen prevalence before and after the introduction of universal vaccination for hepatitis B virus

Ref.	Region	Country		Subjects	Year of the introduction of infant universal vaccination	HBsAg positive rate			
						Before-vaccination program	Year	After vaccination program	Year
95	North America	United States	(Hawai)	School children	1992	1.6%	1989	0.04%	2001-2002
96	South America	Colombia	(Colombian Amazon)	5-9 yr of age	1992	7%	1992	2%	1999
97	Asia	Taiwan	(Taipei)	≤ 12 yr of age	1984	9.8%	1984	1.3%	1994
94	Asia	China		Young children	1992	9%-12%	1992	< 1%	2005
98	Europe	Italy	(Afragola)	General population	1991	13.4%	1978	0.91%	2006
99	Africa	South Africa	(Gauteng Province)	≤ 24 mo after birth	1995	8%-9%	1995-1996	0.9%	2003-2004
100	Africa	Gambia	(Keneba)	≤ 24 yr of age	1984	13.3%	1984	0.6%	2003
100	Africa	Gambia	(Mandar)	≤ 24 yr of age	1984	35%	1984	1%	2003

Table 3 Incidence of acute hepatitis B before and after the introduction of universal vaccination for hepatitis B virus

Ref.	Region	Country	Subjects	Year of the introduction of universal vaccination	Annual incidence of acute hepatitis B cases per 100000 population			
					Before vaccination program	Year	After vaccination program	Year
101	North America	United States	0-19 yr of age	1992	3	1990	0.3	2002
102		United States (Alaska)	Under 20 yr of age	1984	19	1981-1920	0	1993-1994
103	Asia	Taiwan	Infants	1984	5.36	1975-1984	1.71	1993-1994
104	Europe	Italy	General population	1991	5.1	1991	1.3	2005

mothers whose serum HBV DNA levels are $\geq 1 \times 10^6$ IU/mL, and infants whose birth weight is less than 1500 g[91,92]. Japan has a unique schedule of HB vaccines plus HBIG. Babies born to HBeAg-positive mothers are administered HBIG twice (48 h and 2 mo after birth) followed by a three-dose HB vaccine series without a birth dose[93].

RESULTS OF UNIVERSAL VACCINATION

Prevalence of HBsAg

Since the introduction of infant universal vaccination, successful results of universal vaccination for HBsAg have been reported in many countries, although in almost all of the relevant studies the subjects are children and adolescents (Table 2). The report from China noted in Table 2 was a nationwide survey[94], and the remaining data concern limited areas of the United States, Colombia, Taiwan, Italy, South Africa, and Gambia. With the introduction of infant universal vaccination, the prevalence of HBsAg declined from 1.6% to 0.04% in the United States[95], from 7% to 2% in Colombia[96], from 9.8% to 1.3% in Taiwan[97], from 9%-12% to < 1% in China[94], from 13.4% to 0.91% in Italy[98], from 8%-9% to 0.9% in South Africa[99], from 13.3% to 0.6% in Gambia (Keneba), and from 35% to 1% in Gambia (Mandar)[100]. The highly endemic countries in particular showed a remarkable reduction in the prevalence of HBsAg.

Acute hepatitis B

The incidence of acute hepatitis B is shown in Table 3. Almost all cases of acute hepatitis B are asymptomatic. All studies except a study that was performed in Alaska were nationwide surveys[101-104]. The study from Taiwan evaluated the mortality from fulminant hepatitis, whose pathogen was not identified[103]. The incidence of acute hepatitis B per 100000 people declined from 3 to 0.3 in the United States[101], from 19 to 0 in Alaska[102], from 5.36 to 1.71 in Taiwan[103], and from 5.1 to 1.3 in Italy[104]. Clear and sizable reductions in the incidence of acute hepatitis B were achieved after the introduction of universal HB vaccination programs.

HCC

It takes several decades for HCC to develop after an individual becomes an HBV carrier[105]. Only approximately 25 years have passed since the first introduction of universal vaccination for HBV, and thus the number of studies evaluating the incidence of HCC is still limited (Table 4). HCC associated with HBV infection was often observed in young children in Taiwan[106-108]; this finding was very useful for the evaluation of the effectiveness of universal vaccination in a short period. The study from Taiwan showed that nationwide universal vaccination reduced the incidence of HCC in children in 1997[109], which was 13 years after the introduction of the universal vaccine program in that country. The annual incidence of HCC per 100000 people in Taiwan declined from 0.7 to 0.36 in children 6 to 14 years of age and from 0.52 to 0.13 in those 6 to 9 years of age[109]. This was the first report proving that a vaccine could prevent cancer. Moreover, in Alaska, universal newborn vaccination coupled with a simultaneous catch-up vaccination program reduced the annual incidence of HCC

Table 4 Incidence of hepatocellular carcinoma before and after the introduction of universal vaccination for hepatitis B virus

Ref.	Country	Subjects	Year of the introduction of universal vaccination	Annual incidence of HCC cases per 100000 population			
				Before vaccination program	Year	After vaccination program	Year
102	United States (Alaska)	Under 20 yr of age	1984	3	1984-1988	0	1995-1999
109	Taiwan	6-14 yr of age	1984	0.7	1981-1986	0.36	1990-1994
109	Taiwan	6-9 yr of age	1984	0.52	1974-1984	0.13	1984-1986

HCC: Hepatocellular carcinoma.

per 100000 from 3 to 0[102]. Since 1999, no cases of HCC have occurred in Alaska[102].

NEXT STEPS

Further increase in vaccine coverage

The GAVI Alliance estimated that 3.7 million future deaths from HBV infection were averted by vaccine programs conducted during 2000 and 2011[110]. The GAVI Alliance also estimated that 4.9 million future deaths will be averted during the years 2011 to 2020 in the 73 GAVI-eligible countries, compared to no vaccinations[111]. Although the global coverage of HB vaccine lags behind the global coverage levels for DPT, which is an indicator of expanded immunisation programs, the continued introduction of pentavalent vaccines will improve this situation.

In the WHO Africa and South-East Asia regions, however, the coverage of the HB vaccine was still lower than 80% in 2012[50]. Moreover, these two WHO regions had not reached 80% DTP3 coverage in 2012[50], a coverage minimum set by the Global Immunization Vision and Strategy (GIVS) as a target at the national level to form the framework for strengthening national immunisation programs in 2006 and 2015[112,113]. To improve the poor immunisation coverage and achieve the GIVS target, the South-East Asia Regional director declared 2012 as the year for intensifying routine immunisations[67,114]. In the South-East Asia Region, the coverage of a three-dose HB vaccine increased from 56% in 2011 to 72% in 2012[50].

Although inter-country differences exist, the Africa Region is facing a significant hurdle in its efforts to meet the GIVS goal[52,113,115]. Among HIV-infected individuals in sub-Saharan Africa, the prevalence of HBsAg is 15%[116]. HIV sero-positivity increases the risk of failure to respond to vaccines[117-119]. Many researchers have noted that to increase the HB vaccine coverage rate, it is necessary to secure firm commitments from governments (political commitment), financial flow, consistent scheduling of vaccination outreach programs, stable vaccine supplies, strong infrastructures (*e.g.*, cold chain, stock space for new vaccines, delivery points), inter-sectional coordination, adequate human resources (including educated and trained healthcare providers), the education of women and parents, and surveillance systems to determine the impact of vaccines[120-126]. The GAVI Alliance is expected to have substantial public impacts on vaccination coverage in both WHO regions in 2011 and 2020[111,113].

A mathematical model predicts that 90% of the complete HB vaccine series coverage rate, including a birth dose, can achieve an 84% reduction in HBV-related deaths[127]. On the basis of this mathematical model, a pessimistic view is that the HB vaccine alone might be insufficient to eradicate HBV[128]. In addition, there is concern about the quality of immunisation coverage data. Inconsistencies in immunisation data have been observed in many countries. Although there are no data about such inconsistencies regarding the HB vaccine, the proportion of verified DPT-3 doses was found to be lower than 85% (after over-reporting) in 16 of 27 countries[129]. In many cases, the survey-based DPT-3 immunisation coverage rates have not reached the level suggested by countries' official reports or WHO/UNICEF estimates[130]. Immunisation data quality audits are needed to obtain accurate and timely data and to determine how best to improve immunisation programs.

Filling a gap between protocol and routine practice

Not only the coverage rate but also age-appropriate vaccinations *(i.e.*, timely vaccinations) are important to control infections. However, vaccinations are often delayed after the recommended ages[131-134]. In 31 low- and middle-income countries, the median fraction of timely administered vaccinations was 65% for BCG vaccine, 67% for DPT-1, 41% for DTP-3, 68% for polio-1, 38% for polio-3, and 51% for MCV[131]. The median delay in the 31 countries was 2.1 wk for BCG, 2.4 wk for DPT-1, 6.3 wk for DPT 3, 2.0 wk for polio-1, 6.6 wk for polio-3, and 4.1 wk for MCV[131]. Although that study did not discuss the HB vaccine, an HB vaccination delay could presumably be occurring in low- and middle-income countries. For example, in Argentina only 33% of children were vaccinated on time with the HB vaccine by seven months of age in 2002[135]. In Cambodia, the timely birth dose (within 24 h after birth) coverage was 66%[136]. In the 2000-2002 National Immunization Survey for each state in the United States, the timely administration of a three-dose HB vaccine among children aged 24 to 35 mo ranged from 49.4% (Vermont) to 81.6% (Rhode Island)[137]. Although the HB vaccine birth-dose coverage has increased year after year since 2000 in the United States, the nationwide coverage was still only 61.5% in 2007[138]. These data suggest that delayed vaccinations occur frequently in high-income countries just as in low-

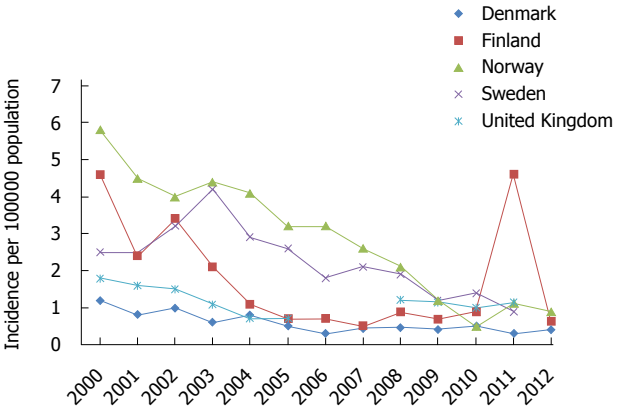


Figure 4 Incidence of acute hepatitis B in northern European countries with selective vaccination (◆: Denmark; ■: Finland; ▲: Norway; ×: Sweden; ✳: United Kingdom). The data for 2006 and 2007 in the United Kingdom were not available.

income countries.

The situation for programs providing for the screening of pregnant women plus HBIG, which is implemented in high-income countries, is more complicated. The screening rate of pregnant women varies from 96.5% to 98.8% in Puerto Rico, the United States, Italy, and Denmark[139-142]. In the United States, perinatal HBsAg test results were documented in 92.6% of maternal medical records; 13.7% of the infants born to HBsAg-positive mothers were not administered an HB vaccine, and 20.1% of infants were born to mothers with unknown HBsAg status[143]. Because the testing and reporting is incomplete in the United States, the true number of perinatal HBV cases per year is likely to be 10 to 20 times higher[144]. In infants born to HBsAg-positive mothers in the United States between 1994 and 2008, the administration rates of HBIG and the HB vaccine at birth remained at the same levels (from 90.3% to 96.4%), and the rate of completed 3-dose series vaccinations by age 12 mo decreased from 86.0% to 77.7%[145]. The reported rates of missed HBIG administration at birth were 4.0% in Denmark, 5.0% in Italy, 19.7% in the United States, and 62.4% in China[141-143,146]. To eradicate HBV infection, closing and/or filling the gaps between the recommended protocol and routine practices are imperative in all countries.

Intensification of approaching high-risk groups

Because the awareness and knowledge of HBV infection and the HB vaccine are insufficient among people who engage in high-risk behaviours[147], it is important to approach high-risk groups using both selective and universal vaccination strategies[148-150]. The European Centre for Disease Prevention and Control reported that heterosexual transmission (23.4%), nosocomial transmission (23.2%), injected drug use (13.4%), and transmission among MSM (10.3%) were the most common routes for acute hepatitis B transmission in 2011[151]. The coverage rates of HB vaccination among high-risk populations were 6% to 39% (MSM, drug users, commercial sex workers, and heterosexuals; > three doses) in the Netherlands in 2007, 50.5% (> one dose), and 41.8% (> three doses) in the United States in 2009, and 22% (prisoners; > one dose) in England and Wales in 2010[66,152,153]. Even among healthcare workers, the coverage rate of the HB vaccine was 84.9% in Belgium, 85.3% in Italy, 87.5% in Poland, 88.0% in Spain, 93.0% in the United Kingdom, and 75.0% in the United States[154,155]. Household exposure and close family contacts of HBV carriers are another high-risk group. According to a survey of European countries, 90% of the countries with a universal vaccination program and all of the countries with a selective vaccination program recommended HB vaccination for individuals with close family contacts with HBV carriers[77]. However, the vaccine coverage was found to be only 25%-34% among the household contacts of HBV carriers in Italy, the United Kingdom, and Denmark[142,156,157].

If high-risk adults are not identified or approached, the health of the children in the area will continue to be threatened. This is a particularly serious problem in the countries implementing selective vaccination policies, where almost all children are susceptible to HBV. Thus, whether these countries with selective vaccination will change their policies has become the focal point of increasing attention[158-163]. The incidence of acute hepatitis B in five northern European countries with selective vaccination is illustrated in Figure 4[164-166]. For the past decade in these countries, the incidence of acute hepatitis B continued to decrease. However, there is no obvious trend of decline in the incidence of acute hepatitis B per 100000 people, which fluctuates approximately 0.3-0.6 in Denmark and 0.7 to 1.0 in the United Kingdom. Because the burden of HBV has remained more or less the same over time since 1990 in the Netherlands despite the use of an intensified targeted approach, the Netherlands recognised the failure of the target vaccination strategy and decided to adopt universal vaccination[89]. Many studies have reported that it is difficult to reach high-risk groups[89,159,167-170], and the targeting of vaccinations for adults belonging to high-risk groups must be strengthened to eliminate HBV infection[171].

Generations miss the benefit of the universal HB immunisation

More than two decades have passed since the first introduction of nationwide universal vaccination, which occurred in Taiwan[172]. Unfortunately, the HB vaccine is not beneficial to individuals who are already chronically infected with HBV, and the majority of HBV carriers might have been born before the introduction of universal vaccination in their country. Some chronically infected individuals might miss the opportunities to receive an infant vaccination or a catch-up vaccination despite the implementation of vaccine programs. Most chronically infected people are unaware of their infection and thus do not receive appropriate treatment.

The WHO global action plan indicated that one of several remaining challenges is the fact that millions of chronically infected individuals do not have timely access to testing, care, and effective treatment to delay the development of the disease and to prevent disability[51]. In the United States, the incidence of acute hepatitis B declined by as much as 80% between 1987 and 2004[173]. However, this decline in acute hepatitis B did not diminish the burden of chronic HBV infection. The burden of HBV infection in the United States, as measured by inpatient and outpatient healthcare utilisation, waitlist registration for liver transplantation, and mortality related to HBV infection, increased substantially throughout the 1990s due to the immigration of a large number of persons from Africa and Asia[173]. In the United States, over half of the individual members of racial/ethnic minority groups have not been tested for HBV, and only one-half of those who tested positive have ever received treatment[174]. Similar to the United States, the United Kingdom also suffers from the burden of chronically infected individuals who were born outside the United Kingdom[175-178]. Chronic HBV infection in migrants has been estimated to account for 96% of all newly added chronic HBV infections in England and Wales between 1996 and 2000[175]. Because screening of the general population is unlikely to be cost-effective, the screening of high-risk populations has been introduced in high-income countries[179-182]. In 2008, the United States Centers for Disease Control updated and expanded the guidelines for testing for chronic HBV infection, in which persons born in geographic regions with an HBsAg-positive prevalence > 2% are recommended for testing[183]. The guidelines recommend the early testing and detection of chronic HBV infection. The 2% screening threshold for the prevalence of chronic HBV infection was demonstrated to be cost-effective[184]. Recent studies in the Netherlands and Canada demonstrated that a selective HBV screening program targeted at all migrants followed by early treatment could be cost-effective[180,185]. This "screen and treat" policy was reported to provide early disease detection, early antiviral treatment, the prevention of HBV-related advanced liver disease, and good quality of life[185,186]. In contrast, low-income countries have no prospect of screening of the high-risk population, diagnosis, or effective treatment[187,188]. The WHO estimates that less than 50% of the blood supply in sub-Saharan Africa is screened for HBV[188]. The budgetary allocation for the implementation of health programs is critically important to identify and treat HBV carriers.

Breakthrough infections

There is no clear definition of "breakthrough infection." Thus, the term is used in several varying contexts[189,190]. In general, "breakthrough infection" means that an HBV infection occurs despite a history of HB vaccination. In the literature, "breakthrough infection" is often characterised by the seroconversion for anti-HBc antibodies or the detection of HBsAg. The pre-existence of anti-HBs antibodies seems to not always be necessary for the diagnosis of "breakthrough infection"[190]. Therefore, primary vaccine failure (non-responder), waning immunity after vaccination (decline of anti-HBsAb levels over time), the emergence of escape mutants, and inappropriate vaccine schedules can all be causes of "breakthrough infection".

Although the evidence is insufficient, non-genotype A could be one of the causes of breakthrough infections[191-193]. In the prophylactic treatment of mother-to-child transmission, a high maternal viral load is the highest risk factor for breakthrough infection[82]. Of course, off-schedule treatment and escape mutants can also cause a breakthrough infection during prophylactic treatment in the perinatal period[194]. The frequency of anti-HBc antibodies in vaccinated children born to HBV carrier mothers was 3.3% (HBsAg-positive carriers: 0.6%) in Italy[195], 8.9% (HBsAg-positive carriers: 3.5%) in China[196], 1.7% in the United Kingdom[197], 25.5% (HBsAg-positive carriers: 2.9%) in Thailand[198], and 6% (HBsAg-positive carriers: 2.9%) in Greenland[199]. In all of these countries except Thailand, HBIG was administered at birth.

In vaccinated adolescent general populations, the frequency of anti-HBc antibodies was 1.8% in Alaska, 13.8% in Gambia, and 4.1% in Taiwan[100,200,201]. Although the rate of breakthrough infection might be influenced by the prevalence of chronic HBV infection in each country, at least a few per cent of vaccinated children could experience a breakthrough infection. Nuclear acid amplification testing for the screening of blood donations, which was introduced for detecting the early window period of HBV infection, identified blood donors with vaccine breakthrough infections who had a history of vaccination and were positive for both HBV DNA and anti-HBs antibodies[192,193,202]. The blood donors with vaccine breakthrough infections developed subclinical acute infection but not chronic infection. Similarly, adolescents and young adults vaccinated in infancy showed transient infection but no chronic infection[203,204]. These findings suggested that a completed series of the HB vaccine cannot guarantee that HBV infection would be completely prevented[205].

Booster doses

The need for booster doses in HB vaccine programmes remains controversial. The duration of vaccine-induced immunity is uncertain, but it is definitely long-term (> 15-20 years)[172,203,206]. Several studies have reported that booster doses of infantile immunisation should be considered in adolescence[207-209]. However, numerous studies have demonstrated that booster doses are not needed in immunocompetent individuals who have received a complete series of HB vaccines[31,172,190,196,203,204,206,210,211]. At present, the WHO does not recommend the universal administration of booster doses. However, immunocompromised hosts, such as hemodialysis patients and HIV-positive patients, are known low responders to vaccines.

Although routine serologic examinations of anti-HBs antibody levels are not needed after HB vaccination, it is recommended that healthcare providers, chronic hemodialysis patients, HIV-infected patients, and other immunocompromised individuals should be monitored and receive booster doses if their anti-HBs antibody levels decrease to less than 10 mIU/mL[190, 211].

Low and non-responders

In 5%-10% of healthy individuals, a three-intramuscular-dose series of the HB vaccine fails to produce protective antibody levels (> 10 mIU/mL)[212-214]. Increasing age, smoking status, male gender, and obesity are the risk factors for poor or no response to the HB vaccine[212,213,215]. Specific human leukocyte antigen (HLA) types have been reported to be associated with the antibody response to the HB vaccine[216,217]. The HLAs are coded by the major histocompatibility complex (MHC) group of genes located on chromosome six in the human genome. The MHC complex plays a central role in the development of the adaptive immune response to HBsAg.

In efforts to overcome the low and non-responsiveness to the HB vaccine, several approaches have been proposed. An additional dose, an additional three-dose series, an increased vaccine dose, changing the route of administration, new adjuvants, and granulocyte-macrophage colony stimulating factor (GM-CSF) have all been proposed to be influential factors for improving the seroprotection rate. The most common strategy for low responders and non-responders is to give an additional vaccine or a series of vaccines. The best injection site was confirmed to be the deltoid muscle, except in infants[218,219]. Of low and non-responders to the initial three-dose series, 39%-91% and 61%-100% showed good responses after one additional dose (4th dose) and an additional three-dose series, respectively[220,221]. An additional three-high-dose series vaccine could further improve the seroprotection rate[222,223]. Moreover, an additional double dose of the combined hepatitis A and hepatitis B vaccine was shown to increase the seroprotection rate to 59% after the first dose and to 95% after the third dose in non-responders[224]. The hepatitis A component might act as an adjuvant for the hepatitis B response. In the United States, chronic hemodialysis patients who had no response to an initial three-dose series are advised to receive a second series using the same dose and schedule[225]. Clearly, the reduction in the numbers of non-responders depends on the number of additional doses[226,227]. Because it is speculated that intradermal inoculation may activate dermal keratinocytes and Langerhan's cells, inducing an effective lymphocyte response[226,228], intradermal vaccination is considered to be superior to intramuscular vaccination[229]. However, a meta-analysis showed that intradermal vaccination was almost equivalent to intramuscular vaccination[229]. The HB vaccine using a new adjuvant system (AS04), which is a combination of a fragment of the bacterial lipopolysaccharide and alum, demonstrated more effective seroprotection rate results than a conventional HB vaccine[215,229]. GM-CSF is a candidate cytokine adjuvant, and it is used for the revaccination of non-responders[230]. A standard dose vaccine plus GM-CSF showed almost the same seroprotection rate as that provided by a high-dose vaccine in healthy non-responders[231]. Although there are several options to overcome poor responsiveness to HB vaccines, no consensus protocol has been established.

CONCLUSION

HB vaccines are very effective against HBV infection and were shown to be the first useful tool for cancer prevention. Financial support for global immunisation, as encouraged by the GAVI Alliance, has become solid and stable. However, the path to the eradication of HBV presents further obstacles. The difficulties to overcome include identifying the best ways to increase coverage rates, closing the gap between recommendations and routine practices, approaching and treating high-risk individuals, screening and treating chronically infected individuals, and preventing breakthrough infections. HBV infection is one of the diseases considered to be a candidate for global eradication, similar to polio, but it is presumed that several decades of effort will be necessary to eradicate HBV. We must acknowledge that the war against HBV will not be over soon. The difference in the prevalence of HBsAg between high- and low-endemic countries is becoming small. Eventually, the countries implementing selective vaccination for HBV will make the wise decision to introduce universal vaccination for global eradication. Although it is uncertain whether the victory against HBV will be gained using only vaccines; at present, vaccines are the most cost-effective method for the control of HBV infections.

REFERENCES

1 World Health Organization (2013). Media Centre. Hepatitis B. Accessed by September 4, 2013. Available from: URL: http://www.who.int/mediacentre/factsheets/fs204/en/

2 **Margolis HS**, Coleman PJ, Brown RE, Mast EE, Sheingold SH, Arevalo JA. Prevention of hepatitis B virus transmission by immunization. An economic analysis of current recommendations. *JAMA* 1995; **274**: 1201-1208 [PMID: 7563509]

3 **Parkin DM**. The global health burden of infection-associated cancers in the year 2002. *Int J Cancer* 2006; **118**: 3030-3044 [PMID: 16404738 DOI: 10.1002/ijc.21731]

4 World Health Organization (2002). Global Alert and Response. Hepatitis B. Prevention and treatment. Accessed by July 24, 2013. Available from: URL: http://www.who.int/csr/disease/hepatitis/whocdscsrlyo20022/en/index5.html

5 **Blumberg BS,** Alter HJ, Visnich S. A "new" antigen in leukemia sera. *JAMA* 1965; **191**: 541-546

6 **Alter H**. Baruch Blumberg (1925-2011). *Nature* 2011; **473**: 155 [PMID: 21562549 DOI: 10.1038/473155a]

7 **Schmid R**. History of viral hepatitis: a tale of dogmas and misinterpretations. *J Gastroenterol Hepatol* 2001; **16**: 718-722 [PMID: 11446877]

8 **Pérez V**. Viral hepatitis: historical perspectives from the 20th to the 21st century. *Arch Med Res* 2007; **38**: 593-605 [PMID: 17613350 DOI: 10.1016/j.arcmed.2006.09.020]
9 **Blumberg BS**, Bernanke D, Allison AC. A human lipoprotein polymorphism. *J Clin Invest* 1962; **41**: 1936-1944 [PMID: 13971873 DOI: 10.1172/jci104651]
10 **Blumberg BS**, Gerstley BJ, Hungerford DA, London WT, Sutnick AI. A serum antigen (Australia antigen) in Down's syndrome, leukemia, and hepatitis. *Ann Intern Med* 1967; **66**: 924-931 [PMID: 4225883]
11 **Blumberg BS**, Sutnick AI, London WT. Hepatitis and leukemia: their relation to Australia antigen. *Bull N Y Acad Med* 1968; **44**: 1566-1586 [PMID: 5247325]
12 **Patlak M**. The hepatitis B story. Accessed July 23, 2013. Available from: URL: http://www.margiepatlak.com/files/QuickSiteImages/hepatitis.pdf
13 **Sutnick AI**, London WT, Gerstley BJ, Cronlund MM, Blumberg BS. Anicteric hepatitis associated with Australia antigen. Occurrence in patients with Down's syndrome. *JAMA* 1968; **205**: 670-674 [PMID: 4233233]
14 **London WT**, Sutnick AI, Blumberg BS. Australia antigen and acute viral hepatitis. *Ann Intern Med* 1969; **70**: 55-59 [PMID: 4236725]
15 **Prince AM**. An antigen detected in the blood during the incubation period of serum hepatitis. *Proc Natl Acad Sci USA* 1968; **60**: 814-821 [PMID: 4970112]
16 **Gitnick GL**, Gleich GJ, Schoenfield LJ, Baggenstoss AH, Sutnick AI, Blumberg BS, London WT, Summerskill WH. Australia antigen in chronic active liver disease with cirrhosis. *Lancet* 1969; **2**: 285-288 [PMID: 4184209]
17 **Dane DS**, Cameron CH, Briggs M. Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. *Lancet* 1970; **1**: 695-698 [PMID: 4190997]
18 **Hilleman MR**. Critical overview and outlook: pathogenesis, prevention, and treatment of hepatitis and hepatocarcinoma caused by hepatitis B virus. *Vaccine* 2003; **21**: 4626-4649 [PMID: 14585670]
19 **Bayer ME**, Blumberg BS, Werner B. Particles associated with Australia antigen in the sera of patients with leukaemia, Down's Syndrome and hepatitis. *Nature* 1968; **218**: 1057-1059 [PMID: 4231935]
20 **Hilleman MR**, Buynak EB, Roehm RR, Tytell AA, Bertland AU, Lampson GP. Purified and inactivated human hepatitis B vaccine: progress report. *Am J Med Sci* 1975; **270**: 401-404 [PMID: 828833]
21 **Buynak EB**, Roehm RR, Tytell AA, Bertland AU, Lampson GP, Hilleman MR. Vaccine against human hepatitis B. *JAMA* 1976; **235**: 2832-2834 [PMID: 819667]
22 **Szmuness W**, Stevens CE, Harley EJ, Zang EA, Oleszko WR, William DC, Sadovsky R, Morrison JM, Kellner A. Hepatitis B vaccine: demonstration of efficacy in a controlled clinical trial in a high-risk population in the United States. *N Engl J Med* 1980; **303**: 833-841 [PMID: 6997738 DOI: 10.1056/nejm198010093031501]
23 **Szmuness W**, Stevens CE, Zang EA, Harley EJ, Kellner A. A controlled clinical trial of the efficacy of the hepatitis B vaccine (Heptavax B): a final report. *Hepatology* 1981; **1**: 377-385
24 **Laura J**, Frost MRR. Access. How do good health technologies get topoor people in poor countires? Harvard Center for Population and Development Studies. 2008: 68-90. Available from: URL: http://www.hsph.harvard.edu/population-development/training/bell-fellowship/
25 **Zanetti AR**, Van Damme P, Shouval D. The global impact of vaccination against hepatitis B: a historical overview. *Vaccine* 2008; **26**: 6266-6273 [PMID: 18848855 DOI: 10.1016/j.vaccine.2008.09.056]
26 **Valenzuela P**, Medina A, Rutter WJ, Ammerer G, Hall BD. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature* 1982; **298**: 347-350 [PMID: 7045698]
27 **McAleer WJ**, Buynak EB, Maigetter RZ, Wampler DE, Miller WJ, Hilleman MR. Human hepatitis B vaccine from recombinant yeast. *Nature* 1984; **307**: 178-180 [PMID: 6318124]
28 **Michel ML**, Pontisso P, Sobczak E, Malpièce Y, Streeck RE, Tiollais P. Synthesis in animal cells of hepatitis B surface antigen particles carrying a receptor for polymerized human serum albumin. *Proc Natl Acad Sci USA* 1984; **81**: 7708-7712 [PMID: 6096851]
29 **Michel ML**, Tiollais P. Hepatitis B vaccines: protective efficacy and therapeutic potential. *Pathol Biol* (Paris) 2010; **58**: 288-295 [PMID: 20382485 DOI: 10.1016/j.patbio.2010.01.006]
30 World Health Organization (2002). Global Alert and Resopnses. Hepatitis B. Surveillance and control. Accessed July 24, 2013. Available from: URL: http://www.who.int/csr/disease/hepatitis/whocdscsrlyo20022/en/index4.html
31 **Poorolajal J**, Mahmoodi M, Majdzadeh R, Nasseri-Moghaddam S, Haghdoost A, Fotouhi A. Long-term protection provided by hepatitis B vaccine and need for booster dose: a meta-analysis. *Vaccine* 2010; **28**: 623-631 [PMID: 19887132 DOI: 10.1016/j.vaccine.2009.10.068]
32 **Tu HA**, Woerdenbag HJ, Kane S, Riewpaiboon A, van Hulst M, Postma MJ. Economic evaluations of hepatitis B vaccination for developing countries. *Expert Rev Vaccines* 2009; **8**: 907-920 [PMID: 19538116 DOI: 10.1586/erv.09.53]
33 **Wong VC**, Ip HM, Reesink HW, Lelie PN, Reerink-Brongers EE, Yeung CY, Ma HK. Prevention of the HBsAg carrier state in newborn infants of mothers who are chronic carriers of HBsAg and HBeAg by administration of hepatitis-B vaccine and hepatitis-B immunoglobulin. Double-blind randomised placebo-controlled study. *Lancet* 1984; **1**: 921-926 [PMID: 6143868]
34 **Lo KJ**, Tsai YT, Lee SD, Wu TC, Wang JY, Chen GH, Yeh CL, Chiang BN, Yeh SH, Goudeau A. Immunoprophylaxis of infection with hepatitis B virus in infants born to hepatitis B surface antigen-positive carrier mothers. *J Infect Dis* 1985; **152**: 817-822 [PMID: 2931490]
35 **Beasley RP**. Rocks along the road to the control of HBV and HCC. *Ann Epidemiol* 2009; **19**: 231-234 [PMID: 19344859 DOI: 10.1016/j.annepidem.2009.01.017]
36 World Health Organization (2013). Global Alert and Response (GAR) Global policy report on the prevention and control of viral hepatitis in WHO Member States. Accessed by July 29, 2013. Available from: URL: http://www.who.int/csr/disease/hepatitis/global_report/en/index.html
37 World Health Organization (2011). Immunization, Vaccines and Biologicals .Viral hepatitis policy and practice: Report of a survey of WHO Member States, 2010. Accessed by July 29, 2013. Available from: URL: http://www.who.int/immunization/topics/hepatitis_b_survey_2010/en/#
38 World Health Organization (2007). Western Pacific regional plan for hepatitis B control through immunization. Accessed by July 30, 2013. Available from: URL: http://www.wpro.who.int/immunization/documents/docs/POA_HepB.pdf
39 UNICEF (2002). Vaccines for children: supply at risk. Accessed by July 27, 2013. Available from: URL: http://www.unicef.org/publications/index_4442.html
40 **DeRoeck D**. Immunization financing in developing countries and the international vaccine market: trends and issues. Accessed by July 27, 2013. Available from: URL: http://www.vaccine-safety-training.org/tl_files/vs/pdf/ADB.pdf
41 **Centers for Disease Control and Prevention (CDC)**. Global progress toward universal childhood hepatitis B vaccination, 2003. *MMWR Morb Mortal Wkly Rep* 2003; **52**: 868-870 [PMID: 12970620]
42 **Glatman-Freedman A**, Cohen ML, Nichols KA, Porges RF, Saludes IR, Steffens K, Rodwin VG, Britt DW. Factors affecting the introduction of new vaccines to poor nations: a comparative study of the Haemophilus influenzae type B and hepatitis B vaccines. *PLoS One* 2010; **5**: e13802 [PMID: 21072192 DOI: 10.1371/journal.pone.0013802]
43 GAVI alliance. Hepatitis B vaccine support. Accessed by July

38, 2013. Available from: URL: http://www.gavialliance.org/support/nvs/hepb/
44 WHO, UNICEF, the World Bank (2009). State of the world's vaccines and immunization. Third edition. Accessed by 28 July, 2013. Available from: URL: http://www.who.int/immunization/sowvi/en/
45 World Health Organization. Millennium Development Goals. Accessed by July 28, 2013. Available from: URL: http://www.un.org/millenniumgoals/
46 World Health Organization (2010). New and under-utilized vaccines implementation. Accessed by July 28, 2013. Available from: URL: http://www.who.int/nuvi/en/
47 World Health Organization (2013). Immunization surveillance, assessment and monitoring.Data, statistics, and graphics. WHO Region and globally. Accessed by Aust 7, 2013. Avaibale from: URL: http://www.who.int/immunization_monitoring/data/gs_gloprofile.pdf
48 GAVI alliance. Investing in immunisation through the GAVI Alliance. The evidence base. Accessed by July 28, 2013. Available from: URL: http://www.gavialliance.org/library/publications/the-evidence-base/
49 GAVI alliance. GAVI role in ever-growing demand for pentavalent. Accesssed by August 6, 2013. Avaibale from: URL: http://www.gavialliance.org/library/news/roi/2009/gavi-role-in-ever-growing-demand-for-pentavalent/
50 Word Health Organaization (2013). Immunization surveillance, assessment and monitoring. Reginal summaries. Regional profile. Accessed by August 10, 2013. Available from: URL: http://www.who.int/immunization_monitoring/data/data_regions/en/index.html
51 World Health Organization (2012). Prevention Control of Viral Hepatitis: Framework for Global Action. Accessed by August 1, 2013. Available from: URL: http://www.who.int/csr/disease/hepatitis/GHP_framework.pdf
52 **Ott JJ**, Stevens GA, Groeger J, Wiersma ST. Global epidemiology of hepatitis B virus infection: new estimates of age-specific HBsAg seroprevalence and endemicity. *Vaccine* 2012; **30**: 2212-2219 [PMID: 22273662 DOI: 10.1016/j.vaccine.2011.12.116]
53 **Kiire CF**. The epidemiology and prophylaxis of hepatitis B in sub-Saharan Africa: a view from tropical and subtropical Africa. *Gut* 1996; **38** Suppl 2: S5-S12 [PMID: 8786055]
54 **Kew MC**. Progress towards the comprehensive control of hepatitis B in Africa: a view from South Africa. *Gut* 1996; **38** Suppl 2: S31-S36 [PMID: 8786050]
55 **Nur YA**, Groen J, Elmi AM, Ott A, Osterhaus AD. Prevalence of serum antibodies against bloodborne and sexually transmitted agents in selected groups in Somalia. *Epidemiol Infect* 2000; **124**: 137-141 [PMID: 10722141]
56 **Ott JJ**, Stevens GA, Wiersma ST. The risk of perinatal hepatitis B virus transmission: hepatitis B e antigen (HBeAg) prevalence estimates for all world regions. *BMC Infect Dis* 2012; **12**: 131 [PMID: 22682147 DOI: 10.1186/1471-2334-12-131]
57 **Guido Françoisa CD**, Jeffrey Mphahlelec M, Burnettd R, Van Hala G, Meheusb A. Hepatitis B vaccination in Africa: mission accomplished? *J Epidemiol Infect* 2008; **23**: 24-28
58 **Ekra D**, Herbinger KH, Konate S, Leblond A, Fretz C, Cilote V, Douai C, Da Silva A, Gessner BD, Chauvin P. A non-randomized vaccine effectiveness trial of accelerated infant hepatitis B immunization schedules with a first dose at birth or age 6 weeks in Côte d'Ivoire. *Vaccine* 2008; **26**: 2753-2761 [PMID: 18436354 DOI: 10.1016/j.vaccine.2008.03.018]
59 **Ropero AM**, Danovaro-Holliday MC, Andrus JK. Progress in vaccination against hepatitis B in the Americas. *J Clin Virol* 2005; **34** Suppl 2: S14-S19 [PMID: 16461235]
60 Pan American Health Organization (2000). Conclusions and Recommendations of the XIV Technical Advisory Group Meeting on Vaccine-preventable Diseases (TAG) of the Pan American Health Organization, was held in Foz do Iguaçu, Brazil, October 2-5, 2000. Accessed by July 30, 2013. Available from: URL: http://www2.paho.org/hq/dmdocuments/2009/tag14_2000_conclusions_Eng.pdf
61 Pan American Health Organization. PAHO's Regional Immunization Vison and Strategy 2007-2015. Accessed by July 30, 2013. Available from: URL: http://www.who.int/immunization/sage/PAHO_RIVS.pdf
62 Pan Americam Health Organization (2012). World Hepatitis Day. Hepatitis vaccination in Latin America and the Caribbean. Accessed by July 30, 2013. Available from: URL: http://www.paho.org/hq/index.php?option=com_docman&task=doc_view&gid=18239&Itemid=
63 World Health Organization. Regional Office for the Eastern Mediterranean (2009).The growing threats of hepatitis B and C in the Eastern Mediterranean Region: a call for action. Accessed by July 31, 2013. Available from: URL: http://applications.emro.who.int/docs/EM_RC56_3_en.pdf
64 World Health Organization. Regional Office for Europe. Hepatitis. What we do. hepatitis. Data and statistics. Hepatitis B. Accessed by September 1, 2013. Available from: URL: http://www.euro.who.int/en/what-we-do/health-topics/communicable-diseases/hepatitis/data-and-statistics/hepatitis-b
65 **Hope VD**, Eramova I, Capurro D, Donoghoe MC. Prevalence and estimation of hepatitis B and C infections in the WHO European Region: a review of data focusing on the countries outside the European Union and the European Free Trade Association. *Epidemiol Infect* 2014; **142**: 270-286 [PMID: 23714072 DOI: 10.1017/s0950268813000940]
66 **Lu PJ**, Byrd KK, Murphy TV, Weinbaum C. Hepatitis B vaccination coverage among high-risk adults 18-49 years, U.S., 2009. *Vaccine* 2011; **29**: 7049-7057 [PMID: 21782873 DOI: 10.1016/j.vaccine.2011.07.030]
67 World Health Organization (2011). Regional Office for South-East Asia.Immunization. Year of Intensification of Routine Immunizatio.n Resolution of the WHO Regional Committee for South-East Asia. Accessed by August 1, 2013. Available from: URL: http://www.searo.who.int/entity/immunization/topics/system_strengthening/sea_rc64_r3_resolution_iri_2011.pdf
68 World Health Organization. Western Pacific Region(2007). Expanded programme on immunization. Western Pacific Regional Plan for Hepatitis B Control Through Immunization. Accessed by July 28, 2013. Available from: URL: http://www.wpro.who.int/immunization/documents/POA_HepB/en/index.html
69 World Health Organization (2009). Hepatitis B control by 2012 in the WHO Western Pacific Region: rationale and implications. Accessed by July 28, 2013. Available from: URL: http://www.who.int/bulletin/volumes/87/9/08-059220/en/
70 **Rani M**, Yang B, Nesbit R. Hepatitis B control by 2012 in the WHO Western Pacific Region: rationale and implications. *Bull World Health Organ* 2009; **87**: 707-713 [PMID: 19784451]
71 **Centers for Disease Control (CDC)**. Recommendation of the Immunization Practices Advisory Committee (ACIP). Inactivated hepatitis B virus vaccine. *MMWR Morb Mortal Wkly Rep* 1982; **31**: 317-322, 327-328 [PMID: 6811846]
72 **Alter MJ**, Hadler SC, Margolis HS, Alexander WJ, Hu PY, Judson FN, Mares A, Miller JK, Moyer LA. The changing epidemiology of hepatitis B in the United States. Need for alternative vaccination strategies. *JAMA* 1990; **263**: 1218-1222 [PMID: 2304237]
73 Hepatitis B virus: a comprehensive strategy for eliminating transmission in the United States through universal childhood vaccination. Recommendations of the Immunization Practices Advisory Committee (ACIP). *MMWR Recomm Rep* 1991; **40**: 1-25 [PMID: 1835756]
74 **Mele A**, Stroffolini T, Zanetti AR. Hepatitis B in Italy: where we are ten years after the introduction of mass vaccination.

J Med Virol 2002; **67**: 440-443 [PMID: 12116041 DOI: 10.1002/jmv.10092]
75 **Bonanni P**, Crovari P. Success stories in the implementation of universal hepatitis B vaccination: an update on Italy. *Vaccine* 1998; **16** Suppl: S38-S42 [PMID: 9915032]
76 **Bonanni P**, Pesavento G, Bechini A, Tiscione E, Mannelli F, Benucci C, Nostro AL. Impact of universal vaccination programmes on the epidemiology of hepatitis B: 10 years of experience in Italy. *Vaccine* 2003; **21**: 685-691 [PMID: 12531340]
77 **Mereckiene J**, Cotter S, Lopalco P, D'Ancona F, Levy-Bruhl D, Giambi C, Johansen K, Dematte L, Salmaso S, Stefanoff P, O'Flanagan D. Hepatitis B immunisation programmes in European Union, Norway and Iceland: where we were in 2009? *Vaccine* 2010; **28**: 4470-4477 [PMID: 20451643 DOI: 10.1016/j.vaccine.2010.04.037]
78 **O'Flanagan D**, Cotter S, Mereckiene J. Hepatitis B vaccination in Europe.November 2008-March 2009. The Health Protection Surveillance Centre European Centre for disease Control VENICE II Project. Accessed by August 3, 2013. Available from: URL: http://venice.cineca.org/Report_Hepatitis_B_Vaccination.pdf
79 EUVAC.NET. National Childhood Vaccination Schedules. Accessed by September 3, 2013. Available from: URL: http://www.euvac.net/graphics/euvac/vaccination/vaccination.html
80 **Mast EE**, Margolis HS, Fiore AE, Brink EW, Goldstein ST, Wang SA, Moyer LA, Bell BP, Alter MJ. A comprehensive immunization strategy to eliminate transmission of hepatitis B virus infection in the United States: recommendations of the Advisory Committee on Immunization Practices (ACIP) part 1: immunization of infants, children, and adolescents. *MMWR Recomm Rep* 2005; **54**: 1-31 [PMID: 16371945]
81 **Chen HL**, Lin LH, Hu FC, Lee JT, Lin WT, Yang YJ, Huang FC, Wu SF, Chen SC, Wen WH, Chu CH, Ni YH, Hsu HY, Tsai PL, Chiang CL, Shyu MK, Lee PI, Chang FY, Chang MH. Effects of maternal screening and universal immunization to prevent mother-to-infant transmission of HBV. *Gastroenterology* 2012; **142**: 773-781.e2 [PMID: 22198276 DOI: 10.1053/j.gastro.2011.12.035]
82 **Wen WH**, Chang MH, Zhao LL, Ni YH, Hsu HY, Wu JF, Chen PJ, Chen DS, Chen HL. Mother-to-infant transmission of hepatitis B virus infection: significance of maternal viral load and strategies for intervention. *J Hepatol* 2013; **59**: 24-30 [PMID: 23485519 DOI: 10.1016/j.jhep.2013.02.015]
83 **Chen SC**, Toy M, Yeh JM, Wang JD, Resch S. Cost-effectiveness of augmenting universal hepatitis B vaccination with immunoglobin treatment. *Pediatrics* 2013; **131**: e1135-e1143 [PMID: 23530168 DOI: 10.1542/peds.2012-1262]
84 UNICEF (2012). The State of the World's Children 2012. Accessed by August 5, 2013. Available from: URL: http://www.unicef.org/sowc2012/statistics.php
85 Global Finance (2013). The Richest Countries in the World. Accessed by August 14, 2013. Available from: URL: http://www.gfmag.com/component/content/article/119-economic-data/12538-the-richest-countries-in-the-world.html#axzz2YEiEtPjQ
86 Ireland's Health Service Executive national immunisation programme. Thornton L (2008). Universal hepatitis B vaccination in Ireland-Why now? Accessed by August 4, 2013. Available from: URL: http://www.immunisation.ie/en/Downloads/PDFFile_15136_en.pdf
87 **Tilson L**, Thornton L, O'Flanagan D, Johnson H, Barry M. Cost effectiveness of hepatitis B vaccination strategies in Ireland: an economic evaluation. *Eur J Public Health* 2008; **18**: 275-282 [PMID: 18160389 DOI: 10.1093/eurpub/ckm123]
88 **Houweling H**, Spaendonck MC, Paulussen T, Verweij M, Ruitenberg EJ. Preparing for the next public debate: universal vaccination against hepatitis B. *Vaccine* 2011; **29**: 8960-8964 [PMID: 21945254 DOI: 10.1016/j.vaccine.2011.09.042]
89 **Houweling H**, Wittevrongel CF, Verweij M, Ruitenberg EJ. Public vaccination programmes against hepatitis B in The Netherlands: assessing whether a targeted or a universal approach is appropriate. *Vaccine* 2010; **28**: 7723-7730 [PMID: 20933042 DOI: 10.1016/j.vaccine.2010.09.068]
90 The Health Council of the Netherlands (2009). General vaccination against hepatitis B revisited. Accessed by August 4, 2013. Available from: URL: http://www.gezondheidsraad.nl/sites/default/files/200903E.pdf
91 Public Health England (2008). Hepatitis B Guildelines. HBIG for babies born to hep b infected mothers. Accessed by July 29, 2013. Available frim: URL: http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/HepatitisB/GuidelinesHepatitisB/hepbGuidelines/
92 Public Health England (2012). Request form for: Issue of Hepatitis B Immunoglobulin for Infants at Risk of Hepatitis B Infection. Version 7 updated January 2012. Accessed by August 4, 2013
93 **Shiraki K**. Perinatal transmission of hepatitis B virus and its prevention. *J Gastroenterol Hepatol* 2000; **15** Suppl: E11-E15 [PMID: 10921375]
94 **Liang X**, Bi S, Yang W, Wang L, Cui G, Cui F, Zhang Y, Liu J, Gong X, Chen Y, Wang F, Zheng H, Wang F, Guo J, Jia Z, Ma J, Wang H, Luo H, Li L, Jin S, Hadler SC, Wang Y. Evaluation of the impact of hepatitis B vaccination among children born during 1992-2005 in China. *J Infect Dis* 2009; **200**: 39-47 [PMID: 19469708 DOI: 10.1086/599332]
95 **Perz JF**, Elm JL, Fiore AE, Huggler JI, Kuhnert WL, Effler PV. Near elimination of hepatitis B virus infections among Hawaii elementary school children after universal infant hepatitis B vaccination. *Pediatrics* 2006; **118**: 1403-1408 [PMID: 17015529 DOI: 10.1542/peds.2006-0724]
96 **de la Hoz F**, Perez L, de Neira M, Hall AJ. Eight years of hepatitis B vaccination in Colombia with a recombinant vaccine: factors influencing hepatitis B virus infection and effectiveness. *Int J Infect Dis* 2008; **12**: 183-189 [PMID: 17913535 DOI: 10.1016/j.ijid.2007.06.010]
97 **Chen HL**, Chang MH, Ni YH, Hsu HY, Lee PI, Lee CY, Chen DS. Seroepidemiology of hepatitis B virus infection in children: Ten years of mass vaccination in Taiwan. *JAMA* 1996; **276**: 906-908 [PMID: 8782640]
98 **Da Villa G**, Romanò L, Sepe A, Iorio R, Paribello N, Zappa A, Zanetti AR. Impact of hepatitis B vaccination in a highly endemic area of south Italy and long-term duration of anti-HBs antibody in two cohorts of vaccinated individuals. *Vaccine* 2007; **25**: 3133-3136 [PMID: 17280750 DOI: 10.1016/j.vaccine.2007.01.044]
99 **Simani OE**, Leroux-Roels G, François G, Burnett RJ, Meheus A, Mphahlele MJ. Reduced detection and levels of protective antibodies to hepatitis B vaccine in under 2-year-old HIV positive South African children at a paediatric outpatient clinic. *Vaccine* 2009; **27**: 146-151 [PMID: 18940220 DOI: 10.1016/j.vaccine.2008.10.004]
100 **van der Sande MA**, Waight P, Mendy M, Rayco-Solon P, Hutt P, Fulford T, Doherty C, McConkey SJ, Jeffries D, Hall AJ, Whittle HC. Long-term protection against carriage of hepatitis B virus after infant vaccination. *J Infect Dis* 2006; **193**: 1528-1535 [PMID: 16652281 DOI: 10.1086/503433]
101 **Shepard CW**, Finelli L, Fiore AE, Bell BP. Epidemiology of hepatitis B and hepatitis B virus infection in United States children. *Pediatr Infect Dis J* 2005; **24**: 755-760 [PMID: 16148839]
102 **McMahon BJ**, Bulkow LR, Singleton RJ, Williams J, Snowball M, Homan C, Parkinson AJ. Elimination of hepatocellular carcinoma and acute hepatitis B in children 25 years after a hepatitis B newborn and catch-up immunization program. *Hepatology* 2011; **54**: 801-807 [PMID: 21618565 DOI: 10.1002/hep.24442]
103 **Kao JH**, Hsu HM, Shau WY, Chang MH, Chen DS. Universal hepatitis B vaccination and the decreased mortality from fulminant hepatitis in infants in Taiwan. *J Pediatr* 2001; **139**:

349-352 [PMID: 11562612 DOI: 10.1067/mpd.2001.116277]
104 **Mele A**, Tosti ME, Mariano A, Pizzuti R, Ferro A, Borrini B, Zotti C, Lopalco P, Curtale F, Balocchini E, Spada E. Acute hepatitis B 14 years after the implementation of universal vaccination in Italy: areas of improvement and emerging challenges. *Clin Infect Dis* 2008; **46**: 868-875 [PMID: 18269332 DOI: 10.1086/528687]
105 **Kao JH**, Chen DS. Global control of hepatitis B virus infection. *Lancet Infect Dis* 2002; **2**: 395-403 [PMID: 12127351]
106 **Hsu HC**, Wu MZ, Chang MH, Su IJ, Chen DS. Childhood hepatocellular carcinoma develops exclusively in hepatitis B surface antigen carriers in three decades in Taiwan. Report of 51 cases strongly associated with rapid development of liver cirrhosis. *J Hepatol* 1987; **5**: 260-267 [PMID: 2828461]
107 **Chen WJ**, Lee JC, Hung WT. Primary malignant tumor of liver in infants and children in Taiwan. *J Pediatr Surg* 1988; **23**: 457-461 [PMID: 2837562]
108 **Chang MH**, Chen DS, Hsu HC, Hsu HY, Lee CY. Maternal transmission of hepatitis B virus in childhood hepatocellular carcinoma. *Cancer* 1989; **64**: 2377-2380 [PMID: 2553245]
109 **Chang MH**, Chen CJ, Lai MS, Hsu HM, Wu TC, Kong MS, Liang DC, Shau WY, Chen DS. Universal hepatitis B vaccination in Taiwan and the incidence of hepatocellular carcinoma in children. Taiwan Childhood Hepatoma Study Group. *N Engl J Med* 1997; **336**: 1855-1859 [PMID: 9197213 DOI: 10.1056/nejm199706263362602]
110 GAVI alliance. GAVI publications. Pledging conference for immunisation. Delivering on the promise. Accessed by August 8, 2013. Available from: URL: http://www.gavialliance.org/library/publications/pledging-conference-for-immunisation/
111 **Lee LA**, Franzel L, Atwell J, Datta SD, Friberg IK, Goldie SJ, Reef SE, Schwalbe N, Simons E, Strebel PM, Sweet S, Suraratdecha C, Tam Y, Vynnycky E, Walker N, Walker DG, Hansen PM. The estimated mortality impact of vaccinations forecast to be administered during 2011-2020 in 73 countries supported by the GAVI Alliance. *Vaccine* 2013; **31** Suppl 2: B61-B72 [PMID: 23598494 DOI: 10.1016/j.vaccine.2012.11.035]
112 World Health Organaization (2005). Immunization, Vaccines and Biologicals. Global Immunization Vision and Strategy. Goals. Accessed by August 10, 2013. Available from: URL: http://www.who.int/immunization/givs/en/index.html
113 **Kamara L**, Lydon P, Bilous J, Vandelaer J, Eggers R, Gacic-Dobo M, Meaney W, Okwo-Bele JM. Global Immunization Vision and Strategy (GIVS): a mid-term analysis of progress in 50 countries. *Health Policy Plan* 2013; **28**: 11-19 [PMID: 22411879 DOI: 10.1093/heapol/czs020]
114 **Vashishtha VM**. Status of immunization and need for intensification of routine immunization in India. *Indian Pediatr* 2012; **49**: 357-361 [PMID: 22700663]
115 **Machingaidze S**, Wiysonge CS, Hussey GD. Strengthening the expanded programme on immunization in Africa: looking beyond 2015. *PLoS Med* 2013; **10**: e1001405 [PMID: 23526886 DOI: 10.1371/journal.pmed.1001405]
116 **Barth RE**, Huijgen Q, Taljaard J, Hoepelman AI. Hepatitis B/C and HIV in sub-Saharan Africa: an association between highly prevalent infectious diseases. A systematic review and meta-analysis. *Int J Infect Dis* 2010; **14**: e1024-e1031 [PMID: 20870439 DOI: 10.1016/j.ijid.2010.06.013]
117 **Bruguera M**, Cremades M, Salinas R, Costa J, Grau M, Sans J. Impaired response to recombinant hepatitis B vaccine in HIV-infected persons. *J Clin Gastroenterol* 1992; **14**: 27-30 [PMID: 1532609]
118 **Arrazola MP**, de Juanes JR, Ramos JT, Aragón AJ, Garcia de Codes A. Hepatitis B vaccination in infants of mothers infected with human immunodeficiency virus. *J Med Virol* 1995; **45**: 339-341 [PMID: 7775958]
119 **Pasricha N**, Datta U, Chawla Y, Singh S, Arora SK, Sud A, Minz RW, Saikia B, Singh H, James I, Sehgal S. Poor responses to recombinant HBV vaccination in patients with HIV infection. *Trop Gastroenterol* 2005; **26**: 178-182 [PMID: 16737046]
120 **Clements CJ**, Nshimirimanda D, Gasasira A. Using immunization delivery strategies to accelerate progress in Africa towards achieving the Millennium Development Goals. *Vaccine* 2008; **26**: 1926-1933 [PMID: 18343540 DOI: 10.1016/j.vaccine.2008.02.032]
121 **Rainey JJ**, Watkins M, Ryman TK, Sandhu P, Bo A, Banerjee K. Reasons related to non-vaccination and under-vaccination of children in low and middle income countries: findings from a systematic review of the published literature, 1999-2009. *Vaccine* 2011; **29**: 8215-8221 [PMID: 21893149 DOI: 10.1016/j.vaccine.2011.08.096]
122 **Chauke-Moagi BE**, Mumba M. New vaccine introduction in the East and Southern African sub-region of the WHO African region in the context of GIVS and MDGs. *Vaccine* 2012; **30** Suppl 3: C3-C8 [PMID: 22939018 DOI: 10.1016/j.vaccine.2012.05.086]
123 **Wiysonge CS**, Uthman OA, Ndumbe PM, Hussey GD. Individual and contextual factors associated with low childhood immunisation coverage in sub-Saharan Africa: a multilevel analysis. *PLoS One* 2012; **7**: e37905 [PMID: 22662247 DOI: 10.1371/journal.pone.0037905]
124 **Lahariya C**, Subramanya BP, Sosler S. An assessment of hepatitis B vaccine introduction in India: Lessons for roll out and scale up of new vaccines in immunization programs. *Indian J Public Health* 2013; **57**: 8-14 [PMID: 23649136 DOI: 10.4103/0019-557x.111357]
125 **Wang SA**, Hyde TB, Mounier-Jack S, Brenzel L, Favin M, Gordon WS, Shearer JC, Mantel CF, Arora N, Durrheim D. New vaccine introductions: assessing the impact and the opportunities for immunization and health systems strengthening. *Vaccine* 2013; **31** Suppl 2: B122-B128 [PMID: 23598473 DOI: 10.1016/j.vaccine.2012.10.116]
126 **Travasso C**. Poor uptake of hepatitis B vaccine in India has several causes, study finds. *BMJ* 2013; **346**: f3596 [PMID: 23744767 DOI: 10.1136/bmj.f3596]
127 **Goldstein ST**, Zhou F, Hadler SC, Bell BP, Mast EE, Margolis HS. A mathematical model to estimate global hepatitis B disease burden and vaccination impact. *Int J Epidemiol* 2005; **34**: 1329-1339 [PMID: 16249217 DOI: 10.1093/ije/dyi206]
128 **Thursz M**, Njie R, Lemoine M. Hepatitis: Global eradication of hepatitis B--feasible or fallacy? *Nat Rev Gastroenterol Hepatol* 2012; **9**: 492-494 [PMID: 22868658 DOI: 10.1038/nrgastro.2012.155]
129 **Ronveaux O**, Rickert D, Hadler S, Groom H, Lloyd J, Bchir A, Birmingham M. The immunization data quality audit: verifying the quality and consistency of immunization monitoring systems. *Bull World Health Organ* 2005; **83**: 503-510 [PMID: 16175824]
130 **Lim SS**, Stein DB, Charrow A, Murray CJ. Tracking progress towards universal childhood immunisation and the impact of global initiatives: a systematic analysis of three-dose diphtheria, tetanus, and pertussis immunisation coverage. *Lancet* 2008; **372**: 2031-2046 [PMID: 19070738 DOI: 10.1016/s0140-6736(08)61869-3]
131 **Akmatov MK**, Mikolajczyk RT. Timeliness of childhood vaccinations in 31 low and middle-income countries. *J Epidemiol Community Health* 2012; **66**: e14 [PMID: 21551179 DOI: 10.1136/jech.2010.124651]
132 **Clark A**, Sanderson C. Timing of children's vaccinations in 45 low-income and middle-income countries: an analysis of survey data. *Lancet* 2009; **373**: 1543-1549 [PMID: 19303633 DOI: 10.1016/s0140-6736(09)60317-2]
133 **Akmatov MK**, Kretzschmar M, Krämer A, Mikolajczyk RT. Timeliness of vaccination and its effects on fraction of vaccinated population. *Vaccine* 2008; **26**: 3805-3811 [PMID: 18565626 DOI: 10.1016/j.vaccine.2008.05.031]

134 **Fadnes LT**, Nankabirwa V, Sommerfelt H, Tylleskär T, Tumwine JK, Engebretsen IM. Is vaccination coverage a good indicator of age-appropriate vaccination? A prospective study from Uganda. *Vaccine* 2011; **29**: 3564-3570 [PMID: 21402043 DOI: 10.1016/j.vaccine.2011.02.093]

135 **Dayan GH**, Shaw KM, Baughman AL, Orellana LC, Forlenza R, Ellis A, Chaui J, Kaplan S, Strebel P. Assessment of delay in age-appropriate vaccination using survival analysis. *Am J Epidemiol* 2006; **163**: 561-570 [PMID: 16421238 DOI: 10.1093/aje/kwj074]

136 **Soeung SC**, Thiep C, Duncan R, Patel M, Hennessey K. Using data to guide policy: next steps for preventing perinatal hepatitis B virus transmission in Cambodia. *Vaccine* 2012; **31**: 149-153 [PMID: 23127515 DOI: 10.1016/j.vaccine.2012.10.079]

137 **Luman ET**, Barker LE, McCauley MM, Drews-Botsch C. Timeliness of childhood immunizations: a state-specific analysis. *Am J Public Health* 2005; **95**: 1367-1374 [PMID: 16043668 DOI: 10.2105/ajph.2004.046284]

138 **Zhao Z**, Murphy TV, Jacques-Carroll L. Progress in newborn hepatitis B vaccination by birth year cohorts-1998-2007, USA. *Vaccine* 2011; **30**: 14-20 [PMID: 22063390 DOI: 10.1016/j.vaccine.2011.10.076]

139 **Dayan GH**, Caquías CR, García Y, Malik T, Copeland J, Bi D, Reef S. Medical practices for prevention of perinatal infections in Puerto Rico. *Paediatr Perinat Epidemiol* 2008; **22**: 31-39 [PMID: 18173782 DOI: 10.1111/j.1365-3016.2007.00873.x]

140 **Schrag SJ**, Arnold KE, Mohle-Boetani JC, Lynfield R, Zell ER, Stefonek K, Noga H, Craig AS, Thomson Sanza L, Smith G, Schuchat A. Prenatal screening for infectious diseases and opportunities for prevention. *Obstet Gynecol* 2003; **102**: 753-760 [PMID: 14551005]

141 **Spada E**, Tosti ME, Zuccaro O, Stroffolini T, Mele A. Evaluation of the compliance with the protocol for preventing perinatal hepatitis B infection in Italy. *J Infect* 2011; **62**: 165-171 [PMID: 21129400 DOI: 10.1016/j.jinf.2010.11.014]

142 **Harder KM**, Cowan S, Eriksen MB, Krarup HB, Christensen PB. Universal screening for hepatitis B among pregnant women led to 96% vaccination coverage among newborns of HBsAg positive mothers in Denmark. *Vaccine* 2011; **29**: 9303-9307 [PMID: 22019756 DOI: 10.1016/j.vaccine.2011.10.028]

143 **Willis BC**, Wortley P, Wang SA, Jacques-Carroll L, Zhang F. Gaps in hospital policies and practices to prevent perinatal transmission of hepatitis B virus. *Pediatrics* 2010; **125**: 704-711 [PMID: 20211952 DOI: 10.1542/peds.2009-1831]

144 **Ward JW**. Time for renewed commitment to viral hepatitis prevention. *Am J Public Health* 2008; **98**: 779-781 [PMID: 18381978 DOI: 10.2105/ajph.2008.136275]

145 **Smith EA**, Jacques-Carroll L, Walker TY, Sirotkin B, Murphy TV. The national Perinatal Hepatitis B Prevention Program, 1994-2008. *Pediatrics* 2012; **129**: 609-616 [PMID: 22451702 DOI: 10.1542/peds.2011-2866]

146 **Hu Y**, Zhang S, Luo C, Liu Q, Zhou YH. Gaps in the prevention of perinatal transmission of hepatitis B virus between recommendations and routine practices in a highly endemic region: a provincial population-based study in China. *BMC Infect Dis* 2012; **12**: 221 [PMID: 22984924 DOI: 10.1186/1471-2334-12-221]

147 **Schenkel K**, Radun D, Bremer V, Bocter N, Hamouda O. Viral hepatitis in Germany: poor vaccination coverage and little knowledge about transmission in target groups. *BMC Public Health* 2008; **8**: 132 [PMID: 18433490 DOI: 10.1186/1471-2458-8-132]

148 **Hope VD**, Ncube F, Hickman M, Judd A, Parry JV. Hepatitis B vaccine uptake among injecting drug users in England 1998 to 2004: is the prison vaccination programme driving recent improvements? *J Viral Hepat* 2007; **14**: 653-660 [PMID: 17697018 DOI: 10.1111/j.1365-2893.2007.00856.x]

149 **Xiridou M**, van Houdt R, Hahné S, Coutinho R, van Steenbergen J, Kretzschmar M. Hepatitis B vaccination of men who have sex with men in the Netherlands: should we vaccinate more men, younger men or high-risk men? *Sex Transm Infect* 2013; **89**: 666-671 [PMID: 23812794 DOI: 10.1136/sextrans-2012-050900]

150 **Judd A**, Hickman M, Hope VD, Sutton AJ, Stimson GV, Ramsay ME, Gill ON, Parry JV. Twenty years of selective hepatitis B vaccination: is hepatitis B declining among injecting drug users in England and Wales? *J Viral Hepat* 2007; **14**: 584-591 [PMID: 17650293 DOI: 10.1111/j.1365-2893.2007.00844.x]

151 European Centre for Disease Prevention and Control (2013). Hepatitis B and C surveillance in Europe 2006-2011. Accessed by August 18, 2013. Available from: URL: http://ecdc.europa.eu/en/publications/Publications/Forms/ECDC_DispForm.aspx?ID=1174

152 **van Houdt R**, Koedijk FD, Bruisten SM, Coul EL, Heijnen ML, Waldhober Q, Veldhuijzen IK, Richardus JH, Schutten M, van Doornum GJ, de Man RA, Hahné SJ, Coutinho RA, Boot HJ. Hepatitis B vaccination targeted at behavioural risk groups in the Netherlands: does it work? *Vaccine* 2009; **27**: 3530-3535 [PMID: 19464531 DOI: 10.1016/j.vaccine.2009.03.072]

153 **Beck CR**, Cloke R, O'Moore É, Puleston R. Hepatitis B vaccination coverage and uptake in prisons across England and Wales 2003-2010: a retrospective ecological study. *Vaccine* 2012; **30**: 1965-1971 [PMID: 22265944 DOI: 10.1016/j.vaccine.2012.01.020]

154 **De Schryver A**, Claesen B, Meheus A, van Sprundel M, François G. European survey of hepatitis B vaccination policies for healthcare workers. *Eur J Public Health* 2011; **21**: 338-343 [PMID: 20817688 DOI: 10.1093/eurpub/ckq122]

155 **Simard EP**, Miller JT, George PA, Wasley A, Alter MJ, Bell BP, Finelli L. Hepatitis B vaccination coverage levels among healthcare workers in the United States, 2002-2003. *Infect Control Hosp Epidemiol* 2007; **28**: 783-790 [PMID: 17564979 DOI: 10.1086/518730]

156 **Scognamiglio P**, Girardi E, Fusco M, Piselli P, Spena SR, Maione C, Pisanti FA, Serraino D. Lack of implementation of Hepatitis B Virus (HBV) vaccination policy in household contacts of HBV carriers in Italy. *BMC Infect Dis* 2009; **9**: 86 [PMID: 19500412 DOI: 10.1186/1471-2334-9-86]

157 **Richardson G**, Evans MR, Westmoreland D. Hepatitis B immunisation of household contacts: retrospective study of vaccine coverage. *J Epidemiol Community Health* 2001; **55**: 934-935 [PMID: 11707490]

158 **Zuckerman J**, van Hattum J, Cafferkey M, Gjørup I, Hoel T, Rummukainen ML, Weiland O. Should hepatitis B vaccination be introduced into childhood immunisation programmes in northern Europe? *Lancet Infect Dis* 2007; **7**: 410-419 [PMID: 17521594 DOI: 10.1016/s1473-3099(07)70136-6]

159 **Dawson AJ**. An ethical argument in favour of routine hepatitis B vaccination in very low-incidence countries. *Lancet Infect Dis* 2005; **5**: 120-125 [PMID: 15680782 DOI: 10.1016/s1473-3099(05)01284-3]

160 **English P**. Should universal hepatitis B immunisation be introduced in the UK? *Arch Dis Child* 2006; **91**: 286-289 [PMID: 16551785 DOI: 10.1136/adc.2005.080028]

161 British Medical Association. Hepatitis B vaccination in childhood (2010). Accessed by September 3, 2013. Available from: URL: http://www.hepb.org.uk/news/current/BritishMedicalAssociationcallsforHBVVaccination.pdf

162 **Louise Heiberg I**, Hogh B. Horizontal transmission of hepatitis B virus--why discuss when we can vaccinate? *J Infect Dis* 2012; **206**: 464-465 [PMID: 22508941 DOI: 10.1093/infdis/jis294]

163 **Dannetun E**, Tegnell A, Giesecke J. Parents' attitudes towards hepatitis B vaccination for their children. A survey comparing paper and web questionnaires, Sweden 2005. *BMC Public Health* 2007; **7**: 86 [PMID: 17511891 DOI: 10.1186/1471-2458-7-86]

164 EpiNorth (2013). NETWORK - A Co-operation Project for Communicable Disease Control in Northern Europe. EpiNorth Data.Epidemiological data. Hepatitis B. Accessed by

August 15, 2013. Available from: URL: http://www.epinorth.org/
165 Public Health England (2012). Epidemiological Data. Hepatitis B annual report 2011. Accessed by August 16, 2013. Available from: URL: http://www.hpa.org.uk/hpr/archives/2012/hpr3412.pdf
166 **Rantala M**, van de Laar MJ. Surveillance and epidemiology of hepatitis B and C in Europe - a review. *Euro Surveill* 2008; **13**: pii: 18880 [PMID: 18761967]
167 **François G**, Hallauer J, Van Damme P. Hepatitis B vaccination: how to reach risk groups. *Vaccine* 2002; **21**: 1-4 [PMID: 12443655]
168 **Rimšelienė G**, Nilsen Ø, Kløvstad H, Blystad H, Aavitsland P. Epidemiology of acute and chronic hepatitis B virus infection in Norway, 1992-2009. *BMC Infect Dis* 2011; **11**: 153 [PMID: 21615904 DOI: 10.1186/1471-2334-11-153]
169 **Papaevangelou G**. Hepatitis B immunization programme: lessons learnt in Greece. *Vaccine* 1998; **16** Suppl: S45-S47 [PMID: 9915034]
170 **Baars JE**, Boon BJ, Garretsen HF, van de Mheen D. The reach of a free hepatitis B vaccination programme: results of a Dutch study among drug users. *Int J Drug Policy* 2010; **21**: 247-250 [PMID: 19700297 DOI: 10.1016/j.drugpo.2009.07.001]
171 **Ioannou GN**. Hepatitis B virus in the United States: infection, exposure, and immunity rates in a nationally representative survey. *Ann Intern Med* 2011; **154**: 319-328 [PMID: 21357909 DOI: 10.7326/0003-4819-154-5-201103010-00006]
172 **Ni YH**, Chang MH, Wu JF, Hsu HY, Chen HL, Chen DS. Minimization of hepatitis B infection by a 25-year universal vaccination program. *J Hepatol* 2012; **57**: 730-735 [PMID: 22668640 DOI: 10.1016/j.jhep.2012.05.021]
173 **Kim WR**. Epidemiology of hepatitis B in the United States. *Hepatology* 2009; **49**: S28-S34 [PMID: 19399791 DOI: 10.1002/hep.22975]
174 **Hu DJ**, Xing J, Tohme RA, Liao Y, Pollack H, Ward JW, Holmberg SD. Hepatitis B testing and access to care among racial and ethnic minorities in selected communities across the United States, 2009-2010. *Hepatology* 2013; **58**: 856-862 [PMID: 23359276 DOI: 10.1002/hep.26286]
175 Public Health England (2006). Health Protection Agency. Migrant Health: Infectious diseases in non-UK born populations in England, Wales and Northern. Ireland. A baseline report - 2006. Accessed by 19 August, 2013. Available from: URL: http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1201767922096
176 **Chu JJ**, Wörmann T, Popp J, Pätzelt G, Akmatov MK, Krämer A, Reintjes R. Changing epidemiology of hepatitis B and migration--a comparison of six Northern and North-Western European countries. *Eur J Public Health* 2013; **23**: 642-647 [PMID: 23132874 DOI: 10.1093/eurpub/cks067]
177 **Uddin G**, Shoeb D, Solaiman S, Marley R, Gore C, Ramsay M, Harris R, Ushiro-Lumb I, Moreea S, Alam S, Thomas HC, Khan S, Watt B, Pugh RN, Ramaiah S, Jervis R, Hughes A, Singhal S, Cameron S, Carman WF, Foster GR. Prevalence of chronic viral hepatitis in people of south Asian ethnicity living in England: the prevalence cannot necessarily be predicted from the prevalence in the country of origin. *J Viral Hepat* 2010; **17**: 327-335 [PMID: 20002307 DOI: 10.1111/j.1365-2893.2009.01240.x]
178 **Williams R**, Holt AP. Screening immigrants for tuberculosis--why not for HBV infection? *Lancet* 2013; **381**: 2164-2165 [PMID: 23791343 DOI: 10.1016/s0140-6736(13)61438-5]
179 European Centre for Disease Prevention and Control (2010). Technical Report. Hepatitis B and C in the EU neighbourhood: prevalence, burden of disease and screening policies. Accessed by August 18, 2013. Available from: URL: http://ecdc.europa.eu/en/publications/Publications/TER_100914_Hep_B_C_EU_neighbourhood.pdf
180 **Veldhuijzen IK**, Toy M, Hahné SJ, De Wit GA, Schalm SW, de Man RA, Richardus JH. Screening and early treatment of migrants for chronic hepatitis B virus infection is cost-effective. *Gastroenterology* 2010; **138**: 522-530 [PMID: 19879275 DOI: 10.1053/j.gastro.2009.10.039]
181 **Wong WW**, Woo G, Heathcote EJ, Krahn M. Disease burden of chronic hepatitis B among immigrants in Canada. *Can J Gastroenterol* 2013; **27**: 137-147 [PMID: 23516678]
182 **Fretz R**, Negro F, Bruggmann P, Lavanchy D, De Gottardi A, Pache I, Masserey Spicher V, Cerny A. Hepatitis B and C in Switzerland - healthcare provider initiated testing for chronic hepatitis B and C infection. *Swiss Med Wkly* 2013; **143**: w13793 [PMID: 23740193 DOI: 10.4414/smw.2013.13793]
183 **Weinbaum CM**, Williams I, Mast EE, Wang SA, Finelli L, Wasley A, Neitzel SM, Ward JW. Recommendations for identification and public health management of persons with chronic hepatitis B virus infection. *MMWR Recomm Rep* 2008; **57**: 1-20 [PMID: 18802412]
184 **Eckman MH**, Kaiser TE, Sherman KE. The cost-effectiveness of screening for chronic hepatitis B infection in the United States. *Clin Infect Dis* 2011; **52**: 1294-1306 [PMID: 21540206 DOI: 10.1093/cid/cir199]
185 **Wong WW**, Woo G, Jenny Heathcote E, Krahn M. Cost effectiveness of screening immigrants for hepatitis B. *Liver Int* 2011; **31**: 1179-1190 [PMID: 21745300 DOI: 10.1111/j.1478-3231.2011.02559.x]
186 **Woo G**, Tomlinson G, Yim C, Lilly L, Therapondos G, Wong DK, Ungar WJ, Einarson TR, Sherman M, Heathcote JE, Krahn M. Health state utilities and quality of life in patients with hepatitis B. *Can J Gastroenterol* 2012; **26**: 445-451 [PMID: 22803020]
187 **Nwokediuko SC**. Chronic hepatitis B: management challenges in resource-poor countries. *Hepat Mon* 2011; **11**: 786-793 [PMID: 22224076 DOI: 10.5812/kowsar.1735143X.757]
188 **Lemoine M**, Nayagam S, Thursz M. Viral hepatitis in resource-limited countries and access to antiviral therapies: current and future challenges. *Future Virol* 2013; **8**: 371-380 [PMID: 23662157 DOI: 10.2217/fvl.13.11]
189 **FitzSimons D**, Hendrickx G, Vorsters A, Van Damme P. Hepatitis B vaccination: a completed schedule enough to control HBV lifelong? Milan, Italy, 17-18 November 2011. *Vaccine* 2013; **31**: 584-590 [PMID: 23142301 DOI: 10.1016/j.vaccine.2012.10.101]
190 **Leuridan E**, Van Damme P. Hepatitis B and the need for a booster dose. *Clin Infect Dis* 2011; **53**: 68-75 [PMID: 21653306 DOI: 10.1093/cid/cir270]
191 **Abushady EA**, Gameel MM, Klena JD, Ahmed SF, Abdel-Wahab KS, Fahmy SM. HBV vaccine efficacy and detection and genotyping of vaccineé asymptomatic breakthrough HBV infection in Egypt. *World J Hepatol* 2011; **3**: 147-156 [PMID: 21860674 DOI: 10.4254/wjh.v3.i6.147]
192 **Stramer SL**, Wend U, Candotti D, Foster GA, Hollinger FB, Dodd RY, Allain JP, Gerlich W. Nucleic acid testing to detect HBV infection in blood donors. *N Engl J Med* 2011; **364**: 236-247 [PMID: 21247314 DOI: 10.1056/NEJMoa1007644]
193 **Seed CR**, Jones NT, Pickworth AM, Graham WR. Two cases of asymptomatic HBV "vaccine breakthrough" infection detected in blood donors screened for HBV DNA. *Med J Aust* 2012; **196**: 651-652 [PMID: 22676882]
194 **Chang MH**. Breakthrough HBV infection in vaccinated children in Taiwan: surveillance for HBV mutants. *Antivir Ther* 2010; **15**: 463-469 [PMID: 20516566 DOI: 10.3851/imp1555]
195 **Mele A**, Tancredi F, Romanò L, Giuseppone A, Colucci M, Sangiuolo A, Lecce R, Adamo B, Tosti ME, Taliani G, Zanetti AR. Effectiveness of hepatitis B vaccination in babies born to hepatitis B surface antigen-positive mothers in Italy. *J Infect Dis* 2001; **184**: 905-908 [PMID: 11509998 DOI: 10.1086/323396]
196 **Young BW**, Lee SS, Lim WL, Yeoh EK. The long-term efficacy of plasma-derived hepatitis B vaccine in babies born to carrier mothers. *J Viral Hepat* 2003; **10**: 23-30 [PMID: 12558908]
197 **Boxall EH**, A Sira J, El-Shuhkri N, Kelly DA. Long-term per-

sistence of immunity to hepatitis B after vaccination during infancy in a country where endemicity is low. *J Infect Dis* 2004; **190**: 1264-1269 [PMID: 15346336 DOI: 10.1086/423818]
198 **Poovorawan Y**, Chongsrisawat V, Theamboonlers A, Srinivasa K, Hutagalung Y, Bock HL, Hoet B. Long-term benefit of hepatitis B vaccination among children in Thailand with transient hepatitis B virus infection who were born to hepatitis B surface antigen-positive mothers. *J Infect Dis* 2009; **200**: 33-38 [PMID: 19473096 DOI: 10.1086/599331]
199 **Børresen ML**, Koch A, Biggar RJ, Ladefoged K, Melbye M, Wohlfahrt J, Krause TG. Effectiveness of the targeted hepatitis B vaccination program in Greenland. *Am J Public Health* 2012; **102**: 277-284 [PMID: 21940914 DOI: 10.2105/ajph.2011.300239]
200 **Dentinger CM**, McMahon BJ, Butler JC, Dunaway CE, Zanis CL, Bulkow LR, Bruden DL, Nainan OV, Khristova ML, Hennessy TW, Parkinson AJ. Persistence of antibody to hepatitis B and protection from disease among Alaska natives immunized at birth. *Pediatr Infect Dis J* 2005; **24**: 786-792 [PMID: 16148845]
201 **Lu CY**, Ni YH, Chiang BL, Chen PJ, Chang MH, Chang LY, Su IJ, Kuo HS, Huang LM, Chen DS, Lee CY. Humoral and cellular immune responses to a hepatitis B vaccine booster 15-18 years after neonatal immunization. *J Infect Dis* 2008; **197**: 1419-1426 [PMID: 18444799 DOI: 10.1086/587695]
202 **Linauts S**, Saldanha J, Strong DM. PRISM hepatitis B surface antigen detection of hepatits B virus minipool nucleic acid testing yield samples. *Transfusion* 2008; **48**: 1376-1382 [PMID: 18422847 DOI: 10.1111/j.1537-2995.2008.01698.x]
203 **Poovorawan Y**, Chongsrisawat V, Theamboonlers A, Leroux-Roels G, Kuriyakose S, Leyssen M, Jacquet JM. Evidence of protection against clinical and chronic hepatitis B infection 20 years after infant vaccination in a high endemicity region. *J Viral Hepat* 2011; **18**: 369-375 [PMID: 20384962 DOI: 10.1111/j.1365-2893.2010.01312.x]
204 **Mendy M**, Peterson I, Hossin S, Peto T, Jobarteh ML, Jeng-Barry A, Sidibeh M, Jatta A, Moore SE, Hall AJ, Whittle H. Observational study of vaccine efficacy 24 years after the start of hepatitis B vaccination in two Gambian villages: no need for a booster dose. *PLoS One* 2013; **8**: e58029 [PMID: 23533578 DOI: 10.1371/journal.pone.0058029]
205 **Boot HJ**, van der Waaij LA, Schirm J, Kallenberg CG, van Steenbergen J, Wolters B. Acute hepatitis B in a healthcare worker: a case report of genuine vaccination failure. *J Hepatol* 2009; **50**: 426-431 [PMID: 19091440 DOI: 10.1016/j.jhep.2008.07.040]
206 **McMahon BJ**, Bruden DL, Petersen KM, Bulkow LR, Parkinson AJ, Nainan O, Khristova M, Zanis C, Peters H, Margolis HS. Antibody levels and protection after hepatitis B vaccination: results of a 15-year follow-up. *Ann Intern Med* 2005; **142**: 333-341 [PMID: 15738452]
207 **Lu CY**, Chiang BL, Chi WK, Chang MH, Ni YH, Hsu HM, Twu SJ, Su IJ, Huang LM, Lee CY. Waning immunity to plasma-derived hepatitis B vaccine and the need for boosters 15 years after neonatal vaccination. *Hepatology* 2004; **40**: 1415-1420 [PMID: 15565627 DOI: 10.1002/hep.20490]
208 **Jan CF**, Huang KC, Chien YC, Greydanus DE, Davies HD, Chiu TY, Huang LM, Chen CJ, Chen DS. Determination of immune memory to hepatitis B vaccination through early booster response in college students. *Hepatology* 2010; **51**: 1547-1554 [PMID: 20209603 DOI: 10.1002/hep.23543]
209 **Wu TW**, Lin HH, Wang LY. Chronic hepatitis B infection in adolescents who received primary infantile vaccination. *Hepatology* 2013; **57**: 37-45 [PMID: 22858989 DOI: 10.1002/hep.25988]
210 **Banatvala JE**, Van Damme P. Hepatitis B vaccine -- do we need boosters? *J Viral Hepat* 2003; **10**: 1-6 [PMID: 12558904]
211 Are booster immunisations needed for lifelong hepatitis B immunity? European Consensus Group on Hepatitis B Immunity. *Lancet* 2000; **355**: 561-565 [PMID: 10683019]
212 **Roome AJ**, Walsh SJ, Cartter ML, Hadler JL. Hepatitis B vaccine responsiveness in Connecticut public safety personnel. *JAMA* 1993; **270**: 2931-2934 [PMID: 8254852]
213 **Wood RC**, MacDonald KL, White KE, Hedberg CW, Hanson M, Osterholm MT. Risk factors for lack of detectable antibody following hepatitis B vaccination of Minnesota health care workers. *JAMA* 1993; **270**: 2935-2939 [PMID: 8254853]
214 **Coates T**, Wilson R, Patrick G, André F, Watson V. Hepatitis B vaccines: assessment of the seroprotective efficacy of two recombinant DNA vaccines. *Clin Ther* 2001; **23**: 392-403 [PMID: 11318074]
215 **Roukens AH**, Visser LG. Hepatitis B vaccination strategy in vaccine low and non-responders: a matter of quantity of quality? *Hum Vaccin* 2011; **7**: 654-657 [PMID: 21508674]
216 **Kubba AK**, Taylor P, Graneek B, Strobel S. Non-responders to hepatitis B vaccination: a review. *Commun Dis Public Health* 2003; **6**: 106-112 [PMID: 12889288]
217 **Li ZK**, Nie JJ, Li J, Zhuang H. The effect of HLA on immunological response to hepatitis B vaccine in healthy people: a meta-analysis. *Vaccine* 2013; **31**: 4355-4361 [PMID: 23887040 DOI: 10.1016/j.vaccine.2013.06.108]
218 Leads from the MMWR. Suboptimal response to hepatitis B vaccine given by injection into the buttock. *JAMA* 1985; **253**: 1705-1707 [PMID: 3156259]
219 **de Lalla F**, Rinaldi E, Santoro D, Pravettoni G. Immune response to hepatitis B vaccine given at different injection sites and by different routes: a controlled randomized study. *Eur J Epidemiol* 1988; **4**: 256-258 [PMID: 2969825]
220 **Clemens R**, Sänger R, Kruppenbacher J, Höbel W, Stanbury W, Bock HL, Jilg W. Booster immunization of low- and non-responders after a standard three dose hepatitis B vaccine schedule--results of a post-marketing surveillance. *Vaccine* 1997; **15**: 349-352 [PMID: 9141203]
221 **Craven DE**, Awdeh ZL, Kunches LM, Yunis EJ, Dienstag JL, Werner BG, Polk BF, Syndman DR, Platt R, Crumpacker CS. Nonresponsiveness to hepatitis B vaccine in health care workers. Results of revaccination and genetic typings. *Ann Intern Med* 1986; **105**: 356-360 [PMID: 2943202]
222 **Bertino JS**, Tirrell P, Greenberg RN, Keyserling HL, Poland GA, Gump D, Kumar ML, Ramsey K. A comparative trial of standard or high-dose S subunit recombinant hepatitis B vaccine versus a vaccine containing S subunit, pre-S1, and pre-S2 particles for revaccination of healthy adult nonresponders. *J Infect Dis* 1997; **175**: 678-681 [PMID: 9041342]
223 **Lin CS**, Xie SB, Liu J, Zhao ZX, Chong YT, Gao ZL. Effect of revaccination using different schemes among adults with low or undetectable anti-HBs titers after hepatitis B virus vaccination. *Clin Vaccine Immunol* 2010; **17**: 1548-1551 [PMID: 20719983 DOI: 10.1128/cvi.00064-10]
224 **Cardell K**, Akerlind B, Sällberg M, Frydén A. Excellent response rate to a double dose of the combined hepatitis A and B vaccine in previous nonresponders to hepatitis B vaccine. *J Infect Dis* 2008; **198**: 299-304 [PMID: 18544037 DOI: 10.1086/589722]
225 Recommendations for preventing transmission of infections among chronic hemodialysis patients. *MMWR Recomm Rep* 2001; **50**: 1-43 [PMID: 11349873]
226 **Fabrizi F**, Dixit V, Magnini M, Elli A, Martin P. Meta-analysis: intradermal vs. intramuscular vaccination against hepatitis B virus in patients with chronic kidney disease. *Aliment Pharmacol Ther* 2006; **24**: 497-506 [PMID: 16886915 DOI: 10.1111/j.1365-2036.2006.03002.x]
227 **Heininger U**, Gambon M, Gruber V, Margelli D. Successful hepatitis B immunization in non- and low responding health care workers. *Hum Vaccin* 2010; **6** [PMID: 20855942 DOI: 10.4161/hv.6.9.12420]
228 **Zuckerman AJ**. Appraisal of intradermal immunisation against hepatitis B. *Lancet* 1987; **1**: 435-436 [PMID: 2880225]
229 **Sangaré L**, Manhart L, Zehrung D, Wang CC. Intradermal hepatitis B vaccination: a systematic review and meta-analysis. *Vaccine* 2009; **27**: 1777-1786 [PMID: 19200451 DOI:

10.1016/j.vaccine.2009.01.043]
230 **Lin R**, Tarr PE, Jones TC. Present status of the use of cytokines as adjuvants with vaccines to protect against infectious diseases. *Clin Infect Dis* 1995; **21**: 1439-1449 [PMID: 8749629]
231 **Kim MJ**, Nafziger AN, Harro CD, Keyserling HL, Ramsey KM, Drusano GL, Bertino JS. Revaccination of healthy nonresponders with hepatitis B vaccine and prediction of seroprotection response. *Vaccine* 2003; **21**: 1174-1179 [PMID: 12559795]

P- Reviewer: Zhao HT
S- Editor: Qi Y **L- Editor**: A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI:10.3748/wjg.v20.i27.9017

World J Gastroenterol 2014 July 21; 20(27): 9017-9025
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Breath volatile organic compounds for the gut-fatty liver axis: Promise, peril, and path forward

Steven Francis Solga

Steven Francis Solga, Solga Gastroenterology, Bethlehem, PA 18015, United States
Author contributions: Solga SF solely contributed to this paper.
Correspondence to: Steven Francis Solga, MD, Solga Gastroenterology, 701 Ostrum St. Suite 604A, Bethlehem, PA 18015, United States. stevesolga@gmail.com
Telephone: +11-610-8663900 Fax: +11-610-8668140
Received: October 25, 2013 Revised: January 15, 2014
Accepted: April 8, 2014
Published online: July 21, 2014

Abstract

The worldwide interest in the gut microbiome and its impact on the upstream liver highlight a critical upside to breath research: it can uniquely measure otherwise unmeasurable biology. Bacteria make gases [volatile organic compounds (VOCs)] that are directly relevant to pathophysiology of the fatty liver and associated conditions, including obesity. Measurement of these VOCs and their metabolites in the exhaled breath, therefore, present an opportunity to safely and easily evaluate, on both a personal and a population level, some of our most pressing public health threats. This is an opportunity that must be pursued. To date, however, breath analysis remains a slowly evolving field which only occasionally impacts clinical research or patient care. One major obstacle to progress is that breath analysis is inherently and emphatically mutli-disciplinary: it connects engineering, chemistry, breath mechanics, biology and medicine. Unbalanced or incomplete teams may produce inconsistent and often unsatisfactory results. A second impediment is the lack of a well-known stepwise structure for the development of non-invasive diagnostics. As a result, the breath research landscape is replete with orphaned single-center pilot studies. Often, important hypotheses and key observations have not been pursued to maturation. This paper reviews the rationale and requirements for breath VOC research applied to the gut-fatty liver axis and offers some suggestions for future development.

Key words: Breath; Volatile organic compounds; Fatty liver; Gut flora; Breath analysis

Core tip: The biology of the gut-liver axis has always been fascinating and exceedingly difficult to study. With the rapidly expanding interest in the gut microbiome, however, finding better measurement techniques to evaluate this biology has never been more relevant. Breath volatile organic compounds (VOCs) measurement presents the unmatched potential to address this critical unmet need. Breath measurement can be challenging, however, and requires coherent teams including engineers, breath chemists, and clinical researchers. It also requires long term vision and strategy. This paper describes the rationale for breath VOCs, critically reviews the history of breath VOC development, and offers suggestions for progress.

Solga SF. Breath volatile organic compounds for the gut-fatty liver axis: Promise, peril, and path forward. *World J Gastroenterol* 2014; 20(27): 9017-9025 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9017.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9017

INTRODUCTION

The gut flora microbiome and the gut-liver axis are exceptionally difficult to evaluate. However, the now universal appreciation of the microbiome's impact on upstream fatty liver and associated disorders such as obesity compels an even greater interest in improved measurement techniques.

Breath researchers have measured gut flora activity in exhaled breath for decades. However, there has been little

Table 1 "Fatty liver" volatile organic compounds candidates

Property	Examples
Fermentation activity[29-31]	Alcohols and their aldehydes
Metabolism[32,33]	Acetone and isoprene
Inflammation[34-36]	Dimethylamine, trimethylamine, hydrogen sulfide, ethane, methylsulfide, methylmercaptan

sustained success. This paper critically reviews the experience to date and offers suggestions for future progress.

Breath analysis still holds the unique and possibly unmatched potential to better measure this challenging and highly significant physiology.

PROMISE: UNPRECEDENTED OPPORTUNITY FOR BREATH VOLATILE ORGANIC COMPOUNDS

The role of gut flora in fatty liver pathogenesis has been studied for decades. Alcohol fatty liver research, for example, demonstrated that gut flora were necessary but not sufficient for liver disease, and explored the use of gut flora therapy (poorly absorbed antibiotics) using animal models[1,2]. Various lines of evidence also pointed to a key role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD)[3-5]. Gut flora, *via* various mechanisms such as altered small bowel motility and impaired mucosal barrier function, have also been long appreciated to affect the clinical course of cirrhosis, regardless of liver disease etiology[6].

Research connecting the gut flora to the liver has been particularly challenging and fascinating because gut bacterial biology and liver disease are distinct disciplines connected anatomically *via* a nearly inaccessible portal venous system. And although there is a history of gut flora therapies (*i.e.*, prebiotics, probiotics, dietary interventions) for liver disease[7,8], progress has been slow because the science, especially the details of the gut flora, is underdeveloped. Nevertheless, the potential impact was evident: non-alcohol fatty liver, alcohol fatty liver, and cirrhosis affect many people.

However, with the now-familiar association of gut flora dysbiosis to obesity[9,10] and insulin resistance[11-13] interest in gut flora biology and, along with it, the gut-liver axis, has grown and today would be difficult to overstate. The gut flora is now regarded as a newly discovered metabolic organ. Many essential questions persist and have triggered a worldwide effort to better understand this new organ[14-16]. Multiple comprehensive reviews have addressed the impact of gut flora on fatty liver and/or obesity[17-20].

The major studies which have propelled these advances have generally used detailed fecal analysis. These analyses can include a variety of techniques including DNA sequencing, culture, and metabolic profiling[21]. The emerging data indicate several possible mechanisms of gut flora influence: fermentation, effects on metabolism, inflammatory signaling, or a combination. Notably, it is understood the gut microbiome is personal; one's gut flora, as well as their metabolic response to diet, and upstream liver effects cannot be predicted a priori[22]. Thus, since exogenous ethanol is metabolized to acetaldehyde at a variable and unpredictable rate[23], the same should follow for endogenous ethanol produced from gut flora. Furthermore, it is acknowledged that there remain many additional unknowns that exist about the gut-liver axis (*i.e.*, motility, mucosal barrier, immune system interactions, molecular mechanisms within the hepatocyte). However, despite both these known differences and true unknowns, there is a rapidly growing interest in the gut flora therapies and dietary interventions premised on these mechanisms[24].

Therefore, notwithstanding the usefulness of fecal analysis to date, is not clear that it will prove as successful for wide scale clinical research[25]. Fecal analysis, by virtually any method, has a number of drawbacks: samples are collected infrequently and episodically, are expensive to run, and result in large amount data that nevertheless remains challenging to interpret in the setting of multiple, interrelated physiologic variables: *i.e.*, gut flora modulate mucosal integrity and immune function with differential impact on the liver, and vice-versa[26,27]. Fecal analysis cannot readily account for a number of factors in the gastrointestinal tract, including transit time, presence or absence of mucosal disease, and the possible differential impact bacterial subpopulations (*e.g.*, distal small bowel *vs* colonic, and so on).

Breath volatile organic compound (VOC) measurement, therefore, may serve to complement fecal analysis[28]. Individual VOCs can be measured for specific hypothesis driven goals tailored to match the present understanding of the role of gut flora in the gut-liver axis.

Since the pathogenesis of fatty liver (Table 1) is multifactorial and there are many variables which impact the gut-liver axis, the most successful research will likely simultaneously measure multiple VOCs.

It is presumed that some of these metabolites (*e.g.*, ethanol) are produced only by gut flora, whereas others (*e.g.*, acetaldehyde) are produced by both gut flora and human metabolism. Notably, some of these VOCs may potentiate others. For example, ethanol and acetaldehyde can increase the growth of gram negative bacteria and intestinal permeability, respectively, and thereby may promote uptake of inflammatory mediators[37]. Hydrogen sulfide may reduce gastrointestinal motility and thereby lead to bacterial stasis and overgrowth[38]. Other VOCs have multiple affects that overlap multiple categories. For example, some gut flora metabolize choline efficiently and their over-abundance can lead both to choline deficiency and an overproduction of the toxic metabolites dimethylamine and trimethylamine[39]. Both mechanisms have been implicated in the pathogenesis of fatty liver and non-alcoholic steatohepatitis[40,41]. Each of these VOCs have been measured in exhaled breath, though

Table 2 Exhaled breath uniquely captures the entire output of the gut liver axis in the context of a person

Gut flora	Lumen factors	Hepatic factors	Host
Bacterial diversity and function	Barrier integrity	Enzyme heterogeneity (*e.g.*, alcohol dehydrogenase)	Diet
Mucosal or lumen associated	Immune defense	Liver disease	Medications
Location (*e.g.*, small bowel, right colon)	Mucosal disease (*e.g.*, celiac, crohns)	Cirrhosis and porto-systemic shunting	Co-morbid conditions (*e.g.*, diabetes)
	Transit time		Age, gender, body mass index

usually separately. However, much like the standard twelve lead electrocardiogram or lipid panels, it is likely that the most meaningful VOC breath data would come from the simultaneous measurement and interpretation of multiple VOCs and/or profiles.

In contrast to fecal analysis, exhaled breath VOC analysis can measure the global activity of the entire gut-liver axis. Because breath measurement is non-invasive, safe, and potentially inexpensive, it easily enables studies with repeated measures. For example, it is simple and highly relevant to envision evaluating the immediate differential effect of various oral challenges (*e.g.*, high/low fiber, high/low fructose) in various subjects (*e.g.*, lean/obesity, fatty liver/cirrhosis) using timed VOC measurements over several hours, days, or longer.

The gut liver axis (Table 2) includes many important, highly variable factors that are difficult to measure physiologically. While fecal analysis is inherently limited, breath VOC measurement may evaluate the global activity of the entire system.

In summary, the microbiome and gut-liver axis are a major research emphasis world-wide, and studies employing fecal analysis are appropriately credited with many advances. However, even if fecal analysis was fully validated, free, easy to perform, and always yielded interpretable results, it still cannot measure many "upstream" factors germane to both fatty liver and the metabolic syndrome and the marked heterogeneity between subjects. Studies using breath VOC analysis, in contrast, can uniquely evaluate the entire organism in real time. The simple capability of repeated measures greatly expands options in clinical research.

PERIL: A HISTORY OF UNMET EXPECTATIONS IN BREATH ANALYSIS

Breath analysis is appealing because it enables the potential for non-invasive, real time, easy to use, point of care measurement of metabolites that are, in some cases, difficult or impossible to measure by blood assays or other means. Previous attempts to apply breath analysis to gut physiology, however, have not been met with great success. Two examples, hydrogen and ammonia, are illustrative.

Hydrogen

Breath hydrogen testing has been available for decades[42]. The monitors are relatively inexpensive, portable, and simple to operate. Aside from the addition of methane (to capture preferential methane producers) and carbon dioxide (for quality control), the instrumentation and breath collection process have not significantly changed in many years. Hydrogen measurement is technically easy: it is relatively inert; its measurement is not affected by background ambient air; and it is present at high concentrations (parts per million)[43]. Breath hydrogen testing has been incorporated into hundreds of published research studies.

The most widely accepted clinical use is in the evaluation of small intestine bacterial overgrowth (SIBO) and carbohydrate mal-absorption. Regarding the former, SIBO has emerged as a possible important and modifiable factor in the pathogenesis of irritable bowel syndrome (IBS) for some patients[44]. As a result, the use of hydrogen breath testing has surged over the last decade to evaluate SIBO in IBS, including responsiveness to putative gut flora therapy (*i.e.*, rifaximin, a poorly absorbed antibiotic)[45,46]. Because SIBO or "gut dysbiosis" is challenging to measure by other means, breath hydrogen testing had the potential to fulfill an important unmet need.

However, there remain serious concerns about its validity. An excellent recent review noted many problems, including lack of standardized instructions regarding testing substrates, doses and time intervals, as well as varying definitions of positive *vs* negative tests persist[47]. Thus, notwithstanding a surging scientific and public interest in the possible role of gut flora in IBS, the American College of Gastroenterology does not endorse routine breath testing[48].

The results of a recent meeting of the United States Food and Drug Administration (FDA) Gastrointestinal Drugs Advisory Committee (GIDAC) provide additional insight[49]. The meeting's purpose was the design of clinical trials to evaluate the safety, efficacy, and durability of response of repeat cycles of Xifaxan (rifaximin). To the author's knowledge, this was the first time a breath test was seriously considered in the drug evaluation and approval process for a gut disease. But despite its long history, lack of technical issues, and the unmet need, GIDAC and the sponsor (Salix) easily agreed that breath hydrogen testing fails to meet criteria as a valid biomarker for any purpose and should not utilized[50]. Future developments seem unlikely.

Ammonia

In contrast to hydrogen, ammonia is highly volatile and difficult to measure by any method[51,52]. Due to its relevance to gut flora and various disease states[53], breath

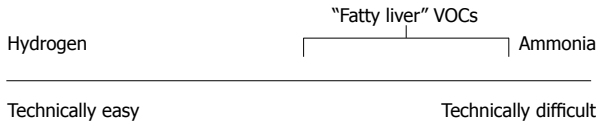


Figure 1 Most volatile organic compounds are challenging to measure. VOCs: Volatile organic compounds.

researchers have aspired to measure it for greater than thirty years[54]. A progression of highly sophisticated measurement platforms (*e.g.*, GC/MS, quantum cascade lasers[55]) have been used in the hopes that ever faster and more precise equipment modifications will finally yield accurate and reproducible results usable for clinical research and patient care. Many technical factors must be considered (*e.g.*, temperature, humidity, flow, and mode of breathing) alongside complex biologic concerns (*e.g.*, contamination from oral bacteria)[56,57]. Despite these major challenges, many small studies were published purporting to demonstrate the utility of breath ammonia measurement for a specific disease or condition (*e.g.*, hepatic encephalopathy, renal dialysis, exercise[58-60]). However, it now appears from work published by highly experienced groups, that exhaled breath may not reflect systemic levels, at least not be by the methods described to date. Aspirations repeatedly exceeded reality. Not surprisingly, therefore, the current ammonia literature has nearly completely ignored breath research[53,61,62].

In summary, breath hydrogen is easy to measure and has an established role in clinical research and patient care. However, it is not a valid biomarker and its impact has not grown with in parallel with the rise in interest in gut flora. Breath ammonia is difficult to measure and, notwithstanding intense efforts by multiple breath research groups, has had little influence on clinical ammonia research. Thus, both the easy and difficult extremes of the breath metabolite spectrum reveal that, at times, the breath enterprise exists as only a tangential contributor to overall human research. The literature is replete with orphaned pilot studies. While hydrogen and ammonia serve as prototypical examples, this pattern has been duplicated with many metabolites (Table 3).

Notably, most of the candidate "fatty liver" VOCs are also quite difficult to measure (Figure 1).

PATH FORWARD

Volatility mandates reproducibility. First, test the test

By definition, VOCs are dynamic and changeable. Furthermore, they are present only in trace quantities and are subject to multiple confounders, including environmental factors. Therefore, studies of VOCs carry an exceptional burden of validation that requires the demonstration of reproducibility. Ideally, this includes at least three kinds of reproducibility: immediate (paired samples back to back), day to day, and location to location. The latter is needed because of ambient air influences, especially if human breath is collected in proximity to medical or research offices and facilities. For example, it must be proven that a subject's breath ethanol at 200 ppb would be measured the same in low (*e.g.*, 50 ppb) and high (*e.g.*, 5000 ppb) ambient air environments. Once established, then other important influences should evaluated, including time of day, mode of breathing, mouth rinses, food intake including composition and timing, and so on.

Table 3 Breath research often failed to meet its potential for multiple reasons

Technical/scientific factors	
Monitor/interface/biology	Too many interrelated unknowns
Unique data: uncertain utility	Relevance difficult to establish
	Relevance may not exist
Non-technical factors	
Inadequate teams	Engineers, chemists, doctors, statisticians
Inadequate synergy	Single center efforts
Lack of focus	Too many diseases, too little strategy
Lack of common languages	Device development is not drug development
Few models of commercial success	Difficult to envision endgame

It must be acknowledged that such studies are often tedious, have poor publication value and short term return on investment. However, they are essential. Historians note that when the United States Food and Drug Administration (FDA) first promoted the basic drug safety expectations that evolved into present day preclinical and phase I studies (*i.e.*, the Food, Drug, and Cosmetic Act of 1938), most pharmaceutical companies simply folded[63]. The survivors, *e.g.*, Merck, not only responded by drastically increasing their research enterprise, their leadership specifically assigned only their best scientists to these early stage efforts in acknowledgement of both their critical importance and tedium.

Breath research has to date failed to uniformly meet these requirements. Breath research papers often detail monitor mechanics and the ability of the monitor to reproducibly measure a targeted VOC against a known laboratory reference gas standard. Without further evaluation, small cross-sectional human studies are then performed purportedly to evaluate a disease state. Unfortunately, this pattern ultimately results in an unconvincing and inherently limited literature, as illustrated above for both breath hydrogen and ammonia. Breath VOC researchers have, therefore, earned skepticism from the broader research community.

Fecal VOC analysis should also meet these standards. For example, a recently published study evaluated fecal VOCs in NAFLD[64]. Using home stool kits, subjects produced samples once, froze them, and later transported them to the lab. Fecal VOCs were then measured and compared to DNA analysis. Given the large number of VOCs measured (two hundred twenty), small sample size (thirty cases and controls) and observational case-control study design, the strength of the study's conclusions is largely determined by the confidence in the measurement

process. However, while the authors and accompanying editorial carefully and appropriately discuss multiple other important influences and limitations of the study, neither substantively addresses this more basic issue[65]. Even for analyses that may be exploratory and descriptive, more complete methods discussion is imperative to build a confidence foundation for additional studies.

Finally, it is noteworthy that while blood VOC analysis may also have important potential, it has similar downsides. For example, Zhu *et al*[66], recently reported that specific gut flora compositions may drive an elevated endogenous ethanol production in a pediatric population with non-alcoholic steatohepatitis. However, blood assays for VOCs can also be challenging[67]; for example, despite the fact that ammonia has been measured in the blood for over one hundred years, the proper blood source (venous *vs* arterial)[68] and state (partial pressure NH_3 *vs* NH_4^+)[69,70] remain debated. Furthermore, phlebotomy makes studies requiring multiple repeated measures difficult.

BIOMARKER DEVELOPMENT: BREATH SUCCESS REQUIRES EXCEPTIONAL TEAMS AND STRATEGY

In the 1950's and 1960's, the United States FDA promulgated a three phase strategy to evaluate the safety and efficacy of new drugs[63]. The phases became familiar worldwide and created a uniform path for drug development. It is relatively easy, therefore, to interpret and compare clinical trials as they evolve through the phases. This is helpful not only for medical researchers, scientists, and regulators, but also for other stakeholders including investors and the broader public. Furthermore, drugs are developed and approved for a specific disease indication. Because this process is slow and highly resource intensive, progression through the phases occurs only after careful and continuous consideration of an unmet need and competing alternatives[71]. As a result of this stepwise structure, regulatory approval, at least in the United States, is a milestone that is almost always associated with at least some commercial potential.

Unfortunately for breath research, an analogous path does not exist for non-invasive diagnostics or biomarker development[72]. While the FDA indeed regulates non-invasive medical devices, the requirements for approval are much different, generally lower, and not as well known. Furthermore, they are not nearly as meaningful. Therefore, while biomarkers researchers may have lower apparent initial development costs and greater latitude than drug researchers, they risk misunderstanding and misdirection amongst members of the development team.

It is essential, however, that an overall strategy exists. This begins with an extensive and thorough validation of a putative biomarker applied to a particular application, *e.g.*, risk estimation, screening, diagnosis, monitoring, and so on. Moreover, biomarkers should also be characterized by purpose, *e.g.*, predictive, prognostic, and so on[73-75]. This compass must guide testing. Poorly designed studies in the wrong population are destined to yield uninterpretable results; this is especially true in breath analysis, where experimental monitors are often touted to measure experimental metabolites *via* experimental interface samplers to describe unknown biology.

Successful breath VOC research requires (1) multiple disciplinary teams; (2) extensive early stage validation studies; and (3) a clear clinical research strategy.

Coherent teams require, at a minimum, the ongoing participation of engineers, breath measurement experts, clinical researchers with experience in gut biology, gastroenterology, hepatology, and statistics. The process should begin with a foundation of knowledge and experience with breath VOCs resulting in focused testable hypotheses that can be transformed into monitors with specific performance specifications and operational capacities. Ideally, multiple monitors are built and are tested clinically side by side first at a single site and then at multiple sites for accuracy and reproducibility. After these are clearly established and normative data are generated, disease specific hypothesis can be pursued. Finally, a clear long term clinical research strategy grounded in the requirements for biomarker development should be articulated. Outside of a few centers of excellence, (*e.g.*, the Austrian Breath Research Institute, ISTM Keele University) such a comprehensive approach would be novel for breath research. The recent publication of comprehensive breath research books[76], growing interest in breath research conferences, and the development and greater use a specially designed interface sampler[77] are positive steps.

BREATH VOC METABOLITES FOR THE GUT-LIVER AXIS: CURRENT STATUS

The breath VOC metabolites of interest shown in Figure 1 are nearly as technically challenging as ammonia. Each of them, however, have been measured in breath with the generation of some normative data[33,78]. Many innovative and useful small, single center studies have been published, as has been recently reviewed[28].

A few studies have specifically focused on the gut liver axis and demonstrated some physiologic insights. For example, Cope *et al*[79], evaluated the effect of an intervention (neomycin, a poorly absorbed antibiotic) on exhaled breath ethanol in an obese murine model of fatty liver compared to lean littermates. In addition to utilizing an intervention, this convincing study also reported repeated measures and thereby accounted for diurnal ethanol variations. The follow up human studies did not have these strengths and were therefore less persuasive[80,81]. At present, though, breath VOCs are most developed not for fatty liver but for use in diabetes monitoring, where multiple groups have many significant recent advances[82,83].

Finally, it must be acknowledged that liver disease, especially fatty liver, is difficult to accurately measure

by any means, including blood assays, imaging, or biopsy[84,85]. Moreover, the pathophysiology of fatty liver, its relationship to steatohepatitis, cirrhosis, and associated conditions like obesity is complex, and there are many important mechanisms that do not involve VOCs. Thus, even if a well validated breath VOC panel existed now, it would be difficult to definitely tie such a profile to a clinical outcome of interest, and multiple measurement modalities are likely needed. As a result, breath research groups might aspire to participate in established long term fatty liver research programs (*e.g.*, the United States based Non-Alcoholic Steatohepatitis Clinical Research Network[86]) as ancillary studies.

ENGINEERS REQUIRED

Many of the pioneers of breath research have creatively adapted existing measurement platforms to breath measurement[87]. Even now, specifically designed breath monitors are usually built as one-of-a-kind prototypes. The obvious legacy has been the small, single center fundamentally limited studies described above. For the same straightforward reasons, clinical researchers need multiple identical monitors that are portable and measure multiple VOCs simultaneously and accurately. Because clinical research is most convincing when multi-center studies involve large numbers of subjects, the need for multiple identical monitors is imperative. This is especially true due to extra reproducibility requirements of breath VOC research. While engineers may be understandably reluctant to commit the resources to build five monitors (especially after the results of a single prototype may have been equivocal), that is the prescription.

Given engineering advances and the right vision, this is achievable. Breath measurement experts, "breathologists," should be involved at every stage, from design through maturation. Like drug development, this process will likely require a collaborative effort between academia and industry, but could occur at a fraction of the cost.

CONCLUSION

Breath analysis continues its infancy, and is almost always discussed in terms of its potential. But the rationale for breath has never been greater: breath affords the almost unique opportunity to quickly, cheaply, and non-invasively measure important markers that reflect the global gut-liver axis biology not measurable in other ways. Engineers, if they are willing, are ever more capable of making fast, portable, ultra-sensitive monitors. Comprehensive breath research teams should thoroughly address reproducibility to build a foundation for specific hypothesis driven goals. Those willing to invest in a long term strategy for breath VOC development may yet transform and revolutionize gut-liver axis research and patient care, with major payoffs in diseases such as fatty liver, obesity, and the metabolic syndrome.

REFERENCES

1 **Thurman RG**, Bradford BU, Iimuro Y, Knecht KT, Arteel GE, Yin M, Connor HD, Wall C, Raleigh JA, Frankenberg MV, Adachi Y, Forman DT, Brenner D, Kadiiska M, Mason RP. The role of gut-derived bacterial toxins and free radicals in alcohol-induced liver injury. *J Gastroenterol Hepatol* 1998; **13** Suppl: S39-S50 [PMID: 9792033]

2 **Ferrier L**, Bérard F, Debrauwer L, Chabo C, Langella P, Buéno L, Fioramonti J. Impairment of the intestinal barrier by ethanol involves enteric microflora and mast cell activation in rodents. *Am J Pathol* 2006; **168**: 1148-1154 [PMID: 16565490 DOI: 10.2353/ajpath.2006.050617]

3 **Ilan Y**. Leaky gut and the liver: a role for bacterial translocation in nonalcoholic steatohepatitis. *World J Gastroenterol* 2012; **18**: 2609-2618 [PMID: 22690069 DOI: 10.3748/wjg.v18.i21.2609]

4 **Compare D**, Coccoli P, Rocco A, Nardone OM, De Maria S, Cartenì M, Nardone G. Gut--liver axis: the impact of gut microbiota on non alcoholic fatty liver disease. *Nutr Metab Cardiovasc Dis* 2012; **22**: 471-476 [PMID: 22546554 DOI: 10.1016/j.numecd.2012.02.007]

5 **Frazier TH**, DiBaise JK, McClain CJ. Gut microbiota, intestinal permeability, obesity-induced inflammation, and liver injury. *JPEN J Parenter Enteral Nutr* 2011; **35**: 14S-20S [PMID: 21807932 DOI: 10.1177/0148607111413772]

6 **Seo YS**, Shah VH. The role of gut-liver axis in the pathogenesis of liver cirrhosis and portal hypertension. *Clin Mol Hepatol* 2012; **18**: 337-346 [PMID: 23323248 DOI: 10.3350/cmh.2012.18.4.337]

7 **Quigley EM**, Monsour HP. Targeting the microbiota in the management of gastrointestinal and liver disease. *Rev Gastroenterol Peru* 2013; **33**: 139-144 [PMID: 23838941]

8 **Kirpich IA**, McClain CJ. Probiotics in the treatment of the liver diseases. *J Am Coll Nutr* 2012; **31**: 14-23 [PMID: 22661622]

9 **Ley RE**, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. *Nature* 2006; **444**: 1022-1023 [PMID: 17183309 DOI: 10.1038/4441022a]

10 **Turnbaugh PJ**, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 2006; **444**: 1027-1031 [PMID: 17183312 DOI: 10.1038/nature05414]

11 **Vanni E**, Bugianesi E. The gut-liver axis in nonalcoholic fatty liver disease: Another pathway to insulin resistance? *Hepatology* 2009; **49**: 1790-1792 [PMID: 19475679 DOI: 10.1002/hep.23036]

12 **Siebler J**, Galle PR, Weber MM. The gut-liver-axis: endotoxemia, inflammation, insulin resistance and NASH. *J Hepatol* 2008; **48**: 1032-1034 [PMID: 18468548 DOI: 10.1016/j.jhep.2008.03.007]

13 **Mehal WZ**. The gut-liver axis: a busy two-way street. *Hepatology* 2012; **55**: 1647-1649 [PMID: 22407752 DOI: 10.1002/hep.25704]

14 **Turnbaugh PJ**, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. *Nature* 2007; **449**: 804-810 [PMID: 17943116 DOI: 10.1038/nature06244]

15 International Human Microbiome Consortium. Available from: URL: http://www.human-microbiome.org/

16 **National Institutes of Health**. Microbiome Project. Available from: URL: http://www.hmpdacc.org/

17 **Duseja A**, Chawla YK. Obesity and NAFLD: The role of bacteria and microbiota. *Clin Liver Dis* 2014; **18**: 59-71 [PMID: 24274865 DOI: 10.1016/j.cld.2013.09.002]

18 **Quigley EM**, Stanton C, Murphy EF. The gut microbiota and the liver. Pathophysiological and clinical implications. *J Hepatol* 2013; **58**: 1020-1027 [PMID: 23183530 DOI: 10.1016/j.jhep.2012.11.023]

19 **Ramakrishna BS**. Role of the gut microbiota in human nutrition and metabolism. *J Gastroenterol Hepatol* 2013; **28** Suppl 4: 9-17 [PMID: 24251697 DOI: 10.1111/jgh.12294]

20 **Park JS**, Seo JH, Youn HS. Gut microbiota and clinical disease: obesity and nonalcoholic Fatty liver disease. *Pediatr Gastroenterol Hepatol Nutr* 2013; **16**: 22-27 [PMID: 24010102 DOI: 10.5223/pghn.2013.16.1.22]

21 **Paliy O**, Agans R. Application of phylogenetic microarrays to interrogation of human microbiota. *FEMS Microbiol Ecol* 2012; **79**: 2-11 [PMID: 22092522 DOI: 10.1111/j.1574-6941.2011.01222.x]

22 **Lozupone CA**, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. *Nature* 2012; **489**: 220-230 [PMID: 22972295 DOI: 10.1038/nature11550]

23 **Cederbaum AI**. Alcohol metabolism. *Clin Liver Dis* 2012; **16**: 667-685 [PMID: 23101976 DOI: 10.1016/j.cld.2012.08.002]

24 **Delzenne NM**, Neyrinck AM, Cani PD. Gut microbiota and metabolic disorders: How prebiotic can work? *Br J Nutr* 2013; **109** Suppl 2: S81-S85 [PMID: 23360884 DOI: 10.1017/S0007114512004047]

25 **Hamer HM**, De Preter V, Windey K, Verbeke K. Functional analysis of colonic bacterial metabolism: relevant to health? *Am J Physiol Gastrointest Liver Physiol* 2012; **302**: G1-G9 [PMID: 22016433 DOI: 10.1152/ajpgi.00048.2011]

26 **Geurts L**, Neyrinck AM, Delzenne NM, Knauf C, Cani PD. Gut microbiota controls adipose tissue expansion, gut barrier and glucose metabolism: novel insights into molecular targets and interventions using prebiotics. *Benef Microbes* 2014; **5**: 3-17 [PMID: 23886976 DOI: 10.3920/BM2012.0065]

27 **Miele L**, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, Mascianà R, Forgione A, Gabrieli ML, Perotti G, Vecchio FM, Rapaccini G, Gasbarrini G, Day CP, Grieco A. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. *Hepatology* 2009; **49**: 1877-1887 [PMID: 19291785 DOI: 10.1002/hep.22848]

28 **Boots AW**, van Berkel JJ, Dallinga JW, Smolinska A, Wouters EF, van Schooten FJ. The versatile use of exhaled volatile organic compounds in human health and disease. *J Breath Res* 2012; **6**: 027108 [PMID: 22621865 DOI: 10.1088/1752-7155/6/2/027108]

29 **Bikov A**, Paschalaki K, Logan-Sinclair R, Horváth I, Kharitonov SA, Barnes PJ, Usmani OS, Paredi P. Standardised exhaled breath collection for the measurement of exhaled volatile organic compounds by proton transfer reaction mass spectrometry. *BMC Pulm Med* 2013; **13**: 43 [PMID: 23837867 DOI: 10.1186/1471-2466-13-43]

30 **Alkhouri N**, Cikach F, Eng K, Moses J, Patel N, Yan C, Hanouneh I, Grove D, Lopez R, Dweik R. Analysis of breath volatile organic compounds as a noninvasive tool to diagnose nonalcoholic fatty liver disease in children. *Eur J Gastroenterol Hepatol* 2014; **26**: 82-87 [PMID: 24284369 DOI: 10.1097/MEG.0b013e3283650669]

31 **Hanouneh IA**, Zein NN, Cikach F, Dababneh L, Grove D, Alkhouri N, Lopez R, Dweik RA. The breathprints in patients with liver disease identify novel breath biomarkers in alcoholic hepatitis. *Clin Gastroenterol Hepatol* 2014; **12**: 516-523 [PMID: 24036050 DOI: 10.1016/j.cgh.2013.08.048]24036050]

32 **Spaněl P**, Dryahina K, Rejšková A, Chippendale TW, Smith D. Breath acetone concentration; biological variability and the influence of diet. *Physiol Meas* 2011; **32**: N23-N31 [PMID: 21725144 DOI: 10.1088/0967-3334/32/8/N01]

33 **Wang T**, Pysanenko A, Dryahina K, Spaněl P, Smith D. Analysis of breath, exhaled via the mouth and nose, and the air in the oral cavity. *J Breath Res* 2008; **2**: 037013 [PMID: 21386174 DOI: 10.1088/1752-7155/2/3/037013]

34 **Wzorek B**, Mochalski P, Sliwka I, Amann A. Application of GC-MS with a SPME and thermal desorption technique for determination of dimethylamine and trimethylamine in gaseous samples for medical diagnostic purposes. *J Breath Res* 2010; **4**: 026002 [PMID: 21383470 DOI: 10.1088/1752-7155/4/2/026002]

35 **Mochalski P**, Wzorek B, Sliwka I, Amann A. Improved preconcentration and detection methods for volatile sulphur breath constituents. *J Chromatogr B Analyt Technol Biomed Life Sci* 2009; **877**: 1856-1866 [PMID: 19493705 DOI: 10.1016/j.jchromb.2009.05.010]

36 **Miekisch W**, Schubert JK, Noeldge-Schomburg GF. Diagnostic potential of breath analysis--focus on volatile organic compounds. *Clin Chim Acta* 2004; **347**: 25-39 [PMID: 15313139 DOI: 10.1016/j.cccn.2004.04.023]

37 **Purohit V**, Bode JC, Bode C, Brenner DA, Choudhry MA, Hamilton F, Kang YJ, Keshavarzian A, Rao R, Sartor RB, Swanson C, Turner JR. Alcohol, intestinal bacterial growth, intestinal permeability to endotoxin, and medical consequences: summary of a symposium. *Alcohol* 2008; **42**: 349-361 [PMID: 18504085 DOI: 10.1016/j.alcohol.2008.03.131]

38 **Gallego D**, Clavé P, Donovan J, Rahmati R, Grundy D, Jiménez M, Beyak MJ. The gaseous mediator, hydrogen sulphide, inhibits in vitro motor patterns in the human, rat and mouse colon and jejunum. *Neurogastroenterol Motil* 2008; **20**: 1306-1316 [PMID: 19019033 DOI: 10.1111/j.1365-2982.2008.01201.x]

39 **Dumas ME**, Barton RH, Toye A, Cloarec O, Blancher C, Rothwell A, Fearnside J, Tatoud R, Blanc V, Lindon JC, Mitchell SC, Holmes E, McCarthy MI, Scott J, Gauguier D, Nicholson JK. Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. *Proc Natl Acad Sci USA* 2006; **103**: 12511-12516 [PMID: 16895997 DOI: 10.1073/pnas.0601056103]

40 **Vance DE**. Role of phosphatidylcholine biosynthesis in the regulation of lipoprotein homeostasis. *Curr Opin Lipidol* 2008; **19**: 229-234 [PMID: 18460912 DOI: 10.1097/MOL.0b013e3282fee935]

41 **Spencer MD**, Hamp TJ, Reid RW, Fischer LM, Zeisel SH, Fodor AA. Association between composition of the human gastrointestinal microbiome and development of fatty liver with choline deficiency. *Gastroenterology* 2011; **140**: 976-986 [PMID: 21129376 DOI: 10.1053/j.gastro.2010.11.049]

42 **Pimentel M**, Mathur R, Chang C. Gas and the microbiome. *Curr Gastroenterol Rep* 2013; **15**: 356 [PMID: 24150797 DOI: 10.1007/s11894-013-0356-y]

43 **Saad RJ**, Chey WD. Breath Testing for Small Intestinal Bacterial Overgrowth: Maximizing Test Accuracy. *Clin Gastroenterol Hepatol* 2013; Epub ahead of print [PMID: 24095975 DOI: 10.1016/j.cgh.2013.09.055]

44 **Simrén M**, Barbara G, Flint HJ, Spiegel BM, Spiller RC, Vanner S, Verdu EF, Whorwell PJ, Zoetendal EG. Intestinal microbiota in functional bowel disorders: a Rome foundation report. *Gut* 2013; **62**: 159-176 [PMID: 22730468 DOI: 10.1136/gutjnl-2012-302167]

45 **Meyrat P**, Safroneeva E, Schoepfer AM. Rifaximin treatment for the irritable bowel syndrome with a positive lactulose hydrogen breath test improves symptoms for at least 3 months. *Aliment Pharmacol Ther* 2012; **36**: 1084-1093 [PMID: 23066911 DOI: 10.1111/apt.12087]

46 **Majewski M**, Reddymasu SC, Sostarich S, Foran P, McCallum RW. Efficacy of rifaximin, a nonabsorbed oral antibiotic, in the treatment of small intestinal bacterial overgrowth. *Am J Med Sci* 2007; **333**: 266-270 [PMID: 17505166 DOI: 10.1097/MAJ.0b013e3180536784]

47 **Simrén M**, Stotzer PO. Use and abuse of hydrogen breath tests. *Gut* 2006; **55**: 297-303 [PMID: 16474100 DOI: 10.1136/gut.2005.075127]

48 **Brandt LJ**, Chey WD, Foxx-Orenstein AE, Schiller LR, Schoenfeld PS, Spiegel BM, Talley NJ, Quigley EM. An evidence-based position statement on the management of irritable bowel syndrome. *Am J Gastroenterol* 2009; **104** Suppl 1: S1-S35 [PMID: 19521341 DOI: 10.1038/ajg.2008.122]

49 GIDAC Rifaximin/IBS Meeting 2011. Available from: URL:

http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/GastrointestinalDrugsAdvisoryCommittee/UCM283448.pdf
50 Salix Briefing Document GIDAC 2011. Available from: URL: http://www.fda.gov/downloads/advisorycommittees/committeesmeetingmaterials/drugs/gastrointestinaldrugsadvisorycommittee/ucm279646.pdf
51 **Goggs R**, Serrano S, Szladovits B, Keir I, Ong R, Hughes D. Clinical investigation of a point-of-care blood ammonia analyzer. *Vet Clin Pathol* 2008; **37**: 198-206 [PMID: 18533920 DOI: 10.1111/j.1939-165X.2008.00024.x]
52 **Blanco Vela CI**, Bosques Padilla FJ. Determination of ammonia concentrations in cirrhosis patients-still confusing after all these years? *Ann Hepatol* 2011; **10** Suppl 2: S60-S65 [PMID: 22228884]
53 **Adeva MM**, Souto G, Blanco N, Donapetry C. Ammonium metabolism in humans. *Metabolism* 2012; **61**: 1495-1511 [PMID: 22921946 DOI: 10.1016/j.metabol.2012.07.007]
54 **Lovett AM**, Reid NM, Buckley JA, French JB, Cameron DM. Real-time analysis of breath using an atmospheric pressure ionization mass spectrometer. *Biomed Mass Spectrom* 1979; **6**: 91-97 [PMID: 369632 DOI: 10.1002/bms.1200060302]
55 **Tittel FK**, Lewicki R. Tunable mid-infrared laser absorption spectroscopy. In: Baranov A, Tournie E, editors. Semiconductor Lasers Fundamentals and Applications, Woodhead Publishing Series in Electronic and Optical Materials. Oxford: Woodhead Publishing Ltd, 2013: 579-629
56 **Solga SF**, Mudalel M, Spacek LA, Lewicki R, Tittel F, Loccioni C, Russo A, Risby TH. Factors influencing breath ammonia determination. *J Breath Res* 2013; **7**: 037101 [PMID: 23774041 DOI: 10.1088/1752-7155/7/3/037101]
57 **Hibbard T**, Crowley K, Killard AJ. Direct measurement of ammonia in simulated human breath using an inkjet-printed polyaniline nanoparticle sensor. *Anal Chim Acta* 2013; **779**: 56-63 [PMID: 23663672 DOI: 10.1016/j.aca.2013.03.051]
58 **Adrover R**, Cocozzella D, Ridruejo E, García A, Rome J, Podestá JJ. Breath-ammonia testing of healthy subjects and patients with cirrhosis. *Dig Dis Sci* 2012; **57**: 189-195 [PMID: 21842240 DOI: 10.1007/s10620-011-1858-9]
59 **Endre ZH**, Pickering JW, Storer MK, Hu WP, Moorhead KT, Allardyce R, McGregor DO, Scotter JM. Breath ammonia and trimethylamine allow real-time monitoring of haemodialysis efficacy. *Physiol Meas* 2011; **32**: 115-130 [PMID: 21149927 DOI: 10.1088/0967-3334/32/1/008]
60 **Senthilmohan ST**, Milligan DB, McEwan MJ, Freeman CG, Wilson PF. Quantitative analysis of trace gases of breath during exercise using the new SIFT-MS technique. *Redox Rep* 2000; **5**: 151-153 [PMID: 10939300]
61 **Walker V**. Severe hyperammonaemia in adults not explained by liver disease. *Ann Clin Biochem* 2012; **49**: 214-228 [PMID: 22349554 DOI: 10.1258/acb.2011.011206]
62 **Tranah TH**, Vijay GK, Ryan JM, Shawcross DL. Systemic inflammation and ammonia in hepatic encephalopathy. *Metab Brain Dis* 2013; **28**: 1-5 [PMID: 23224356 DOI: 10.1007/s11011-012-9370-2]
63 **Carpenter D**. Repuation and Power. Organizational Image and Pharmaceutical Regulation at the FDA. Princeton: Princeton University Press, 2010
64 **Raman M**, Ahmed I, Gillevet PM, Probert CS, Ratcliffe NM, Smith S, Greenwood R, Sikaroodi M, Lam V, Crotty P, Bailey J, Myers RP, Rioux KP. Fecal microbiome and volatile organic compound metabolome in obese humans with nonalcoholic fatty liver disease. *Clin Gastroenterol Hepatol* 2013; **11**: 868-75.e1-3 [PMID: 23454028 DOI: 10.1016/j.cgh.2013.02.015]
65 **Boursier J**, Rawls JF, Diehl AM. Obese humans with nonalcoholic fatty liver disease display alterations in fecal microbiota and volatile organic compounds. *Clin Gastroenterol Hepatol* 2013; **11**: 876-878 [PMID: 23628504 DOI: 10.1016/j.cgh.2013.04.016]
66 **Zhu L**, Baker SS, Gill C, Liu W, Alkhouri R, Baker RD, Gill SR. Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: a connection between endogenous alcohol and NASH. *Hepatology* 2013; **57**: 601-609 [PMID: 23055155 DOI: 10.1002/hep.26093]
67 **Aylward LL**, Kirman CR, Blount BC, Hays SM. Chemical-specific screening criteria for interpretation of biomonitoring data for volatile organic compounds (VOCs)--application of steady-state PBPK model solutions. *Regul Toxicol Pharmacol* 2010; **58**: 33-44 [PMID: 20685286 DOI: 10.1016/j.yrtph.2010.05.011]
68 **Ong JP**, Aggarwal A, Krieger D, Easley KA, Karafa MT, Van Lente F, Arroliga AC, Mullen KD. Correlation between ammonia levels and the severity of hepatic encephalopathy. *Am J Med* 2003; **114**: 188-193 [PMID: 12637132 DOI: 10.1016/S0002-9343(02)01477-8]
69 **He Y**, Li G, Song H, Luo T, Gao B, Xu J. Partial pressure of NH_3 in cirrhotic patients with and without hepatic encephalopathy. *J Gastrointestin Liver Dis* 2011; **20**: 169-174 [PMID: 21725514]
70 **Lin CH**, Chi CH, Wu SY, Hsu HC, Chang YH, Huang YY, Chang CJ, Hong MY, Chan TY, Shih HI. Prognostic values of blood ammonia and partial pressure of ammonia on hospital arrival in out-of-hospital cardiac arrests. *Am J Emerg Med* 2013; **31**: 8-15 [PMID: 22795429 DOI: 10.1016/j.ajem.2012.04.037]
71 **Friedhoff LT**. New Drugs. Glasgow: PSPG Publishing, 2009
72 **Kramer DB**, Xu S, Kesselheim AS. Regulation of medical devices in the United States and European Union. *N Engl J Med* 2012; **366**: 848-855 [PMID: 22332952 DOI: 10.1056/NEJMhle1113918]
73 **Lee JW**, Devanarayan V, Barrett YC, Weiner R, Allinson J, Fountain S, Keller S, Weinryb I, Green M, Duan L, Rogers JA, Millham R, O'Brien PJ, Sailstad J, Khan M, Ray C, Wagner JA. Fit-for-purpose method development and validation for successful biomarker measurement. *Pharm Res* 2006; **23**: 312-328 [PMID: 16397743 DOI: 10.1007/s11095-005-9045-3]
74 **Colburn WA**, Lee JW. Biomarkers, validation and pharmacokinetic-pharmacodynamic modelling. *Clin Pharmacokinet* 2003; **42**: 997-1022 [PMID: 12959633 DOI: 10.2165/00003088-200342120-00001]
75 **Pepe MS**, Etzioni R, Feng Z, Potter JD, Thompson ML, Thornquist M, Winget M, Yasui Y. Phases of biomarker development for early detection of cancer. *J Natl Cancer Inst* 2001; **93**: 1054-1061 [PMID: 11459866]
76 **Amann A**, Smith D. Volatile Biomarkers. 1st ed. Amsterdam: Elsevier, 2013
77 Loccioni Sampler. Available from: URL: http://humancare.loccioni.com/about-us/breath/breath-analysis-sampler/
78 **Spaněl P**, Dryahina K, Smith D. A quantitative study of the influence of inhaled compounds on their concentrations in exhaled breath. *J Breath Res* 2013; **7**: 017106 [PMID: 23445832 DOI: 10.1088/1752-7155/7/1/017106]
79 **Cope K**, Risby T, Diehl AM. Increased gastrointestinal ethanol production in obese mice: implications for fatty liver disease pathogenesis. *Gastroenterology* 2000; **119**: 1340-1347 [PMID: 11054393]
80 **Nair S**, Cope K, Risby TH, Diehl AM. Obesity and female gender increase breath ethanol concentration: potential implications for the pathogenesis of nonalcoholic steatohepatitis. *Am J Gastroenterol* 2001; **96**: 1200-1204 [PMID: 11316170 DOI: 10.1111/j.1572-0241.2001.03702.x]
81 **Solga SF**, Alkhuraishe A, Cope K, Tabesh A, Clark JM, Torbenson M, Schwartz P, Magnuson T, Diehl AM, Risby TH. Breath biomarkers and non-alcoholic fatty liver disease: preliminary observations. *Biomarkers* 2006; **11**: 174-183 [PMID: 16766393 DOI: 10.1080/13547500500421070]
82 **Minh Tdo C**, Blake DR, Galassetti PR. The clinical potential of exhaled breath analysis for diabetes mellitus. *Diabetes Res Clin Pract* 2012; **97**: 195-205 [PMID: 22410396 DOI: 10.1016/j.diabres.2012.02.006]

83 **Dowlaty N**, Yoon A, Galassetti P. Monitoring states of altered carbohydrate metabolism via breath analysis: are times ripe for transition from potential to reality? *Curr Opin Clin Nutr Metab Care* 2013; **16**: 466-472 [PMID: 23739629 DOI: 10.1097/MCO.0b013e328361f91f]
84 **Baršić N**, Lerotić I, Smirčić-Duvnjak L, Tomašić V, Duvnjak M. Overview and developments in noninvasive diagnosis of nonalcoholic fatty liver disease. *World J Gastroenterol* 2012; **18**: 3945-3954 [PMID: 22912545 DOI: 10.3748/wjg.v18.i30.3945]
85 **Heeringa M**, Hastings A, Yamazaki S, de Koning P. Serum biomarkers in nonalcoholic steatohepatitis: value for assessing drug effects? *Biomark Med* 2012; **6**: 743-757 [PMID: 23227839 DOI: 10.2217/bmm.12.87]
86 National Institute of Diabetes and Digestive and Kidney Diseases. NASH CRN. Available from: UPL: http://jhuccs1.us/nash/
87 **Konvalina G**, Haick H. Sensors for breath testing: from nanomaterials to comprehensive disease detection. *Acc Chem Res* 2014; **47**: 66-76 [PMID: 23926883 DOI: 10.1021/ar400070m]

P- Reviewers: Assy N, Kawaguchi T, Rosa H **S- Editor**: Ma YJ
L- Editor: A **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9026

World J Gastroenterol 2014 July 21; 20(27): 9026-9037
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Role of liver biopsy in nonalcoholic fatty liver disease

ILKe Nalbantoglu, Elizabeth M Brunt

ILKe Nalbantoglu, Elizabeth M Brunt, Department of Pathology and Immunology, Washington University School of Medicine, Saint Louis, MO 63110, United States
Author contributions: Nalbantoglu I and Brunt EM contributed to this manuscript.
Correspondence to: ILKe Nalbantoglu, MD, Department of Pathology and Immunology, Washington University School of Medicine, Campus Box 8118, 660 S Euclid Ave, Saint Louis, MO 63110, United States. inalbantoglu@path.wustl.edu
Telephone: +1-314-3620101 Fax: +1-314-3628950
Received: December 25, 2013 Revised: February 11, 2014
Accepted: May 12, 2014
Published online: July 21, 2014

Abstract

Nonalcoholic fatty liver disease (NAFLD), defined as abnormal accumulation (> 5%) of hepatic triglyceride without excess alcohol intake, is the most common form of chronic liver disease in adults and children in the United States. NAFLD encompasses a spectrum of histologic findings including uncomplicated steatosis, steatosis with inflammation and steatohepatitis [nonalcoholic steatohepatitis (NASH)]; the latter can advance to cirrhosis and hepatocellular carcinoma. NASH is currently accepted as the hepatic manifestation of the set of cardiovascular risk factors collectively known as metabolic syndrome. In 1999 a system for histologic grading and staging for NASH was proposed; this was revised by the NASH Clinical Research Network in 2005 for the entire spectrum of lesions in NAFLD, including the lesions and patterns of pediatric NAFLD, and for application in clinical research trials. Diagnosis remains distinct from grade and stage. A recent European proposal separates steatosis from activity to derive a numeric diagnosis of NASH. Even though there have been promising advancements in non-invasive testing, these tests are not yet detailed enough to replace the full range of findings provided by liver biopsy evaluation. Limitations of biopsy are acknowledged, but liver biopsy remains the "gold standard" for diagnosis and determination of amounts of necroinflammatory activity, and location of fibrosis, as well as remodeling of the parenchyma in NASH. This review focuses on the specific histologic lesions of NAFLD and NASH, grading and staging, differential diagnoses to be considered, and the continuing role of the liver biopsy in this important liver disease.

Key words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Liver biopsy; Histopathology; Grading and staging

Core tip: Nonalcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease; no clinical or serologic tests have yet replaced liver biopsy for definitive diagnosis. The histologic spectrum includes steatosis, steatohepatitis, and cirrhosis with or without active steatohepatitis. Hepatocellular carcinoma may occur in cirrhosis, or prior to cirrhosis. Liver biopsy provides vital data for patient care, clinical trials, and for ongoing research into nuances of the disease process. The histologic spectrum of NAFLD, features with coexistent diseases, differential diagnoses, grading and staging methods and the role of liver biopsy, as well as a brief description of non-invasive alternatives, are discussed.

Nalbantoglu I, Brunt EM. Role of liver biopsy in nonalcoholic fatty liver disease. *World J Gastroenterol* 2014; 20(27): 9026-9037 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9026.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9026

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), defined as abnormal accumulation of triglycerides in the liver (*i.e.*, > 5%) in the absence of significant alcohol intake, is recog-

nized as the most common cause of chronic liver disease, and is estimated to affect 30% of adults and 10% of children in United States[1]. The prevalence of NAFLD parallels that of metabolic syndrome, insulin resistance, type 2 diabetes and central obesity[2]. It is currently recognized that mortality in the majority of individuals with NAFLD is more likely from cardiovascular diseases than from liver disease[3], thus, even though recent studies have documented similar epidemiologic and histologic features of NAFLD and nonalcoholic steatohepatitis (NASH) in the geriatric population[4,5], this age group will not be further discussed in this review. The prevalence is highest in the ethnic Hispanic population, followed by non-Hispanic whites, Asians and African Americans[6,7]. The prevalence increases significantly up to 80%-90% in obese adults, 60% in patients with hyperlipidemia, and 30%-50% in diabetics[2]. Only a minority of subjects with steatohepatitis actually progress to fibrosis or cirrhosis[8]. Currently, however, NAFLD is the third most common cause of liver transplantation in United States[9], and is projected to be the leading cause of liver transplantation in the United States by 2020[1]. In addition, approximately 4%-22% of hepatocellular carcinomas in the Western world are attributed to NAFLD[10]. Hepatocellular carcinoma may develop in either cirrhosis or non-cirrhotic NAFLD, as recently reviewed[10,11].

NAFLD is a spectrum of histologic lesions of steatosis at one end, and steatohepatitis and cirrhosis at the other[12]. However, whether in a given patient there is actual "progression" from steatosis to steatohepatitis without concurrent clinical complications such as weight gain, or new onset diabetes, has not been adequately studied. What is accepted is that steatosis itself is considered "non-progressive" whereas steatohepatitis is the constellation of lesions with potential to progress; this was first shown in a seminal series of Matteoni *et al*[13]. Thus, the ability of the liver biopsy is to separate individuals with steatohepatitis from those with "only" steatosis (which includes steatosis with inflammation); this is an important goal that any diagnostic test should meet. NASH is defined histopathologically by the presence of a constellation of features: steatosis, lobular and portal inflammation and liver cell injury in the form of hepatocyte ballooning. Initially, in adults, the ballooning and fibrosis are in a zone 3 distribution; once abnormal matrix is deposited (*i.e.*, fibrosis), and architectural remodeling occurs, the zonality of injury is less apparent.

Many advances have occurred since the initial recognition that fatty liver disease could occur in overweight and/or diabetic subjects who weren't over-exposed to alcohol by several researchers including Thaler[14], Klatskin *et al*[15], Schaffner *et al*[16] and Ludwig *et al*[17] in the late 1970's and early 1980's. These include histopathologic work in humans with careful clinical correlations discerning the prevalence of NAFLD and NASH[18], the roles of progenitor cells and the ductular reaction in fibrogenesis[18-21], and the role of innate and adaptive immune-mediated mechanisms in steatohepatitis[22], progression of steatohepatitis to cirrhosis and hepatocellular carcinoma[10,23,24] and genetic and genomic underpinnings of disease susceptibility and progression[25,26].

LIVER BIOPSY: ROLE, INDICATIONS, AND DRAWBACKS

The general indications for performing a liver biopsy in patients with NAFLD are to confirm or exclude the diagnosis, diagnose other liver diseases, and to determine amounts of damage to the liver for treatment and prognosis. The last includes necroinflammatory activity, which is potentially reversible, and collagen deposition with varying degrees of remodeling, which is potentially less reversible. More specific indications have been recently stated. According to the 2012 guidelines from American Association for the Study of Liver Disease (AASLD), liver biopsy should be reserved for subjects who will "benefit", for subjects with potentially competing diagnoses, and for children with either an unclear diagnosis or in whom consideration is being given for medication[27]. The European Association for the Study of Liver Disease position statement on liver biopsy differed slightly and recommended liver biopsy in all bariatric surgery subjects, and as an endpoint in all clinical trials[28]. Liver biopsy remains the standard against which noninvasive (serologic and imaging) methods are judged in order to assess these features. By histologic evaluation, one is able to distinguish between NASH, a lesion with progressive potential, and no NASH, lesions without potential to progress[28-33].

Performing a liver biopsy on every patient with suspected NAFLD remains a controversial subject in daily practice, and clearly is not a practical consideration as a "screening" tool. There are studies, however, that support the value of liver biopsy. A frequently cited albeit older study by Skelly *et al*[34] showed that of 354 biopsied patients with otherwise unexplained abnormal liver tests 66% had fatty liver, 50% of those had steatohepatitis, approximately 19% of the remaining biopsies had other treatable causes diagnosed by the pathology evaluation including autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), hemochromatosis and alcoholic liver disease (ALD).

A more recent study used a modeling system to show that obtaining a liver biopsy could provide survival benefit to patients with NAFLD. Gaidos *et al*[35] evaluated the long-term benefit of biopsy *vs* no biopsy. The study showed that performing an early liver biopsy resulted in a higher percentage of having mild liver disease ultimately in NAFLD patients. Early diagnosis and treatment resulted in decrease in progression to severe disease or transplant and a predicted survival advantage in NAFLD patients. In addition, the risk of death related to liver biopsy did not offset the survival advantage. Others have demonstrated the benefits of liver biopsy in NAFLD by demonstrating the presence of NAFLD or NASH in "atypical settings" such as concurrent disease processes such as Hepatitis C[36-38], AIH, PBC, hereditary hemochromatosis (HH)[36], as well as drugs and occupational

exposures[39-41]. Liver biopsy studies proved the validity of the concept of the presence of all ranges of necro-inflammatory activity and fibrosis, including cirrhosis, in the presence of normal alanine aminotransferase values in adults[42-44], and children[45]. The current state of investigation for pathologists is to evaluate which subjects will develop fibrosis and why, and which findings on early biopsies, if any, may be predictive of future outcomes.

Liver biopsy studies have given insight into natural history of NASH, albeit in a selected group of primarily adult subjects. The prevalence of NASH, 12.2% of over 300 multi-ethnic adult subjects, was established in a Texas city by liver biopsy in an unbiased community who were willing to undergo ultrasound and subsequent liver biopsy. Nearly 30% had evidence of NAFLD by ultrasound[18]. This was the first study of its kind in adults, as all prior studies had been done either in living donors, who did not have evidence of steatohepatitis, or in autopsies. Liver pathology evaluation was the first to establish the remarkable incidence of fatty liver of up to 13% in an autopsy study of children who died from accidental deaths[46]. Liver biopsy further characterized the association of cardiovascular disease and biopsy proven NAFLD in 150 overweight children compared with 150 overweight children without NAFLD[47].

At the current time, it remains common practice to attribute cases of cryptogenic cirrhosis to burned out "NASH". This is not altogether appropriate unless there is prior biopsy proof of NASH because clinically it is known that other forms of liver injury can "burn out", in particular alcoholic liver disease and autoimmune liver disease. It was liver biopsy studies that indicated that NASH may be one of the underlying causes of cryptogenic cirrhosis, which, by definition, has no serologic markers for determination of cause of liver disease. This was shown in a small case series[48] then larger case studies comparing NAFLD-related cirrhosis to cirrhosis related to other forms of serologically diagnosed chronic liver disease[49], and finally in kindred studies[50]. Liver biopsy in cirrhosis of otherwise unknown etiology can show evidence of ongoing steatohepatitis, or burned out disease without any activity or steatosis in subjects with clinical features of metabolic syndrome. Additionally, not only will NASH progress to advanced fibrosis and cirrhosis in a subset of individuals if the underlying metabolic conditions are unchanged, but several studies have verified that NASH may also regress spontaneously, as reviewed[51]. This phenomenon has largely been observed in either placebo arms or in the non-responders of treatment trials[52-54].

In 2009, an AASLD sponsored workshop raised important questions for advancement in the field of NAFLD, providing guidance for investigators for clinical design and end points including pathologic and laboratory data[55]. As evidenced by published recommendations, liver biopsy is an important tool in clinical trials. Outcomes remain based on histologic features which provide information regarding the effects of intervention on extent and severity of hepatocellular injury, inflammation, and architectural alterations as well as the ultimate diagnosis. To date, histologic evaluation remains the "hard endpoint" that can be measured with the most reliability[12]. The most commonly used tool for histologic evaluation is the *n*onalcoholic fatty liver disease *a*ctivity *s*core (NAS) (Details will be discussed in later in the text). The NAS was not intended to replace the diagnosis but to provide a sensitive tool to assess the changes that might occur with treatment[56]. However, unintended use of this score has been noted by the investigators[56]. A study by NASH clinical research network (CRN), including 976 adult liver biopsies, challenged this concept and demonstrated that while the NAS correlated with the diagnosis, it was in the lower and higher ends of the spectrum. Therefore, if NAS were to reflect the diagnosis, a significant portion of these patients would be misclassified[56]. Most importantly diagnosis of NASH was strongly associated with the presence of diabetes, quantitative insulin sensitivity check index (QUICKi) and homeostasis model assessment-estimated insulin resistance (HOMA-IR)[56] while the diagnosis and the NAS both correlated with aminotransferases. This data further supported the concept that scoring lesions and diagnosing patterns of injury are different processes for a pathologist[29,56].

LIVER BIOPSY LIMITATIONS

Liver biopsy, as useful as it is, however, does have limitations. The major limitation of liver biopsy is the invasive nature of the procedure. Though considered "minimal", liver biopsy is an invasive procedure and can have complications even in the ideal clinical conditions, including pain, minor and major bleeding (0.3%). Organ perforation is uncommon, but more likely in blind biopsy. Death albeit rare, has been reported at 0.01%[57]. Currently, the majority of liver biopsies are performed under ultrasound guidance. As with liver biopsy interpretation, operator experience is an important factor in success[58].

As in other chronic liver diseases, biopsy size is an important, but often unrecognized consideration in diagnostic accuracy[12,59,60]. A biopsy, at least 1.6 cm in length with 1.2-1.8 mm diameter, containing approximately 10 portal tracts is considered adequate[61,62]. Even then an adequate liver biopsy represents approximately 1:50.000 of the entire organ[63].

Some limitations of liver biopsy are due to variability of the disease process itself, as with all other forms of chronic liver disease. NAFLD, while a diffuse process of the liver, can have differences particularly in fibrosis due to the location of the samples under evaluation. The subcapsular liver tissue is generally more fibrotic, and the left lobe has larger portal areas near the capsule than the right. Thus, it is important, particularly for studies, that both pre and post study biopsies are done in a similar fashion and from the same region of the liver[12]. Authors have not always agreed on the amounts of histologic sampling variability in NAFLD. Larson *et al*[62] found minimal variability in steatosis, NAS $\geqslant$ 5 and fibrosis between two samples in bariatric subjects. The study also emphasized the need for not only appropriate length, but also width of liver bi-

opsy needle. Another study in morbidly obese individuals undergoing bariatric surgery found moderate histologic variability between lobes[64]. In two additional studies, one in bariatrics and one in non-morbidly obese subjects, two separate biopsies from the same location in the same lobe were graded and staged independently and showed one or more points in discordance in fibrosis stage[59,60]. In addition hepatocyte ballooning, one of the diagnostic requirements of NASH, was not present in 24% of patients in one study in one set of the biopsies[60]. The implications for clinical studies for evaluating pre and post treatment biopsies are apparent.

The experience of the pathologist also plays a significant role in making the diagnosis of NAFLD; this is similar to other liver diseases[58]. The interobserver agreements on steatosis, ballooning and fibrosis were good amongst pathologists in the study of Kleiner *et al*[65] that included 32 adult biopsies and 9 pathologists but not so strong for location of steatosis and for inflammation[66]. Similar observations were reported in a study of 21 liver biopsies read by eight experienced Japanese hepatopathologists with good agreement on fibrosis and extent of steatosis. Younossi *et al*[67], showed good concordance for extent of steatosis and degree of fibrosis along with ballooned hepatocytes in 53 liver biopsies interpreted by 4 experienced liver pathologists.

The high prevalence of NAFLD in the population and the limitations, risks and cost of liver biopsy have led investigators to seek for non-invasive methods to diagnose, and stage NALFD. The ideal test would be cheap, reproducible, and would be able to diagnose the full spectrum of NAFLD, predict fibrosis, and also reflect changes that occur with treatment[31,32]. Several different methodologies including imaging modalities, serum markers and combined tests are currently being investigated. Even though advancements are being made in these fields, none of these can provide detailed and accurate enough information to replace the liver biopsy. For a more comprehensive summary of recent non-invasive tests, the reader is referred to current reviews[31,32].

In summary an adequate liver biopsy, with appropriate clinical history, interpreted by a trained liver pathologist, is not only pivotal for an accurate and complete diagnosis (or exclusion) of NAFLD (or NASH), but also is optimal for obtaining detailed information regarding disease pattern, severity and fibrosis. It provides important information with respect to subtypes, potential future risks, possible etiology, and natural history of disease, and sets the ground work for future molecular studies and clinical trials, assisting clinical colleagues and patients with treatments and follow-up.

HISTOLOGIC FEATURES, GRADING, AND STAGING OF NAFLD: ADULTS

In NAFLD, 5% or more macrovesicular steatosis is required for the diagnosis. This may occur with or without other findings, but without it, the diagnosis cannot be entertained. Steatosis initially is found predominantly in hepatocytes around the terminal hepatic venule (zone 3); when abundant, steatosis can be panacinar, and when resolving, may be irregular, or azonal. The type of triglyceride accumulation that is observed in NAFLD is predominantly macrovesicular and is typically characterized by a single or a few lipid droplets within the affected hepatocytes, displacing the nucleus peripherally within the cell (Figure 1A). When the droplets are smaller, *i.e.,* "small droplet" steatosis, they are well-defined and easily delineated from one another. Small droplets often form droplets around the larger droplets and may coalesce to form the macrodroplet. Albeit small, these types of droplets should not be confused for true "microvesicular" steatosis, as the pathophysiology and thus implications for liver function differ (Figure 1B). Microvesicular steatosis may occur in a patchy distribution in up to 10 % of NASH cases[68,69], and has been shown in a large study to be associated with ballooned hepatocytes, and advanced fibrosis[69].

There are a constellation of histopathologic lesions required for the diagnosis of steatohepatitis. These include steatosis, inflammation (lobular and portal) and hepatocyte injury, commonly in the form of ballooning. Ballooned hepatocytes appear as enlarged hepatocytes with a flocculent, cleared appearance of the cytoplasm with irregular cytoplasmic borders[30,70] (Figure 1C). Often the nucleus is hyperchromatic. Hepatocyte ballooning has been shown to correlate with oxidative damage[71] and microtubule disruption[30,70], and loss of normal structure of the cytoskeleton[71,72]. In adult NASH, ballooned hepatocytes are most commonly seen in zone 3 and if fibrosis is present, they are intermixed with perisinusoidal collagen fibers. Keratins 8/18, present throughout the cytoplasm of all normal hepatocytes[72], are damaged in ballooned hepatocytes; this is manifest with loss of cytoplasmic immunoexpression (Figure 1D), movement to submembranous location, and highlighting of Mallory-Denk bodies (MDB)[73]. MDB are ubiquitinated keratins and cytoskeletal structures within the proteosome (Figure 1E). p62 and ubiquitin are immunomarkers that label MDB[74]. By trichrome stain, MDB can be blue or green (Figure 1F). In NASH, MDBs are often less well-formed than in alcoholic hepatitis or alcoholic steatohepatitis[75]. Finally, ballooning has been associated with several relevant clinical markers such as measures of insulin resistance[76], increased serum cholesterol[76,77] and serum markers of necroinflammation[78]. Similar correlations have also been noted in the pediatric population[79].

Lobular inflammation, typically more prominent than portal inflammation in uncomplicated adult NAFLD, is mostly mononuclear, but also includes Kupffer cell clusters, microgranulomas with or without lipid droplets, and larger lipogranulomas (Figure 1G). Lefkowitch noted prominent enlargement and aggregation of Kupffer cells around terminal hepatic venules in NASH, in contrast to the even distribution in normal livers and uncomplicated steatosis[80]. Kupffer cells, the largest resident macrophage

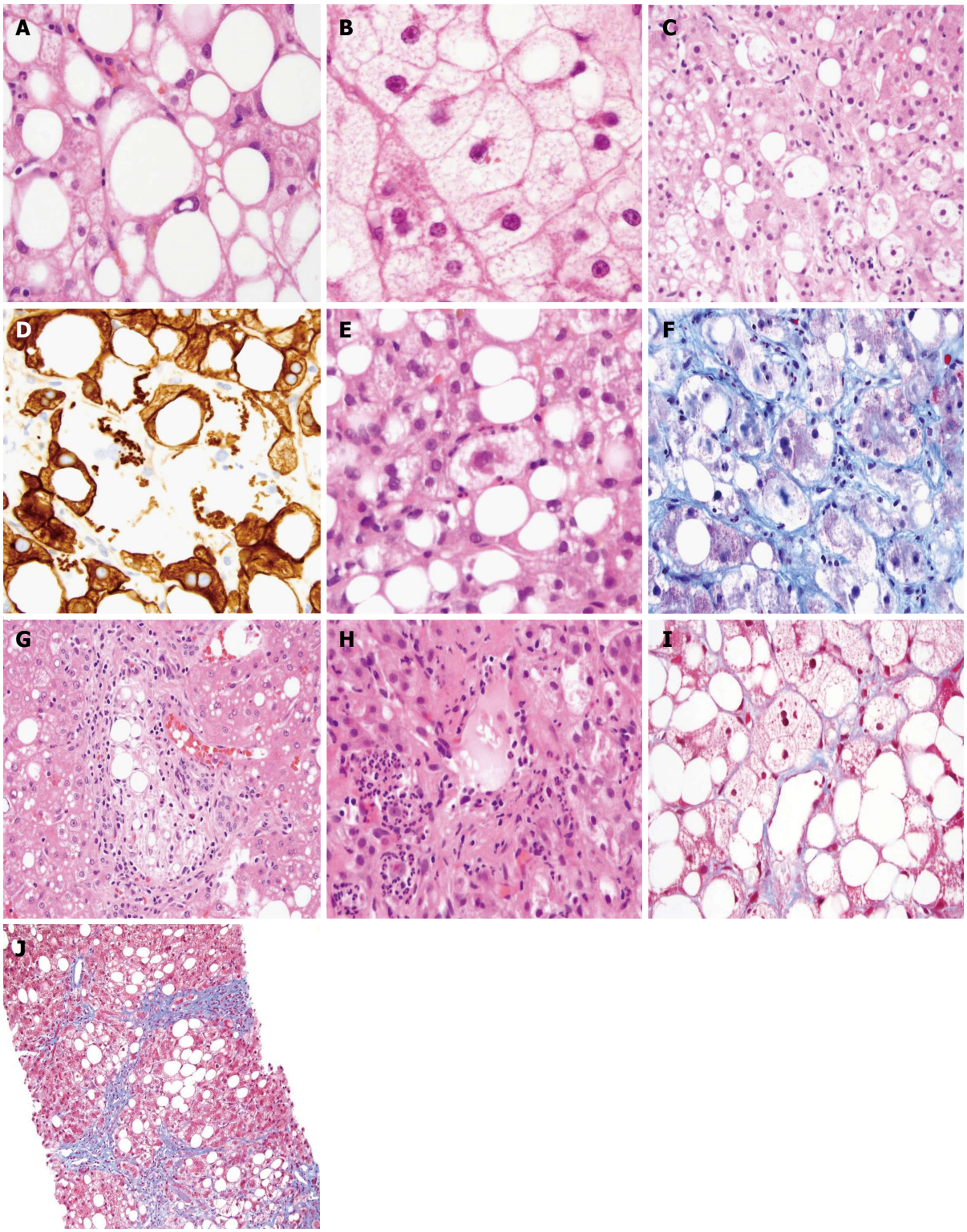


Figure 1 Histologic features, grading, and staging of nonalcoholic fatty liver disease. A: Mixed large and small droplet steatosis, single droplet, with nucleus pushed to one side, HE stain, 600 ×; B: Microvesicular steatosis, nuclei in the center with foamy cytoplasm, and megamitochondria HE stain, 600 ×; C: Ballooned hepatocytes with flocculent cytoplasm, HE stain, 600 ×; D: Loss of cytoplasmic expression of keratin 8/18 in ballooned hepatocytes, 600 ×; E: Mallory-Denk body, HE stain, 600 ×; F: Mallory-Denk body in blue-green color and dense perisinusoidal fibrosis, Trichrome stain, 600 ×; G: Portal lipogranuloma, HE stain, 400 ×; H: Mallory-Denk bodies and satellitosis HE stain, 600 ×; I: Delicate perisinusoidal fibrosis, Trichrome stain, 600 ×; J: Bridging fibrosis, Trichrome stain, 200 ×.

population in liver, are an important component of the innate immune system[81] and are implicated in the development and the progression of steatohepatitis[82,83] as well as in fibrosis[83].

Occasional polymorphonuclear leukocytes may also occur as a part of lobular inflammation. When intense and encircling hepatocytes (*i.e.*, satellitosis), one should consider alcoholic hepatitis (Figure 1H). The intensity

and distribution of the inflammation varies within the lobule. In some cases, the intense inflammation in zone 3 may be confused with a portal area with the duct obscured by inflammation. This may be particularly true in the cases when an artery branch is readily appreciated in zone 3[84].

Portal inflammation in NAFLD/NASH can be seen in 4 situations in increased amounts. In most cases, however, it is usually milder than lobular inflammation, and mononuclear cells are typically predominant[85]. Increased portal inflammation in active NASH has been associated with increased steatosis, ballooning and fibrosis in a series of 728 adult and 205 pediatric biopsies[86]. Additionally, portal inflammation was noted to predominate along with portal fibrosis in a study of 100 pediatric NAFLD biopsies[87]. When portal inflammation is unusually prevalent, or when lymphoid aggregates occur in adult NAFLD or NASH, one should consider the possibility of a concurrent liver disease such as viral hepatitis, autoimmune liver disease, as examples[36,88]. Liver biopsy data has shown that pathologists are capable of diagnosing NASH concurrently with another serologically positive liver disease, in particular, HCV, but the criteria may differ. This concept was shown by 3 separate studies; 2 retrospective reviews of large biopsy series[36,38] and a prospective study[89]. One group emphasized the necessity of stricter histologic criteria when evaluating NASH with other diseases by focusing on the characteristic zone 3 perisinusoidal fibrosis of the former that does not occur in the latter[36]. The fourth consideration is increased portal inflammation compared to lobular in adult patients following otherwise effective intervention[29]. Finally, a different type of portal inflammation, *i.e.*, polymorphonuclear leukocytes accompanying periportal ductular reaction may be indicative of ALD with pancreatitis or other forms of biliary obstruction.

Studies have demonstrated expansion of the periportal progenitor cell compartment in NASH. Hepatic progenitor cells (HPC) reside within the canal of Hering, along the limiting plate. They are rarely visualized by light microscopy unless activated. HPC are characterized by high N:C, round to spindled cytoplasm, ovoid nuclei and positivity for keratin 7 and 19; these characteristics are altered with progressive stages of development towards hepatocellular or biliary epithelium. In certain circumstances of liver injury and repair, stem cell markers, Hedgehog pathway markers and others are also reported in activated HPC[90]. Roskams *et al*[91] demonstrated increased number of progenitor cells in patients with NASH and ALD; the study further showed correlation between numbers of hepatic progenitor cells and fibrosis stage. In a multi-center study of subjects before and after various forms of treatment, the investigators demonstrated expansion of progenitor cell component, correlation between ductular reaction, steatosis, amount of portal inflammation, and NASH activity grade[19]. The group went on to demonstrate that the ductular reaction correlated with p21 positive replicative arrest in hepatocytes which was also associated with NASH activity and with insulin resistance. This area of epithelial-mesenchymal communication is one of ongoing interest in NASH.

Apoptotic hepatocytes are common both in NASH and ALD. Investigators have found that increased apoptosis is associated with disease severity[92,93], as well as fibrosis in NASH[92].

Isolated arteries observed in zone 3 correlated with advanced fibrosis in NASH[84]. Care must be taken to not confuse this region for a portal tract when there is marked inflammation.

Iron deposition, typically mild, can be noted as punctate granules within reticuloendothelial lining cells and as granules or blush within hepatocytes. It has been reported in 15%-55% of cases[94]. Reticuloendothelial iron deposition was associated with steatosis, ballooning, portal inflammation, and fibrosis in a study of 849 patient biopsies from the NASH CRN[95,96]. The relationships of iron deposition, hepcidin, iron regulatory genetics, advanced fibrosis and insulin resistance in fatty liver disease are complex and under intense investigation[96-99].

The typical pattern of fibrosis in adult NASH is initially located in zone 3 in the perisinusoidal spaces in a pattern that is described as pericellular. When delicate, it is best appreciated with Masson trichrome or other collagen stains (Figure 1I). As the disease progresses, the fibrosis becomes denser in zone 3 perisinusoidal spaces and, with further progression, portal and periportal fibrosis can be appreciated. At that point, ductular reaction is often present. In time, central-central, central-portal, or portal-portal bridging, architectural remodeling and finally cirrhosis may occur (Figure 1J). In the advanced stages of fibrosis and remodeled architecture, perisinusoidal fibrosis may no longer be present. Cartoon depiction of progression may be seen in texts[100].

GRADING AND STAGING THE LESIONS OF NAFLD/NASH

In 1999, a semi-quantitative grading and staging system to describe and unify the approach of pathologists to the histopathologic lesions of NASH and fibrosis along with architectural alterations, was proposed by Brunt *et al*[101]. The system was developed from evaluation of 51 liver biopsies of NASH, and followed the broad method recently developed for chronic hepatitis of separating activity (grade) from fibrosis (stage), with the recognition that NASH was not a portal-based process[102]. A semi-quantitative activity grade was assigned by a combination of parameters including steatosis, lobular and portal inflammation, and hepatocyte ballooning (Table 1). Fibrosis staging was based on fibrosis patterns of adult NASH, and reflects the progression of fibrosis as well as subsequent architectural remodeling. Figures 1I and J illustrate Stages 1 and 3. The details of staging system can be seen in Table 2.

In 2002 the Brunt grading and staging was revised by

Table 1 Brunt grading system

Grade	Steatosis	Ballooning	Inflammation
Mild (1)	1-2 (< 66%)	Minimal	L: 1-2 P: 0-1
Moderate (2)	2-3	Present-zone 3	L: 2 P: 1-2
Severe (3)	2-3	Marked-zone 3	L: 3 P: 1-2

Reproduced with permission[101]. Steatosis: grade 1: < 33%; grade 2: > 33%-66%; grade 3: > 66%. Lobular inflammation: grade 1: < 2 foci per 200 × field; grade 2: 2-4 foci per 200 × field; grade 3: > 4 foci per 200 × field. Portal inflammation: grade 0: None; grade 1: Mild; grade 2: Moderate; grade 3: Severe. Ballooning: grade 1: Rare; grade 2: Prominent ballooning. L: Lobular/acinar inflammation; P: Portal inflammation.

Table 2 Brunt staging system

Stage	Zone 3 PSF, focal or extensive	Portal, periportal	Bridging	Cirrhosis
1	+	0	0	0
2	+	+	0	0
3	+/-	+/-	+	0
4	+/-	+/-	+/-	+

Reproduced with permission[101]. PSF: Perisinusoidal fibrosis.

NASH CRN for use as a feature-based system in clinical trials. The system was published in 2005, and has come to be known as "NAFLD Activity Score (NAS)"[65]. The NASH CRN Scoring system includes the entire spectrum of lesions that can be seen in the full range of NAFLD and NASH, including pediatric liver disease (Table 3). The disease activity score represents the unweighted sum of scores for steatosis, hepatocyte ballooning, and lobular inflammation. The fibrosis stage is an expansion of the Brunt scoring, with additional subdivisions to stage 1 (1a-mild perisinusoidal fibrosis, 1b-moderate perisinusoidal fibrosis, and 1c-portal fibrosis only, as occurs in pediatric NAFLD).

Alkhouri *et al*[103] recently published a calculated pediatric NAFLD histological score (PNHS), using the histologic parameters in NAS. The PNHS consists of weighted sum of steatosis, hepatocyte ballooning, lobular inflammation and portal inflammation. The "borderline NASH" category has been eliminated from the pediatric scoring system and high scores are associated with a diagnosis of NASH in this patient group[103]. The reader is referred to the reference for the calculation.

Most recently, an algorithmic approach to scoring has been proposed by Bedossa *et al*[104] based on over 600 bariatric patient liver biopsies. The SAF (steatosis, activity, fibrosis) system is a sum of scores of steatosis, activity (hepatocyte ballooning + lobular inflammation) and fibrosis. Many details are modeled on the criteria by NASH CRN[65]. The SAF system differs from NAS in three major areas: it includes fibrosis into the final score, it excludes steatosis from the activity score and one is able to derive a diagnosis of NASH from a numeric value of SAF. It is noteworthy that the proponents of this system do not take patterns of any of the lesions or any assessments of portal inflammation into account. Further validation of this method in non-morbidly obese subjects is awaited.

Table 3 Nonalcoholic steatohepatitis clinical research network nonalcoholic fatty liver disease scoring system

Steatosis grade	Lobular inflammation	Liver cell ballooning
0: < 5%	0: No foci	0: None
1: 5%-33%	1: < 2 foci per 200 × field	1: Few ballooned hepatocytes
2: 34%-66%	2: 2-4 foci per 200 × field	2: Many ballooned hepatocytes
3: > 66%	3: > 4 foci per 200 × field	

Reproduced with permission[65]. Nonalcoholic fatty liver disease activity score (NAS): Steatosis + inflammation + ballooning. Fibrosis, 0: None; 1: Perisinusoidal or periportal; 1A: Mild, zone 3 perisinusoidal; 1B: Moderate, zone 3 perisinusoidal; 1C: Portal, periportal; 2: Perisinusoidal and portal/periportal; 3: Bridging fibrosis; 4: Cirrhosis.

In summary, grading and staging systems are useful tools in terms of providing a standard in pathology reporting, monitoring response to treatment and/or progression of disease both in patient care and clinical trials. However, the diagnosis of NAFLD depends on interpreting a variety of histologic findings and patterns and, cannot be replaced or reflected with a single number or score.

PEDIATRIC NAFLD

One of the most consistent observations of pediatric NAFLD that differed from adults was the difference in distribution of the fat, and the preferential accentuation of portal inflammation and fibrosis (Figure 2A, B). Steatosis is commonly either panacinar or distinctly periportal (zone 1)[87,105-107]. From a study that resulted in a hierarchical analysis of 100 pediatric biopsies, Schwimmer proposed two dominant subtypes of pediatric NAFLD: Type 1, the least common, but seen in Caucasian girls and with the similar zone 3 accentuation as with adult NAFLD, and Type 2, the most common, seen more often in boys and characterized by either panacinar or periportal steatosis, portal-predominant inflammation and portal-based fibrosis, and most commonly encountered in Asian, Hispanic or Native American ethnic groups. The remainder of the biopsies were "overlap" or steatosis[87]. Carter-Kent *et al*[108] studied a large multi-ethnic overweight biopsy population from several North American centers and found less ability to clearly separate the biopsies into discrete patterns with an overlap of the two main patterns in 82% of cases. Nobili *et al*[109] also noted more overlap (52.4%) than either Type 1 or 2 in a series of 84 Italian overweight subjects. The NASH CRN refers to zone 1 (periportal) pattern as "borderline, zone 1", and zone 3 pattern as "borderline, zone 3", and has found similar ethnic correlations to Patton *et al*[110]. Both patterns have been seen in blinded biopsy reviews by the Central Pathology Committee in adult biopsies, albeit in small numbers. Interestingly, to date, there is yet to be a definitive agreement amongst expert

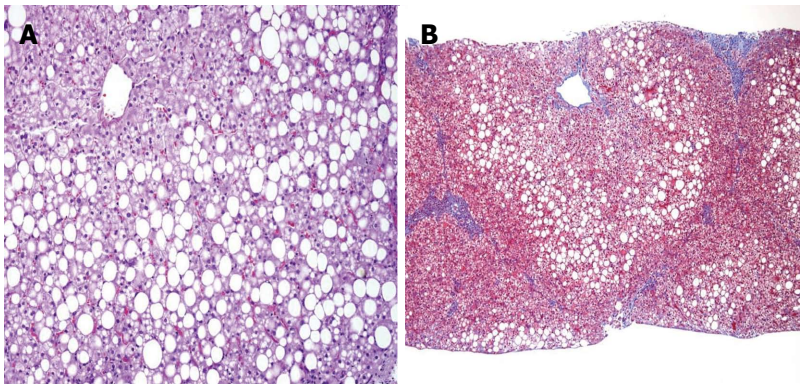


Figure 2 Pediatric nonalcoholic fatty liver disease. A: Periportal accentuation of steatosis with sparing of zone 3, pediatric nonalcoholic fatty liver disease (NAFLD), HE stain, 200 ×; B: Portal fibrosis without zone 3 perisinusoidal fibrosis, pediatric NAFLD, Trichrome stain, 100 ×.

pathologists in the field for pediatric NASH histology, unless there are the very same characteristics found in adult NASH, as described above. There is also no knowledge about when or how a transition may occur from pediatric patterns to adult patterns of disease, but an initial retrospective cross-sectional review of 186 NASH CRN biopsies has shown that comparing biopsies from children during prepuberty, puberty and post puberty, there is less steatosis and portal inflammation, but increased steatohepatitis and Mallory-Denk bodies with the change in age[111]. These findings are strongly suggestive that with the changes of aging and associated hormonal alterations and shifts of insulin sensitivity, the liver is more susceptible to the injury of increased free fatty acids and lipotoxicity. Natural history studies with prospective biopsies are needed in this growing population.

DIFFERENTIATION FROM ALCOHOLIC LIVER DISEASE

Some histologic features of NASH and ALD such as steatosis, hepatocyte injury (including ballooning, necrosis and apoptosis, MDBs), and lobular inflammation are shared[112]. However, in ALD lobular inflammation may show clusters of PMNs; when present, the lesion is known as "satellitosis". The lesion is a clue to the presence of MDB, often in apoptotic hepatocytes (Figure 1H). Steatosis is not a diagnostic requirement for ALD. An unusual form of ALD is nearly all microvesicular steatosis and is referred as "alcoholic foamy degeneration"; there is no equivalent described in NASH. Canalicular cholestasis and features of pancreatitis or biliary obstruction (ductular reaction accompanied by marked acute inflammation and edema) can occur in ALD; these lesions have not been described in NASH. Thickening and perivenular fibrosis of terminal hepatic venules and, veno-occlusive lesions are described in ALD[113]. Sclerosing hyaline necrosis (obliteration of terminal hepatic venules, hepatocyte necrosis and MDBs) is exclusive to severe alcoholic hepatitis[112,113].

REFERENCES

1 **Wree A**, Broderick L, Canbay A, Hoffman HM, Feldstein AE. From NAFLD to NASH to cirrhosis-new insights into disease mechanisms. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 627-636 [PMID: 23958599 DOI: 10.1038/nrgastro.2013.149]
2 **Loomba R**, Sanyal AJ. The global NAFLD epidemic. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 686-690 [PMID: 24042449 DOI: 10.1038/nrgastro.2013.171]
3 **Rafiq N**, Bai C, Fang Y, Srishord M, McCullough A, Gramlich T, Younossi ZM. Long-term follow-up of patients with nonalcoholic fatty liver. *Clin Gastroenterol Hepatol* 2009; **7**: 234-238 [PMID: 19049831 DOI: 10.1016/j.cgh.2008.11.005]
4 **Koehler EM**, Schouten JN, Hansen BE, van Rooij FJ, Hofman A, Stricker BH, Janssen HL. Prevalence and risk factors of non-alcoholic fatty liver disease in the elderly: results from the Rotterdam study. *J Hepatol* 2012; **57**: 1305-1311 [PMID: 22871499]
5 **Zois CD**, Baltayiannis GH, Bekiari A, Goussia A, Karayiannis P, Doukas M, Demopoulos D, Mitsellou A, Vougiouklakis T, Mitsi V, Tsianos EV. Steatosis and steatohepatitis in postmortem material from Northwestern Greece. *World J Gastroenterol* 2010; **16**: 3944-3949 [PMID: 20712056 DOI: 10.3748/wjg.v16.i31.3944]
6 **Weston SR**, Leyden W, Murphy R, Bass NM, Bell BP, Manos MM, Terrault NA. Racial and ethnic distribution of nonalcoholic fatty liver in persons with newly diagnosed chronic liver disease. *Hepatology* 2005; **41**: 372-379 [PMID: 15723436]
7 **Browning JD**, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004; **40**: 1387-1395 [PMID: 15565570]
8 **Anstee QM**, Targher G, Day CP. Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 330-344 [PMID: 23507799 DOI: 10.1038/nrgastro.2013.41]
9 **Charlton MR**, Burns JM, Pedersen RA, Watt KD, Heimbach JK, Dierkhising RA. Frequency and outcomes of liver transplantation for nonalcoholic steatohepatitis in the United States. *Gastroenterology* 2011; **141**: 1249-1253 [PMID: 21726509 DOI: 10.1053/j.gastro.2011.06.061]
10 **Michelotti GA**, Machado MV, Diehl AM. NAFLD, NASH and liver cancer. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 656-665 [PMID: 24080776 DOI: 10.1038/nrgastro.2013.183]
11 **Baffy G**, Brunt EM, Caldwell SH. Hepatocellular carcinoma in non-alcoholic fatty liver disease: an emerging menace. *J*

Hepatol 2012; **56**: 1384-1391 [PMID: 22326465]
12 **Kleiner DE**, Brunt EM. Nonalcoholic fatty liver disease: pathologic patterns and biopsy evaluation in clinical research. *Semin Liver Dis* 2012; **32**: 3-13 [PMID: 22418883 DOI: 10.1055/s-0032-1306421]
13 **Matteoni CA**, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999; **116**: 1413-1419 [PMID: 10348825]
14 **Thaler H**. Relation of steatosis to cirrhosis. *Clin Gastroenterol* 1975; **4**: 273-280 [PMID: 1126047]
15 **Miller DJ**, Ishimaru H, Klatskin G. Non-Alcoholic liver disease mimicking alcoholic hepatitis. *Gastroenterology* 1979; **77**: A27
16 **Adler M**, Schaffner F. Fatty liver hepatitis and cirrhosis in obese patients. *Am J Med* 1979; **67**: 811-816 [PMID: 507094]
17 **Ludwig J**, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980; **55**: 434-438 [PMID: 7382552]
18 **Williams CD**, Stengel J, Asike MI, Torres DM, Shaw J, Contreras M, Landt CL, Harrison SA. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. *Gastroenterology* 2011; **140**: 124-131 [PMID: 20858492 DOI: 10.1053/j.gastro.2010.09.038]
19 **Richardson MM**, Jonsson JR, Powell EE, Brunt EM, Neuschwander-Tetri BA, Bhathal PS, Dixon JB, Weltman MD, Tilg H, Moschen AR, Purdie DM, Demetris AJ, Clouston AD. Progressive fibrosis in nonalcoholic steatohepatitis: association with altered regeneration and a ductular reaction. *Gastroenterology* 2007; **133**: 80-90 [PMID: 17631134]
20 **Carpino G**, Renzi A, Onori P, Gaudio E. Role of hepatic progenitor cells in nonalcoholic fatty liver disease development: cellular cross-talks and molecular networks. *Int J Mol Sci* 2013; **14**: 20112-20130 [PMID: 24113587 DOI: 10.3390/ijms141020112]
21 **Nobili V**, Carpino G, Alisi A, Franchitto A, Alpini G, De Vito R, Onori P, Alvaro D, Gaudio E. Hepatic progenitor cells activation, fibrosis, and adipokines production in pediatric nonalcoholic fatty liver disease. *Hepatology* 2012; **56**: 2142-2153 [PMID: 22467277 DOI: 10.1002/hep.25742]
22 **Radwan MM**, Radwan BM, Nandipati KC, Hunter WJ, Agrawal DK. Immunological and molecular basis of nonalcoholic steatohepatitis and nonalcoholic fatty liver disease. *Expert Rev Clin Immunol* 2013; **9**: 727-738 [PMID: 23971751 DOI: 10.1586/1744666X.2013.816484]
23 **Jou J**, Choi SS, Diehl AM. Mechanisms of disease progression in nonalcoholic fatty liver disease. *Semin Liver Dis* 2008; **28**: 370-379 [PMID: 18956293 DOI: 10.1055/s-0028-1091981]
24 **Tian Y**, Wong VW, Chan HL, Cheng AS. Epigenetic regulation of hepatocellular carcinoma in non-alcoholic fatty liver disease. *Semin Cancer Biol* 2013; **23**: 471-482 [PMID: 24018165 DOI: 10.1016/j.semcancer.2013.08.010]
25 **Anstee QM**, Daly AK, Day CP. Genetics of alcoholic and nonalcoholic fatty liver disease. *Semin Liver Dis* 2011; **31**: 128-146 [PMID: 21538280]
26 **Sookoian S**, Pirola CJ. The genetic epidemiology of nonalcoholic fatty liver disease: toward a personalized medicine. *Clin Liver Dis* 2012; **16**: 467-485 [PMID: 22824476 DOI: 10.1016/j.cld.2012.05.011]
27 **Chalasani N**, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, Charlton M, Sanyal AJ. The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. *Gastroenterology* 2012; **142**: 1592-1609 [PMID: 22656328 DOI: 10.1053/j.gastro.2012.04.001]
28 **Ratziu V**, Bellentani S, Cortez-Pinto H, Day C, Marchesini G. A position statement on NAFLD/NASH based on the EASL 2009 special conference. *J Hepatol* 2010; **53**: 372-384 [PMID: 20494470 DOI: 10.1016/j.jhep.2010.04.008]
29 **Brunt EM**. Nonalcoholic fatty liver disease: what the pathologist can tell the clinician. *Dig Dis* 2012; **30** Suppl 1: 61-68 [PMID: 23075870]
30 **Brunt EM**, Neuschwander-Tetri BA, Burt A. Fatty liver disease: alcoholic and non-alcoholic. In: Burt A, Portmann B, Ferrell L, editors. MacSween's Pathology of the Liver. 6th ed. Amsterdam: Elsevier, 2012: 1020
31 **Grandison GA**, Angulo P. Can NASH be diagnosed, graded, and staged noninvasively? *Clin Liver Dis* 2012; **16**: 567-585 [PMID: 22824481 DOI: 10.1016/j.cld.2012.05.001]
32 **Torres DM**, Harrison SA. Noninvasive methods of assessing nonalcoholic fatty liver disease: what the clinician needs to know. *Clin Gastroenterol Hepatol* 2013; **11**: 1205-1207 [PMID: 23811242 DOI: 10.1016/j.cgh.2013.05.040]
33 **Neuschwander-Tetri BA**, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. *Hepatology* 2003; **37**: 1202-1219 [PMID: 12717402]
34 **Skelly MM**, James PD, Ryder SD. Findings on liver biopsy to investigate abnormal liver function tests in the absence of diagnostic serology. *J Hepatol* 2001; **35**: 195-199 [PMID: 11580141]
35 **Gaidos JK**, Hillner BE, Sanyal AJ. A decision analysis study of the value of a liver biopsy in nonalcoholic steatohepatitis. *Liver Int* 2008; **28**: 650-658 [PMID: 18331241 DOI: 10.1111/j.1478-3231.2008.01693.x]
36 **Brunt EM**, Ramrakhiani S, Cordes BG, Neuschwander-Tetri BA, Janney CG, Bacon BR, Di Bisceglie AM. Concurrence of histologic features of steatohepatitis with other forms of chronic liver disease. *Mod Pathol* 2003; **16**: 49-56 [PMID: 12527713]
37 **Ong JP**, Younossi ZM, Speer C, Olano A, Gramlich T, Boparai N. Chronic hepatitis C and superimposed nonalcoholic fatty liver disease. *Liver* 2001; **21**: 266-271 [PMID: 11454190]
38 **Sanyal AJ**, Contos MJ, Sterling RK, Luketic VA, Shiffman ML, Stravitz RT, Mills AS. Nonalcoholic fatty liver disease in patients with hepatitis C is associated with features of the metabolic syndrome. *Am J Gastroenterol* 2003; **98**: 2064-2071 [PMID: 14499789]
39 **Farrell GC**, Liddle C. Drugs and the liver updated, 2002. *Semin Liver Dis* 2002; **22**: 109-113 [PMID: 12064318]
40 **Cotrim HP**, Andrade ZA, Parana R, Portugal M, Lyra LG, Freitas LA. Nonalcoholic steatohepatitis: a toxic liver disease in industrial workers. *Liver* 1999; **19**: 299-304 [PMID: 10459628]
41 **Cotrim HP**, Carvalho F, Siqueira AC, Lordelo M, Rocha R, De Freitas LA. Nonalcoholic fatty liver and insulin resistance among petrochemical workers. *JAMA* 2005; **294**: 1618-1620 [PMID: 16204660]
42 **Sorrentino P**, Tarantino G, Conca P, Perrella A, Terracciano ML, Vecchione R, Gargiulo G, Gennarelli N, Lobello R. Silent non-alcoholic fatty liver disease-a clinical-histological study. *J Hepatol* 2004; **41**: 751-757 [PMID: 15519647]
43 **Fracanzani AL**, Valenti L, Bugianesi E, Andreoletti M, Colli A, Vanni E, Bertelli C, Fatta E, Bignamini D, Marchesini G, Fargion S. Risk of severe liver disease in nonalcoholic fatty liver disease with normal aminotransferase levels: a role for insulin resistance and diabetes. *Hepatology* 2008; **48**: 792-798 [PMID: 18752331 DOI: 10.1002/hep.22429]
44 **Mofrad P**, Contos MJ, Haque M, Sargeant C, Fisher RA, Luketic VA, Sterling RK, Shiffman ML, Stravitz RT, Sanyal AJ. Clinical and histologic spectrum of nonalcoholic fatty liver disease associated with normal ALT values. *Hepatology* 2003; **37**: 1286-1292 [PMID: 12774006]
45 **A-Kader HH**, Henderson J, Vanhoesen K, Ghishan F, Bhattacharyya A. Nonalcoholic fatty liver disease in children: a single center experience. *Clin Gastroenterol Hepatol* 2008; **6**:

799-802 [PMID: 18486560 DOI: 10.1016/j.cgh.2008.03.001]
46 **Schwimmer JB**, Deutsch R, Kahen T, Lavine JE, Stanley C, Behling C. Prevalence of fatty liver in children and adolescents. *Pediatrics* 2006; **118**: 1388-1393 [PMID: 17015527]
47 **Schwimmer JB**, Pardee PE, Lavine JE, Blumkin AK, Cook S. Cardiovascular risk factors and the metabolic syndrome in pediatric nonalcoholic fatty liver disease. *Circulation* 2008; **118**: 277-283 [PMID: 18591439 DOI: 10.1161/CIRCULATIONAHA.107.739920]
48 **Abdelmalek M**, Ludwig J, Lindor KD. Two cases from the spectrum of nonalcoholic steatohepatitis. *J Clin Gastroenterol* 1995; **20**: 127-130 [PMID: 7769192]
49 **Caldwell SH**, Lee VD, Kleiner DE, Al-Osaimi AM, Argo CK, Northup PG, Berg CL. NASH and cryptogenic cirrhosis: a histological analysis. *Ann Hepatol* 2009; **8**: 346-352 [PMID: 20009134]
50 **Struben VM**, Hespenheide EE, Caldwell SH. Nonalcoholic steatohepatitis and cryptogenic cirrhosis within kindreds. *Am J Med* 2000; **108**: 9-13 [PMID: 11059435]
51 **Angulo P**. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231 [PMID: 11961152]
52 **Neuschwander-Tetri BA**, Brunt EM, Wehmeier KR, Oliver D, Bacon BR. Improved nonalcoholic steatohepatitis after 48 weeks of treatment with the PPAR-gamma ligand rosiglitazone. *Hepatology* 2003; **38**: 1008-1017 [PMID: 14512888]
53 **Lindor KD**, Kowdley KV, Heathcote EJ, Harrison ME, Jorgensen R, Angulo P, Lymp JF, Burgart L, Colin P. Ursodeoxycholic acid for treatment of nonalcoholic steatohepatitis: results of a randomized trial. *Hepatology* 2004; **39**: 770-778 [PMID: 14999696]
54 **Adams LA**, Sanderson S, Lindor KD, Angulo P. The histological course of nonalcoholic fatty liver disease: a longitudinal study of 103 patients with sequential liver biopsies. *J Hepatol* 2005; **42**: 132-138 [PMID: 15629518]
55 **Sanyal AJ**, Brunt EM, Kleiner DE, Kowdley KV, Chalasani N, Lavine JE, Ratziu V, McCullough A. Endpoints and clinical trial design for nonalcoholic steatohepatitis. *Hepatology* 2011; **54**: 344-353 [PMID: 21520200 DOI: 10.1002/hep.24376]
56 **Brunt EM**, Kleiner DE, Wilson LA, Belt P, Neuschwander-Tetri BA. Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings. *Hepatology* 2011; **53**: 810-820 [PMID: 21319198 DOI: 10.1002/hep.24127]
57 **Piccinino F**, Sagnelli E, Pasquale G, Giusti G. Complications following percutaneous liver biopsy. A multicentre retrospective study on 68,276 biopsies. *J Hepatol* 1986; **2**: 165-173 [PMID: 3958472]
58 **Rousselet MC**, Michalak S, Dupré F, Croué A, Bedossa P, Saint-André JP, Calès P. Sources of variability in histological scoring of chronic viral hepatitis. *Hepatology* 2005; **41**: 257-264 [PMID: 15660389]
59 **Janiec DJ**, Jacobson ER, Freeth A, Spaulding L, Blaszyk H. Histologic variation of grade and stage of non-alcoholic fatty liver disease in liver biopsies. *Obes Surg* 2005; **15**: 497-501 [PMID: 15946428]
60 **Ratziu V**, Charlotte F, Heurtier A, Gombert S, Giral P, Bruckert E, Grimaldi A, Capron F, Poynard T. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. *Gastroenterology* 2005; **128**: 1898-1906 [PMID: 15940625 DOI: 10.1053/j.gastro.2005.03.084]
61 **Goldstein NS**, Hastah F, Galan MV, Gordon SC. Fibrosis heterogeneity in nonalcoholic steatohepatitis and hepatitis C virus needle core biopsy specimens. *Am J Clin Pathol* 2005; **123**: 382-387 [PMID: 15716234]
62 **Larson SP**, Bowers SP, Palekar NA, Ward JA, Pulcini JP, Harrison SA. Histopathologic variability between the right and left lobes of the liver in morbidly obese patients undergoing Roux-en-Y bypass. *Clin Gastroenterol Hepatol* 2007; **5**: 1329-1332 [PMID: 17702661]
63 **Theise ND**, Bodenheimer HC, Ferrel L. Acute and chronic viral hepatitis. In: Burt AD, Portmann B, Ferrel L, editors. MacSween's pathology of the liver. 6th ed. Amsterdam: Elsevier, 2012: 361-402
64 **Merriman RB**, Ferrell LD, Patti MG, Weston SR, Pabst MS, Aouizerat BE, Bass NM. Correlation of paired liver biopsies in morbidly obese patients with suspected nonalcoholic fatty liver disease. *Hepatology* 2006; **44**: 874-880 [PMID: 17006934]
65 **Kleiner DE**, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313-1321 [PMID: 15915461]
66 **Fukusato T**, Fukushima J, Shiga J, Takahashi Y, Nakano T, Maeyama S, Masayuki U, Ohbu M, Matsumoto T, Matsumoto K, Hano H, Sakamoto M, Kondo F, Komatsu A, Ishikawa T, Ohtake H, Takikawa H, Yoshimura K. Interobserver variation in the histopathological assessment of nonalcoholic steatohepatitis. *Hepatol Res* 2005; **33**: 122-127 [PMID: 16890173]
67 **Younossi ZM**, Gramlich T, Liu YC, Matteoni C, Petrelli M, Goldblum J, Rybicki L, McCullough AJ. Nonalcoholic fatty liver disease: assessment of variability in pathologic interpretations. *Mod Pathol* 1998; **11**: 560-565 [PMID: 9647594]
68 **Aly FZ**, Kleiner DE. Update on fatty liver disease and steatohepatitis. *Adv Anat Pathol* 2011; **18**: 294-300 [PMID: 21654360]
69 **Tandra S**, Yeh MM, Brunt EM, Vuppalanchi R, Cummings OW, Unalp-Arida A, Wilson LA, Chalasani N. Presence and significance of microvesicular steatosis in nonalcoholic fatty liver disease. *J Hepatol* 2011; **55**: 654-659 [PMID: 21172393 DOI: 10.1016/j.jhep.2010.11.021]
70 **Brunt EM**. Nonalcoholic steatohepatitis. *Semin Liver Dis* 2004; **24**: 3-20 [PMID: 15085483]
71 **Caldwell S**, Ikura Y, Dias D, Isomoto K, Yabu A, Moskaluk C, Pramoonjago P, Simmons W, Scruggs H, Rosenbaum N, Wilkinson T, Toms P, Argo CK, Al-Osaimi AM, Redick JA. Hepatocellular ballooning in NASH. *J Hepatol* 2010; **53**: 719-723 [PMID: 20624660 DOI: 10.1016/j.jhep.2010.04.031]
72 **Ku NO**, Strnad P, Zhong BH, Tao GZ, Omary MB. Keratins let liver live: Mutations predispose to liver disease and crosslinking generates Mallory-Denk bodies. *Hepatology* 2007; **46**: 1639-1649 [PMID: 17969036]
73 **Lackner C**, Gogg-Kamerer M, Zatloukal K, Stumptner C, Brunt EM, Denk H. Ballooned hepatocytes in steatohepatitis: the value of keratin immunohistochemistry for diagnosis. *J Hepatol* 2008; **48**: 821-828 [PMID: 18329127]
74 **Stumptner C**, Fuchsbichler A, Heid H, Zatloukal K, Denk H. Mallory body--a disease-associated type of sequestosome. *Hepatology* 2002; **35**: 1053-1062 [PMID: 11981755]
75 **Ludwig J**, McGill DB, Lindor KD. Review: nonalcoholic steatohepatitis. *J Gastroenterol Hepatol* 1997; **12**: 398-403 [PMID: 9195388]
76 **Guy CD**, Suzuki A, Burchette JL, Brunt EM, Abdelmalek MF, Cardona D, McCall SJ, Ünalp A, Belt P, Ferrell LD, Diehl AM. Costaining for keratins 8/18 plus ubiquitin improves detection of hepatocyte injury in nonalcoholic fatty liver disease. *Hum Pathol* 2012; **43**: 790-800 [PMID: 22036053 DOI: 10.1016/j.humpath.2011.07.007]
77 **Law K**, Brunt EM. Nonalcoholic fatty liver disease. *Clin Liver Dis* 2010; **14**: 591-604 [PMID: 21055684 DOI: 10.1016/j.cld.2010.07.006]
78 **Brunt EM**, Neuschwander-Tetri BA, Oliver D, Wehmeier KR, Bacon BR. Nonalcoholic steatohepatitis: histologic features and clinical correlations with 30 blinded biopsy specimens. *Hum Pathol* 2004; **35**: 1070-1082 [PMID: 15343508]
79 **Patton HM**, Yates K, Unalp-Arida A, Behling CA, Huang

TT, Rosenthal P, Sanyal AJ, Schwimmer JB, Lavine JE. Association between metabolic syndrome and liver histology among children with nonalcoholic Fatty liver disease. *Am J Gastroenterol* 2010; **105**: 2093-2102 [PMID: 20372110 DOI: 10.1038/ajg.2010.152]

80 **Lefkowitch JH**, Haythe JH, Regent N. Kupffer cell aggregation and perivenular distribution in steatohepatitis. *Mod Pathol* 2002; **15**: 699-704 [PMID: 12118106]

81 **Vonghia L**, Michielsen P, Francque S. Immunological mechanisms in the pathophysiology of non-alcoholic steatohepatitis. *Int J Mol Sci* 2013; **14**: 19867-19890 [PMID: 24084730 DOI: 10.3390/ijms141019867]

82 **Maher JJ**, Leon P, Ryan JC. Beyond insulin resistance: Innate immunity in nonalcoholic steatohepatitis. *Hepatology* 2008; **48**: 670-678 [PMID: 18666225 DOI: 10.1002/hep.22399]

83 **Rivera CA**, Adegboyega P, van Rooijen N, Tagalicud A, Allman M, Wallace M. Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. *J Hepatol* 2007; **47**: 571-579 [PMID: 17644211]

84 **Gill RM**, Belt P, Wilson L, Bass NM, Ferrell LD. Centrizonal arteries and microvessels in nonalcoholic steatohepatitis. *Am J Surg Pathol* 2011; **35**: 1400-1404 [PMID: 21836480 DOI: 10.1097/PAS.0b013e3182254283]

85 **Brunt EM**. Nonalcoholic steatohepatitis: definition and pathology. *Semin Liver Dis* 2001; **21**: 3-16 [PMID: 11296695]

86 **Brunt EM**, Kleiner DE, Wilson LA, Unalp A, Behling CE, Lavine JE, Neuschwander-Tetri BA. Portal chronic inflammation in nonalcoholic fatty liver disease (NAFLD): a histologic marker of advanced NAFLD-Clinicopathologic correlations from the nonalcoholic steatohepatitis clinical research network. *Hepatology* 2009; **49**: 809-820 [PMID: 19142989 DOI: 10.1002/hep.22724]

87 **Schwimmer JB**, Behling C, Newbury R, Deutsch R, Nievergelt C, Schork NJ, Lavine JE. Histopathology of pediatric nonalcoholic fatty liver disease. *Hepatology* 2005; **42**: 641-649 [PMID: 16116629 DOI: 10.1002/hep.20842]

88 **Clouston AD**, Powell EE. Interaction of non-alcoholic fatty liver disease with other liver diseases. *Best Pract Res Clin Gastroenterol* 2002; **16**: 767-781 [PMID: 12406444]

89 **Bedossa P**, Moucari R, Chelbi E, Asselah T, Paradis V, Vidaud M, Cazals-Hatem D, Boyer N, Valla D, Marcellin P. Evidence for a role of nonalcoholic steatohepatitis in hepatitis C: a prospective study. *Hepatology* 2007; **46**: 380-387 [PMID: 17659580]

90 **Roskams TA**, Theise ND, Balabaud C, Bhagat G, Bhathal PS, Bioulac-Sage P, Brunt EM, Crawford JM, Crosby HA, Desmet V, Finegold MJ, Geller SA, Gouw AS, Hytiroglou P, Knisely AS, Kojiro M, Lefkowitch JH, Nakanuma Y, Olynyk JK, Park YN, Portmann B, Saxena R, Scheuer PJ, Strain AJ, Thung SN, Wanless IR, West AB. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. *Hepatology* 2004; **39**: 1739-1745 [PMID: 15185318]

91 **Roskams T**, Yang SQ, Koteish A, Durnez A, DeVos R, Huang X, Achten R, Verslype C, Diehl AM. Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease. *Am J Pathol* 2003; **163**: 1301-1311 [PMID: 14507639]

92 **Feldstein AE**, Canbay A, Angulo P, Taniai M, Burgart LJ, Lindor KD, Gores GJ. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. *Gastroenterology* 2003; **125**: 437-443 [PMID: 12891546]

93 **Wieckowska A**, Zein NN, Yerian LM, Lopez AR, McCullough AJ, Feldstein AE. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. *Hepatology* 2006; **44**: 27-33 [PMID: 16799979]

94 **Yeh MM**, Brunt EM. Pathology of nonalcoholic fatty liver disease. *Am J Clin Pathol* 2007; **128**: 837-847 [PMID: 17951208]

95 **Nelson JE**, Wilson L, Brunt EM, Yeh MM, Kleiner DE, Unalp-Arida A, Kowdley KV. Relationship between the pattern of hepatic iron deposition and histological severity in nonalcoholic fatty liver disease. *Hepatology* 2011; **53**: 448-457 [PMID: 21274866 DOI: 10.1002/hep.24038]

96 **Turlin B**, Mendler MH, Moirand R, Guyader D, Guillygomarc'h A, Deugnier Y. Histologic features of the liver in insulin resistance-associated iron overload. A study of 139 patients. *Am J Clin Pathol* 2001; **116**: 263-270 [PMID: 11488074]

97 **O'Brien J**, Powell LW. Non-alcoholic fatty liver disease: is iron relevant? *Hepatol Int* 2011; Epub ahead of print [PMID: 22020821]

98 **Kowdley KV**, Belt P, Wilson LA, Yeh MM, Neuschwander-Tetri BA, Chalasani N, Sanyal AJ, Nelson JE. Serum ferritin is an independent predictor of histologic severity and advanced fibrosis in patients with nonalcoholic fatty liver disease. *Hepatology* 2012; **55**: 77-85 [PMID: 21953442 DOI: 10.1002/hep.24706]

99 **Nelson JE**, Brunt EM, Kowdley KV. Lower serum hepcidin and greater parenchymal iron in nonalcoholic fatty liver disease patients with C282Y HFE mutations. *Hepatology* 2012; **56**: 1730-1740 [PMID: 22611049 DOI: 10.1002/hep.25856]

100 **Farrell GC**, Larter CZ. Nonalcoholic fatty liver disease: from steatosis to cirrhosis. *Hepatology* 2006; **43**: S99-S112 [PMID: 16447287 DOI: 10.1002/hep.20973]

101 **Brunt EM**, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999; **94**: 2467-2474 [PMID: 10484010]

102 **Brunt EM**. Grading and staging the histopathological lesions of chronic hepatitis: the Knodell histology activity index and beyond. *Hepatology* 2000; **31**: 241-246 [PMID: 10613753]

103 **Alkhouri N**, De Vito R, Alisi A, Yerian L, Lopez R, Feldstein AE, Nobili V. Development and validation of a new histological score for pediatric non-alcoholic fatty liver disease. *J Hepatol* 2012; **57**: 1312-1318 [PMID: 22871498 DOI: 10.1016/j.jhep.2012.07.027]

104 **Bedossa P**, Poitou C, Veyrie N, Bouillot JL, Basdevant A, Paradis V, Tordjman J, Clement K. Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients. *Hepatology* 2012; **56**: 1751-1759 [PMID: 22707395 DOI: 10.1002/hep.25889]

105 **Baldridge AD**, Perez-Atayde AR, Graeme-Cook F, Higgins L, Lavine JE. Idiopathic steatohepatitis in childhood: a multicenter retrospective study. *J Pediatr* 1995; **127**: 700-704 [PMID: 7472819]

106 **Rashid M**, Roberts EA. Nonalcoholic steatohepatitis in children. *J Pediatr Gastroenterol Nutr* 2000; **30**: 48-53 [PMID: 10630439]

107 **Molleston JP**, White F, Teckman J, Fitzgerald JF. Obese children with steatohepatitis can develop cirrhosis in childhood. *Am J Gastroenterol* 2002; **97**: 2460-2462 [PMID: 12358273]

108 **Carter-Kent C**, Yerian LM, Brunt EM, Angulo P, Kohli R, Ling SC, Xanthakos SA, Whitington PF, Charatcharoenwitthaya P, Yap J, Lopez R, McCullough AJ, Feldstein AE. Nonalcoholic steatohepatitis in children: a multicenter clinicopathological study. *Hepatology* 2009; **50**: 1113-1120 [PMID: 19637190 DOI: 10.1002/hep.23133]

109 **Nobili V**, Marcellini M, Devito R, Ciampalini P, Piemonte F, Comparcola D, Sartorelli MR, Angulo P. NAFLD in children: a prospective clinical-pathological study and effect of lifestyle advice. *Hepatology* 2006; **44**: 458-465 [PMID: 16871574 DOI: 10.1002/hep.21262]

110 **Patton HM**, Lavine JE, Van Natta ML, Schwimmer JB, Kleiner D, Molleston J. Clinical correlates of histopathology in pediatric nonalcoholic steatohepatitis. *Gastroenterology* 2008; **135**: 1961-1971.e2 [PMID: 19013463 DOI: 10.1053/j.gastro.2008.08.050]

111 **Suzuki A**, Abdelmalek MF, Schwimmer JB, Lavine JE, Schei-

mann AO, Unalp-Arida A, Yates KP, Sanyal AJ, Guy CD, Diehl AM. Association between puberty and features of nonalcoholic fatty liver disease. *Clin Gastroenterol Hepatol* 2012; **10**: 786-794 [PMID: 22343513 DOI: 10.1016/j.cgh.2012.01.020]

112 **Brunt EM**. Alcoholic and nonalcoholic steatohepatitis. *Clin Liver Dis* 2002; **6**: 399-420, vii [PMID: 12122863]

113 **Yip WW**, Burt AD. Alcoholic liver disease. *Semin Diagn Pathol* 2006; **23**: 149-160 [PMID: 17355088]

P- Reviewers: Penkova-Radicheva MP, Sanal MG, Streba LAM
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI:10.3748/wjg.v20.i27.9038

World J Gastroenterol 2014 July 21; 20(27): 9038-9049
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Translational approaches: From fatty liver to non-alcoholic steatohepatitis

Natalia Rosso, Norberto C Chavez-Tapia, Claudio Tiribelli, Stefano Bellentani

Natalia Rosso, Claudio Tiribelli, Stefano Bellentani, Norberto C Chavez-Tapia, Fondazione Italiana Fegato, 34149 Trieste, Italy
Norberto C Chavez-Tapia, Obesity and Digestive Diseases Unit, Médica Sur Clinic and Foundation, Mexico 14050, México
Claudio Tiribelli, Department of Medical Sciences, University of Trieste, 34149 Trieste, Italy
Stefano Bellentani, Department of Gastroenterology and Endoscopy, Azienda USL di Modena, Ospedale "Ramazzini", 41012 Carpi (Modena), Italy
Author contributions: Rosso N planned the research of the most relevant papers, reviewed the contents and wrote this work; Chavez-Tapia NC contributed to the research and the analysis of the available *in vitro* models; Bellentani S contributed to the study of the epidemiological data and revised the final version of the paper; Tiribelli C and Bellentani S contributed with the correction of the work and participated in the analysis of the contents of the present study; Tiribelli C edited the text.
Supported by European Union Seventh Framework Program (FP7/2007-2013) under grant agreement, No. Health-F2-2009-241762, for the project FLIP; Italian National Grant MIUR (Art.13 D.LGS 297/99 - Progetto Nutrizione e Salute); and an in house grant from Fondazione Italiana Fegato, ONLUS
Correspondence to: Stefano Bellentani, MD, PhD, Department of Gastroenterology and Endoscopy, Azienda USL di Modena, Ospedale "Ramazzini", Via Guido Molinari, 41012 Carpi (Modena), Italy. bellentanistefano@gmail.com
Telephone: +39-59-371102 Fax: +39-40-3757832
Received: October 21, 2013 Revised: March 3, 2014
Accepted: April 21, 2014
Published online: July 21, 2014

Abstract

Over the past few decades, non-alcoholic fatty liver disease (NAFLD) has become one, if not the most common, cause of chronic liver disease affecting both adults and children. The increasing number of cases at an early age is the most worrying aspect of this pathology, since it provides more time for its evolution. The spectrum of this disease ranges from liver steatosis to steatohepatitis, fibrosis and in some cases, hepatocellular carcinoma. NAFLD may not always be considered a benign disease and hepatologists must be cautious in the presence of fatty liver. This should prompt the use of the available experimental models to understand better the pathogenesis and to develop a rational treatment of a disease that is dangerously increasing. In spite of the growing efforts, the pathogenesis of NAFLD is still poorly understood. In the present article we review the most relevant hypotheses and evidence that account for the progression of NAFLD to non-alcoholic steatohepatitis (NASH) and fibrosis. The available *in vitro* and *in vivo* experimental models of NASH are discussed and revised in terms of their validity in translational studies. These studies must be aimed at the discovery of the still unknown triggers or mediators that induce the progression of hepatic inflammation, apoptosis and fibrosis.

Key words: Fatty Liver; Obesity; Metabolic syndrome; Inflammation; *In vitro*; Experimental model

Core tip: The molecular mechanism associated with the accumulation of fatty acids in the liver cells and the resulting molecular cascade leading to hepatic damage is far from being understood. Due to the development of reliable *in vitro* and *in vivo* models, we are starting to open the "black box". This will lead to a better understanding of the active clinical condition and hopefully to a more effective treatment. This article critically reviews what is known and what has still to be discovered about the link between the accumulation of fat within the liver and the resulting damage.

Rosso N, Chavez-Tapia NC, Tiribelli C, Bellentani S. Translational approaches: From fatty liver to non-alcoholic steatohepatitis. *World J Gastroenterol* 2014; 20(27): 9038-9049 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9038.htm

DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9038

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a complex spectrum of diseases ranging from benign steatosis (usually asymptomatic) to more severe alterations like non-alcoholic steatohepatitis (NASH), cirrhosis and, in some cases, hepatocellular carcinoma (HCC). The most serious aspect of the disease is the high incidence in pediatric and adolescent populations, providing longer time for evolution[1]. Day by day, social and medical operators witness the dramatic increase in the incidence of this phenomenon. The "global society" is driving us towards a global epidemic of obesity, type 2 diabetes mellitus (T2DM) and metabolic syndrome (MS). NAFLD and NASH are strictly linked to the presence of insulin resistance (IR) and are nowadays considered the hepatic manifestation of the MS[2]. Although most typical forms of NAFLD are overwhelmingly associated with IR and MS, it cannot be said that IR and MS are invariably associated with fatty liver[3]. Interestingly, NAFLD markers have also been associated with IR in type 1 diabetes[4], which is not closely related to the MS. Hepatology and Gastroenterology communities are facing a great challenge since within few years, NAFLD will be the most important chronic liver disease worldwide (Figure 1).

Lifestyle changes have occurred in the industrialized societies due to the introduction of modern technologies resulting in eating more and more importantly, moving less. According to the Food and Agriculture Organization of the United Nations (FAO http://www.fao.org/docrep/x0262e/x0262e23.htm), in the next 40 years the daily caloric requirements will decrease by 350 calories. Several epidemiological studies have linked NAFLD to unhealthy diet and sedentary behaviors[5-7], and the only effective treatment for NAFLD and NASH is to guide the patient to a healthier lifestyle[8] with lifestyle coaching including personalized diet, physical activity and cognitive-behaviour therapy[9]. However, the lack of patient compliance is the main limitation of this approach. Although to a lesser extent, NAFLD can also occur in non-obese populations[10], suggesting that dietary composition is not the only cause of fatty liver. Several sets of data reviewed by Caldwell *et al*[3] showed that both ethnicity and genetic polymorphisms play a major role in the development and progression of the disease, and different genetic profiles might be also responsible for the variations of steatosis in the MS.

It is therefore of pivotal importance to further develop a strong translational approach to understand the pathophysiology of this new disease and to translate it into clinical practice. In the present paper, we review the most recently published data on the pathophysiology of NAFLD in an attempt to amalgamate the available information in order to contribute to the understanding of the factors involved, including a critical analysis of the *in vitro* and *in vivo* models.

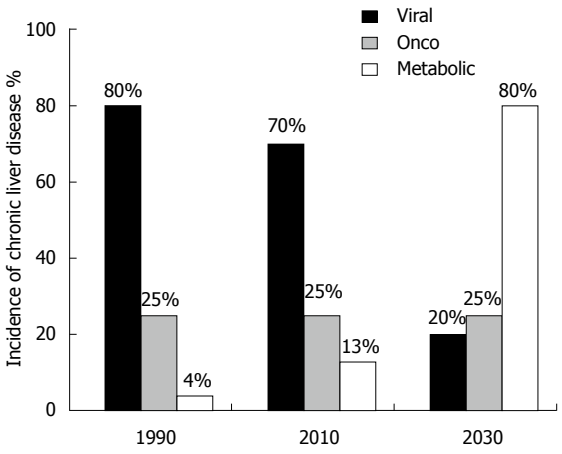


Figure 1 Estimation of the main etiological incidence of past, present and future chronic liver diseases according to the available data from the United States[130] and Europe[131,132].

PATHOGENESIS

The most accepted scheme to explain the development of NAFLD and the progression from simple steatosis to NASH is still based on theories. In 1998, Day[11] proposed the "two hits" theory. The "first hit" is characterized by the accumulation of lipids in hepatocytes due to an altered intrahepatic lipid metabolism, where insulin resistance seems to be the key pathogenic factor for the development of hepatic steatosis[12], while the "second hit" leads to hepatocyte injury, inflammation and fibrosis. Several factors were suggested to initiate the second hit such as: (1) proinflammatory cytokines and adipokines[13]; (2) mitochondrial dysfunction[13,14]; (3) oxidative stress; and (4) endoplasmic reticulum (ER) stress[15] with subsequent apoptosis. In 2010, a more complex, global and realistic model, the "multiparallel hits" hypothesis, was proposed to explain the pathogenesis of NAFLD[16]. In this model, the adipose tissue and gut-related factors play a key role in the initiation of hepatic inflammation, suggesting that simple steatosis and NASH might be two different disorders and pointing to new, non-hepatic players in the mechanisms of NAFLD and its progression.

Contributors to the development of insulin resistance

During the last few years, the interplay among gut microbiota, obesity and the metabolic consequences (liver sensitization) has become important. Some of the main factors involved in this process are summarized in Figure 2. Several clinical and experimental studies recently reviewed in detail[17] suggest that microbiotal factors may be the driving forces of IR[18], hepatic steatosis and subsequent inflammatory state. Changes in the composition of the gut microbiota might induce an increased permeability and translocation of bacterial endotoxins promoting a chronic inflammatory state. This condition can alter pathways such as insulin signaling, promoting the devel-

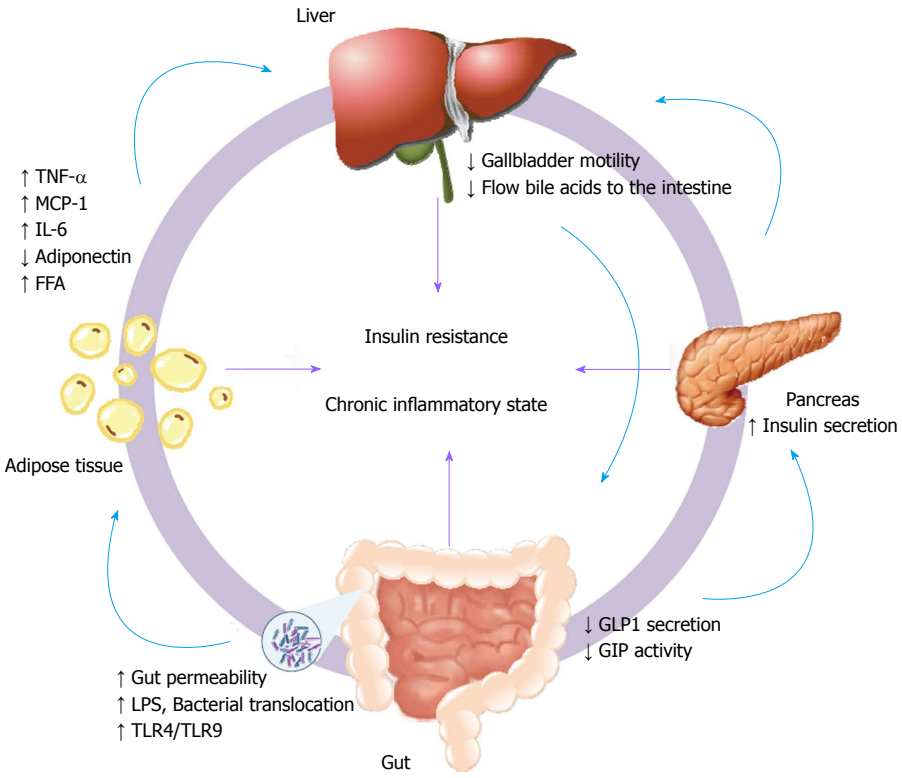


Figure 2 Extrahepatic factors involved in the pathogenesis of non-alcoholic fatty liver disease. The affected organs and their response are represented in a dynamic circle; in the center are indicated the main factors that contribute to the initiation/perpetuation of the hepatic injury (insulin resistance and chronic inflammatory state). The light blue arrows represent the organ-specific effects of each response. TNF-α: Tumor necrosis factor-α; MCP-1: Monocyte chemotactic protein 1; IL-6: Interleukin-6; FFA: Free fatty acids; LPS: Lipopolysaccharides; TLR: Toll-like receptor; GIP: Glucose-dependent insulinotropic peptide; GLP: Glucagon-like-peptide.

opment of IR. The molecular basis of IR is the result of multiple genetic[19] and non-genetic mechanisms. IR can initiate a dangerous vicious circle, involving inflammation and hypercoagulability, which increases atherogenesis[20]. Data from animal models indicate that IR develops in the vasculature well before these responses are detected in muscle, liver, or adipose tissue[21]. These findings could explain the high cardiovascular risk observed in subjects with MS. Moreover, disruption in the endothelial insulin signaling can promote the development of atherosclerosis in the absence of diabetes-related risk factors including hyperglycemia and hyperinsulinemia. The development of atherosclerosis is associated with a reduced bioavailability of nitric oxide and an excessive production of reactive oxygen species[22]. The endothelial dysfunction might be mediated by FoxOs transcription factors; FoxOs inhibition in endothelial cells has been shown to have promising atheroprotective effects[23]. Altogether these findings are in agreement with previous data[24], reinforcing the idea that hepatic IR and hepatic steatosis might precede the development of T2DM. Epidemiological evidence (reviewed in detail elsewhere[25]) also suggests an association between MS and the risk of developing chronic kidney disorders beyond the contribution of hyperglycemia and high blood pressure. The increased rates of chronic kidney disease (CKD) and cardiovascular disease are the most important clinical features associated with NAFLD. To date, there is a mounting body of evidence (reviewed extensively by Targher *et al*[26]) suggesting that patients with NAFLD have multiple risk factors of CKD and that NAFLD is associated with an increased prevalence and incidence of CKD both in patients with and without diabetes. Renal dysfunction may be promoted by a mosaic of effects such as: (1) inflammatory cytokines released by the adipose tissue (TNF-α (tumor necrosis factor-α), IL6, adiponectin[27], leptin[28]); (2) obesity-related mechanisms such as altered renal hemodynamics; (3) excess of renal sodium reabsorption; (4) activation of renin-angiotensin and sympathetic nervous systems; and (5) physical compression of kidneys by adipose tissue.

Over the last 14 years there has been a surge in the number of studies confirming that NAFLD is associated with IR (the "IR dogma"). Based on such studies, one could expect that, by correcting IR, NAFLD could be healed. Unfortunately, therapeutic studies[29] failed to confirm this expectation, suggesting a more complex interplay of factors involved in the pathogenic process.

Role of incretin hormones

Incretin hormones, such as glucose-dependent insulinotropic peptide (GIP) and glucagon-like-peptide 1 (GLP-1), are released by the gastrointestinal tract in response to nutrients that increase the glucose-mediated insulin secretion[31]. In patients with T2DM the incretin effect is severely reduced[32], due to an impaired secretion of GLP-1 and a decreased activity of GIP[33]. Recent *in*

vitro[34] and *in vivo*[35] data clearly show that hepatocytes express GLP-1 receptors, and the exposure to GLP-1 agonists leads to: (1) a reduction of intracellular fat load[36,37]; (2) enhanced fat oxidation[38]; and (3) an induction of macroautophagy[39], which is a critical process for the removal of toxic fatty acids from cells. Other important regulators of glucose homeostasis are the bile acids, which through various signaling pathways regulate cholesterol, fasting and mealtime glucose, and metabolism/energy homeostasis, as well as their own synthesis and blood levels in the enterohepatic circulation[40,41]. The composition of bile acids in T2DM has been shown to be altered[42] as a consequence of a reduced gallbladder motility resulting in a reduced secretion of bile acids to the intestine. A low bile acid concentration is associated with a reduction in the secretion of GLP-1 and consequently, an impaired glucose homeostasis with a decreased insulin secretion[43]. Paradoxically, patients with NAFLD can often present a hyperinsulinemic state. However, instead of a regulation of gluconeogenesis, insulin promotes *de novo* lipogenesis that exacerbates hepatic lipid deposition and accelerates the development of the disease. One possible mechanism to explain this situation could be the activation of sterol regulatory transcription factor element-binding protein-1c (SREBP1c), a master transcription factor regulator of lipid synthesis, through the stimulation of the target of rapamycin complex 1 (mTORC1)[44]. The regulation of incretin hormones represents a promising strategy for NAFLD. Therapy with GLP-1 agonists (like exenatide) in T2DM patients promotes a positive effect in the liver[45], since hepatocytes express GLP-1 receptor[34]. This compound might reduce or even reverse hepatic fat accumulation and reduce the triglyceride (TG) levels, most probably as a consequence of a reduced caloric intake, which is one of the main therapeutic contributions of this kind of drug[46]. Unfortunately, the success of bile acid interventions is limited in clinical practice and the results obtained are discordant from those observed in experimental models[47]. Hyperinsulinemia in NAFLD leads to upregulation of the production of insulin-like growth factor-1 (IGF-1) and activation of insulin receptor substrate (IRS)-1. This may activate several molecules and signaling pathways including p53, mitogen-activated protein kinases (MAPK), and phosphatidylinositol-3 kinase/Akt[48]. These pathways play a significant role in carcinogenesis by inducing cell proliferation and inhibition of cell apoptosis[49,50]. Thus, NAFLD and HCC appear to be regulated by similar signaling molecules and pathways related to inflammation. This evidence is particularly interesting to support the idea that NAFLD itself could promote HCC development in earlier stages, even in the absence of cirrhosis[51,52].

Alterations in hepatic lipid metabolism

TGs are the preferred nutritional storage to buffer fluctuations in energy demand and availability. TG physical properties allow their accumulation without adverse osmotic or colloidal effects. In higher organisms, TGs are stored mainly in adipocytes, and can be accumulated in other cell types only under particular circumstances. In this regard, an interesting example was presented by Cohen *et al*[53] in migratory birds that store large quantities of TGs in the liver as an energy source in preparation for prolonged seasonal flights. Like migratory birds, some humans who consume excess calories deposit fat in the liver, as a maladaptive process. The moiety of the intracellular fat has distinct toxic effects. As mentioned before, hepatic accumulation of neutral cholesterol esters and TG appears not to be a threat[54,55] (though this is still an open question[56]); however, the presence of the intermediate products seems to have a more deleterious effect on liver cells. An altered lipid metabolism leads to the accumulation of intermediate products such as diacylglycerol (DG) and phospholipids (sphingolipids and ceramides)[57-59], and these compounds account for the fatty acid-induced toxicity and for the hepatic IR (Figure 3). Moreover, these metabolites promote the activation of numerous kinases, including nPKC isoforms, MAPK, ERKs and c-Jun N-terminal kinase (JNK), S6K and inhibitor kappa beta kinase beta (IKKβ), that participate in the phosphorylation of the IRS inducing positive or negative effects on the insulin pathway[60]. Recent data suggest a connection between altered cholesterol homeostasis and hepatic free cholesterol (FC) accumulation as a trigger for the pathogenesis of NASH[61,62]. Most probably, FC accumulates within the ER membrane impairing its fluidity. The resulting stiffening of the ER membrane leads to an impaired activity triggering the ER stress and eventual unfolded protein response, cell apoptosis[63,64] *via* JNK signaling and to the release of RE Ca^{2+} stores. Adjacent mitochondria readily take up the released Ca^{2+}, and the acute Ca^{2+} overload results in changes in mitochondrial potential and opening of the permeability transition pores (PTPs)[65] ensuring a potent cellular cell signal[66]. Dysregulation in nuclear transcription factors SREBP-2[67], liver X-receptor (LXR)-α and farnesoid X receptor (FXR) might be the cause of cholesterol altered homoeostasis (extensively reviewed by Musso[68]). Interestingly, the incidence of NAFLD in the non-obese population has been associated with a high dietary cholesterol intake rather than intake of polyunsaturated fatty acids[69].

Chronic inflammatory state

Persistent IR associated with an excessive caloric diet and sedentary life style lead to obesity, now recognized as a chronic inflammatory disorder. Thus, inflammation is considered the major risk of obesity and is associated with white adipose tissue dysfunction. An altered adipokine profile has been suggested to play a pivotal role in the initiation and perpetuation of the pathological events[70,71]. In NAFLD, adipose tissue contributes to the systemic production of TNF-α[72], MCP1, IL6 and adiponectin; these mediators modify the hepatic inflammatory/immune system[56-59]. Furthermore, it has been reported that the adipose tissue of obese subjects presents an increased number of macrophages[73], and they

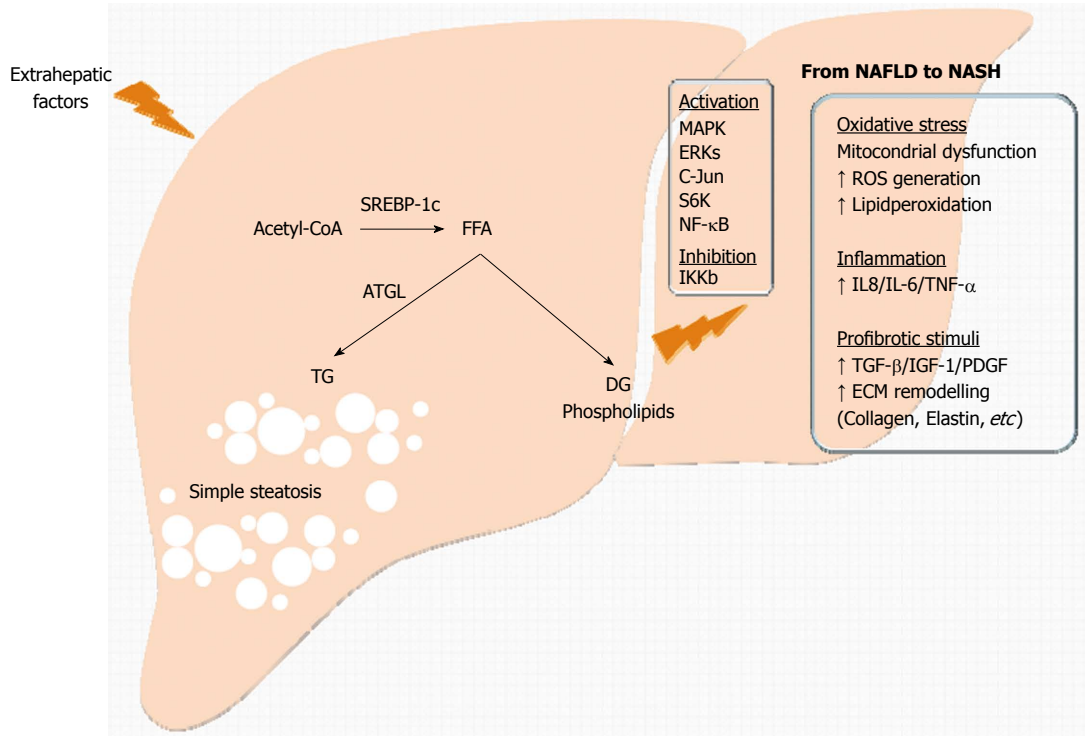


Figure 3 Effect of intracellular fat accumulation within the liver. Liver sensitization induces an alteration of the normal hepatic liver metabolism leading to simple steatosis with neutral triglyceride (TG) accumulation or in the more severe cases, to the production of intermediate products (DG and phospholipids) responsible for lipotoxicity. Alteration of several mediators of signaling pathways leads to the events observed during the progression from non-alcoholic fatty liver disease (NAFLD) to non-alcoholic steatohepatitis (NASH) (hepatic insulin resistance, oxidative stress, inflammation, and fibrosis). IKKb: Protein Kinase-1-mediated IB Kinase; ROS: Reactive oxygen species; IL-6: Interleukin-6; TNFα: Tumor necrosis factor-α; IGF-1: Insulin-like growth factor-1; PDGF: Platelet-derivedgrowth factor; ECM: Extracellular matrix; MAPK: Mitogen-activated protein kinases; ERKs: Extracellular signal-regulated kinases; NF-κB: Nuclear factor κB.

might account for much of the adipose tissue inflammatory cytokine secretion. These cells presumably arise from peripheral blood monocytes that become activated by hyperinsulinemia and the abnormal levels of FFA encountered in individuals with IR. Monocytes have also been shown to be activated in poorly controlled type 1 diabetes, showing an increased ability to attach to the endothelial cells[74], one of the early stages in atherosclerosis. Moreover, it has been reported that monocytes are strongly correlated with glycated hemoglobin (HbA1c), explaining the association between monocytes and IR in type 1 diabetes[75]. Activation of these cells produces abundant quantities of cytokines such as TNF-α and IL6. Studies performed in human monocytes suggest that these cells might respond to the increased concentrations of saturated non-esterified fatty acids observed in IR conditions by producing high levels of IL6. This increased secretion of IL6 could prime these cells to generate a robust local or systemic inflammatory response contributing to the development of complications such as T2DM and atherosclerosis[76]. In the liver, fatty acid accumulation induces mainly the up-regulation of IL8, produced both by hepatocytes and non parenchymal cells[77-79]. It was reported that IL6 and TNF-α signaling *via* TNF-α receptor-1 are important in NASH-related development of HCC, and that hypoadiponectinemia accelerated hepatic tumor formation in the mouse model of NASH[80,81]. A detailed study of the role of the main cytokines in humans and animal models can be found in a recently published work from Braunersreuther *et al*[82]. Collectively, these data confirm the close relationship between lipid metabolism and liver cancer in animal experimental models, although there are still many doubts regarding human studies.

Contribution of oxidative stress

Several papers demonstrate that oxidative stress occurs during NAFLD, especially due to mitochondrial dysfunction[14,83-86]. It has been reported that activated hepatic mitochondrial metabolism[87] is a common characteristic of NAFLD in both human subjects[88] and animal models[89]. However, the regulatory connection linking FFA to altered mitochondrial function is still undefined. Currently, there are two competing views on the role of lipid beta-oxidation in the development of NAFLD[88,89]. One view holds that impaired or incomplete beta-oxidation leads to hepatic steatosis and accumulation of lipid intermediates that inhibit insulin signaling. The other view holds that increased supply of FFA to the liver results in excessive beta-oxidation that fuels reactive oxygen species (ROS) accumulation and inflammation. The loss of electrons from complexes I and Ⅲ in the mitochondrial electron transport chain can combine with oxygen to generate ROS, powerful oxidizing agents that indiscriminately damage many important components of the cell including DNA, lipid membranes and proteins. ROS are

known to activate pro-apoptotic pathways and initiate programmed cell death. However, it has also been reported that ROS-related lipoapoptosis appears to be cell-type dependent[90,91]. Altogether, the role of specific FFA metabolic pathways in promoting ROS accumulation and damage remain largely unclear. The oxidative stress observed in NAFLD subjects might probably be a bystander consequence of a sensitized liver, rather than the main cause of the disease.

Progression of NAFLD to NASH

The progression from simple steatosis to NASH is determined by the initiation of the fibrotic response. Understanding the regulation of the initiation, progression and perpetuation of fibrosis will be very important, particularly from a therapeutic viewpoint. Hepatic stellate cells (HSC) are the main regulators of extracellular matrix (ECM) production and play an essential role in the development of fibrosis (extensively reviewed elsewhere[92,93]). Under normal conditions, HSC have a quiescent phenotype and constitute a third of the non-parenchymal cell population; 85% of hepatic vitamin A is dissolved and stored within quiescent HSC[94]. However, these cells can be activated by noxious stimuli triggered by damaged hepatocytes. When activated, HSC undergo several phenotypic and functional changes. A decrease in the retinoid content is accompanied by a strong increase in the production of extracellular components and cell proliferation. During the initial fibrogenic process, there is a cross-talk between injured hepatocytes and HSC, which is further stimulated in a paracrine mode by the infiltrated leukocytes and activated Kupffer cells (KC). The initial process is followed by the perpetuation of the fibrogenic response. The master regulator of this process is TGF-β[95], a pro-fibrotic cytokine released by almost all the involved cells, whose effect is cell-type dependent. For instance, in mature hepatocytes TGF-β is responsible for inhibition of cell proliferation and participates in the induction of apoptosis[96], while in HSC it promotes cell activation[97] and enhanced production of ECM (collagen, elastin, proteogycans, among others) associated with a decreased degradation by inhibition of the activity of matrix metalloproteinases.

From 1980 when Ludwig *et al*[98] first defined the condition, great efforts have been dedicated to elucidate the underlying mechanisms involved in this multifactorial and frequent disorder. In spite of data obtained in clinical settings, animal models and *in vitro* systems, the molecular causes of NASH remains mostly speculative, and further investigations are needed.

In vivo and in vitro experimental models

Due to ethical considerations, mechanistic studies are difficult (or impossible) to be conducted in humans. Consequently, the development of experimental models able to mimic the human condition becomes a necessary tool in the study of the pathophysiology and progression from NAFLD to NASH. Over the last two decades, several animal models have been established and proposed as preclinical platforms for the study of NASH development and the definition of therapeutic options. Table 1 summarizes the most used animal models and their characteristics. The main advantage of this approach is the possibility to define pathogenic pathways in a cause-effect response. The goals these models need to fulfill are: (1) that the pathological pattern of liver injury reflects human steatohepatitis; and (2) that the model should reproduce the context in which human NASH develops. The most used models are genetically modified animals (see for detailed reviews[99-102]) such as the *ob/ob* mouse with a mutation in the leptin gene[73] or the *db/db* mouse which lacks the leptin receptor. However, to develop fibrosis and consequent NASH, both models require a methionine and choline deficient (MCD) diet[54]. A controversy still exists about the validity of this diet, since MCD feeding in normal animals induces weight loss and insulin sensitivity[103] despite the impairment of hepatic receptor signaling[104]. Moreover, few human diets are deficient in methionine and choline. Another genetic model consists of animals with deletions in acyl-CoA oxidase (ACOX). Although at an initial stage these animals present severe steatosis and liver inflammatory infiltration with hepatocyte apoptosis, after 6-8 mo they become resistant to steatosis with (PPAR)-α dependent liver regeneration, limiting the utility of this model for the study of steatohepatitis. Deletions in methionine adenosyltransferase (MAT)-1A (MATO mice)[105] and liver-specific *pten* lead to the development of steatohepatitis but without MS. Sterol regulatory element binding protein (SREBP)-1c transgenic mice, which present an overexpression of this protein in adipose tissue, show IR secondary to impaired adipose differentiation leading to severe hepatic steatosis with the histological features of steatohepatitis[106]. Conversely, these transgenic mice exhibit decreased adipose mass limiting its application to NAFLD/NASH, where adipose tissue is the storage compartment that contributes to perturbations of whole-body lipid homeostasis. An alternative genetically modified animal model is the KK-A^y mouse in which there is a heterozygous mutation of the agouti gene (*KK-Ay/a*). Interestingly, these animals present impaired hypothalamic appetite suppression[107] and consequently, they are hyperphagic and develop an obese phenotype. They also present hepatic steatosis in conjunction with IR. However, the main limitation of this model is that NASH does not occur spontaneously, and a MCD diet is required for the induction.

The use of diet-induced models, extensively reviewed elsewhere[102], is another strategy in the study of NASH development. Different diets for small animals have been characterized[108-110] with good results in the development of steatosis and inflammation, but marginal results in generating fibrosis. Different effects depending on the composition of the diet have been reported. High-carbohydrate diets stimulate moderate hepatic lipogenesis in rats, whereas animals fed with high-fat diets present a strong inhibition of this anabolic pathway. The plasma TG levels

Table 1 Summary of the major findings obtained among the most widespread *in vivo* models

Model	Genetic manipulation	Diet modifications	Obesity	Metabolic syndrome (IR)	Hepatosteatosis	Steatohepatitis	Fibrosis
ob/*ob* mice	Leptin Deficient	No Yes MCD	Yes Variable (loss weight in some)	Yes Yes	Yes Yes	Yes (in males) Yes	No (protected) Yes
db/*db* mice	Mutation on leptin receptor	No Yes MCD	Yes Variable (age-related weight gain)	Yes No	Yes Yes	No Yes	No Yes
AOX null mice	Nullizygous for acycil - CoA oxidase	No	No	No	Yes [before 6-8 mo Resistant (after 8 mo)]	Yes (before 6-8 mo) Resistant (after 8 mo)	No
MATO null mice	Nullizygous for (MAT)-1A	No	No	No	Yes	Yes	Yes
pten null mice	Liver specific pten deletion	No	No	No	Yes	Yes	Yes
(SREBP)-1c transgenic mice	SREBP-1c overexpressed in adipose tissue	No	No	Yes	Yes	Yes	Yes
KK-Ay mice	Heterozygous mutation on agouti gene (KK-Ay/a)	No Yes MCD	Yes Yes	Yes Yes	Yes Yes	No Yes	No No
LIRKO mice	Liver-specific Leptin receptor KO	No	No	Hepatic IR	No	-	-
C57Bl/6J	No	Yes HFHC HF	Yes	Yes	Yes	Yes	Yes (mild)
Cholesterol-Cholate (Atherogenic diet)	No	Yes Cholesterol Cholate	No	No (only hepatic IR)	Yes (over 1-6 mo)	Yes (over 1-6 mo)	Yes (over 1-6 mo)

MCD: Methionine choline deficient diet; HFHC: High fat-high carbohydrate diet; HF: High fructose diet; KO: Knock-out; IR: Insulin resistance.

are higher in the high-carbohydrates diets, whereas the high-fat diet determines an accumulation of TG in the liver. However, both diets induce an increase of plasmatic levels of glucose and insulin[111]. Regarding the generation of fibrosis, promising evidence has emerged from mice fed with an atherogenic diet containing 1.25% cholesterol and 0.5% cholate[112]. Under these dietary conditions, a progressive formation of steatosis is observed associated with an evident inflammatory response, induction of oxidative stress and development of fibrosis in 6-24 wk. However, these animals are systematically insulin-sensitive, albeit they develop hepatic IR and surprisingly, they show a weight loss. This makes the cholesterol-cholate model substantially different from human NASH, severely limiting its application. A valid tool for the study of hepatic IR and the effect of insulin on leptin homeostasis is represented by LIRKO mice, a liver-specific insulin receptor knock-out[113]. These animals present abnormal glucose metabolism and progressive liver dysfunction, and display focal dysplasia and hyperplastic nodules. However, serum TG levels are decreased, most probably by the inability of insulin to promote TG synthesis in the liver and by reduced lipolysis in adipose tissue. In spite of the hyperinsulinemia and IR, these animals are not obese[114]. A promising approach is the administration of a high-fat diet associated with high fructose to male C57Bl/6J mice, which induces results similar to those observed in human NASH[115]. In spite of the promising results, substantial objections remain: (1) the long term exposure required for observing the pathological phenotype[116]; (2) the inclusion of only male animals excluding the application of this approach to the female population; and (3) rodents might adapt to high-fat feeding and become resistant to the development of obesity[117].

Worthy of attention is the fact that under specific experimental settings, animals can develop NASH from simple steatosis. However, the data fail to explain why in humans only some individuals develop NASH while others can live with NAFLD with no complications[118]. This crucial issue is still an open question, and most probably may be related to a different response of the cell to fat storage[119,120].

Contrary to other liver diseases in which *in vitro* models are important tools in research, convincing data are still missing in NAFLD and NASH. One of the reasons may be related to the use of a rather simplistic set-up to tackle the multistep process of the development of NASH. The use of an *in vitro* approach presents several advantages and disadvantages, as recently reviewed in detail[121]. A broad spectrum of *in vitro* validated possibilities is available, such as the use of primary cell culture, immortalized cell lines, or an even more sophisticated

system such as precision-cut slices of perfused liver. The main obstacle of the *in vitro* system is the extrapolation of the results to the much more complex human environment. A good example of this limitation is the choice of free fatty acids (FFA), since it has been reported that individual FFA have distinct inherent steatotic and toxic activities, the saturated FFA presenting the highest toxicity[122]. In normal and in NAFLD subjects, the most abundant FFAs in liver triglycerides are oleic acid (18:1) and palmitoleic acid (16:1) for unsaturated, and palmitic acid (16:0) and stearic acid (18:0) for saturated FFAs[123]. The relative concentration has been demonstrated to be a determinant in their hepatic accumulation and toxicity[124]. For instance, different effects of oleic and palmitic acid were reported on lipid accumulation and on the induction of apoptosis. Oleic acid was shown in several hepatic cell lines to be more steatogenic than palmitic acid[125] but less toxic that the latter. Long chain FFAs are highly insoluble in the aqueous phase, and for this reason are carried in blood associated with serum albumin. Whereas under physiological conditions the FFA: albumin ratio is around 2:1[78] under pathological states, the ratio can be as high as 7.5:1[126]. This simple, but fundamental, detail is often disregarded in several studies. In addition, since the development of NAFLD and the progression to NASH involve several cell types, another crucial point is the cell type used in the experimental system. The vast majority of the published data has been obtained in hepatocyte cultures, but for the study of the progression to fibrosis, other cell types such as HSC and KC must be considered. The crucial role played by the interaction among the different cell types points to the need of much more controlled experimental setups to provide a comprehensive approach to the molecular mechanisms involved. For this reason, the establishment of co-culture systems has been acknowledged to be promising in the last few years[77,127-129] with regard to the study of the different intracellular mechanisms.

In any case, in spite of the progress in the molecular biology of NAFLD/NASH, the main limitation of these *in vitro* approaches remains the different models and experimental variables used in the different laboratories. This makes each study somewhat unique and independent from the others. A better definition of the experimental conditions and standardized models would greatly contribute to improving the possibility of achieving solid results.

CONCLUSION

A translational approach to NAFLD and NASH is just at the beginning. The disease is rather new and is still based on a negative definition, but we now know that it is linked to a metabolic dysfunction of the glucose and/or lipid hepatic pathways. The number of patients affected by this disorder is exponentially growing worldwide, and NAFLD diagnosis must be performed early to prevent the progression to NASH, cirrhosis and HCC or to cardiovascular diseases, and to adopt effective preventive strategies. We now have the experimental models to investigate the still unknown reasons why only some types of sugars and lipids induce progressive hepatic inflammation, apoptosis and fibrosis. We hope they will help us to understand the inner mechanism of the damage and design better drugs that will combine with a much healthier lifestyle to fight this plague.

REFERENCES

1 **Sartorio A**, Del Col A, Agosti F, Mazzilli G, Bellentani S, Tiribelli C, Bedogni G. Predictors of non-alcoholic fatty liver disease in obese children. *Eur J Clin Nutr* 2007; **61**: 877-883 [PMID: 17151586 DOI: 10.1038/sj.ejcn.1602588]

2 **Chen SH**, He F, Zhou HL, Wu HR, Xia C, Li YM. Relationship between nonalcoholic fatty liver disease and metabolic syndrome. *J Dig Dis* 2011; **12**: 125-130 [PMID: 21401898 DOI: 10.1111/j.1751-2980.2011.00487.x]

3 **Caldwell SH**, Ikura Y, Iezzoni JC, Liu Z. Has natural selection in human populations produced two types of metabolic syndrome (with and without fatty liver)? *J Gastroenterol Hepatol* 2007; **22** Suppl 1: S11-S19 [PMID: 17567458 DOI: 10.1111/j.1440-1746.2006.04639.x]

4 **Bulum T**, Kolarić B, Duvnjak L, Duvnjak M. Nonalcoholic fatty liver disease markers are associated with insulin resistance in type 1 diabetes. *Dig Dis Sci* 2011; **56**: 3655-3663 [PMID: 21735081 DOI: 10.1007/s10620-011-1807-7]

5 **Marchesini G**, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R, Natale S, Vanni E, Villanova N, Melchionda N, Rizzetto M. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology* 2003; **37**: 917-923 [PMID: 12668987 DOI: 10.1053/jhep.2003.50161]

6 **Zelber-Sagi S**, Nitzan-Kaluski D, Goldsmith R, Webb M, Blendis L, Halpern Z, Oren R. Long term nutritional intake and the risk for non-alcoholic fatty liver disease (NAFLD): a population based study. *J Hepatol* 2007; **47**: 711-717 [PMID: 17850914 DOI: 10.1016/j.jhep.2007.06.020]

7 **Zelber-Sagi S**, Nitzan-Kaluski D, Goldsmith R, Webb M, Zvibel I, Goldiner I, Blendis L, Halpern Z, Oren R. Role of leisure-time physical activity in nonalcoholic fatty liver disease: a population-based study. *Hepatology* 2008; **48**: 1791-1798 [PMID: 18972405 DOI: 10.1002/hep.22525]

8 **Centis E**, Moscatiello S, Bugianesi E, Bellentani S, Fracanzani AL, Calugi S, Petta S, Dalle Grave R, Marchesini G. Stage of change and motivation to healthier lifestyle in non-alcoholic fatty liver disease. *J Hepatol* 2013; **58**: 771-777 [PMID: 23201248 DOI: 10.1016/j.jhep.2012.11.031]

9 **Scaglioni F**, Marino M, Ciccia S, Procaccini A, Busacchi M, Loria P, Lonardo A, Malavolti M, Battistini NC, Pellegrini M, Carubbi F, Bellentani S. Short-term multidisciplinary non-pharmacological intervention is effective in reducing liver fat content assessed non-invasively in patients with nonalcoholic fatty liver disease (NAFLD). *Clin Res Hepatol Gastroenterol* 2013; **37**: 353-358 [PMID: 23273500 DOI: 10.1016/j.clinre.2012.10.009]

10 **Liu CJ**. Prevalence and risk factors for non-alcoholic fatty liver disease in Asian people who are not obese. *J Gastroenterol Hepatol* 2012; **27**: 1555-1560 [PMID: 22741595 DOI: 10.1111/j.1440-1746.2012.07222.x]

11 **Day CP**, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998; **114**: 842-845 [PMID: 9547102]

12 **Bugianesi E**, Moscatiello S, Ciaravella MF, Marchesini G. Insulin resistance in nonalcoholic fatty liver disease. *Curr Pharm Des* 2010; **16**: 1941-1951 [PMID: 20370677]

13 **Diehl AM**, Li ZP, Lin HZ, Yang SQ. Cytokines and the pathogenesis of non-alcoholic steatohepatitis. *Gut* 2005; **54**: 303-306 [PMID: 15647199 DOI: 10.1136/gut.2003.024935]

14 **Begriche K**, Igoudjil A, Pessayre D, Fromenty B. Mitochondrial dysfunction in NASH: causes, consequences and pos-

sible means to prevent it. *Mitochondrion* 2006; **6**: 1-28 [PMID: 16406828 DOI: 10.1016/j.mito.2005.10.004]
15 **Bachar E**, Ariav Y, Ketzinel-Gilad M, Cerasi E, Kaiser N, Leibowitz G. Glucose amplifies fatty acid-induced endoplasmic reticulum stress in pancreatic beta-cells via activation of mTORC1. *PLoS One* 2009; **4**: e4954 [PMID: 19305497 DOI: 10.1371/journal.pone.0004954]
16 **Tilg H**, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology* 2010; **52**: 1836-1846 [PMID: 21038418 DOI: 10.1002/hep.24001]
17 **Moschen AR**, Kaser S, Tilg H. Non-alcoholic steatohepatitis: a microbiota-driven disease. *Trends Endocrinol Metab* 2013; **24**: 537-545 [PMID: 23827477 DOI: 10.1016/j.tem.2013.05.009]
18 **Vrieze A**, Van Nood E, Holleman F, Salojärvi J, Kootte RS, Bartelsman JF, Dallinga-Thie GM, Ackermans MT, Serlie MJ, Oozeer R, Derrien M, Druesne A, Van Hylckama Vlieg JE, Bloks VW, Groen AK, Heilig HG, Zoetendal EG, Stroes ES, de Vos WM, Hoekstra JB, Nieuwdorp M. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology* 2012; **143**: 913-916.e7 [PMID: 22728514 DOI: 10.1053/j.gastro.2012.06.031]
19 **Sookoian S**, Pirola CJ. Meta-analysis of the influence of I148M variant of patatin-like phospholipase domain containing 3 gene (PNPLA3) on the susceptibility and histological severity of nonalcoholic fatty liver disease. *Hepatology* 2011; **53**: 1883-1894 [PMID: 21381068 DOI: 10.1002/hep.24283]
20 **Montecucco F**, Bertolotto M, Vuilleumier N, Franciosi U, Puddu A, Minetti S, Delrio A, Quercioli A, Bergamini E, Ottonello L, Pende A, Lenglet S, Pelli G, Mach F, Dallegri F, Viviani GL. Acipimox reduces circulating levels of insulin and associated neutrophilic inflammation in metabolic syndrome. *Am J Physiol Endocrinol Metab* 2011; **300**: E681-E690 [PMID: 21266669 DOI: 10.1152/ajpendo.00527.2010]
21 **Kim F**, Pham M, Maloney E, Rizzo NO, Morton GJ, Wisse BE, Kirk EA, Chait A, Schwartz MW. Vascular inflammation, insulin resistance, and reduced nitric oxide production precede the onset of peripheral insulin resistance. *Arterioscler Thromb Vasc Biol* 2008; **28**: 1982-1988 [PMID: 18772497 DOI: 10.1161/ATVBAHA.108.169722]
22 **Gage MC**, Yuldasheva NY, Viswambharan H, Sukumar P, Cubbon RM, Galloway S, Imrie H, Skromna A, Smith J, Jackson CL, Kearney MT, Wheatcroft SB. Endothelium-specific insulin resistance leads to accelerated atherosclerosis in areas with disturbed flow patterns: a role for reactive oxygen species. *Atherosclerosis* 2013; **230**: 131-139 [PMID: 23958265 DOI: 10.1016/j.atherosclerosis.2013.06.017]
23 **Tsuchiya K**, Tanaka J, Shuiqing Y, Welch CL, DePinho RA, Tabas I, Tall AR, Goldberg IJ, Accili D. FoxOs integrate pleiotropic actions of insulin in vascular endothelium to protect mice from atherosclerosis. *Cell Metab* 2012; **15**: 372-381 [PMID: 22405072 DOI: 10.1016/j.cmet.2012.01.018]
24 **Davis RC**, Castellani LW, Hosseini M, Ben-Zeev O, Mao HZ, Weinstein MM, Jung DY, Jun JY, Kim JK, Lusis AJ, Péterfy M. Early hepatic insulin resistance precedes the onset of diabetes in obese C57BLKS-db/db mice. *Diabetes* 2010; **59**: 1616-1625 [PMID: 20393148 DOI: 10.2337/db09-0878]
25 **Carbone F**, Montecucco F, Mach F, Pontremoli R, Viazzi F. The liver and the kidney: two critical organs influencing the atherothrombotic risk in metabolic syndrome. *Thromb Haemost* 2013; **110**: 940-958 [PMID: 23966104 DOI: 10.1160/TH13-06-0499]
26 **Targher G**, Chonchol M, Pichiri I, Zoppini G. Risk of cardiovascular disease and chronic kidney disease in diabetic patients with non-alcoholic fatty liver disease: just a coincidence? *J Endocrinol Invest* 2011; **34**: 544-551 [PMID: 21427524 DOI: 10.3275/7614]
27 **Wisse BE**. The inflammatory syndrome: the role of adipose tissue cytokines in metabolic disorders linked to obesity. *J Am Soc Nephrol* 2004; **15**: 2792-2800 [PMID: 15504932 DOI: 10.1097/01.ASN.0000141966.69934.21]
28 **Wolf G**, Chen S, Han DC, Ziyadeh FN. Leptin and renal disease. *Am J Kidney Dis* 2002; **39**: 1-11 [PMID: 11774095 DOI: 10.1053/ajkd.2002.29865]
29 **Lonardo A**, Bellentani S, Ratziu V, Loria P. Insulin resistance in nonalcoholic steatohepatitis: necessary but not sufficient - death of a dogma from analysis of therapeutic studies? *Expert Rev Gastroenterol Hepatol* 2011; **5**: 279-289 [PMID: 21476922 DOI: 10.1586/egh.11.19]
30 **Rask E**, Olsson T, Söderberg S, Johnson O, Seckl J, Holst JJ, Ahrén B. Impaired incretin response after a mixed meal is associated with insulin resistance in nondiabetic men. *Diabetes Care* 2001; **24**: 1640-1645 [PMID: 11522713]
31 **Holst JJ**, Gromada J. Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic humans. *Am J Physiol Endocrinol Metab* 2004; **287**: E199-E206 [PMID: 15271645 DOI: 10.1152/ajpendo.00545.2003]
32 **Nauck M**, Stöckmann F, Ebert R, Creutzfeldt W. Reduced incretin effect in type 2 (non-insulin-dependent) diabetes. *Diabetologia* 1986; **29**: 46-52 [PMID: 3514343]
33 **Gautier JF**, Choukem SP, Girard J. Physiology of incretins (GIP and GLP-1) and abnormalities in type 2 diabetes. *Diabetes Metab* 2008; **34** Suppl 2: S65-S72 [PMID: 18640588 DOI: 10.1016/S1262-3636(08)73397-4]
34 **Gupta NA**, Mells J, Dunham RM, Grakoui A, Handy J, Saxena NK, Anania FA. Glucagon-like peptide-1 receptor is present on human hepatocytes and has a direct role in decreasing hepatic steatosis in vitro by modulating elements of the insulin signaling pathway. *Hepatology* 2010; **51**: 1584-1592 [PMID: 20225248 DOI: 10.1002/hep.23569]
35 **Ding X**, Saxena NK, Lin S, Gupta NA, Anania FA. Exendin-4, a glucagon-like protein-1 (GLP-1) receptor agonist, reverses hepatic steatosis in ob/ob mice. *Hepatology* 2006; **43**: 173-181 [PMID: 16374859 DOI: 10.1002/hep.21006]
36 **Trevaskis JL**, Griffin PS, Wittmer C, Neuschwander-Tetri BA, Brunt EM, Dolman CS, Erickson MR, Napora J, Parkes DG, Roth JD. Glucagon-like peptide-1 receptor agonism improves metabolic, biochemical, and histopathological indices of nonalcoholic steatohepatitis in mice. *Am J Physiol Gastrointest Liver Physiol* 2012; **302**: G762-G772 [PMID: 22268099 DOI: 10.1152/ajpgi.00476.2011]
37 **Ben-Shlomo S**, Zvibel I, Shnell M, Shlomai A, Chepurko E, Halpern Z, Barzilai N, Oren R, Fishman S. Glucagon-like peptide-1 reduces hepatic lipogenesis via activation of AMP-activated protein kinase. *J Hepatol* 2011; **54**: 1214-1223 [PMID: 21145820 DOI: 10.1016/j.jhep.2010.09.032]
38 **Svegliati-Baroni G**, Saccomanno S, Rychlicki C, Agostinelli L, De Minicis S, Candelaresi C, Faraci G, Pacetti D, Vivarelli M, Nicolini D, Garelli P, Casini A, Manco M, Mingrone G, Risaliti A, Frega GN, Benedetti A, Gastaldelli A. Glucagon-like peptide-1 receptor activation stimulates hepatic lipid oxidation and restores hepatic signalling alteration induced by a high-fat diet in nonalcoholic steatohepatitis. *Liver Int* 2011; **31**: 1285-1297 [PMID: 21745271 DOI: 10.1111/j.1478-3231.2011.02462.x]
39 **Sharma S**, Mells JE, Fu PP, Saxena NK, Anania FA. GLP-1 analogs reduce hepatocyte steatosis and improve survival by enhancing the unfolded protein response and promoting macroautophagy. *PLoS One* 2011; **6**: e25269 [PMID: 21957486 DOI: 10.1371/journal.pone.0025269]
40 **Staels B**, Fonseca VA. Bile acids and metabolic regulation: mechanisms and clinical responses to bile acid sequestration. *Diabetes Care* 2009; **32** Suppl 2: S237-S245 [PMID: 19875558 DOI: 10.2337/dc09-S355]
41 **Lefebvre P**, Cariou B, Lien F, Kuipers F, Staels B. Role of bile acids and bile acid receptors in metabolic regulation. *Physiol Rev* 2009; **89**: 147-191 [PMID: 19126757 DOI: 10.1152/physrev.00010.2008]
42 **Andersén E**, Karlaganis G, Sjövall J. Altered bile acid pro-

files in duodenal bile and urine in diabetic subjects. *Eur J Clin Invest* 1988; **18**: 166-172 [PMID: 3133222]
43 **Knop FK**. Bile-induced secretion of glucagon-like peptide-1: pathophysiological implications in type 2 diabetes? *Am J Physiol Endocrinol Metab* 2010; **299**: E10-E13 [PMID: 20424139 DOI: 10.1152/ajpendo.00137.2010]
44 **Laplante M**, Sabatini DM. mTORC1 activates SREBP-1c and uncouples lipogenesis from gluconeogenesis. *Proc Natl Acad Sci USA* 2010; **107**: 3281-3282 [PMID: 20167806 DOI: 10.1073/pnas.1000323107]
45 **Blaslov K**, Zibar K, Bulum T, Duvnjak L. Effect of exenatide therapy on hepatic fat quantity and hepatic biomarkers in type 2 diabetic patients. *Clin Res Hepatol Gastroenterol* 2013; Epub ahead of print [PMID: 24315013 DOI: 10.1016/j.clinre.2013.10.013]
46 **Holst JJ**, Vilsbøll T, Deacon CF. The incretin system and its role in type 2 diabetes mellitus. *Mol Cell Endocrinol* 2009; **297**: 127-136 [PMID: 18786605 DOI: 10.1016/j.mce.2008.08.012]
47 **Mudaliar S**, Henry RR, Sanyal AJ, Morrow L, Marschall HU, Kipnes M, Adorini L, Sciacca CI, Clopton P, Castelloe E, Dillon P, Pruzanski M, Shapiro D. Efficacy and safety of the farnesoid X receptor agonist obeticholic acid in patients with type 2 diabetes and nonalcoholic fatty liver disease. *Gastroenterology* 2013; **145**: 574-582.e1 [PMID: 23727264 DOI: 10.1053/j.gastro.2013.05.042]
48 **Tanaka S**, Wands JR. Insulin receptor substrate 1 overexpression in human hepatocellular carcinoma cells prevents transforming growth factor beta1-induced apoptosis. *Cancer Res* 1996; **56**: 3391-3394 [PMID: 8758899]
49 **Ish-Shalom D**, Christoffersen CT, Vorwerk P, Sacerdoti-Sierra N, Shymko RM, Naor D, De Meyts P. Mitogenic properties of insulin and insulin analogues mediated by the insulin receptor. *Diabetologia* 1997; **40** Suppl 2: S25-S31 [PMID: 9248698]
50 **Page JM**, Harrison SA. NASH and HCC. *Clin Liver Dis* 2009; **13**: 631-647 [PMID: 19818310 DOI: 10.1016/j.cld.2009.07.007]
51 **Stickel F**, Hellerbrand C. Non-alcoholic fatty liver disease as a risk factor for hepatocellular carcinoma: mechanisms and implications. *Gut* 2010; **59**: 1303-1307 [PMID: 20650925 DOI: 10.1136/gut.2009.199661]
52 **Yu J**, Shen J, Sun TT, Zhang X, Wong N. Obesity, insulin resistance, NASH and hepatocellular carcinoma. *Semin Cancer Biol* 2013; **23**: 483-491 [PMID: 23876851 DOI: 10.1016/j.semcancer.2013.07.003]
53 **Cohen JC**, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. *Science* 2011; **332**: 1519-1523 [PMID: 21700865 DOI: 10.1126/science.1204265]
54 **Yamaguchi K**, Yang L, McCall S, Huang J, Yu XX, Pandey SK, Bhanot S, Monia BP, Li YX, Diehl AM. Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. *Hepatology* 2007; **45**: 1366-1374 [PMID: 17476695 DOI: 10.1002/hep.21655]
55 **Malhi H**, Gores GJ. Molecular mechanisms of lipotoxicity in nonalcoholic fatty liver disease. *Semin Liver Dis* 2008; **28**: 360-369 [PMID: 18956292 DOI: 10.1055/s-0028-1091980]
56 **Bass NM**. Lipidomic dissection of nonalcoholic steatohepatitis: moving beyond foie gras to fat traffic. *Hepatology* 2010; **51**: 4-7 [PMID: 20034031 DOI: 10.1002/hep.23458]
57 **Choi SS**, Diehl AM. Hepatic triglyceride synthesis and nonalcoholic fatty liver disease. *Curr Opin Lipidol* 2008; **19**: 295-300 [PMID: 18460922 DOI: 10.1097/MOL.0b013e3282ff5e55]
58 **Bikman BT**, Summers SA. Ceramides as modulators of cellular and whole-body metabolism. *J Clin Invest* 2011; **121**: 4222-4230 [PMID: 22045572 DOI: 10.1172/JCI57144]
59 **Neuschwander-Tetri BA**. Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. *Hepatology* 2010; **52**: 774-788 [PMID: 20683968 DOI: 10.1002/hep.23719]
60 **Tanti JF**, Jager J. Cellular mechanisms of insulin resistance: role of stress-regulated serine kinases and insulin receptor substrates (IRS) serine phosphorylation. *Curr Opin Pharmacol* 2009; **9**: 753-762 [PMID: 19683471 DOI: 10.1016/j.coph.2009.07.004]
61 **Wouters K**, van Bilsen M, van Gorp PJ, Bieghs V, Lütjohann D, Kerksiek A, Staels B, Hofker MH, Shiri-Sverdlov R. Intrahepatic cholesterol influences progression, inhibition and reversal of non-alcoholic steatohepatitis in hyperlipidemic mice. *FEBS Lett* 2010; **584**: 1001-1005 [PMID: 20114046 DOI: 10.1016/j.febslet.2010.01.046]
62 **Zhao L**, Chen Y, Tang R, Chen Y, Li Q, Gong J, Huang A, Varghese Z, Moorhead JF, Ruan XZ. Inflammatory stress exacerbates hepatic cholesterol accumulation via increasing cholesterol uptake and de novo synthesis. *J Gastroenterol Hepatol* 2011; **26**: 875-883 [PMID: 21488946 DOI: 10.1111/j.1440-1746.2010.06560.x]
63 **Li L**, Hossain MA, Sadat S, Hager L, Liu L, Tam L, Schroer S, Huogen L, Fantus IG, Connelly PW, Woo M, Ng DS. Lecithin cholesterol acyltransferase null mice are protected from diet-induced obesity and insulin resistance in a gender-specific manner through multiple pathways. *J Biol Chem* 2011; **286**: 17809-17820 [PMID: 21454561 DOI: 10.1074/jbc.M110.180893]
64 **Hager L**, Li L, Pun H, Liu L, Hossain MA, Maguire GF, Naples M, Baker C, Magomedova L, Tam J, Adeli K, Cummins CL, Connelly PW, Ng DS. Lecithin: cholesterol acyltransferase deficiency protects against cholesterol-induced hepatic endoplasmic reticulum stress in mice. *J Biol Chem* 2012; **287**: 20755-20768 [PMID: 22500017 DOI: 10.1074/jbc.M112.340919]
65 **Bernardi P**. Mitochondrial transport of cations: channels, exchangers, and permeability transition. *Physiol Rev* 1999; **79**: 1127-1155 [PMID: 10508231]
66 **Scorrano L**, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T, Korsmeyer SJ. BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. *Science* 2003; **300**: 135-139 [PMID: 12624178 DOI: 10.1126/science.1081208]
67 **Musso G**, Cassader M, Bo S, De Michieli F, Gambino R. Sterol regulatory element-binding factor 2 (SREBF-2) predicts 7-year NAFLD incidence and severity of liver disease and lipoprotein and glucose dysmetabolism. *Diabetes* 2013; **62**: 1109-1120 [PMID: 23274901 DOI: 10.2337/db12-0858]
68 **Musso G**, Gambino R, Cassader M. Cholesterol metabolism and the pathogenesis of non-alcoholic steatohepatitis. *Prog Lipid Res* 2013; **52**: 175-191 [PMID: 23206728 DOI: 10.1016/j.plipres.2012.11.002]
69 **Yasutake K**, Nakamuta M, Shima Y, Ohyama A, Masuda K, Haruta N, Fujino T, Aoyagi Y, Fukuizumi K, Yoshimoto T, Takemoto R, Miyahara T, Harada N, Hayata F, Nakashima M, Enjoji M. Nutritional investigation of non-obese patients with non-alcoholic fatty liver disease: the significance of dietary cholesterol. *Scand J Gastroenterol* 2009; **44**: 471-477 [PMID: 19058085 DOI: 10.1080/00365520802588133]
70 **Asrih M**, Jornayvaz FR. Inflammation as a potential link between nonalcoholic fatty liver disease and insulin resistance. *J Endocrinol* 2013; **218**: R25-R36 [PMID: 23833274 DOI: 10.1530/JOE-13-0201]
71 **Marra F**, Bertolani C. Adipokines in liver diseases. *Hepatology* 2009; **50**: 957-969 [PMID: 19585655 DOI: 10.1002/hep.23046]
72 **Tilg H**, Diehl AM. Cytokines in alcoholic and nonalcoholic steatohepatitis. *N Engl J Med* 2000; **343**: 1467-1476 [PMID: 11078773 DOI: 10.1056/NEJM200011163432007]
73 **Weisberg SP**, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003; **112**: 1796-1808 [PMID: 14679176 DOI: 10.1172/JCI19246]
74 **Kunt T**, Forst T, Früh B, Flohr T, Schneider S, Harzer O, Pfützner A, Engelbach M, Löbig M, Beyer J. Binding of

monocytes from normolipidemic hyperglycemic patients with type 1 diabetes to endothelial cells is increased in vitro. *Exp Clin Endocrinol Diabetes* 1999; **107**: 252-256 [PMID: 10433064 DOI: 10.1055/s-0029-1212108]
75 **Bulum** T, KolariÄ B, Duvnjak L. Decreased serum monocytes and elevated neutrophils as additional markers of insulin resistance in type 1 diabetes. *Int J Diabetes Dev Ctries* 2013; 1-6 [DOI: 10.1007/s13410-013-0176-5]
76 **Bunn RC**, Cockrell GE, Ou Y, Thrailkill KM, Lumpkin CK, Fowlkes JL. Palmitate and insulin synergistically induce IL-6 expression in human monocytes. *Cardiovasc Diabetol* 2010; **9**: 73 [PMID: 21054880 DOI: 10.1186/1475-2840-9-73]
77 **Chavez-Tapia NC**, Rosso N, Tiribelli C. Effect of intracellular lipid accumulation in a new model of non-alcoholic fatty liver disease. *BMC Gastroenterol* 2012; **12**: 20 [PMID: 22380754 DOI: 10.1186/1471-230X-12-20]
78 **Joshi-Barve S**, Barve SS, Amancherla K, Gobejishvili L, Hill D, Cave M, Hote P, McClain CJ. Palmitic acid induces production of proinflammatory cytokine interleukin-8 from hepatocytes. *Hepatology* 2007; **46**: 823-830 [PMID: 17680645 DOI: 10.1002/hep.21752]
79 **Chávez-Tapia NC**, Rosso N, Uribe M, Bojalil R, Tiribelli C. Kinetics of the inflammatory response induced by free fatty acid accumulation in hepatocytes. *Ann Hepatol* 2013; **13**: 113-120 [PMID: 24378274]
80 **Bråkenhielm E**, Veitonmäki N, Cao R, Kihara S, Matsuzawa Y, Zhivotovsky B, Funahashi T, Cao Y. Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. *Proc Natl Acad Sci USA* 2004; **101**: 2476-2481 [PMID: 14983034]
81 **Kamada Y**, Matsumoto H, Tamura S, Fukushima J, Kiso S, Fukui K, Igura T, Maeda N, Kihara S, Funahashi T, Matsuzawa Y, Shimomura I, Hayashi N. Hypoadiponectinemia accelerates hepatic tumor formation in a nonalcoholic steatohepatitis mouse model. *J Hepatol* 2007; **47**: 556-564 [PMID: 17459514 DOI: 10.1016/j.jhep.2007.03.020]
82 **Braunersreuther V**, Viviani GL, Mach F, Montecucco F. Role of cytokines and chemokines in non-alcoholic fatty liver disease. *World J Gastroenterol* 2012; **18**: 727-735 [PMID: 22371632 DOI: 10.3748/wjg.v18.i8.727]
83 **Sanyal AJ**, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, Luketic VA, Shiffman ML, Clore JN. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 2001; **120**: 1183-1192 [PMID: 11266382 DOI: 10.1053/gast.2001.23256]
84 **Cortez-Pinto H**, Chatham J, Chacko VP, Arnold C, Rashid A, Diehl AM. Alterations in liver ATP homeostasis in human nonalcoholic steatohepatitis: a pilot study. *JAMA* 1999; **282**: 1659-1664 [PMID: 10553793]
85 **Serviddio G**, Sastre J, Bellanti F, Viña J, Vendemiale G, Altomare E. Mitochondrial involvement in non-alcoholic steatohepatitis. *Mol Aspects Med* 2008; **29**: 22-35 [PMID: 18061659 DOI: 10.1016/j.mam.2007.09.014]
86 **Teodoro JS**, Rolo AP, Duarte FV, Simões AM, Palmeira CM. Differential alterations in mitochondrial function induced by a choline-deficient diet: understanding fatty liver disease progression. *Mitochondrion* 2008; **8**: 367-376 [PMID: 18765303 DOI: 10.1016/j.mito.2008.07.008]
87 **Leamy AK**, Egnatchik RA, Young JD. Molecular mechanisms and the role of saturated fatty acids in the progression of non-alcoholic fatty liver disease. *Prog Lipid Res* 2013; **52**: 165-174 [PMID: 23178552 DOI: 10.1016/j.plipres.2012.10.004]
88 **Sunny NE**, Parks EJ, Browning JD, Burgess SC. Excessive hepatic mitochondrial TCA cycle and gluconeogenesis in humans with nonalcoholic fatty liver disease. *Cell Metab* 2011; **14**: 804-810 [PMID: 22152305 DOI: 10.1016/j.cmet.2011.11.004]
89 **Satapati S**, Sunny NE, Kucejova B, Fu X, He TT, Méndez-Lucas A, Shelton JM, Perales JC, Browning JD, Burgess SC. Elevated TCA cycle function in the pathology of diet-induced hepatic insulin resistance and fatty liver. *J Lipid Res* 2012; **53**: 1080-1092 [PMID: 22493093 DOI: 10.1194/jlr.M023382]
90 **Borradaile NM**, Han X, Harp JD, Gale SE, Ory DS, Schaffer JE. Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. *J Lipid Res* 2006; **47**: 2726-2737 [PMID: 16960261 DOI: 10.1194/jlr.M600299-JLR200]
91 **Hickson-Bick DL**, Sparagna GC, Buja LM, McMillin JB. Palmitate-induced apoptosis in neonatal cardiomyocytes is not dependent on the generation of ROS. *Am J Physiol Heart Circ Physiol* 2002; **282**: H656-H664 [PMID: 11788415 DOI: 10.1152/ajpheart.00726.2001]
92 **Friedman SL**. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev* 2008; **88**: 125-172 [PMID: 18195085 DOI: 10.1152/physrev.00013.2007]
93 **Friedman SL**. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008; **134**: 1655-1669 [PMID: 18471545 DOI: 10.1053/j.gastro.2008.03.003]
94 **Gressner OA**, Weiskirchen R, Gressner AM. Evolving concepts of liver fibrogenesis provide new diagnostic and therapeutic options. *Comp Hepatol* 2007; **6**: 7 [PMID: 17663771 DOI: 10.1186/1476-5926-6-7]
95 **Dooley S**, Delvoux B, Streckert M, Bonzel L, Stopa M, ten Dijke P, Gressner AM. Transforming growth factor beta signal transduction in hepatic stellate cells via Smad2/3 phosphorylation, a pathway that is abrogated during in vitro progression to myofibroblasts. TGFbeta signal transduction during transdifferentiation of hepatic stellate cells. *FEBS Lett* 2001; **502**: 4-10 [PMID: 11478938]
96 **Cavin LG**, Romieu-Mourez R, Panta GR, Sun J, Factor VM, Thorgeirsson SS, Sonenshein GE, Arsura M. Inhibition of CK2 activity by TGF-beta1 promotes IkappaB-alpha protein stabilization and apoptosis of immortalized hepatocytes. *Hepatology* 2003; **38**: 1540-1551 [PMID: 14647065 DOI: 10.1016/j.hep.2003.09.019]
97 **Proell V**, Carmona-Cuenca I, Murillo MM, Huber H, Fabregat I, Mikulits W. TGF-beta dependent regulation of oxygen radicals during transdifferentiation of activated hepatic stellate cells to myofibroblastoid cells. *Comp Hepatol* 2007; **6**: 1 [PMID: 17311678 DOI: 10.1186/1476-5926-6-1]
98 **Ludwig J**, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980; **55**: 434-438 [PMID: 7382552]
99 **Anstee QM**, Goldin RD. Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. *Int J Exp Pathol* 2006; **87**: 1-16 [PMID: 16436109 DOI: 10.1111/j.0959-9673.2006.00465.x]
100 **Koteish A**, Mae Diehl A. Animal models of steatohepatitis. *Best Pract Res Clin Gastroenterol* 2002; **16**: 679-690 [PMID: 12406439]
101 **Larter CZ**, Yeh MM. Animal models of NASH: getting both pathology and metabolic context right. *J Gastroenterol Hepatol* 2008; **23**: 1635-1648 [PMID: 18752564 DOI: 10.1111/j.1440-1746.2008.05543.x]
102 **Takahashi Y**, Soejima Y, Fukusato T. Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World J Gastroenterol* 2012; **18**: 2300-2308 [PMID: 22654421 DOI: 10.3748/wjg.v18.i19.2300]
103 **Rinella ME**, Green RM. The methionine-choline deficient dietary model of steatohepatitis does not exhibit insulin resistance. *J Hepatol* 2004; **40**: 47-51 [PMID: 14672613]
104 **Schattenberg JM**, Wang Y, Singh R, Rigoli RM, Czaja MJ. Hepatocyte CYP2E1 overexpression and steatohepatitis lead to impaired hepatic insulin signaling. *J Biol Chem* 2005; **280**: 9887-9894 [PMID: 15632182 DOI: 10.1074/jbc.M410310200]
105 **Martínez-Chantar ML**, Corrales FJ, Martínez-Cruz LA, García-Trevijano ER, Huang ZZ, Chen L, Kanel G, Avila MA, Mato JM, Lu SC. Spontaneous oxidative stress and liver tumors in mice lacking methionine adenosyltransferase 1A. *FASEB J* 2002; **16**: 1292-1294 [PMID: 12060674 DOI: 10.1096/fj.02-0078fje]

106 **Nakayama H**, Otabe S, Ueno T, Hirota N, Yuan X, Fukutani T, Hashinaga T, Wada N, Yamada K. Transgenic mice expressing nuclear sterol regulatory element-binding protein 1c in adipose tissue exhibit liver histology similar to nonalcoholic steatohepatitis. *Metabolism* 2007; **56**: 470-475 [PMID: 17379003 DOI: 10.1016/j.metabol.2006.11.004]

107 **Schattenberg JM**, Galle PR. Animal models of non-alcoholic steatohepatitis: of mice and man. *Dig Dis* 2010; **28**: 247-254 [PMID: 20460919 DOI: 10.1159/000282097]

108 **Tetri LH**, Basaranoglu M, Brunt EM, Yerian LM, Neuschwander-Tetri BA. Severe NAFLD with hepatic necroinflammatory changes in mice fed trans fats and a high-fructose corn syrup equivalent. *Am J Physiol Gastrointest Liver Physiol* 2008; **295**: G987-G995 [PMID: 18772365 DOI: 10.1152/ajpgi.90272.2008]

109 **Kawasaki T**, Igarashi K, Koeda T, Sugimoto K, Nakagawa K, Hayashi S, Yamaji R, Inui H, Fukusato T, Yamanouchi T. Rats fed fructose-enriched diets have characteristics of nonalcoholic hepatic steatosis. *J Nutr* 2009; **139**: 2067-2071 [PMID: 19776184]

110 **Lieber CS**, Leo MA, Mak KM, Xu Y, Cao Q, Ren C, Ponomarenko A, DeCarli LM. Model of nonalcoholic steatohepatitis. *Am J Clin Nutr* 2004; **79**: 502-509 [PMID: 14985228]

111 **Ferramosca A**, Conte A, Damiano F, Siculella L, Zara V. Differential effects of high-carbohydrate and high-fat diets on hepatic lipogenesis in rats. *Eur J Nutr* 2014; **53**: 1103-1114 [PMID: 24197180 DOI: 10.1007/s00394-013-0613-8]

112 **Matsuzawa N**, Takamura T, Kurita S, Misu H, Ota T, Ando H, Yokoyama M, Honda M, Zen Y, Nakanuma Y, Miyamoto K, Kaneko S. Lipid-induced oxidative stress causes steatohepatitis in mice fed an atherogenic diet. *Hepatology* 2007; **46**: 1392-1403 [PMID: 17929294 DOI: 10.1002/hep.21874]

113 **Michael MD**, Kulkarni RN, Postic C, Previs SF, Shulman GI, Magnuson MA, Kahn CR. Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol Cell* 2000; **6**: 87-97 [PMID: 10949030]

114 **Cohen SE**, Kokkotou E, Biddinger SB, Kondo T, Gebhardt R, Kratzsch J, Mantzoros CS, Kahn CR. High circulating leptin receptors with normal leptin sensitivity in liver-specific insulin receptor knock-out (LIRKO) mice. *J Biol Chem* 2007; **282**: 23672-23678 [PMID: 17556363 DOI: 10.1074/jbc.M704053200]

115 **Kohli R**, Kirby M, Xanthakos SA, Softic S, Feldstein AE, Saxena V, Tang PH, Miles L, Miles MV, Balistreri WF, Woods SC, Seeley RJ. High-fructose, medium chain trans fat diet induces liver fibrosis and elevates plasma coenzyme Q9 in a novel murine model of obesity and nonalcoholic steatohepatitis. *Hepatology* 2010; **52**: 934-944 [PMID: 20607689 DOI: 10.1002/hep.23797]

116 **Ito M**, Suzuki J, Tsujioka S, Sasaki M, Gomori A, Shirakura T, Hirose H, Ito M, Ishihara A, Iwaasa H, Kanatani A. Longitudinal analysis of murine steatohepatitis model induced by chronic exposure to high-fat diet. *Hepatol Res* 2007; **37**: 50-57 [PMID: 17300698 DOI: 10.1111/j.1872-034X.2007.00008.x]

117 **Romestaing C**, Piquet MA, Bedu E, Rouleau V, Dautresme M, Hourmand-Ollivier I, Filippi C, Duchamp C, Sibille B. Long term highly saturated fat diet does not induce NASH in Wistar rats. *Nutr Metab* (Lond) 2007; **4**: 4 [PMID: 17313679 DOI: 10.1186/1743-7075-4-4]

118 **Yilmaz Y**. Review article: is non-alcoholic fatty liver disease a spectrum, or are steatosis and non-alcoholic steatohepatitis distinct conditions? *Aliment Pharmacol Ther* 2012; **36**: 815-823 [PMID: 22966992 DOI: 10.1111/apt.12046]

119 **Lonardo A**, Loria P, Argo C, Caldwell S. Perspectives on cellular dysfunction in nonalcoholic steatohepatitis: a case of 'multiorganelle failure'? Proceedings of a virtual workshop on nonalcoholic steatohepatitis. *Expert Rev Gastroenterol Hepatol* 2011; **5**: 135-139 [PMID: 21476906 DOI: 10.1586/egh.11.24]

120 **Arrese M**. Burning hepatic fat: therapeutic potential for liver-specific thyromimetics in the treatment of nonalcoholic fatty liver disease. *Hepatology* 2009; **49**: 348-351 [PMID: 19177580 DOI: 10.1002/hep.22783]

121 **Chavez-Tapia NC**, Rosso N, Tiribelli C. In vitro models for the study of non-alcoholic fatty liver disease. *Curr Med Chem* 2011; **18**: 1079-1084 [PMID: 21254970]

122 **Malhi H**, Bronk SF, Werneburg NW, Gores GJ. Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. *J Biol Chem* 2006; **281**: 12093-12101 [PMID: 16505490 DOI: 10.1074/jbc.M510660200]

123 **Araya J**, Rodrigo R, Videla LA, Thielemann L, Orellana M, Pettinelli P, Poniachik J. Increase in long-chain polyunsaturated fatty acid n - 6/n - 3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clin Sci* (Lond) 2004; **106**: 635-643 [PMID: 14720121 DOI: 10.1042/CS20030326]

124 **Gómez-Lechón MJ**, Donato MT, Martínez-Romero A, Jiménez N, Castell JV, O'Connor JE. A human hepatocellular in vitro model to investigate steatosis. *Chem Biol Interact* 2007; **165**: 106-116 [PMID: 17188672 DOI: 10.1016/j.cbi.2006.11.004]

125 **Ricchi M**, Odoardi MR, Carulli L, Anzivino C, Ballestri S, Pinetti A, Fantoni LI, Marra F, Bertolotti M, Banni S, Lonardo A, Carulli N, Loria P. Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *J Gastroenterol Hepatol* 2009; **24**: 830-840 [PMID: 19207680 DOI: 10.1111/j.1440-1746.2008.05733.x]

126 **Listenberger LL**, Ory DS, Schaffer JE. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. *J Biol Chem* 2001; **276**: 14890-14895 [PMID: 11278654 DOI: 10.1074/jbc.M010286200]

127 **Krause P**, Saghatolislam F, Koenig S, Unthan-Fechner K, Probst I. Maintaining hepatocyte differentiation in vitro through co-culture with hepatic stellate cells. *In Vitro Cell Dev Biol Anim* 2009; **45**: 205-212 [PMID: 19184253 DOI: 10.1007/s11626-008-9166-1]

128 **Pradere JP**, Kluwe J, De Minicis S, Jiao JJ, Gwak GY, Dapito DH, Jang MK, Guenther ND, Mederacke I, Friedman R, Dragomir AC, Aloman C, Schwabe RF. Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice. *Hepatology* 2013; **58**: 1461-1473 [PMID: 23553591 DOI: 10.1002/hep.26429]

129 **Coulouarn C**, Corlu A, Glaise D, Guénon I, Thorgeirsson SS, Clément B. Hepatocyte-stellate cell cross-talk in the liver engenders a permissive inflammatory microenvironment that drives progression in hepatocellular carcinoma. *Cancer Res* 2012; **72**: 2533-2542 [PMID: 22419664 DOI: 10.1158/0008-5472.CAN-11-3317]

130 **Kim WR**, Brown RS, Terrault NA, El-Serag H. Burden of liver disease in the United States: summary of a workshop. *Hepatology* 2002; **36**: 227-242 [PMID: 12085369 DOI: 10.1053/jhep.2002.34734]

131 **Blachier M**, Leleu H, Peck-Radosavljevic M, Valla DC, Roudot-Thoraval F. The burden of liver disease in Europe: a review of available epidemiological data. *J Hepatol* 2013; **58**: 593-608 [PMID: 23419824 DOI: 10.1016/j.jhep.2012.12.005]

132 **Bellentani S**, Scaglioni F, Marino M, Bedogni G. Epidemiology of non-alcoholic fatty liver disease. *Dig Dis* 2010; **28**: 155-161 [PMID: 20460905 DOI: 10.1159/000282080]

P- Reviewers: Bulum T, Kayadibi H, Lonardo A, Montecucco F, Perazzo H, Takahashi Y, Zara V
S- Editor: Ma YJ **L- Editor**: Logan S **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9050

World J Gastroenterol 2014 July 21; 20(27): 9050-9054
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Epidemiology of fatty liver: An update

Giorgio Bedogni, Valerio Nobili, Claudio Tiribelli

Giorgio Bedogni, Claudio Tiribelli, Liver Research Center, Fondazione Italiana Fegato-ONLUS, 34013 Trieste, Italy
Giorgio Bedogni, International Center for the Assessment of Nutritional Status, University of Milan, 20126 Milan, Italy
Valerio Nobili, Liver Research Unit, Bambino Gesù Children's Hospital, IRCCS, 00149 Rome, Italy
Claudio Tiribelli, Department of Medical Sciences, University of Trieste, 34013 Trieste, Italy
Author contributions: All the authors contributed to this paper equally.
Correspondence to: Giorgio Bedogni, MD, Clinical Epidemiology Unit, Liver Research Center, Fondazione Italiana Fegato-ONLUS, Building Q, Strada Statale 14 - km 163.5, Basovizza, 34013 Trieste, Italy. giorgiobedogni@gmail.com
Telephone: +39-522-1714445 Fax: +39-522-841949
Received: November 4, 2013 Revised: January 14, 2014
Accepted: February 17, 2014
Published online: July 21, 2014

Abstract

We provide a concise review of the main epidemiological literature on fatty liver (FL) published between January 2011 and October 2013. The findings from the literature will be considered in light of the already available knowledge. We discuss the limitations inherent in the categorization of FL into non-alcoholic and alcoholic FL, the potential relevance of FL as an independent predictor of cardiometabolic disease, and recent research addressing the role of FL as an independent predictor of mortality. This review is organized as a series of answers to relevant questions about the epidemiology of FL.

Key words: Fatty liver; Epidemiology

Core tip: We discuss the limitations inherent in the division of fatty liver into non-alcoholic and alcoholic FL, the potential relevance of FL as an independent predictor of cardiometabolic disease, and recent research addressing the role of FL as an independent predictor of mortality.

Bedogni G, Nobili V, Tiribelli C. Epidemiology of fatty liver: An update. *World J Gastroenterol* 2014; 20(27): 9050-9054 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9050.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9050

INTRODUCTION

The aim of this paper is to provide a concise review of the main epidemiological literature on fatty liver (FL) published between January 2011 and October 2013. The findings from such literature will be considered in light of the already available knowledge[1,2]. Our main focus will be on the general population though we will also consider selected clinical studies. We have organized this paper as a series of answers to relevant questions about the epidemiology of FL. It is our hope that this format will attract the interest of practicing physicians as did our previous review on FL that was presented in this manner[3].

WHAT IS FATTY LIVER?

A liver is said to be "fatty" when its hepatocytes contain more than 5% of triglycerides[4,5].

The reference method for the diagnosis of FL is liver biopsy (LB), which is presently used to classify steatosis as light (5% to 33%), moderate (> 33% and < 66%) or severe (> 66%)[4,5]. Although LB is the reference method for the diagnosis of FL, it is an imperfect gold-standard because of sampling error[6,7]. More importantly, LB cannot be employed outside Liver Centers, and less invasive methods are needed to study the epidemiology of FL in the general population[8].

Liver ultrasonography (LUS) is the method most commonly employed to assess FL in the general population[8-11]. Compared with LB, LUS has a sensitivity of

84.8%, a specificity of 93.6%, a positive likelihood ratio of 13.3, and a negative likelihood ratio of 0.16 for the detection of moderate to severe FL[11]. LUS offers an accurate assessment of FL starting from an intrahepatic triglyceride content of 10%[11]. We have found LUS to agree well with LB for the assessment of moderate to severe FL in children[12] but there are presently not enough data to draw definitive conclusions about the interchangeability of LUS and LB in pediatric age[13,14].

Magnetic resonance spectroscopy of the liver (LMRS) has also been used to perform population studies of FL[15] but is less portable and more expensive than LUS[8]. However, a clear advantage of LMRS over LUS is that it offers a continuous rather than an ordinal measure of FL[16].

A further option to study FL in the general population is the use of surrogate markers. A discussion of such markers is beyond the scope of this article, and the interested reader is referred to a recent review on this topic[8]. We wish however to briefly mention the fatty liver index (FLI), which we developed in about 500 adult citizens of Campogalliano (Modena, Northern Italy) during the Dionysos Nutrition and Liver Study[9,17]. FLI is based on four common anthropometric and biochemical measures (body mass index, waist circumference, gamma-glutamyltransferase and triglycerides) and has gained much attention because of its association with prevalent cardiovascular disease, incident type 2 diabetes mellitus (T2DM), and liver-related mortality[18-22]. More importantly for its ability to serve as surrogate marker of FL, FLI has been successfully cross-validated in external populations[23,24].

WHAT IS NON-ALCOHOLIC FATTY LIVER (DISEASE)?

FL is usually divided into alcoholic fatty liver (AFL) and non-alcoholic fatty liver (NAFL)[3,25].

NAFL is however just one part of the spectrum of liver disease that falls under the umbrella term of non-alcoholic fatty liver disease (NAFLD)[3]. It should be noted that we are using the term NAFL in a broader sense than that recently suggested by the American Gastroenterological Association (AGA), *i.e.*, the finding of 'steatosis without steatohepatitis' at LB[26].

Besides NAFL, the NAFLD spectrum includes steato-hepatitis (NASH), fibrosis, cirrhosis and hepatocarcinoma (HCC). The idea behind NAFLD as a spectrum of liver disease was that simple steatosis might progress to NASH and then to chronic liver disease. However, this idea has been increasingly challenged in the last decade[27]. Studies performed in Liver Centers have shown that, whereas about 20% of cases of NASH will develop liver fibrosis, simple steatosis will virtually never progress to NASH[1,2,28]. There is indeed the possibility that NAFL and NASH are twin but independent conditions and that triglyceride accumulation alone is protective, at least up to a certain degree, as far as liver outcomes are concerned[27,29].

NAFL(D) and AFL(D) cannot be distinguished at LB and their differentiation is based on the assessment of ethanol intake[3,25]. After exclusion of other causes of FL (mostly hepatitis B or hepatitis C virus infection and use of steatogenic drugs), the guidelines of the European Association for the Study of the Liver (EASL) suggest that NAFLD should be diagnosed when ethanol intake is less than or equal to 20 g/d in women and less than or equal to 30 g/d in men[30]. AGA guidelines suggest that NAFLD should be diagnosed when men consume less than or equal to 21 drinks per week and women consume less than or equal to 14 drinks per week[26]. Although the EASL and AGA cut-points are roughly equivalent, the former have the advantage of focusing on actual ethanol intake, possibly avoiding the problems associated with the choice of different "drink units"[31].

The NAFL(D) *vs* AFL(D) categorization is vulnerable to many criticisms[25]. Besides the obvious loss of information[32], the most important criticism is that such categorization hides the fact that obesity and alcohol interact in determining the prevalence and incidence of FL[25,33,34]. From a public health perspective, it is more useful to study the effect of alcohol intake on FL-related outcomes independently from other risk factors rather than dividing FL more or less arbitrarily into NAFL and AFL[10,17,25]. Another problem is that such a categorization assumes the use of an instrument accurate enough to detect small differences in ethanol intake. Even the 7-d weighted food record method that we employed in the Dionysos Nutrition and Liver study may not be accurate enough to detect such differences[9].

WHAT IS THE PREVALENCE OF FATTY LIVER?

FL is the most common liver disease in Western countries, and NAFLD is the most common reason for altered liver enzymes in primary care[30].

In the general population of the Dionysos Nutrition and Liver Study, 45% of individuals had any degree of FL at LUS[9,17]. Using a cut-point of 20 g/d for ethanol intake, 25% had NAFLD and 20% had AFLD[9]. A recent study performed in a large primary care practice has shown that nearly one in every three patients with persistently elevated alanine transaminase has NAFLD[35,36].

Systematic reviews estimate that about 20%-30% of individuals in Western countries have NAFLD[26] and similar figures are being increasingly provided for Eastern countries[37]. The prevalence of NAFLD increases with age, is highest in males between 40 and 65 years and is higher in Hispanics and lower in African-Americans[26,30,38]. The prevalence of NAFLD is increasing rapidly among children in parallel with the current epidemic of obesity[39].

LUS data from the third edition of the National Health and Nutrition Examination Survey (NHANES Ⅲ) (1988-1994) have recently been used to provide an estimate of the prevalence of FL in the general United

States population[40]. Although these data were collected more than 20 years ago and may underestimate the present prevalence of FL, they are unique because they were obtained in a representative sample of the general population. The age-adjusted prevalence of FL in NHANES Ⅲ, defined as moderate to severe FL at LUS, was 21% while that of NAFLD was 20%[40].

Because LB can be performed only in Liver Centers, it is unknown how many individuals in the general population have NASH or liver fibrosis. Projections made mostly on the basis of autopsy data suggest that 3%-5% of individuals in the general population might have NASH[2,41]. Using surrogate markers of liver fibrosis, it has been postulated that about 3% of individuals in the general population might have liver fibrosis[42].

WHAT IS THE INCIDENCE OF FATTY LIVER?

The incidence of LUS-determined FL (any degree) in the Dionysos Study was 2 per 1000 person-years[10] but values of up to 10 per 1000 person-years have been reported by other studies employing the same method[2,30].

WHAT IS THE NATURAL HISTORY OF FATTY LIVER?

Systematic reviews of studies performed in tertiary care centers have clearly shown that NASH is a risk factor for liver fibrosis, cirrhosis and HCC[1,2,28]. However, as determined by LUS, most cases of FL in the general population regress, especially in the presence of weight loss[10,43,44].

A recent longitudinal analysis of about 11000 individuals from NHANES Ⅲ has shown that LUS-determined NAFLD alone is not an independent predictor of mortality[45]. However, when considered together with advanced fibrosis - as detected by surrogate markers - NAFLD was associated with increased mortality independently of known risk factors[45]. Another recent analysis of the same NHANES Ⅲ data (with a different number of subjects because of different inclusion criteria) has shown that NAFLD may be an independent predictor of liver-related mortality in Whites[46]. Considering the different effect measures and statistical methods employed by these studies[45,46], their results are not necessarily at odds if one considers that the effect size of the 'positive' study was highly variable (relative risk of death attributable to NAFLD = 10.74, 95%CI: 1.17-98.54).

WHAT IS THE RELATIONSHIP BETWEEN FATTY LIVER AND METABOLIC SYNDROME?

There is no doubt that NAFLD is more common among obese individuals and those with metabolic syndrome (MS)[26,30]. Because of this association, it has become common to state that NAFLD is the "hepatic component" of MS[46]. However, this hypothesis has not undergone formal testing until very recently[47]. A confirmatory factor analysis of NHANES III cross-sectional data has indeed shown that NAFLD is more likely to be a separate entity rather than an additional component of MS[47]. Even if NAFLD is not the "hepatic component" of MS, however, it remains to be tested whether MS and NAFLD contribute independently to 'hard outcomes' in the general population. This is important also in view of the ongoing controversy about the clinical relevance of the MS concept[48-50].

Although NAFLD is most commonly associated with obesity, it is by no means uncommon in lean individuals. A recent analysis of NHANES Ⅲ data has shown that the prevalence of NAFLD in lean individuals, defined as those with body mass index $\leqslant$ 25 kg/m^2, is a quarter of that observed in overweight-obese individuals (7% *vs* 28%)[35]. Compared with its overweight-obese counterpart, 'lean NAFLD' is characterized by younger age, higher insulin sensitivity and lower frequency of MS[35].

WHAT IS THE RELATIONSHIP BETWEEN FATTY LIVER AND CARDIOMETABOLIC DISEASE?

Much of the interest in NAFLD among researchers and clinicians outside the field of Hepatology stems from its association with cardiometabolic disease[51-53].

In the last few years, an increasing number of cohort studies performed in the general population of Western and Eastern countries has shown that NAFLD, diagnosed by LUS or by surrogate markers such as FLI, is independently associated with incident T2DM[18,21,54,55]. The available evidence pointing to an association between NAFLD and incident cardiovascular disease (CVD) is presently of lower quality than that available for incident T2DM[54]. In a recent study performed in a tertiary CVD care center, NAFLD was associated with coronary artery disease but not with cardiovascular mortality[56]. Likewise, a recent analysis of NHANES Ⅲ cohort data showed that NAFLD was associated with incident CVD but not with CVD mortality[57].

The availability of long-term follow-up data in more or less representative samples of the general population will be central in coming years to improve our understanding of the NAFLD-CVD relationship.

REFERENCES

1 **Musso G**, Gambino R, Cassader M, Pagano G. Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. *Ann Med* 2011; **43**: 617-649 [PMID: 21039302 DOI: 10.3109/07853890.2010.518623]

2 **Vernon G**, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Ali-*

ment Pharmacol Ther 2011; **34**: 274-285 [PMID: 21623852 DOI: 10.1111/j.1365-2036.2011.04724.x]
3 **Bedogni G**, Bellentani S. Fatty liver: how frequent is it and why? *Ann Hepatol* 2004; **3**: 63-65 [PMID: 15257248]
4 **Brunt EM**. Nonalcoholic fatty liver disease: what the pathologist can tell the clinician. *Dig Dis* 2012; **30** Suppl 1: 61-68 [PMID: 23075870 DOI: 10.1159/000341127]
5 **Kleiner DE**, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313-1321 [PMID: 15915461 DOI: 10.1002/hep.20701]
6 **Ratziu V**, Bugianesi E, Dixon J, Fassio E, Ekstedt M, Charlotte F, Kechagias S, Poynard T, Olsson R. Histological progression of non-alcoholic fatty liver disease: a critical reassessment based on liver sampling variability. *Aliment Pharmacol Ther* 2007; **26**: 821-830 [PMID: 17767466 DOI: 10.1111/j.1365-2036.2007.03425.x]
7 **Ratziu V**, Charlotte F, Heurtier A, Gombert S, Giral P, Bruckert E, Grimaldi A, Capron F, Poynard T. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. *Gastroenterology* 2005; **128**: 1898-1906 [PMID: 15940625 DOI: 10.1053/j.gastro.2005.03.084]
8 **Festi D**, Schiumerini R, Marzi L, Di Biase AR, Mandolesi D, Montrone L, Scaioli E, Bonato G, Marchesini-Reggiani G, Colecchia A. Review article: the diagnosis of non-alcoholic fatty liver disease -- availability and accuracy of non-invasive methods. *Aliment Pharmacol Ther* 2013; **37**: 392-400 [PMID: 23278163 DOI: 10.1111/apt.12186]
9 **Bedogni G**, Miglioli L, Masutti F, Tiribelli C, Marchesini G, Bellentani S. Prevalence of and risk factors for nonalcoholic fatty liver disease: the Dionysos nutrition and liver study. *Hepatology* 2005; **42**: 44-52 [PMID: 15895401 DOI: 10.1002/hep.20734]
10 **Bedogni G**, Miglioli L, Masutti F, Castiglione A, Crocè LS, Tiribelli C, Bellentani S. Incidence and natural course of fatty liver in the general population: the Dionysos study. *Hepatology* 2007; **46**: 1387-1391 [PMID: 17685472 DOI: 10.1002/hep.21827]
11 **Hernaez R**, Lazo M, Bonekamp S, Kamel I, Brancati FL, Guallar E, Clark JM. Diagnostic accuracy and reliability of ultrasonography for the detection of fatty liver: a meta-analysis. *Hepatology* 2011; **54**: 1082-1090 [PMID: 21618575 DOI: 10.1002/hep.24452]
12 **Shannon A**, Alkhouri N, Carter-Kent C, Monti L, Devito R, Lopez R, Feldstein AE, Nobili V. Ultrasonographic quantitative estimation of hepatic steatosis in children With NAFLD. *J Pediatr Gastroenterol Nutr* 2011; **53**: 190-195 [PMID: 21788761 DOI: 10.1097/MPG.0b013e31821b4b61]
13 **Vajro P**, Lenta S, Socha P, Dhawan A, McKiernan P, Baumann U, Durmaz O, Lacaille F, McLin V, Nobili V. Diagnosis of nonalcoholic fatty liver disease in children and adolescents: position paper of the ESPGHAN Hepatology Committee. *J Pediatr Gastroenterol Nutr* 2012; **54**: 700-713 [PMID: 22395188 DOI: 10.1097/MPG.0b013e318252a13f]
14 **Awai HI**, Newton KP, Sirlin CB, Behling C, Schwimmer JB. Evidence and recommendations for imaging liver fat in children, based on systematic review. *Clin Gastroenterol Hepatol* 2014; **12**: 765-773 [PMID: 24090729 DOI: 10.1016/j.cgh.2013.09.050]
15 **Browning JD**, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004; **40**: 1387-1395 [PMID: 15565570 DOI: 10.1002/hep.20466]
16 **Qayyum A**. MR spectroscopy of the liver: principles and clinical applications. *Radiographics* 2009; **29**: 1653-1664 [PMID: 19959513 DOI: 10.1148/rg.296095520]
17 **Bedogni G**, Bellentani S, Miglioli L, Masutti F, Passalacqua M, Castiglione A, Tiribelli C. The Fatty Liver Index: a simple and accurate predictor of hepatic steatosis in the general population. *BMC Gastroenterol* 2006; **6**: 33 [PMID: 17081293 DOI: 10.1186/1471-230X-6-33]
18 **Jung CH**, Lee WJ, Hwang JY, Yu JH, Shin MS, Lee MJ, Jang JE, Leem J, Park JY, Kim HK. Assessment of the fatty liver index as an indicator of hepatic steatosis for predicting incident diabetes independently of insulin resistance in a Korean population. *Diabet Med* 2013; **30**: 428-435 [PMID: 23278318 DOI: 10.1111/dme.12104]
19 **Kozakova M**, Palombo C, Eng MP, Dekker J, Flyvbjerg A, Mitrakou A, Gastaldelli A, Ferrannini E. Fatty liver index, gamma-glutamyltransferase, and early carotid plaques. *Hepatology* 2012; **55**: 1406-1415 [PMID: 22334565 DOI: 10.1002/hep.25555]
20 **Calori G**, Lattuada G, Ragogna F, Garancini MP, Crosignani P, Villa M, Bosi E, Ruotolo G, Piemonti L, Perseghin G. Fatty liver index and mortality: the Cremona study in the 15th year of follow-up. *Hepatology* 2011; **54**: 145-152 [PMID: 21488080 DOI: 10.1002/hep.24356]
21 **Balkau B**, Lange C, Vol S, Fumeron F, Bonnet F. Nine-year incident diabetes is predicted by fatty liver indices: the French D.E.S.I.R. study. *BMC Gastroenterol* 2010; **10**: 56 [PMID: 20529259 DOI: 10.1186/1471-230X-10-56]
22 **Gastaldelli A**, Kozakova M, Højlund K, Flyvbjerg A, Favuzzi A, Mitrakou A, Balkau B. Fatty liver is associated with insulin resistance, risk of coronary heart disease, and early atherosclerosis in a large European population. *Hepatology* 2009; **49**: 1537-1544 [PMID: 19291789 DOI: 10.1002/hep.22845]
23 **Zelber-Sagi S**, Webb M, Assy N, Blendis L, Yeshua H, Leshno M, Ratziu V, Halpern Z, Oren R, Santo E. Comparison of fatty liver index with noninvasive methods for steatosis detection and quantification. *World J Gastroenterol* 2013; **19**: 57-64 [PMID: 23326163 DOI: 10.3748/wjg.v19.i1.57]
24 **Koehler EM**, Schouten JN, Hansen BE, Hofman A, Stricker BH, Janssen HL. External validation of the fatty liver index for identifying nonalcoholic fatty liver disease in a population-based study. *Clin Gastroenterol Hepatol* 2013; **11**: 1201-1204 [PMID: 23353640 DOI: 10.1016/j.cgh.2012.12.031]
25 **Völzke H**. Multicausality in fatty liver disease: is there a rationale to distinguish between alcoholic and non-alcoholic origin? *World J Gastroenterol* 2012; **18**: 3492-3501 [PMID: 22826613 DOI: 10.3748/wjg.v18.i27.3492]
26 **Chalasani N**, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, Charlton M, Sanyal AJ. The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* 2012; **55**: 2005-2023 [PMID: 22488764 DOI: 10.1002/hep.25762]
27 **Yilmaz Y**. Review article: is non-alcoholic fatty liver disease a spectrum, or are steatosis and non-alcoholic steatohepatitis distinct conditions? *Aliment Pharmacol Ther* 2012; **36**: 815-823 [PMID: 22966992]
28 **Argo CK**, Northup PG, Al-Osaimi AM, Caldwell SH. Systematic review of risk factors for fibrosis progression in non-alcoholic steatohepatitis. *J Hepatol* 2009; **51**: 371-379 [PMID: 19501928 DOI: 10.1016/j.jhep.2009.03.019]
29 **Tilg H**, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology* 2010; **52**: 1836-1846 [PMID: 21038418 DOI: 10.1002/hep.24001]
30 **Ratziu V**, Bellentani S, Cortez-Pinto H, Day C, Marchesini G. A position statement on NAFLD/NASH based on the EASL 2009 special conference. *J Hepatol* 2010; **53**: 372-384 [PMID: 20494470 DOI: 10.1016/j.jhep.2010.04.008]
31 **MacDonald I**. Health issues related to alcohol consumption. Malden, MA: Blackwell Science Ltd., 1999
32 **Kuss O**. The danger of dichotomizing continuous variables: A visualization. *Teaching Statistics* 2013; **35**: 78-79 [DOI: 10.1111/test.12006]

33 **Bellentani S**, Tiribelli C, Bedogni G. Alcohol and Nutrition as Risk Factors for Chronic Liver Disease. In: Alcohol, Nutrition, and Health Consequences. Totowa, NJ: Humana Press, 2013: 497-506

34 **Bellentani S**, Saccoccio G, Masutti F, Crocè LS, Brandi G, Sasso F, Cristanini G, Tiribelli C. Prevalence of and risk factors for hepatic steatosis in Northern Italy. *Ann Intern Med* 2000; **132**: 112-117 [PMID: 10644271 DOI: 10.7326/0003-4819-132-2-200001180-00004]

35 **Armstrong MJ**, Houlihan DD, Bentham L, Shaw JC, Cramb R, Olliff S, Gill PS, Neuberger JM, Lilford RJ, Newsome PN. Presence and severity of non-alcoholic fatty liver disease in a large prospective primary care cohort. *J Hepatol* 2012; **56**: 234-240 [PMID: 21703178 DOI: 10.1016/j.jhep.2011.03.020]

36 **Ratziu V**, Voiculescu M, Poynard T. Touching some firm ground in the epidemiology of NASH. *J Hepatol* 2012; **56**: 23-25 [PMID: 21875499 DOI: 10.1016/j.jhep.2011.08.002]

37 **Fan JG**. Epidemiology of alcoholic and nonalcoholic fatty liver disease in China. *J Gastroenterol Hepatol* 2013; **28** Suppl 1: 11-17 [PMID: 23855290 DOI: 10.1111/jgh.12036]

38 **Younossi ZM**, Stepanova M, Negro F, Hallaji S, Younossi Y, Lam B, Srishord M. Nonalcoholic fatty liver disease in lean individuals in the United States. *Medicine* (Baltimore) 2012; **91**: 319-327 [PMID: 23117851 DOI: 10.1097/MD.0b013e3182779d49]

39 **Giorgio V**, Prono F, Graziano F, Nobili V. Pediatric non alcoholic fatty liver disease: old and new concepts on development, progression, metabolic insight and potential treatment targets. *BMC Pediatr* 2013; **13**: 40 [PMID: 23530957 DOI: 10.1186/1471-2431-13-40]

40 **Lazo M**, Hernaez R, Eberhardt MS, Bonekamp S, Kamel I, Guallar E, Koteish A, Brancati FL, Clark JM. Prevalence of nonalcoholic fatty liver disease in the United States: the Third National Health and Nutrition Examination Survey, 1988-1994. *Am J Epidemiol* 2013; **178**: 38-45 [PMID: 23703888 DOI: 10.1093/aje/kws448]

41 **Scaglioni F**, Ciccia S, Marino M, Bedogni G, Bellentani S. ASH and NASH. *Dig Dis* 2011; **29**: 202-210 [PMID: 21734385 DOI: 10.1159/000323886]

42 **Poynard T**, Lebray P, Ingiliz P, Varaut A, Varsat B, Ngo Y, Norha P, Munteanu M, Drane F, Messous D, Bismut FI, Carrau JP, Massard J, Ratziu V, Giordanella JP. Prevalence of liver fibrosis and risk factors in a general population using non-invasive biomarkers (FibroTest). *BMC Gastroenterol* 2010; **10**: 40 [PMID: 20412588 DOI: 10.1186/1471-230X-10-40]

43 **Zelber-Sagi S**, Ratziu V, Oren R. Nutrition and physical activity in NAFLD: an overview of the epidemiological evidence. *World J Gastroenterol* 2011; **17**: 3377-3389 [PMID: 21876630 DOI: 10.3748/wjg.v17.i29.3377]

44 **Zelber-Sagi S**, Lotan R, Shlomai A, Webb M, Harrari G, Buch A, Nitzan Kaluski D, Halpern Z, Oren R. Predictors for incidence and remission of NAFLD in the general population during a seven-year prospective follow-up. *J Hepatol* 2012; **56**: 1145-1151 [PMID: 22245895 DOI: 10.1016/j.jhep.2011.12.011]

45 **Kim D**, Kim WR, Kim HJ, Therneau TM. Association between noninvasive fibrosis markers and mortality among adults with nonalcoholic fatty liver disease in the United States. *Hepatology* 2013; **57**: 1357-1365 [PMID: 23175136 DOI: 10.1002/hep.26156]

46 **Otgonsuren M**, Stepanova M, Gerber L, Younossi ZM. Anthropometric and clinical factors associated with mortality in subjects with nonalcoholic fatty liver disease. *Dig Dis Sci* 2013; **58**: 1132-1140 [PMID: 23143735 DOI: 10.1007/s10620-012-2446-3]

47 **Smits MM**, Ioannou GN, Boyko EJ, Utzschneider KM. Non-alcoholic fatty liver disease as an independent manifestation of the metabolic syndrome: results of a US national survey in three ethnic groups. *J Gastroenterol Hepatol* 2013; **28**: 664-670 [PMID: 23286209 DOI: 10.1111/jgh.12106]

48 **Reaven GM**. The metabolic syndrome: time to get off the merry-go-round? *J Intern Med* 2011; **269**: 127-136 [PMID: 21129047 DOI: 10.1111/j.1365-2796.2010.02325.x]

49 **Simmons RK**, Alberti KG, Gale EA, Colagiuri S, Tuomilehto J, Qiao Q, Ramachandran A, Tajima N, Brajkovich Mirchov I, Ben-Nakhi A, Reaven G, Hama Sambo B, Mendis S, Roglic G. The metabolic syndrome: useful concept or clinical tool? Report of a WHO Expert Consultation. *Diabetologia* 2010; **53**: 600-605 [PMID: 20012011 DOI: 10.1007/s00125-009-1620-4]

50 **Beckstead JW**, Beckie TM. How much information can metabolic syndrome provide? An application of information theory. *Med Decis Making* 2011; **31**: 79-92 [PMID: 20729508 DOI: 10.1177/0272989X10373401]

51 **Nestel PJ**, Mensink RP. Perspective: nonalcoholic fatty liver disease and cardiovascular risk. *Curr Opin Lipidol* 2013; **24**: 1-3 [PMID: 23298957 DOI: 10.1097/MOL.0b013e32835c0834]

52 **Williams KH**, Shackel NA, Gorrell MD, McLennan SV, Twigg SM. Diabetes and nonalcoholic Fatty liver disease: a pathogenic duo. *Endocr Rev* 2013; **34**: 84-129 [PMID: 23238855 DOI: 10.1210/er.2012-1009]

53 **Anstee QM**, Targher G, Day CP. Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 330-344 [PMID: 23507799 DOI: 10.1038/nrgastro.2013.41]

54 **Ghouri N**, Preiss D, Sattar N. Liver enzymes, nonalcoholic fatty liver disease, and incident cardiovascular disease: a narrative review and clinical perspective of prospective data. *Hepatology* 2010; **52**: 1156-1161 [PMID: 20658466 DOI: 10.1002/hep.23789]

55 **Sung KC**, Wild SH, Byrne CD. Resolution of fatty liver and risk of incident diabetes. *J Clin Endocrinol Metab* 2013; **98**: 3637-3643 [PMID: 23873989 DOI: 10.1210/jc.2013-1519]

56 **Wong VW**, Wong GL, Yip GW, Lo AO, Limquiaco J, Chu WC, Chim AM, Yu CM, Yu J, Chan FK, Sung JJ, Chan HL. Coronary artery disease and cardiovascular outcomes in patients with non-alcoholic fatty liver disease. *Gut* 2011; **60**: 1721-1727 [PMID: 21602530 DOI: 10.1136/gut.2011.242016]

57 **Stepanova M**, Younossi ZM. Independent association between nonalcoholic fatty liver disease and cardiovascular disease in the US population. *Clin Gastroenterol Hepatol* 2012; **10**: 646-650 [PMID: 22245962 DOI: 10.1016/j.cgh.2011.12.039]

P- Reviewers: Sanal MG, Shen WJ **S- Editor**: Zhai HH
L- Editor: Cant MR **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9055

World J Gastroenterol 2014 July 21; 20(27): 9055-9071
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease and the heart in children and adolescents

Lucia Pacifico, Claudio Chiesa, Caterina Anania, Antonio De Merulis, John Frederick Osborn, Sara Romaggioli, Eugenio Gaudio

Lucia Pacifico, Caterina Anania, Antonio De Merulis, Sara Romaggioli, Department of Pediatrics and Child Neuropsychiatry, Sapienza University of Rome, 00161 Rome, Italy
Claudio Chiesa, Institute of Translational Pharmacology, National Research Council, 00133 Rome, Italy
John Frederick Osborn, Department of Public Health and Infectious Diseases, Sapienza University of Rome, 00161 Rome, Italy
Eugenio Gaudio, Department of Human Anatomy, Histology, Forensic Medicine and Orthopedics Sciences, Sapienza University of Rome, 00161 Rome, Italy
Author contributions: Pacifico L, Chiesa C, Osborn JF and Gaudio E designed the study, analyzed the data and wrote the manuscript; Anania C, De Merulis A and Romaggioli S collected the data; all the authors participated in the critical review and in the final approval of the manuscript.
Correspondence to: Claudio Chiesa, MD, Institute of Translational Pharmacology, National Research Council, Via Fosso del Cavaliere, 100, 00133-Rome, Italy. claudio.chiesa@ift.cnr.it
Telephone: +39-6-49979215 Fax: +39-6-49979216
Received: November 26, 2013 Revised: February 7, 2014
Accepted: May 12, 2014
Published online: July 21, 2014

Abstract

Over the last two decades, the rise in the prevalence rates of overweight and obesity explains the emergence of nonalcoholic fatty liver disease (NAFLD) as the leading cause of chronic liver disease worldwide. As described in adults, children and adolescents with fatty liver display insulin resistance, glucose intolerance, and dyslipidemia. Thus NAFLD has emerged as the hepatic component of the metabolic syndrome (MetS) and a strong cardiovascular risk factor even at a very early age. Several studies, including pediatric populations, have reported independent associations between NAFLD and markers of subclinical atherosclerosis including impaired flow-mediated vasodilation, increased carotid artery intima-media thickness, and arterial stiffness, after adjusting for cardiovascular risk factors and MetS. Also, it has been shown that NAFLD is associated with cardiac alterations, including abnormal left ventricular structure and impaired diastolic function. The duration of these subclinical abnormalities may be important, because treatment to reverse the process is most likely to be effective earlier in the disease. In the present review, we examine the current evidence on the association between NAFLD and atherosclerosis as well as between NAFLD and cardiac dysfunction in the pediatric population, and discuss briefly the possible biological mechanisms linking NAFLD and cardiovascular changes. We also address the approach to treatment for this increasingly prevalent disease, which is likely to have an important future global impact on the burden of ill health, to prevent not only end-stage liver disease but also cardiovascular disease.

Key words: Nonalcoholic fatty liver disease; Children; Atherosclerosis; Cardiac structure; Cardiac function

Core tip: Nonalcoholic fatty liver disease (NAFLD) is an important and emerging health problem in childhood. It is recognized as part of the metabolic syndrome and especially the necroinflammatory form is associated with a high risk for the development of functional and structural vascular changes as well as left ventricular dysfunction at an early age. In addition, there seems to be a complex bidirectional relationship between the progression to nonalcoholic steatohepatitis (NASH) and the development of insulin resistance and cardiovascular abnormalities. Early intervention during childhood to recognize NAFLD, as well as to prevent its progression to NASH, may be a crucial step in averting an unfavorable cardiac phenotype.

Pacifico L, Chiesa C, Anania C, De Merulis A, Osborn JF, Romaggioli S, Gaudio E. Nonalcoholic fatty liver disease and the heart in children and adolescents. *World J Gastroenterol* 2014; 20(27): 9055-9071 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9055.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9055

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of fat-associated liver conditions that can result in end-stage liver disease and the need for liver transplantation[1]. Simple steatosis, or fatty liver, occurs early in NAFLD and may progress to nonalcoholic steatohepatitis (NASH), fibrosis and cirrhosis with increased risk of hepatocellular carcinoma[1]. Over the last two decades, the rise in the prevalence rates of overweight and obesity explains the emergence of NAFLD as the leading cause of chronic liver disease in pediatric populations worldwide[2,3]. The liver is one of the main ectopic sites where lipids may accumulate in obese subjects. Ectopic fat disposition occurs particularly when the energy storage capacity of the adipose tissue is exceeded, leading to increased net lipid flux to non-adipose organs, thereby causing lipotoxiciy and insulin resistance[4,5]. As described in adults, children and adolescents with fatty liver display insulin resistance, glucose intolerance, and dyslipidemia[6,7]. Thus NAFLD has emerged as the hepatic component of the metabolic syndrome (MetS)[8] and a strong cardiovascular risk factor even at a very early age[9,10].

Several studies (including pediatric populations) have reported independent associations between NAFLD and markers of subclinical atherosclerosis such as impaired flow-mediated vasodilation (FMD), increased carotid artery intima-media thickness (cIMT) and arterial stiffness, after adjusting for cardiovascular risk factors and MetS[9-14]. Also, it has been shown that NAFLD is associated with cardiac alterations, including myocardial insulin resistance[15], altered cardiac energy metabolism[16], abnormal left ventricular (LV) structure and impaired diastolic function[17,18]. The duration of these subclinical abnormalities may be important, because treatment to reverse the process is most likely to be effective earlier in the disease. In the present review, we examine the current evidence on the association between NAFLD and subclinical atherosclerosis as well as between NAFLD and cardiac dysfunction in the pediatric population, and discuss briefly the possible biological mechanisms linking NAFLD and cardiovascular changes. We also address the approach to treatment for this increasingly prevalent disease, which is likely to have an important future global impact on the burden of ill health, to prevent not only end-stage liver disease but also cardiovascular disease (CVD).

This is a clinical, narrative review and not a systematic review and meta-analysis. PubMed was extensively searched for articles using keywords and mesh terms: "nonalcoholic fatty liver disease", "fatty liver", "cardiovascular risk", "atherosclerosis", "endothelial dysfunction", arterial stiffness", "cardiac structure", "cardiac dysfunction", and "children".

NAFLD AND MARKERS OF SUBCLINICAL ATHEROSCLEROSIS

Pathologic studies have shown that atherosclerosis is an early process beginning in childhood, with fatty streaks observed in the aorta and the coronary and carotid arteries of children and adolescents[19,20]. Early assessment of the arterial damage is therefore important to prevent future vascular risk since subclinical atherosclerosis can be reversible if detected early and intervention is provided[10].

Several studies have focused on the relation between NAFLD and atherosclerosis in the pediatric population (Table 1)[9,14,21-36]. In the earliest study, involving 817 children (aged 2 to 19 years) who died of external causes (accident, homicide, suicide) from 1993 to 2003, Schwimmer *et al*[21] showed that the prevalence of atherosclerosis was increased by a factor of 2 among those with NAFLD. Fatty liver was present in 15% of the children. Mild atherosclerosis was present in 21% of the children, and moderate to severe atherosclerosis in 2%. Atherosclerosis was significantly more common in children with fatty liver than those without. Body mass index (BMI) was not independently associated with the presence of atherosclerosis, but fatty liver status and BMI did interact significantly ($P < 0.01$). Consequently, for obese subjects, the odds of having atherosclerosis was more than 6 times higher in children with fatty liver than those without[21]. Despite this, there are few data regarding the possible association between liver histopathologic changes and atherogenic risk in children.

Atherogenic dyslipidemia

In the Bogalusa Heart study in children, investigators found that the extent to which the intimal surface was covered with atherosclerotic lesions was significantly associated with elevation of concentrations of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), triglycerides (TG), and lower concentration of high-density lipoprotein cholesterol (HDL-c)[37]. In particular, the TC/HDL-c, LDL-c/HDL-c, and TG/HDL-c ratios have been reported as useful markers of atherogenic lipid abnormalities, as well as of insulin resistance, MetS, and high cardiovascular risk[38,39]. Schwimmer *et al*[9] in a case-control study involving a large clinical sample of overweight and obese children and adolescents, showed that children with a biopsy-proven NAFLD had a significantly higher fasting glucose, insulin, TC, LDL-c, TG, systolic and diastolic blood pressure than age-, gender-, and BMI-matched peers without NAFLD. Thus, obese children and adolescents with a definitive diagnosis of NAFLD had a more severe cardiovascular risk profile. Nobili *et al*[27], in a large group of consecutively recruited

Table 1 Studies of the association between nonalcoholic fatty liver disease and markers of atherosclerosis in children and adolescents

Ref.	Study population and sample size	Diagnosis	Outcome	Main results
Schwimmer *et al*[21]	Children (n = 817) who died of external causes from 1993 to 2003; 15% with NAFLD	Autoptic liver biopsy	Atherosclerosis was assessed as absent, mild (aorta only), moderate (coronary artery streaks/plaques), or severe (coronary artery narrowing)	For the entire cohort, mild atherosclerosis was present in 21% and moderate to severe in 2%. Atherosclerosis was significantly more common in children with fatty liver than those without (30% *vs* 19%, P < 0.001)
Schwimmer *et al*[9]	Overweight children with (n = 150), and without (n = 150) NAFLD, matched for gender, age, and severity of obesity	Liver biopsy	Prevalence of cardiovascular risk factors (abdominal obesity, dyslipidemia, hypertension, IR, and glucose abnormalities)	NAFLD was strongly associated with multiple cardiovascular risk factors independently of both BMI and hyperinsulinemia
Pacifico *et al*[22]	Obese children with (n = 29), and without NAFLD (n = 33); healthy lean controls (n = 30)	Liver ultrasound	cIMT, mean (95%CI)	NAFLD *vs* no NAFLD and controls: 0.58 (0.54-0.62) mm *vs* 0.49 (0.46-0.52) mm and 0.40 (0.36-0.43) mm; P < 0.01 and P < 0.0005, respectively Log cIMT was associated with NAFLD severity in a multiple linear regression analysis adjusted for age, gender, Tanner stage, and cardiovascular risk factors Coefficient b, 0.08; P < 0.0005
Demircioğlu *et al*[23]	Obese children with mild (n = 32), moderate-severe NAFLD (n = 22), and without NAFLD (n = 26); healthy lean controls (n = 30) matched for age and gender	Liver ultrasound	cIMT, mean ± SD	All obese *vs* controls: Left CCA, 0.414 ± 0.071 mm *vs* 0.352 ± 0.054 mm, P < 0.0001 Left CB, 0.412 ± 0.067 mm *vs* 0.350 ± 0.058 mm, P < 0.0001 Left ICA, 0.324 ± 0.068 mm *vs* 0.266 ± 0.056 mm, P < 0.0001 NAFLD was significantly associated with left CCA, CB, ICA in multiple regression linear analyses adjusted for age, gender, weight, mean ALT level, TC, obesity, and grade of hepatosteatosis CCA = standardized β, 0.451; P = 0.01 CB = standardized β, 0.627; P < 0.0001 ICA = standardized β, 0.501; P = 0.020
Kelishadi *et al*[24]	Obese adolescents with (n = 25), and without (n = 25) components of MetS; normal weight adolescents with (n = 25) and without (n = 25) components of MetS	Liver ultrasound and elevated ALT	cIMT, mean ± SD	NWMN *vs* NWMA *vs* POMN *vs* POMA: 0.29 ± 0.02 mm *vs* 0.37 ± 0.04 mm *vs* 0.41 ± 0.05 mm; the differences were significant between groups with the exception of NWMA *vs* POMN cIMT was significantly associated with NAFLD in a logistic regression analysis after adjustment for age, gender and pubertal status Odds ratio, 1.2 (95%CI: 1.03-2.1)
Manco *et al*[25]	Overweight and obese children with (n = 31), and without (n = 49) NAFLD, matched for age, gender, and BMI	Liver biopsy	cIMT, median (IQR)	NAFLD *vs* no NAFLD: Right cIMT, 0.47 (0.07) mm *vs* 0.48 (0.05) mm, P = 0.659 Left cIMT, 0.49 (0.12) mm *vs* 0.47 (0.05) mm, P = 0.039 NAFLD was not associated with cIMT in a multivariate analysis
Caserta *et al*[26]	Randomly selected adolescents (n = 642) of whom 30.5% and 13.5% were, respectively, overweight and obese. Overall prevalence of NAFLD, 12.5%	Liver ultrasound	cIMT, mean (95%CI)	NAFLD *vs* no NAFLD: 0.417 (0.409-0.425) mm *vs* 0.395 (0.392-0.397) mm, P < 0.001 NAFLD was significantly associated with cIMT in a multivariate analysis after adjustment for age, BP, BMI, TG, c-HDL, TC, IR, MetS, grade of steatosis Standardized β, 0.0147 (95%CI: 0.0054-0.0240); P = 0.002
Pacifico *et al*[14]	Obese children with (n = 100), and without (n = 150) NAFLD; healthy lean controls (n = 150)	Liver ultrasound and elevated ALT	cIMT and FMD, mean (95%CI)	Controls and no NAFLD *vs* NAFLD: cIMT, 0.47 (0.46-0.48) mm and 0.52 (0.50-0.54) mm *vs* 0.55 (0.53-0.54) mm, P < 0.0001 and P < 0.01, respectively FMD, 15.0 (13.9-17.3) and 11.8 (10.1-13.7) *vs* 6.7 (5.0-8.6) %, P < 0.01 and P < 0.001 respectively NAFLD was associated with low FMD and increased cIMT in a multiple logistic regression analysis after adjustment for age, gender, Tanner stage, and MetS Odds ratio, 2.31 (95%CI: 1.35-3.97); P = 0.002 and 1.99 (95%CI: 1.18-3.38); P = 0.010, respectively
Nobili *et al*[27]	Children with NAFLD (n = 118)	Liver biopsy	Atherogenic lipid profile (TG/HDL-c, TC/HDL-c and LDL-c/HDL-c ratios)	The severity of liver injury was strongly associated with a more atherogenic profile, independently of BMI, insulin resistance, and the presence of MetS

Weghuber *et al*[28]	Obese children with (*n* = 14), and without (*n* = 14) NAFLD	Proton MR spectroscopy	FMD, mean ± SD	NAFLD *vs* no NAFLD: 108.6% ± 11.8% *vs* 110.7% ± 9.0%; *P* = 0.41
El-Koofy *et al*[29]	Overweight/obese children (*n* = 33)	Liver biopsy	Atherogenic lipid profile (TC, LDL-c, HDL-c, TG)	Children with NAFLD had significantly higher TC, LDL-c, TG and lower HDL-c compared to patients with normal liver histology (*P* < 0.05)
Sert *et al*[30]	Obese children with (*n* = 44), and without (*n* = 36) NAFLD; lean subjects (*n* = 37)	Liver ultrasound and elevated ALT	cIMT, mean ± SD	Lean and no NAFLD *vs* NAFLD: 0.0359 ± 0.012 mm *vs* 0.378 ± 0.017 mm *vs* 0.440 ± 0.026 mm, *P* < 0.05 and *P* < 0.05, respectively
Akın *et al*[31]	Obese children with (*n* = 56), and without (*n* = 101) NAFLD	Liver ultrasound	cIMT, mean (95%CI)	NAFLD *vs* no NAFLD: 0.48 (0.47-0.49) mm *vs* 0.45 (0.44-0.45) mm, *P* < 0.001 NAFLD was the only variable associated with increased cIMT in a multiple regression adjusted for age and gender β, 0.031 [SE (β) = 0.008]; *P* < 0.001
Gökçe *et al*[32]	Obese children with (*n* = 50), and without (*n* = 30) NAFLD; healthy lean controls (*n* = 30)	Liver ultrasound	cIMT, mean ± SD	NAFLD *vs* no NAFLD *vs* control group: Right cIMT, 0.46 ± 0.21 mm *vs* 0.35 ± 0.09 mm *vs* 0.30 ± 0.13 mm, *P* < 0.01 Left cIMT, 0.44 ± 0.09 mm *vs* 0.35 ± 0.08 mm *vs* 0.27 ± 0.04 mm, *P* < 0.01 NAFLD was the only variable associated with increased cIMT in a multiple regression adjusted for age, gender, BMI, BP, TG, HDL-c, IR and MetS Right cIMT = β, 0.241; *P* < 0.05 Left cIMT = β, 0.425; *P* < 0.01
Sert *et al*[33]	Obese children with (*n* = 97), and without (*n* = 83) NAFLD; lean subjects (*n* = 68)	Liver ultrasound and elevated ALT	cIMT, mean ± SD	Lean and no NAFLD *vs* NAFLD: 0.354 ± 0.009 mm *vs* 0.383 ± 0.019 mm *vs* 0.437 ± 0.028 mm; *P* < 0.05 and *P* < 0.05, respectively
Alp *et al*[34]	Obese children with (*n* = 93), and without (*n* = 307) NAFLD; healthy lean controls (*n* = 150)	Liver ultrasound	cIMT, mean ± SD	Severe NAFLD *vs* mild NAFLD *vs* no NAFLD *vs* controls: 0.09 ± 0.01 cm *vs* 0.10 ± 0.01 cm *vs* 0.09 ± 0.01 cm *vs* 0.06 ± 0.01 cm, *P* < 0.001
Huang *et al*[35]	Adolescents (*n* = 964)	Liver ultrasound	PWV, mean ± SD	No NAFLD, low metabolic risk *vs* NAFLD, low metabolic risk *vs* no NAFLD, high metabolic risk *vs* NAFLD, high metabolic risk: males, 6.6 ± 0.7 m/s *vs* 6.7 ± 0.6 m/s *vs* 6.9 ± 1.0 m/s; females, 6.2 ± 0.7 m/s *vs* 6.3 ± 0.7 m/s *vs* 6.5 ± 0.7 m/s *vs* 6.4 ± 0.6 m/s Males and females who had NAFLD in the presence of the metabolic cluster had greater PWV b, 0.20 (95%CI: 0.01-0.38); *P* = 0.037
Jin *et al*[36]	Obese children (*n* = 71), and healthy controls (*n* = 47)	Liver ultrasound	PWV, mean ± SD	Obese *vs* controls: 4.54 ± 0.66 m/s *vs* 3.70 ± 0.66 m/s, *P* < 0.001 Fatty liver was positively correlated with PWV (*P* < 0.01)

NAFLD: Nonalcoholic fatty liver disease; IR: Insulin resistance; BMI: Body mass index; cIMT: Carotid intima media thickness; CCA: Common carotid artery; CB: Carotid bulbus; ICA: Internal carotid artery; ALT: Alanine aminotransferase; TC: Total cholesterol; MetS: Metabolic syndrome; NWMN: Normal weight metabolically normal; NWMA: Normal weight metabolically abnormal; POMN: Phenotipically obese metabolically normal; POMA: Phenotipically obese metabolically abnormal; IQR: Interquartile range; BP: Blood pressure; TG: Triglycerides; FMD: Flow-mediated dilation; HDL-c: High-density lipoprotein cholesterol; LDL-c: Low-density lipoprotein cholesterol; MR: Magnetic resonance; PWV: Pulse wave velocity.

children with liver biopsy-proven NAFLD, found that the NAFLD activity and fibrosis scores had significant positive correlations with TG/HDL-c, TC/HDL-c, and LDL-c/HDL-c ratios. After adjusting for potential confounders including BMI, insulin resistance, impaired glucose tolerance, and presence of MetS, both NAFLD activity score and stage of fibrosis remained independent predictors of an atherogenic lipid profile. The lipid ratios were found to be markedly higher in children with established NASH compared with those with simple steatosis or borderline disease, indicating that severity of liver injury in children with NAFLD is strongly associated with increased atherogenic risk. Very recently, El-Koofy *et al*[29] studied the prevalence of MetS, insulin resistance, and NAFLD in a small group of overweight/obese children presenting with hepatomegaly and/or raised alanine aminotransferase. Laboratory analysis included fasting blood glucose, serum insulin, TG, HDL-c, LDL-c and liver biochemical profile in addition to liver biopsy. They found a close association between obesity, MetS, insulin resistance and NAFLD. Children with NAFLD had significantly higher TC, LDL-c, TG, fasting insulin, and lower HDL-c

compared to patients with normal liver histology.

Vascular imaging

Recent improvements in imaging technology have identified early vascular changes that can be assessed noninvasively using ultrasonography[40-42]. These early changes include impairment of FMD, arterial stiffness, and increased cIMT. Measurement of these changes have been shown to be good surrogate markers for atherosclerosis disease identification and progression as well as of future clinical cardiovascular events[43,44].

Carotid intima-media thickness and endothelial dysfunction

Pacifico *et al*[22] first showed that severity of ultrasonographycally diagnosed NAFLD in obese children was significantly associated with cIMT, independently of anthropometric and metabolic features. Similarly, Demircioğlu *et al*[23], in a subsequent study, observed an association between ultrasonographically detected NAFLD and cIMT measured at the common artery, carotid bulb and internal carotid artery. Moreover, there was an increase in cIMT values of each segment with the increase in grades of hepatosteatosis. Kelishadi *et al*[24] reported a significant association between cIMT and NAFLD in children and adolescents, suggesting that the liver and the vessels share common mediators. In a case-control study involving a mixed population of 80 overweight and mildly obese children of whom 31 had biopsy-proven NAFLD, and 49 had no ultrasound evidence of NAFLD as well as no abnormal levels of aminotransferases, Manco *et al*[25] found that cIMT were significantly higher on the left side in NAFLD cases, though there was a substantial overlap of cIMT values between cases and controls. No association was found between cIMT and histologic severity of steatosis, NAFLD activity score, and fibrosis.

The association between NAFLD and carotid atherosclerosis has also been determined in a large, randomly selected adolescent population from Reggio Calabria, a town in southern Italy[26]. The authors found that NAFLD, as well as BMI, waist circumference, and systolic blood pressure were independent markers of increased cIMT. In a study involving a large sample size, Pacifico *et al*[14] showed that obese children with ultrasound-diagnosed NAFLD have a significantly lower FMD response and increased cIMT compared to obese children without NAFLD independently of other cardiovascular risk factors and MetS, and that obese children exibit more functional and morphologic vascular changes than healthy lean controls, regardless of liver involvement. The large number of subjects in that study may in part account for the associations the authors were able to identify between NAFLD and functional vascular changes, in contrast to the study by Weghuber *et al*[28] in which a very small sample of obese children with NAFLD [diagnosed by nuclear magnetic resonance spectroscopy (MRS)] had a FMD response similar to those without NAFLD. In a recent study, Akın *et al*[31] demonstrated that obese children and adolescents with ultrasonographycally detected NAFLD had higher cIMT than those without NAFLD regardless of association with elevated liver enzymes. Moreover, there was a statistically significant correlation between cIMT values and the grade of NAFLD. Similar results were obtained by Gökçe *et al*[32] and by Sert *et al*[30,33] in obese children with ultrasound diagnosed NAFLD. Gökçe *et al*[32] found that cIMT was significantly higher in obese with NAFLD than in obese children without NAFLD and in a control group. After adjusting for potential confounders (age, blood pressure, BMI, TG, HDL-c, insulin resistance, and MetS), NAFLD was observed to be strongly correlated with cIMT. Sert and colleagues[30,33] confirmed that children with NAFLD have significantly higher cIMT values than those without NAFLD and the lean group. Components of MetS, such as dyslipidemia, elevated fasting glucose levels and insulin resistance did not show a significant association with increased cIMT. Finally, in a very recent study, Alp *et al*[34] demonstrated that cIMT was significantly higher in children with ultrasonographycally detected NAFLD than the control subjects. Additionally, there was an increase in cIMT values with the increase in grades of liver steatosis.

Arterial stiffness

A few pediatric studies have analyzed the relationship between NAFLD and carotid artery stiffness. In a well-defined community-based cohort of Australian adolescents, Huang *et al*[35] aimed to examine the association between NAFLD (diagnosed by ultrasound), MetS, and arterial stiffness as measured by applanation tonometry. The enrolled subjects were identified at "high metabolic risk" and at "low metabolic risk" according to systolic blood pressure, homeostasis model assessment of insulin resistance (HOMA-IR), TG, and BMI. The authors found that NAFLD is associated with increased arterial stiffness only in the presence of the "high risk" metabolic cluster. Jin *et al*[36], in an attempt to identify a marker of early vascular functional change in obese children, compared carotid artery stiffness parameters (*i.e.*, compliance coefficient, stiffness index, and pulse wave velocity, obtained with ultrasound radiofrequency technology) in obese children and healthy controls. Arterial stiffness was higher in obese children than in healthy subjects; in addition the authors demonstrated that in obese children the carotid arterial stiffness parameters, especially the pulse wave velocity, were correlated with obesity-related risk factors, the systolic blood pressure, and the presence of fatty liver.

NAFLD AND CARDIAC DYSFUNCTION

Information regarding abnormalities in cardiac function among NAFLD patients is limited in both adults and children. Moreover, the data are conflicting.

Adult studies

Table 2 summarizes the studies on the effects of NAFLD on

cardiac metabolism, structure and function in adults[15-17,45-50]. Goland *et al*[17] have shown a markedly impaired diastolic function and mild alterations in LV structure in 38 adult patients with (ultrasound-diagnosed, n = 27; biopsy-proven, n = 11) NAFLD, in the absence of diabetes, hypertension, and morbid obesity. Using multivariate analysis, early diastolic myocardial velocity on tissue Doppler imaging (TDI) was the only independent index able to characterize patients with NAFLD. Similar findings have been later reported by Fotbolcu *et al*[18] in 35 non-diabetic, normotensive adult patients with ultrasound-diagnosed NAFLD. However, independent predictors of LV impairment were not determined. Recently, in a study examining cardiac status by high resolution magnetic resonance imaging (MRI) in a clinical group of 19 adult patients with NAFLD (defined as > 5% intrahepatic lipid on MRS), Hallsworth *et al*[49] demonstrated significant changes in cardiac structure and evidence of early diastolic dysfunction in the 19 patients with NAFLD compared to the 19 age-, gender-, and BMI-matched controls, in the absence of cardiac metabolic changes or overt cardiac disease. There was no correlation between blood pressure and cardiac parameters. Finally, Karabay *et al*[50] found that patients with biopsy-proven NAFLD have evidence of subclinical myocardial dysfunction. However, no significant differences were found among NAFLD groups (*i.e.*, simple steatosis, borderline NASH, and definite NASH). The absence of differences in cardiac function between subgroup patients may be explained by similar HOMA-IR values. Conversely, Perseghin *et al*[16] showed that 21 men with higher intrahepatic fat content, as measured by MRS, had excessive fat accumulation in the epicardial area and abnormal LV energy metabolism compared to the 21 men matched for anthropometric features with lower intrahepatic fat content. These alterations were detected despite normal LV morphology and function by cardiac MRI. Similarly, in a study using cardiac MRI and involving 61 diabetic male subjects, Rijzewijk *et al*[46] found that, compared with the 29 men with lower intrahepatic fat content on MRS, the 32 patients with higher intrahepatic fat content had decreased myocardial perfusion, glucose uptake, and high-energy phosphate metabolism but similar values of LV function and morphology.

Pediatric studies

In the pediatric population, information on the relationship between NAFLD and cardiac structure and function is very scant (Table 3)[30,33,34,51-53]. In the earliest study by Sert *et al*[30], increased LV mass was found in adolescents with NAFLD compared to both lean controls and obese subjects without liver involvement. Similar results were obtained in a subsequent study by the same authors[33]. In a study including 93 obese children with ultrasound-diagnosed NAFLD, 307 obese subjects without liver involvement, and 150 age- and gender-matched healthy controls, Alp *et al*[34] showed that subclinical systolic and diastolic impairment could be detected by TDI in obese children with NAFLD. Also, cardiac dysfunction was correlated with the increase in grades of liver steatosis. Recently, Singh *et al*[51] measured by 2-dimensional speckle tracking echocardiography myocardial function in 3 groups of age-, gender-, and Tanner stage-matched adolescents [lean (n = 14); obese with normal (n = 15) or increased (n = 15) intrahepatic triglyceride (IHTG) content (≥ 5.6%)]. The authors showed that obese adolescents with increased IHTG had greater impairment of systolic and diastolic function, manifested by decreased systolic and diastolic myocardial strain and strain rate than BMI-SD score matched obese adolescents with normal IHTG content. The cardiac functional abnormalities were independently associated only with HOMA-IR, after adjustment for BMI, conventional cardiovascular risk factors, and intra-abdominal, intra-cardiac, and intrahepatic fat content. However, given the small number of adolescents included in their study, the possibility of a type 2 error raised by the authors themselves is possible. In a more recent study, Pacifico *et al*[52] showed that obese children with NAFLD have features of early LV diastolic and systolic dysfunction, as measured by two-dimensional echocardiography using TDI, compared to obese children without NAFLD and lean controls. Notably, when the group of obese subjects was divided according to the presence of NASH, it was evident that some functional cardiac differences were more pronounced in the group of NASH. A major finding of this study was that the echocardiographic features of early LV diastolic and systolic dysfunction were significantly associated with NAFLD independently of several metabolic variables.

PATHOGENESIS

The pathophysiological mechanisms of CVD in NAFLD are still poorly understood. Probably they involve insulin resistance, an abnormal ectopic fat storage as well as atherogenic dyslipidemia, and a low-grade inflammatory state in the presence of genetic susceptibility (Figure 1).

Insulin resistance

The expansion and inflammation of visceral adipose tissue mass, with consequent release of multiple molecules, is one of the earliest steps in the chain of events involved in the development of insulin resistance and NAFLD, as well as atherosclerosis, including free fatty acids (FFAs), hormones and proinflammatory adipocytokines, particularly in obese and overweight persons[54-57]. In this situation, the liver might function both as the target organ and the source of the resulting systemic abnormalities that can promote increased risk of CVD. Increased insulin resistance occurs when the advanced forms of NAFLD develop, which potentially sets up a vicious cycle of insulin resistance, increased influx of FFAs into the liver and increased hepatic steatosis[11]. In turn, hepatic steatosis (Figure 2A) leads to subacute intrahepatic inflammation through activation of nuclear factor-κB (NF-κB) pathways (Figure 2B) that exacerbate insulin resistance both locally in the liver and systematically including

Table 2 Studies of the association between nonalcoholic fatty liver disease and alterations in cardiac structure and function in the adult population

Ref.	Study population and sample size	Diagnosis	Outcomes	Main results
Lautamäki *et al*[15]	T2DM and coronary artery disease patients with (n = 27), and without (n = 28) fatty liver. The 2 groups were matched for age, BMI, and fasting plasma glucose	Hepatic MRS	Myocardial insulin resistance and perfusion (PET)	In patients with T2DM and coronary artery disease, liver fat is an indicator of myocardial insulin resistance and reduced coronary functional capacity
Goland *et al*[17]	Nondiabetic, normotensive patients with NAFLD (n = 38), and age and gender-matched controls (n = 25)	Liver ultrasound and liver biopsy in a subgroup of 11 NAFLD patients	LV structure and function (M-mode echocardiography; and pulsed Doppler echocardiography)	Patients with NAFLD had mild changes in cardiac geometry (thickening of the interventricular septum and posterior wall, and increased LV mass) as well as significant differences in parameters of diastolic function compared with the control group
Perseghin *et al*[16]	Young nondiabetic men matched for anthropometric features with (n = 21) or without (n = 21) fatty liver	MRS	LV morphology and function; Intrapericardial and extrapericardial fat content; and resting LV energy metabolism (Cardiac MRI and cardiac ^{31}P-MRS)	Newly found young individuals with fatty liver had excessive fat accumulation in the epicardial area and abnormal LV energy metabolism despite normal LV morphological features and systolic and diastolic functions
Fallo *et al*[45]	Never-treated essential hypertensive patients with (n = 48) or without (n = 38) fatty liver. The 2 groups were similar as to gender, age and blood pressure levels	Liver ultrasound	LV structure and function (M-mode echocardiography; and pulsed Doppler echocardiography)	NAFLD patients had similar prevalence of LV hypertrophy compared to subjects without NAFLD, but a higher prevalence of LV diastolic dysfunction
Rijzewijk *et al*[46]	T2DM patients with (n = 32) and without (n = 29) fatty liver	MRS	Cardiac perfusion and substrate metabolism; LV morphology and function (PET, cardiac MRI and cardiac ^{31}P-MRS)	T2DM patients with fatty liver showed decreased myocardial perfusion, glucose uptake, high-energy phosphate metabolism compared with similar patients without hepatic steatosis
Fotbolcu *et al*[18]	Nondiabetic, normotensive patients with NAFLD (n = 35) and control subjects (n = 30). The 2 groups were similar as to gender and age	Liver ultrasound	LV structure and function (M-mode echocardiography; Pulsed and Tissue Doppler echocardiography)	Patients with NAFLD had changes in cardiac geometry (thickening of the interventricular septum and posterior wall, and increased LV mass) as well as significant differences in parameters of systolic and diastolic function compared with the control group
Bonapace *et al*[47]	T2DM patients with (n = 32) and without (n = 18) fatty liver. The 2 groups were similar as to gender, age, BMI, waist circumference, and diabetes duration	Liver ultrasound	LV structure and function (M-mode echocardiography; Pulsed and Tissue Doppler echocardiography)	T2DM patients with fatty liver showed LV diastolic dysfunction, even if the LV morphology and systolic function were preserved
Mantovani *et al*[48]	Hypertensive T2DM patients with (n = 59) and without (n = 57) fatty liver	Liver ultrasound	LV structure (M-mode echocardiography)	Hypertensive T2DM patients with NAFLD have a remarkably higher frequency of LV hypertrophy than hypertensive diabetic patients without NAFLD
Hallsworth *et al*[49]	Adult subjects matched for anthropometric features with (n = 19) or without (n = 19) fatty liver	MRS	Cardiac structure, function, and metabolism (cardiac MRI, cardiac tagging, and cardiac ^{31}P-MRS)	The major findings in NAFLD patients compared to controls were: thickening of the cardiac wall, independent of changes in LV mass; altered myocardial strains; concentric remodeling; evidence of diastolic dysfunction; but no significant difference in cardiac energetics
Karabay *et al*[50]	NAFLD patients (n = 55) and healthy controls (n = 21; normal laboratory values and liver ultrasound)	Liver biopsy	LV structure and function (M-mode echocardiography; Pulsed and Tissue Doppler echocardiography; and speckle tracking echocardiography)	Patients with NAFLD had changes in cardiac geometry (thickening of the interventricular septum and posterior wall, and increased LV mass) as well as significant differences in parameters of diastolic function compared with the control group LV global longitudinal strain and strain rate in systole were lower in NAFLD group as compared to controls; however no significant differences were found among NAFLD groups (*i.e.*, simple steatosis, borderline NASH, and definite NASH)

T2DM: Type 2 diabetes mellitus; BMI: Body mass index; MRS: Magnetic resonance spectroscopy; PET: Positron emission tomography; NAFLD: Nonalcoholic fatty liver disease; LV: Left ventricular; NASH: Nonalcoholic steatohepatitis.

Table 3 Studies of the association between nonalcoholic fatty liver disease and alterations in cardiac structure and function in children and adolescents

Ref.	Study population and sample size	Diagnosis	Outcomes	Main results
Sert *et al*[30]	Obese adolescents with (n = 44), and without (n = 36) NAFLD; and control subjects (n = 37)	Liver ultrasound and elevated serum alanine aminotransferase	LV structure (M-mode echocardiography)	Increased LV mass was found in NAFLD group compared to both lean controls and obese subjects without NAFLD
Alp *et al*[34]	Obese children and adolescents with (n = 93), and without (n = 307) NAFLD matched for gender and age; and control subjects (n = 150)	Liver ultrasound	LV structure and function; Epicardial fat (M-mode echocardiography; Pulsed and Tissue Doppler echocardiography)	Increased end-systolic thickness of the interventricular septum, and larger LV mass, as well as LV systolic and diastolic dysfunction were found in NAFLD group. In addition, obese children with NAFLD had increased epicardial fat thickness
Singh *et al*[51]	Obese children and adolescents with (n = 15), and without (n = 15) NAFLD matched for age, gender, Tanner stage, and BMI z score; and control subjects (n = 15) matched for gender, age, and Tanner stage	Hepatic MRS	LV structure and function; Intracardiac triglyceride content (Integrated backscatter ultrasonography and speckle tracking echocardiography; cardiac MRS)	LV global longitudinal strain and early diastolic strain rates were significantly decreased in obese children with NAFLD compared to both lean controls and obese subjects without NAFLD. Intracardiac triglyceride content was not different among the 3 groups
Sert *et al*[33]	Obese adolescents with (n = 97), and without (n = 83) NAFLD; and control subjects (n = 68)	Liver ultrasound and elevated serum alanine aminotransferase	LV structure and function (M-mode echocardiography; Pulsed and Tissue Doppler echocardiography)	Obese adolescents with NAFLD exhibited increased LV dimensions and mass, as well as LV diastolic dysfunction
Pacifico *et al*[52]	Obese children and adolescents with (n = 54), and without (n = 54) NAFLD matched for age, gender, pubertal status, and BMI-SD score; and healthy control subjects (n = 18) matched for gender, age, and pubertal status	Hepatic magnetic resonance imaging; and liver biopsy in a subgroup of 41 NAFLD patients	LV structure and function; Epicardial fat (M-mode echocardiography; Pulsed and Tissue Doppler echocardiography)	Increased interventricular septum thickness at end-diastole and at end-systole, as well as LV systolic and diastolic dysfunction were found in NAFLD group. Children with more severe liver histology had worse LV dysfunction than those with more mild liver changes. NAFLD group had also increased epicardial fat thickness
Fintini *et al*[53]	Children with biopsy-proven NAFLD (n = 50). No patients without NAFLD, and no healthy control children were included	Liver biopsy	LV structure and function (M-mode echocardiography; and pulsed Doppler echocardiography)	About 35% (n = 18) of the 50 children with NAFLD had LV hypertrophy. Children with NASH showed, almost invariably, the presence of clear cut LV hypertrophy

NAFLD: Nonalcoholic fatty liver disease; LV: Left ventricular; BMI: Body mass index; MRS: Magnetic resonance spectroscopy; NASH: Nonalcoholic steatohepatitis.

cardiac insulin resistance[55-58]. Indeed, Lautamäki *et al*[15] demonstrated that, in patients with type 2 diabetes and coronary artery disease, liver fat content was an independent indicator of myocardial insulin resistance and reduced coronary functional capacity. The consequences of insulin resistance in the heart are incompletely understood. Using genetically engineered mice with deletion of insulin receptors in cardiomyocites, Boudina *et al*[59] have shown that impaired myocardial insulin signaling leads to multiple mitochondrial defects that include reduced oxygen consumption and adenosine triphosphate synthesis, reduced levels of mitochondrial enzymes that regulate pyruvate and fatty acid metabolism, and decreased content of citric acid cycle proteins. Insulin signaling also regulates the expression of genes such as peroxisome proliferator-activated receptor-α in the heart, which controls the capacity of mitochondria to oxidize fatty acids. In addition, mitochondria from hearts with defective insulin signaling demonstrate evidence of oxidative stress. These mechanisms could potentially contribute to myocardial dysfunction when the heart becomes insulin resistant.

Abnormal ectopic fat storage and atherogenic dyslipidemia

In patients with NAFLD, the increased FFAs may induce myocardial lipid accumulation, which is detrimental to LV function[60-63]. In fact, myocardial steatosis may cause alterations in myocardial substrate metabolism and efficiency (cardiac work/myocardial oxygen consumption) that occur early in the cascade of events leading to impaired LV contractility. Rijzewijk *et al*[46] showed that intramyocardial fat content, as detected by ^{1}H-MRS was significantly higher in uncomplicated type 2 diabetic men than in nondiabetic control subjects and was associated with impaired cardiac metabolism[46]. Moreover, using cardiac MRI and ^{31}P-MRS, Perseghin *et al*[16] demonstrated that individuals with fatty liver had an increased amount of fat in the epicardial area and displayed abnormal cardiac metabolism. Epicardial fat is a metabolically active organ that generates proatherogenic, proinflammatory and prothrombotic adipo-cytokines[64-67]. Its anatomic location, without any barrier to the adjacent myocardium, enables local paracrine interaction between epicardial fat and the myocardium[64]. Thus, epicardial

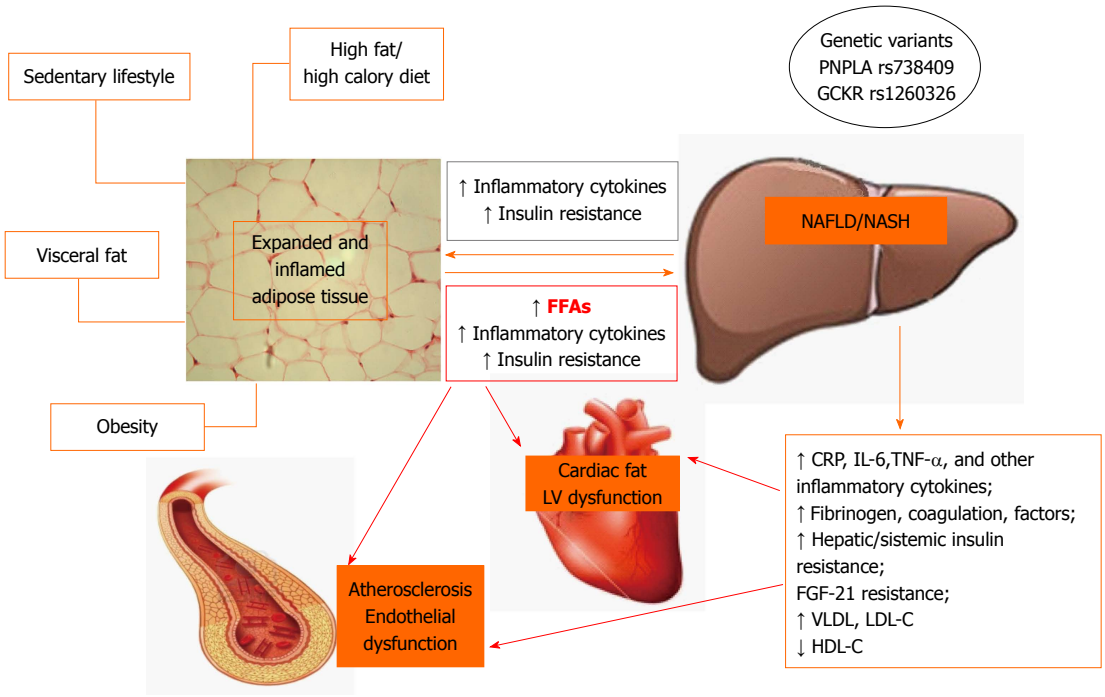


Figure 1 Suggested pathophysiological mechanisms linking nonalcoholic fatty liver disease to atherosclerosis and cardiac abnormalities in obese subjects. FFAs: Fatty free acids; CRP: C-reactive protein; IL-6: Interleukin-6; TNF-α: Tumor necrosis factor-α; FGF-21: Fibroblast growth factor-21; VLDL: Very low density lipoprotein; LDL-c: Low density lipoprotein cholesterol; HDL-c: High density lipoprotein cholesterol; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

and myocardial fat represent abnormal ectopic fat storage and may be a marker of the cumulative effects of NAFLD and insulin resistance in the setting of pathological adiposity, with consequent adverse associated cardiovascular outcome[65,68].

Cardiac lipotoxicity is a well-described phenomenon in insulin resistance, and is generally attributed to products of FFA excess metabolism[62,69]. Ceramide is a sphingolipid that is a key mediator of cellular stress pathways that induce apoptosis and mitochondrial dysfunction. In normal physiology, ceramide is derived from *de novo* synthesis or can be derived from sphingomyielin hydrolysis. Ceramide acts as a lipotoxic intermediate when it builds up as a result of elevated circulating FFAs. Structural alterations in mitochondria can reduce cardiac function by providing an insufficient supply of ATP to cardiac myocytes or by increasing reactive oxygen species production, which has been associated with increased apoptosis, DNA damage, and DNA repair[70]. Recently, it has been shown that ceramide also plays an important role in the pathogenesis of obesity-mediated vascular dysfunction *via* a mechanism that involves protein phosphatase 2A-mediated dephosphorylation of nitric oxide synthase Ⅲ[71]. In addition, the treatment of mice with lipotoxic cardiomyopathy with the inhibitor of ceramide synthesis myriocin reversed contractile dysfunction in a mouse model of lipotoxic cardiomyopathy[72]. Taken together, it is reasonable that ceramide accumulation may contribute to the pathogenesis of cardiac and vascular dysfunction in insulin-resistant states.

NAFLD is also characterized by an atherogenic lipid profile, consisting of high TG levels, low HDL-c, an increase in small, dense LDL-c particles, increased very low-density lipoprotein (VLDL) cholesterol levels and elevated apolipoprotein B100 concentration[58]. This type of atherogenic dyslipidemia is strongly linked to adverse cardiovascular outcome[37,58]. The increased hepatic production of TG-rich VLDL provides a limited compensatory mechanism for IHTG[58,73]. However, this also results in abnormal HDL-c metabolism causing HDL-c reduction as well as compositional alterations. In fact, the amount of liver fat has a significant negative correlation with subfractions of HDL-c known to be antiatherogenic, which are reduced in NAFLD independently of peripheral insulin sensitivity[74].

Inflammation

In the presence of increased FFA flux and chronic, low grade inflammation, the liver is again both the target of and a contributor to systemic inflammatory changes. The steatotic and inflamed liver releases several mediators including C reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and other inflammatory cytokines that amplify the systemic, low-grade inflammation[11,55-57]. Increased intrahepatic cytokine expression results from local activation of the NF-κB pathway (Figure 2B), as mediated by hepatocellular damage and fat-derived factors, and is likely to play a key role in the progression of NAFLD and CVD[9,55-57]. Several studies have shown that a number of genes involved in fatty acid metabolism, lipolysis, monocyte and macrophage recruitment, coagulation, and inflammation are overexpressed in pa-

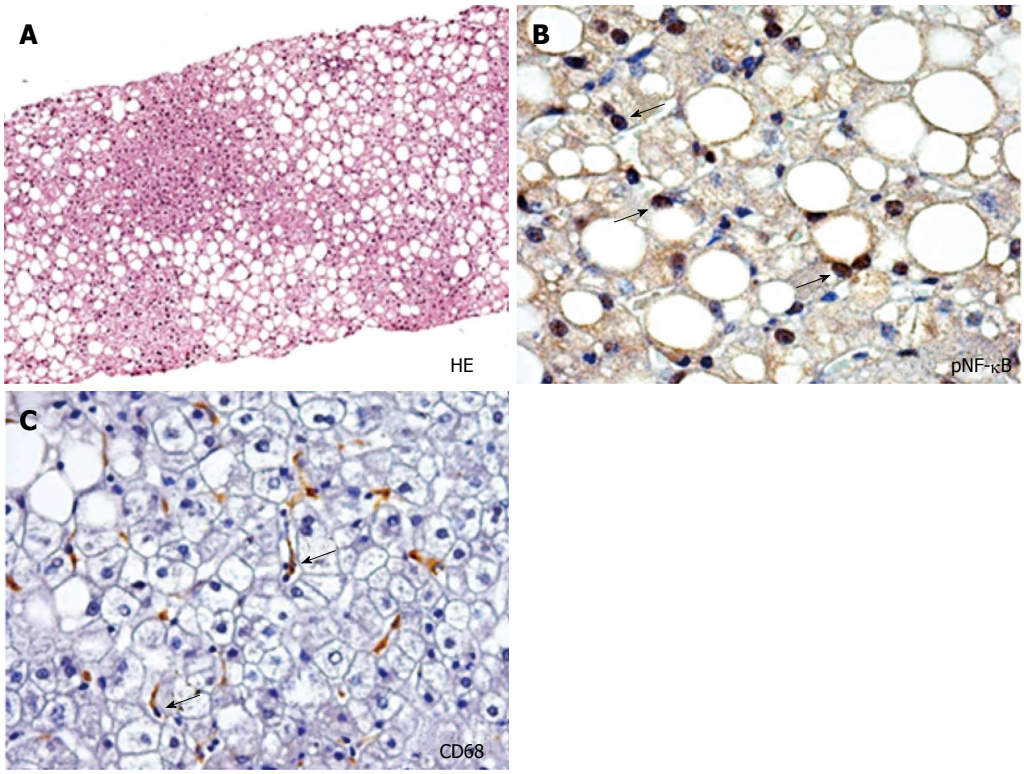


Figure 2 Histological and immunohistochemical features of nonalcoholic fatty liver disease. A: Hematoxylin-Eosin (HE) in nonalcoholic fatty liver disease (NAFLD) biopsy shows hepatic steatosis (fatty liver). Original Magnification: 10 ×; B: Immunohistochemistry for phosphorylated (p) nuclear factor κB shows the nuclear expression by hepatocytes in NAFLD (arrows). Original Magnification: 40 ×; C: Macrophages in NAFLD. Immunohistochemistry for CD68 shows the presence of macrophages in nonalcoholic steatohepatitis (yellow arrows). Original Magnification: 40 ×. Photos were obtained from a liver biopsy of a 60-year-old male affected by NAFLD. Photos are original and taken in Prof. Gaudio's Laboratory.

tients with NAFLD[75,76]. In particular, NASH presents a distinct panel of regulatory genes which are dysregulated compared to controls and subjects with simple steatosis. Indeed, circulating levels of several inflammatory markers (CRP, IL-6, monocyte chemotactic protein 1, and TNF-α), procoagulant factors (plasminogen activator inhibitor 1, fibrinogen, and factor Ⅶ), and oxidative stress markers are highest in patients with NASH, intermediate in those with simple steatosis, and lowest in control subjects without steatosis, and the differences are independent of obesity and other potentially confounding factors[76,77].

There is much evidence to suggest that macrophage infiltration (Figure 2C) could play an essential role in the pathogenesis of NAFLD and atherosclerosis by communicating inflammatory signals by scavenging modified lipids[78]. In this light, the systemic inflammation, which is exacerbated by steatohepatitis, could have a dual role on the progression of atherosclerotic plaque and steatohepatitis. Macrophages were the first inflammatory cells to be associated with atherosclerosis[79]; recently, the process of macrophage polarization has been a subject of interest[80,81]. Two distinct modes of macrophage activation were proposed to differentiate between inflammatory M1 and anti-inflammatory M2 macrophages[82]. M1-macrophages exert definitive pro-inflammatory roles and M1-derived cytokines may be involved in further activating myofibroblasts and fibrogenetic cells; M2-macrophages have been described as wound-healing, based on their ability to promote wound healing through matrix remodeling and the recruitment of fibroblasts[83]. The process of macrophage polarization during atherogenesis has been a subject of interest as macrophage subsets have been demonstrated to display some degree of plasticity and heterogeneity within atherosclerotic lesions[80,81]. In parallel, in NAFLD, the exacerbated release of M1 macrophages derived mediators contributes to the pathogenesis of several liver lesions, namely hepatocyte steatosis and apoptosis, inflammatory cell recruitment, and activation of fibrogenesis[84,85].

Adipocyte-derived hormones

The term "adipokines" (adipose tissue cytokines) comprises polypeptide factors which are expressed significantly, although not exclusively, by adipose tissue in a regulated manner. Besides adipocytes, accounting for one-third of the cells, adipose tissue is composed of stromal cells, including macrophages, fibroblasts, and infiltrating monocytes, all of which contribute to adipokine production[78,86]. The major adipokines (leptin, adiponectin, resistin) exert several metabolic actions and have a role in cellular and animal models of liver injury[87]. Leptin has several immune and metabolic functions. Obesity is associated with high circulating leptin levels and leptin resistance in the central nervous system as leptin fails to correct hyperglycemia in patients with obesity[86]. Several

in vitro and *in vivo* studies have identified a close connection between leptin and liver fibrosis. These show that leptin modulates the biology of different cell types participating in the response to liver injury, such as Kupffer cells, sinusoidal endothelial cells, and myofibroblast-like cells[88]. Leptin is also a potential mediator of cardiac hypertrophy in obesity, possibly by causing an increase in sympathetic vasoconstrictor tone and arterial blood pressure, or through direct stimulation of protein synthesis in cardiomyocytes[89].

Adiponectin exerts insulin-sensitizing effects in the liver, skeletal muscle, and adipose tissue. Adiponectin improves insulin signaling and profoundly affects glucose metabolism. Adiponectin and leptin have divergent effects on inflammation; adiponectin reduces inflammation, stimulating secretion of anti-inflammatory cytokines (*i.e.*, IL-10), and inhibiting release of TNF-α, IL-6, and chemokines[86,87]. Resistin may represent a link between obesity and insulin resistance; its action determinates reduction of peripheral insulin sensitivity, increase in endogenous glucose production by the liver, induction of insulin resistance and stimulation of proinflammatory cytokines (*i.e.*, IL-6 and TNF-α)[87].

Recently, hepatocytes and hepatic stem/progenitor cells (HPC) have been indicated as a source of adiponectin and resistin in the course of NAFLD[90,91]. In NASH, the expression of adiponectin by liver parenchymal cells (hepatocytes and HPCs) was down-regulated and it was inversely correlated with steatohepatitis grade. This is in agreement with the current understanding of this adipokine. In fact, adiponectin has anti-inflammatory and anti-fibrogenic properties and, in steatotic liver, has been shown to ameliorate necroinflammation and steatosis when administered in experimental NASH[87,92]. On the other hand, HPCs up-regulated their expression of resistin in correlation with progression towards NASH and fibrosis[93]. Several lines of evidence link the biology of resistin with hepatic inflammation, fibrogenesis and macrophage polarization. In rats, resistin administration significantly worsens inflammation after lipopolysaccharide injection[94], and activates fibrogenetic cells through the activation of NF-κB pathway[93,94]. Moreover, hepatic resistin expression increases in NASH; is correlated with inflammatory cell infiltration, and has been associated with macrophage recruitment within the liver[91].

Widespread research has been conducted on the relationship of adiponectin, as well as of resistin, with cardiovascular risk. Adiponectin exerts a protective effect against endothelial dysfunction induced by advanced glycation end-products[95]. This process is, in part, mediated by a decrease in the expression of adhesion molecules, and provides evidence of the protective role of adiponectin in the pathogenesis of the vascular complications of obesity/MetS. Low adiponectin levels may impair the ability of the heart to adapt to acute and chronic stress, as suggested by studies of adiponectin deficiency in mice[61]. On the other hand, resistin can act as an effector molecule that leads to an atherosclerotic state, possibly through several mechanisms. It has been shown that resistin has direct effects on endothelial cell activation by inducing the expression of cell adhesion molecules, thereby enhancing leukocyte adhesion[96,97]. Previous "*in vitro*" experimental studies on endothelial cells and atherosclerotic plaque progression also showed that resistin can impair endothelium-dependent relaxation, promote angiogenesis[98], and induce vascular inflammation[99]. An increase in resistin concentration significantly decreases endothelial nitric oxide synthase expression and nitric oxide production through oxidative stress in cultured human coronary artery endothelial cells[100], suggesting that the effects of resistin can be mediated by oxidative stress. However, the precise role of resistin in the clinical scenario remains to be fully elucidated.

Fibroblast growth factor-21 (FGF-21) has emerged as important endocrine factor involved in glucose and lipid metabolism and energy regulation[101]. FGF-21 is primarily expressed by liver, adipose tissue, and pancreas. FGF-21 stimulates glucose uptake in adipocytes and regulates energy metabolism and enhanced mitochondrial oxidative function through the activation of AMP-activated protein kinase and sirtuin 1[101]. FGF-21 has a hepato-protective action, but subjects with NAFLD show a condition of "FGF-21 resistance", which worsens in subjects with NASH[102]. A recent study has demonstrated that FGF-21 knockout mice exhibit an increased relative heart weight and develop enhanced signs of dilation and cardiac dysfunction[103]. In addition, *in vitro* treatment of cardiomyocytes with FGF-21 reverses these cardiac alterations[103]. Thus, FGF-21 resistance or reduced levels observed in subjects with NAFLD/NASH, might play a role in cardiac anatomic and functional abnormalities.

Genetic factors

In recent years, genetic studies have highlighted several single nucleotide polymorphisms (SNPs) that may characterize children with a high risk for NAFLD development and progression[104-110]. In particular, a common missense variant (rs738409), characterized by a C-to-G substitution encoding an isoleucine-to-methionine substitution at amino acid position 148 (I148M), in the patatin-like phospholipase 3 (*PNPLA3*) gene has been associated not only with hepatic fat content and increased serum liver enzymes but also with increased risk of NASH and fibrosis progression[104,111-114]. The I148M PNPLA3 variant influences liver triglyceride content without apparently affecting body mass, serum lipid levels and systemic insulin resistance[113,115]. The association between I148M variant and both liver enzymes and steatosis has been confirmed in obese children of different ethnicities[111,112,116,117]. More recently, a SNP (rs1260326) in the glucokinase regulatory protein (*GCKR*) gene has been associated with fatty liver and with higher serum triglycerides and large VLDL levels in obese children and adolescents[109]. This association was independent of ethnicity, age, gender, z-score BMI,

and glucose tolerance[109].

PREVENTION AND TREATMENT

Because of the limited knowledge of the molecular pathogenesis of NAFLD, the current therapies consist of strategies aimed at decreasing the incidence of the known risk factors. Prevention and control of modifiable risk factors such as overweight and unhealthy lifestyle can have an impact on the overall health of children and adolescents as well as on the prevention and control of pediatric NAFLD and the related MetS[2,3,10]. Lifestyle changes and pharmacological treatment of pediatric NAFLD have extensively been discussed elsewhere[2,3,10]. However, it is not known how treatment of NAFLD modulates the risk of CVD.

It has been established that preclinical atherosclerosis is not an irreversible but rather a dynamic process. Different interventions on cardiovascular risk factors (dyslipidemia, hypertension, diabetes mellitus, and obesity) have been shown to slow or even regress the progression of atherosclerosis[118-123]. However, data on the reversibility of subclinical atherosclerotic markers in children with NAFLD are scant. In a study evaluating a 1-year intervention program with diet and physical exercise in children and adolescents with NAFLD, Pacifico *et al*[124] showed favorable changes in vascular function as estimated by FMD. In the same study, the authors failed to demonstrate a significant regression of cIMT, though there was a trend after the lifestyle intervention. Studies in adults and children have shown that lifestyle interventions can halt the progression of cIMT[118-120,122,123], but others have shown no such effect[125,126]. Possible reasons for such conflicting results include the age of population (prepubertal children, adolescents, or adults), the type of population (healthy, otherwise healthy obese, or obese subjects with obesity related comorbidities), the length of intervention, the type and intensity of lifestyle intervention, the degree of weight and visceral fat loss, and different analyses of cIMT measurements (*i.e.*, maximum or mean value of cIMT). In this context, of great interest is the study by Koskinen *et al*[127] who showed that in young adults recovery from the MetS was associated with reduced cIMT progression during a 6-year follow-up period. Thus, it is possible that a longer lifestyle intervention may be necessary to regress cIMT in children with NAFLD. Of note, Pacifico *et al*[124] demonstrated that higher cIMT values in obese children with fatty liver as well as in those with MetS were related to impaired brachial FMD, supporting the idea that endothelial dysfunction is a necessary step before the development of structural arterial disease. The restoration of FMD observed in such patients might be the initial step to halt the progression of the atherosclerotic disease.

In a recent systematic review on the effect of current non-surgical treatments on liver disease and cardio-metabolic risk in NAFLD, Musso *et al*[128] found that weight loss is safe and may ameliorate both liver and cardio-metabolic disease in NAFLD. Although a $\geqslant$ 5% weight loss improves steatosis and cardio-metabolic variables, a $\geqslant$ 7% weight loss improves also histological disease activity in NASH; however, the latter goal was achieved by < 50% individuals even in randomized controlled studies adopting intensive multidisciplinary lifestyle interventions, making patient compliance a concern. No studies have yet examined the effect of reducing liver fat and inflammation on cardiac function and geometry both in children and adults.

CONCLUSION

Although cross-sectional studies have shown that children with NAFLD are at risk for early atherosclerotic changes and cardiac abnormalities, long-term longitudinal studies are required to determine more definitively the extent to which pediatric NAFLD and its severity influence long-term cardiovascular outcomes in the general population. In particular, follow-up studies may clarify whether the increased risk of atherosclerotic changes and cardiac alterations might reflect the clustering of underlying metabolic risk factors, or NAFLD *per se*, especially NASH, might confer a risk of adverse cardiovascular outcome above and beyond that associated with the individual components of MetS. In children, the cardiovascular system remains plastic and damage-reversible if early and appropriate interventions are established effectively. Therapeutic goals for NAFLD should address nutrition, physical activity and avoidance of smoking to prevent not only end-stage liver disease but also CVD. Future studies should also examine the long-term effect of reducing liver fat and inflammation on vascular functional and structural changes as well as on cardiac function and geometry in children.

REFERENCES

1 **Angulo P**. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231 [PMID: 11961152 DOI: 10.1056/NEJMra011775]

2 **Loomba R**, Sirlin CB, Schwimmer JB, Lavine JE. Advances in pediatric nonalcoholic fatty liver disease. *Hepatology* 2009; **50**: 1282-1293 [PMID: 19637286]

3 **Mencin AA**, Lavine JE. Advances in pediatric nonalcoholic fatty liver disease. *Pediatr Clin North Am* 2011; **58**: 1375-1392, x [PMID: 22093857 DOI: 10.1016/j.pcl.2011]

4 **Byrne CD**. Ectopic fat, insulin resistance and non-alcoholic fatty liver disease. *Proc Nutr Soc* 2013; **72**: 412-419 [PMID: 23668723 DOI: 10.1017/S0029665113001249]

5 **Samuel VT**, Petersen KF, Shulman GI. Lipid-induced insulin resistance: unravelling the mechanism. *Lancet* 2010; **375**: 2267-2277 [PMID: 20609972 DOI: 10.1016/S0140-6736(10)60408-4]

6 **Cali AM**, De Oliveira AM, Kim H, Chen S, Reyes-Mugica M, Escalera S, Dziura J, Taksali SE, Kursawe R, Shaw M, Savoye M, Pierpont B, Constable RT, Caprio S. Glucose dysregulation and hepatic steatosis in obese adolescents: is there a link? *Hepatology* 2009; **49**: 1896-1903 [PMID: 19434725 DOI: 10.1002/hep.22858]

7 **D'Adamo E**, Cali AM, Weiss R, Santoro N, Pierpont B, Northrup V, Caprio S. Central role of fatty liver in the patho-

genesis of insulin resistance in obese adolescents. *Diabetes Care* 2010; **33**: 1817-1822 [PMID: 20668154 DOI: 10.2337/dc10-0284]
8 **Kotronen A**, Yki-Järvinen H. Fatty liver: a novel component of the metabolic syndrome. *Arterioscler Thromb Vasc Biol* 2008; **28**: 27-38 [PMID: 17690317 DOI: 10.1161/ATVBAHA.107.147538]
9 **Schwimmer JB**, Pardee PE, Lavine JE, Blumkin AK, Cook S. Cardiovascular risk factors and the metabolic syndrome in pediatric nonalcoholic fatty liver disease. *Circulation* 2008; **118**: 277-283 [PMID: 18591439 DOI: 10.1161/CIRCULATIONAHA]
10 **Pacifico L**, Nobili V, Anania C, Verdecchia P, Chiesa C. Pediatric nonalcoholic fatty liver disease, metabolic syndrome and cardiovascular risk. *World J Gastroenterol* 2011; **17**: 3082-3091 [PMID: 21912450 DOI: 10.3748/wjg.v17.i26.3082]
11 **Targher G**, Day CP, Bonora E. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. *N Engl J Med* 2010; **363**: 1341-1350 [PMID: 20879883 DOI: 10.1056/NEJMra0912063]
12 **Villanova N**, Moscatiello S, Ramilli S, Bugianesi E, Magalotti D, Vanni E, Zoli M, Marchesini G. Endothelial dysfunction and cardiovascular risk profile in nonalcoholic fatty liver disease. *Hepatology* 2005; **42**: 473-480 [PMID: 15981216]
13 **Anstee QM**, Targher G, Day CP. Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 330-344 [PMID: 23507799 DOI: 10.1038/nrgastro]
14 **Pacifico L**, Anania C, Martino F, Cantisani V, Pascone R, Marcantonio A, Chiesa C. Functional and morphological vascular changes in pediatric nonalcoholic fatty liver disease. *Hepatology* 2010; **52**: 1643-1651 [PMID: 20890890 DOI: 10.1002/hep.23890]
15 **Lautamäki R**, Borra R, Iozzo P, Komu M, Lehtimäki T, Salmi M, Jalkanen S, Airaksinen KE, Knuuti J, Parkkola R, Nuutila P. Liver steatosis coexists with myocardial insulin resistance and coronary dysfunction in patients with type 2 diabetes. *Am J Physiol Endocrinol Metab* 2006; **291**: E282-E290 [PMID: 16478772 DOI: 10.1152/ajpendo.00604.2005]
16 **Perseghin G**, Lattuada G, De Cobelli F, Esposito A, Belloni E, Ntali G, Ragogna F, Canu T, Scifo P, Del Maschio A, Luzi L. Increased mediastinal fat and impaired left ventricular energy metabolism in young men with newly found fatty liver. *Hepatology* 2008; **47**: 51-58 [PMID: 17955548 DOI: 10.1002/hep.21983]
17 **Goland S**, Shimoni S, Zornitzki T, Knobler H, Azoulai O, Lutaty G, Melzer E, Orr A, Caspi A, Malnick S. Cardiac abnormalities as a new manifestation of nonalcoholic fatty liver disease: echocardiographic and tissue Doppler imaging assessment. *J Clin Gastroenterol* 2006; **40**: 949-955 [PMID: 17063117]
18 **Fotbolcu H**, Yakar T, Duman D, Karaahmet T, Tigen K, Cevik C, Kurtoglu U, Dindar I. Impairment of the left ventricular systolic and diastolic function in patients with nonalcoholic fatty liver disease. *Cardiol J* 2010; **17**: 457-463 [PMID: 20865675]
19 **Bland J**, Skordalaki A, Emery JL. Early intimal lesions in the common carotid artery. *Cardiovasc Res* 1986; **20**: 863-868 [PMID: 3621287 DOI: 10.1093/cvr/20]
20 **Stary HC**. Lipid and macrophage accumulations in arteries of children and the development of atherosclerosis. *Am J Clin Nutr* 2000; **72** (Suppl 5): 1297S-1306S [PMID: 11063472]
21 **Schwimmer JB**, Deutsch R, Behling C, Lavine JE. Fatty liver as a determinant of atherosclerosis. *Hepatology* 2005; **42** (Suppl 1): 610A
22 **Pacifico L**, Cantisani V, Ricci P, Osborn JF, Schiavo E, Anania C, Ferrara E, Dvisic G, Chiesa C. Nonalcoholic fatty liver disease and carotid atherosclerosis in children. *Pediatr Res* 2008; **63**: 423-427 [PMID: 18356751 DOI: 10.1203/PDR.0b013e318165b8e7]
23 **Demircioğlu F**, Koçyiğit A, Arslan N, Cakmakçi H, Hizli S, Sedat AT. Intima-media thickness of carotid artery and susceptibility to atherosclerosis in obese children with nonalcoholic fatty liver disease. *J Pediatr Gastroenterol Nutr* 2008; **47**: 68-75 [PMID: 18607271 DOI: 10.1097/MPG]
24 **Kelishadi R**, Cook SR, Amra B, Adibi A. Factors associated with insulin resistance and non-alcoholic fatty liver disease among youths. *Atherosclerosis* 2009; **204**: 538-543 [PMID: 19013572 DOI: 10.1016/j.atherosclerosis.2008.09.034]
25 **Manco M**, Bedogni G, Monti L, Morino G, Natali G, Nobili V. Intima-media thickness and liver histology in obese children and adolescents with non-alcoholic fatty liver disease. *Atherosclerosis* 2010; **209**: 463-468 [PMID: 19897197]
26 **Caserta CA**, Pendino GM, Amante A, Vacalebre C, Fiorillo MT, Surace P, Messineo A, Surace M, Alicante S, Cotichini R, Zuin M, Rosmini F, Mele A, Marcucci F. Cardiovascular risk factors, nonalcoholic fatty liver disease, and carotid artery intima-media thickness in an adolescent population in southern Italy. *Am J Epidemiol* 2010; **171**: 1195-1202 [PMID: 20457571 DOI: 10.1093/aje/kwq073]
27 **Nobili V**, Alkhouri N, Bartuli A, Manco M, Lopez R, Alisi A, Feldstein AE. Severity of liver injury and atherogenic lipid profile in children with nonalcoholic fatty liver disease. *Pediatr Res* 2010; **67**: 665-670 [PMID: 20496475 DOI: 10.1203/PDR]
28 **Weghuber D**, Roden M, Franz C, Chmelik M, Torabia S, Nowotny P, Gruber S, Waldhäusl W, Klingler A, Bieglmayer C, Bischof M, Wolzt M, Schaller G, Widhalm K. Vascular function in obese children with non-alcoholic fatty liver disease. *Int J Pediatr Obes* 2011; **6**: 120-127 [PMID: 20950125 DOI: 10.3109/17477161003792580]
29 **El-Koofy NM**, Anwar GM, El-Raziky MS, El-Hennawy AM, El-Mougy FM, El-Karaksy HM, Hassanin FM, Helmy HM. The association of metabolic syndrome, insulin resistance and non-alcoholic fatty liver disease in overweight/obese children. *Saudi J Gastroenterol* 2012; **18**: 44-49 [PMID: 22249092 DOI: 10.4103/1319-3767.91738]
30 **Sert A**, Pirgon O, Aypar E, Yilmaz H, Odabas D. Relationship between left ventricular mass and carotid intima media thickness in obese adolescents with non-alcoholic fatty liver disease. *J Pediatr Endocrinol Metab* 2012; **25**: 927-934 [PMID: 23426822 DOI: 10.1515/jpem-2012-0187]
31 **Akın L**, Kurtoglu S, Yikilmaz A, Kendirci M, Elmalı F, Mazicioglu M. Fatty liver is a good indicator of subclinical atherosclerosis risk in obese children and adolescents regardless of liver enzyme elevation. *Acta Paediatr* 2013; **102**: e107-e113 [PMID: 23190373 DOI: 10.1111/apa.12099]
32 **Gökçe S**, Atbinici Z, Aycan Z, Cınar HG, Zorlu P. The relationship between pediatric nonalcoholic fatty liver disease and cardiovascular risk factors and increased risk of atherosclerosis in obese children. *Pediatr Cardiol* 2013; **34**: 308-315 [PMID: 22875138 DOI: 10.1007/s00246-012-0447-9]
33 **Sert A**, Aypar E, Pirgon O, Yilmaz H, Odabas D, Tolu I. Left ventricular function by echocardiography, tissue Doppler imaging, and carotid intima-media thickness in obese adolescents with nonalcoholic fatty liver disease. *Am J Cardiol* 2013; **112**: 436-443 [PMID: 23642511 DOI: 10.1016/j.amjcard.2013.03.056]
34 **Alp H**, Karaarslan S, Selver Eklioğlu B, Atabek ME, Altın H, Baysal T. Association between nonalcoholic fatty liver disease and cardiovascular risk in obese children and adolescents. *Can J Cardiol* 2013; **29**: 1118-1125 [PMID: 23040432 DOI: 10.1016/j.cjca.2012.07.846]
35 **Huang RC**, Beilin LJ, Ayonrinde O, Mori TA, Olynyk JK, Burrows S, Hands B, Adams LA. Importance of cardiometabolic risk factors in the association between nonalcoholic fatty liver disease and arterial stiffness in adolescents. *Hepatology* 2013; **58**: 1306-1314 [PMID: 23703776]
36 **Jin Y**, Chen Y, Tang Q, Xue M, Li W, Jiang J. Evaluation of carotid artery stiffness in obese children using ultrasound radiofrequency data technology. *J Ultrasound Med* 2013; **32**:

105-113 [PMID: 23269715]
37 **Stampfer MJ**, Sacks FM, Salvini S, Willett WC, Hennekens CH. A prospective study of cholesterol, apolipoproteins, and the risk of myocardial infarction. *N Engl J Med* 1991; **325**: 373-381 [PMID: 2062328 DOI: 10.1056/NEJM199108083250601]
38 **McLaughlin T**, Reaven G, Abbasi F, Lamendola C, Saad M, Waters D, Simon J, Krauss RM. Is there a simple way to identify insulin-resistant individuals at increased risk of cardiovascular disease? *Am J Cardiol* 2005; **96**: 399-404 [PMID: 16054467 DOI: 10.1016/j.amjcard.2005.03.085]
39 **Quijada Z**, Paoli M, Zerpa Y, Camacho N, Cichetti R, Villarroel V, Arata-Bellabarba G, Lanes R. The triglyceride/HDL-cholesterol ratio as a marker of cardiovascular risk in obese children; association with traditional and emergent risk factors. *Pediatr Diabetes* 2008; **9**: 464-471 [PMID: 18507788 DOI: 10.1111/j.1399-5448.2008.00406.x]
40 **Raitakari OT**. Imaging of subclinical atherosclerosis in children and young adults. *Ann Med* 1999; **31** (Suppl 1): 33S-40S [PMID: 10342498]
41 **Corretti MC**, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol* 2002; **39**: 257-265 [PMID: 11788217 DOI: 10.1016/S0735-1097(01)01746-6]
42 **Anderson TJ**, Uehata A, Gerhard MD, Meredith IT, Knab S, Delagrange D, Lieberman EH, Ganz P, Creager MA, Yeung AC. Close relation of endothelial function in the human coronary and peripheral circulations. *J Am Coll Cardiol* 1995; **26**: 1235-1241 [PMID: 7594037 DOI: 10.1016/0735-1097(95)00327-4]
43 **Raitakari OT**, Juonala M, Kähönen M, Taittonen L, Laitinen T, Mäki-Torkko N, Järvisalo MJ, Uhari M, Jokinen E, Rönnemaa T, Akerblom HK, Viikari JS. Cardiovascular risk factors in childhood and carotid artery intima-media thickness in adulthood: the Cardiovascular Risk in Young Finns Study. *JAMA* 2003; **290**: 2277-2283 [PMID: 14600186 DOI: 10.1001/jama.290.17.2277]
44 **O'Leary DH**, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. *N Engl J Med* 1999; **340**: 14-22 [PMID: 9878640]
45 **Fallo F**, Dalla Pozza A, Sonino N, Lupia M, Tona F, Federspil G, Ermani M, Catena C, Soardo G, Di Piazza L, Bernardi S, Bertolotto M, Pinamonti B, Fabris B, Sechi LA. Non-alcoholic fatty liver disease is associated with left ventricular diastolic dysfunction in essential hypertension. *Nutr Metab Cardiovasc Dis* 2009; **19**: 646-653 [PMID: 19278843 DOI: 10.1016/j.numecd.2008.12.007]
46 **Rijzewijk LJ**, Jonker JT, van der Meer RW, Lubberink M, de Jong HW, Romijn JA, Bax JJ, de Roos A, Heine RJ, Twisk JW, Windhorst AD, Lammertsma AA, Smit JW, Diamant M, Lamb HJ. Effects of hepatic triglyceride content on myocardial metabolism in type 2 diabetes. *J Am Coll Cardiol* 2010; **56**: 225-233 [PMID: 20620743 DOI: 10.1016/j.jacc.2010.02.049]
47 **Bonapace S**, Perseghin G, Molon G, Canali G, Bertolini L, Zoppini G, Barbieri E, Targher G. Nonalcoholic fatty liver disease is associated with left ventricular diastolic dysfunction in patients with type 2 diabetes. *Diabetes Care* 2012; **35**: 389-395 [PMID: 22210573 DOI: 10.2337/dc11-1820]
48 **Mantovani A**, Zoppini G, Targher G, Golia G, Bonora E. Non-alcoholic fatty liver disease is independently associated with left ventricular hypertrophy in hypertensive Type 2 diabetic individuals. *J Endocrinol Invest* 2012; **35**: 215-218 [PMID: 22490991]
49 **Hallsworth K**, Hollingsworth KG, Thoma C, Jakovljevic D, MacGowan GA, Anstee QM, Taylor R, Day CP, Trenell MI. Cardiac structure and function are altered in adults with non-alcoholic fatty liver disease. *J Hepatol* 2013; **58**: 757-762 [PMID: 23178979 DOI: 10.1016/j.jhep.2012.11.015]
50 **Karabay CY**, Kocabay G, Kalayci A, Colak Y, Oduncu V, Akgun T, Kalkan S, Guler A, Kirma C. Impaired left ventricular mechanics in nonalcoholic fatty liver disease: a speckle-tracking echocardiography study. *Eur J Gastroenterol Hepatol* 2014; **26**: 325-331 [PMID: 24161963 DOI: 10.1097/MEG]
51 **Singh GK**, Vitola BE, Holland MR, Sekarski T, Patterson BW, Magkos F, Klein S. Alterations in ventricular structure and function in obese adolescents with nonalcoholic fatty liver disease. *J Pediatr* 2013; **162**: 1160-1168 [PMID: 23260104 DOI: 10.1016/j.jpeds.2012.11.024]
52 **Pacifico L**, Di Martino M, De Merulis A, Bezzi M, Osborn JF, Catalano C, Chiesa C. Left ventricular dysfunction in obese children and adolescents with nonalcoholic fatty liver disease. *Hepatology* 2014; **59**: 461-470 [PMID: 23843206 DOI: 10.1002/hep.26610]
53 **Fintini D**, Chinali M, Cafiero G, Esposito C, Giordano U, Turchetta A, Pescosolido S, Pongiglione G, Nobili V. Early left ventricular abnormality/dysfunction in obese children affected by NAFLD. *Nutr Metab Cardiovasc Dis* 2014; **24**: 72-74 [PMID: 24119987 DOI: 10.1016/j.numecd]
54 **Badman MK**, Flier JS. The adipocyte as an active participant in energy balance and metabolism. *Gastroenterology* 2007; **132**: 2103-2115 [PMID: 17498506 DOI: 10.1053/j.gastro.2007]
55 **Tilg H**, Moschen AR. Insulin resistance, inflammation, and non-alcoholic fatty liver disease. *Trends Endocrinol Metab* 2008; **19**: 371-379 [PMID: 18929493 DOI: 10.1016/j.tem.2008.08.005]
56 **Shoelson SE**, Herrero L, Naaz A. Obesity, inflammation, and insulin resistance. *Gastroenterology* 2007; **132**: 2169-2180 [PMID: 17498510 DOI: 10.1053/j.gastro]
57 **Stefan N**, Kantartzis K, Häring HU. Causes and metabolic consequences of Fatty liver. *Endocr Rev* 2008; **29**: 939-960 [PMID: 18723451 DOI: 10.1210/er.2008-0009]
58 **Bhatia LS**, Curzen NP, Calder PC, Byrne CD. Non-alcoholic fatty liver disease: a new and important cardiovascular risk factor? *Eur Heart J* 2012; **33**: 1190-1200 [PMID: 22408036 DOI: 10.1093/eurheartj/ehr453]
59 **Boudina S**, Bugger H, Sena S, O'Neill BT, Zaha VG, Ilkun O, Wright JJ, Mazumder PK, Palfreyman E, Tidwell TJ, Theobald H, Khalimonchuk O, Wayment B, Sheng X, Rodnick KJ, Centini R, Chen D, Litwin SE, Weimer BE, Abel ED. Contribution of impaired myocardial insulin signaling to mitochondrial dysfunction and oxidative stress in the heart. *Circulation* 2009; **119**: 1272-1283 [PMID: 19237663 DOI: 10.1161/CIRCULATIONAHA.108.792101]
60 **Bugianesi E**. Nonalcoholic fatty liver disease (NAFLD) and cardiac lipotoxicity: Another piece of the puzzle. *Hepatology* 2008; **47**: 2-4 [PMID: 18161723 DOI: 10.1002/hep.22105]
61 **Bugianesi E**, Gastaldelli A. Hepatic and cardiac steatosis: are they coupled? *Heart Fail Clin* 2012; **8**: 663-670 [PMID: 22999247 DOI: 10.1016/j.hfc.2012.06.010]
62 **Abel ED**, O'Shea KM, Ramasamy R. Insulin resistance: metabolic mechanisms and consequences in the heart. *Arterioscler Thromb Vasc Biol* 2012; **32**: 2068-2076 [PMID: 22895668 DOI: 10.1161/ATVBAHA.111.241984]
63 **Boden G**. Obesity, insulin resistance and free fatty acids. *Curr Opin Endocrinol Diabetes Obes* 2011; **18**: 139-143 [PMID: 21297467 DOI: 10.1097/MED]
64 **Iacobellis G**, Corradi D, Sharma AM. Epicardial adipose tissue: anatomic, biomolecular and clinical relationships with the heart. *Nat Clin Pract Cardiovasc Med* 2005; **2**: 536-543 [PMID: 16186852]
65 **Kankaanpää M**, Lehto HR, Pärkkä JP, Komu M, Viljanen A, Ferrannini E, Knuuti J, Nuutila P, Parkkola R, Iozzo P. Myocardial triglyceride content and epicardial fat mass in human obesity: relationship to left ventricular function and serum free fatty acid levels. *J Clin Endocrinol Metab* 2006; **91**:

4689-4695 [PMID: 16926257 DOI: 10.1210/jc.2006-0584]
66 **Dutour A**, Achard V, Sell H, Naour N, Collart F, Gaborit B, Silaghi A, Eckel J, Alessi MC, Henegar C, Clément K. Secretory type II phospholipase A2 is produced and secreted by epicardial adipose tissue and overexpressed in patients with coronary artery disease. *J Clin Endocrinol Metab* 2010; **95**: 963-967 [PMID: 20008021 DOI: 10.1210/jc.2009-1222]
67 **Mazurek T**, Zhang L, Zalewski A, Mannion JD, Diehl JT, Arafat H, Sarov-Blat L, O'Brien S, Keiper EA, Johnson AG, Martin J, Goldstein BJ, Shi Y. Human epicardial adipose tissue is a source of inflammatory mediators. *Circulation* 2003; **108**: 2460-2466 [PMID: 14581396 DOI: 10.1161/01.CIR]
68 **Iacobellis G**, Pellicelli AM, Grisorio B, Barbarini G, Leonetti F, Sharma AM, Barbaro G. Relation of epicardial fat and alanine aminotransferase in subjects with increased visceral fat. *Obesity* (Silver Spring) 2008; **16**: 179-183 [PMID: 18223632 DOI: 10.1038/oby.2007.50]
69 **Unger RH**, Orci L. Lipotoxic diseases of nonadipose tissues in obesity. *Int J Obes Relat Metab Disord* 2000; **24** (Suppl 4): 28S-32S [PMID: 11126236]
70 **Boudina S**, Abel ED. Diabetic cardiomyopathy revisited. *Circulation* 2007; **115**: 3213-3223 [PMID: 17592090 DOI: 10.1161/CIRCULATIONAHA.106.679597]
71 **Zhang QJ**, Holland WL, Wilson L, Tanner JM, Kearns D, Cahoon JM, Pettey D, Losee J, Duncan B, Gale D, Kowalski CA, Deeter N, Nichols A, Deesing M, Arrant C, Ruan T, Boehme C, McCamey DR, Rou J, Ambal K, Narra KK, Summers SA, Abel ED, Symons JD. Ceramide mediates vascular dysfunction in diet-induced obesity by PP2A-mediated dephosphorylation of the eNOS-Akt complex. *Diabetes* 2012; **61**: 1848-1859 [PMID: 22586587 DOI: 10.2337/db11-1399]
72 **Park TS**, Hu Y, Noh HL, Drosatos K, Okajima K, Buchanan J, Tuinei J, Homma S, Jiang XC, Abel ED, Goldberg IJ. Ceramide is a cardiotoxin in lipotoxic cardiomyopathy. *J Lipid Res* 2008; **49**: 2101-2112 [PMID: 18515784 DOI: 10.1194/jlr.M800147-JLR200]
73 **Fabbrini E**, Magkos F, Mohammed BS, Pietka T, Abumrad NA, Patterson BW, Okunade A, Klein S. Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity. *Proc Natl Acad Sci USA* 2009; **106**: 15430-15435 [PMID: 19706383 DOI: 10.1073/pnas.0904944106]
74 **Kantartzis K**, Rittig K, Cegan A, Machann J, Schick F, Balletshofer B, Fritsche A, Schleicher E, Häring HU, Stefan N. Fatty liver is independently associated with alterations in circulating HDL2 and HDL3 subfractions. *Diabetes Care* 2008; **31**: 366-368 [PMID: 18000185 DOI: 10.2337/dc07-1558]
75 **Sookoian S**, Gianotti TF, Rosselli MS, Burgueño AL, Castaño GO, Pirola CJ. Liver transcriptional profile of atherosclerosis-related genes in human nonalcoholic fatty liver disease. *Atherosclerosis* 2011; **218**: 378-385 [PMID: 21664615 DOI: 10.1016/j.atherosclerosis.2011.05.014]
76 **Targher G**, Bertolini L, Rodella S, Lippi G, Franchini M, Zoppini G, Muggeo M, Day CP. NASH predicts plasma inflammatory biomarkers independently of visceral fat in men. *Obesity* (Silver Spring) 2008; **16**: 1394-1399 [PMID: 18369343 DOI: 10.1038/oby.2008.64]
77 **Targher G**, Chonchol M, Miele L, Zoppini G, Pichiri I, Muggeo M. Nonalcoholic fatty liver disease as a contributor to hypercoagulation and thrombophilia in the metabolic syndrome. *Semin Thromb Hemost* 2009; **35**: 277-287 [PMID: 19452403 DOI: 10.1055/s-0029-1222606]
78 **Gaudio E**, Nobili V, Franchitto A, Onori P, Carpino G. Nonalcoholic fatty liver disease and atherosclerosis. *Intern Emerg Med* 2012; **7** (Suppl 3): 297S-305S [PMID: 23073871 DOI: 10.1007/s11739-012-0826-5]
79 **Butcher MJ**, Galkina EV. Phenotypic and functional heterogeneity of macrophages and dendritic cell subsets in the healthy and atherosclerosis-prone aorta. *Front Physiol* 2012; **3**: 44 [PMID: 22457649 DOI: 10.3389/fphys.2012.00044]
80 **Ley K**, Miller YI, Hedrick CC. Monocyte and macrophage dynamics during atherogenesis. *Arterioscler Thromb Vasc Biol* 2011; **31**: 1506-1516 [PMID: 21677293 DOI: 10.1161/ATVBAHA.110.221127]
81 **Moore KJ**, Tabas I. Macrophages in the pathogenesis of atherosclerosis. *Cell* 2011; **145**: 341-355 [PMID: 21529710 DOI: 10.1016/j.cell.2011.04.005]
82 **Gordon S**, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 2005; **5**: 953-964 [PMID: 16322748 DOI: 10.1038/nri1733]
83 **Gordon S**, Martinez FO. Alternative activation of macrophages: mechanism and functions. *Immunity* 2010; **32**: 593-604 [PMID: 20510870 DOI: 10.1016/j.immuni.2010.05.007]
84 **Van Hul N**, Lanthier N, Español Suñer R, Abarca Quinones J, van Rooijen N, Leclercq I. Kupffer cells influence parenchymal invasion and phenotypic orientation, but not the proliferation, of liver progenitor cells in a murine model of liver injury. *Am J Pathol* 2011; **179**: 1839-1850 [PMID: 21854752 DOI: 10.1016/j.ajpath.2011.06.042]
85 **Wan J**, Benkdane M, Teixeira-Clerc F, Bonnafous S, Louvet A, Lafdil F, Pecker F, Tran A, Gual P, Mallat A, Lotersztajn S, Pavoine C. M2 Kupffer cells promote M1 Kupffer cell apoptosis: a protective mechanism against alcoholic and nonalcoholic fatty liver disease. *Hepatology* 2014; **59**: 130-142 [PMID: 23832548 DOI: 10.1002/hep.26607]
86 **Tilg H**. Adipocytokines in nonalcoholic fatty liver disease: key players regulating steatosis, inflammation and fibrosis. *Curr Pharm Des* 2010; **16**: 1893-1895 [PMID: 20370678]
87 **Marra F**, Bertolani C. Adipokines in liver diseases. *Hepatology* 2009; **50**: 957-969 [PMID: 19585655 DOI: 10.1002/hep.23046]
88 **Bertolani C**, Marra F. The role of adipokines in liver fibrosis. *Pathophysiology* 2008; **15**: 91-101 [PMID: 18602801 DOI: 10.1016/j.pathophys.2008.05.001]
89 **Paolisso G**, Tagliamonte MR, Galderisi M, Zito GA, Petrocelli A, Carella C, de Divitiis O, Varricchio M. Plasma leptin level is associated with myocardial wall thickness in hypertensive insulin-resistant men. *Hypertension* 1999; **34**: 1047-1052 [PMID: 10567180 DOI: 10.1161/01.HYP.34.5.1047]
90 **Nobili V**, Carpino G, Alisi A, Franchitto A, Alpini G, De Vito R, Onori P, Alvaro D, Gaudio E. Hepatic progenitor cells activation, fibrosis, and adipokines production in pediatric nonalcoholic fatty liver disease. *Hepatology* 2012; **56**: 2142-2153 [PMID: 22467277 DOI: 10.1002/hep.25742]
91 **Carpino G**, Renzi A, Onori P, Gaudio E. Role of hepatic progenitor cells in nonalcoholic fatty liver disease development: cellular cross-talks and molecular networks. *Int J Mol Sci* 2013; **14**: 20112-20130 [PMID: 24113587 DOI: 10.3390/ijms141020112]
92 **Lumeng CN**, Saltiel AR. Inflammatory links between obesity and metabolic disease. *J Clin Invest* 2011; **121**: 2111-2117 [PMID: 21633179 DOI: 10.1172/JCI57132]
93 **Bertolani C**, Sancho-Bru P, Failli P, Bataller R, Aleffi S, DeFranco R, Mazzinghi B, Romagnani P, Milani S, Ginés P, Colmenero J, Parola M, Gelmini S, Tarquini R, Laffi G, Pinzani M, Marra F. Resistin as an intrahepatic cytokine: overexpression during chronic injury and induction of proinflammatory actions in hepatic stellate cells. *Am J Pathol* 2006; **169**: 2042-2053 [PMID: 17148667 DOI: 10.2353/ajpath]
94 **Beier JI**, Guo L, von Montfort C, Kaiser JP, Joshi-Barve S, Arteel GE. New role of resistin in lipopolysaccharide-induced liver damage in mice. *J Pharmacol Exp Ther* 2008; **325**: 801-808 [PMID: 18339969 DOI: 10.1124/jpet.108.136721]
95 **Del Turco S**, Navarra T, Gastaldelli A, Basta G. Protective role of adiponectin on endothelial dysfunction induced by AGEs: a clinical and experimental approach. *Microvasc Res* 2011; **82**: 73-76 [PMID: 21397613 DOI: 10.1016/j.mvr.2011.03.003]
96 **Verma S**, Li SH, Wang CH, Fedak PW, Li RK, Weisel RD, Mickle DA. Resistin promotes endothelial cell activation: further evidence of adipokine-endothelial interaction. *Circulation* 2003; **108**: 736-740 [PMID: 12874180]

97 **Kawanami D**, Maemura K, Takeda N, Harada T, Nojiri T, Imai Y, Manabe I, Utsunomiya K, Nagai R. Direct reciprocal effects of resistin and adiponectin on vascular endothelial cells: a new insight into adipocytokine-endothelial cell interactions. *Biochem Biophys Res Commun* 2004; **314**: 415-419 [PMID: 14733921]

98 **Mu H**, Ohashi R, Yan S, Chai H, Yang H, Lin P, Yao Q, Chen C. Adipokine resistin promotes in vitro angiogenesis of human endothelial cells. *Cardiovasc Res* 2006; **70**: 146-157 [PMID: 16515776 DOI: 10.1016/j.cardiores.2006.01.015]

99 **Cho Y**, Lee SE, Lee HC, Hur J, Lee S, Youn SW, Lee J, Lee HJ, Lee TK, Park J, Hwang SJ, Kwon YW, Cho HJ, Oh BH, Park YB, Kim HS. Adipokine resistin is a key player to modulate monocytes, endothelial cells, and smooth muscle cells, leading to progression of atherosclerosis in rabbit carotid artery. *J Am Coll Cardiol* 2011; **57**: 99-109 [PMID: 21185508 DOI: 10.1016/j.jacc.2010.07.035]

100 **Chen C**, Jiang J, Lü JM, Chai H, Wang X, Lin PH, Yao Q. Resistin decreases expression of endothelial nitric oxide synthase through oxidative stress in human coronary artery endothelial cells. *Am J Physiol Heart Circ Physiol* 2010; **299**: H193-H201 [PMID: 20435848 DOI: 10.1152/ajpheart.00431.2009]

101 **Badman MK**, Pissios P, Kennedy AR, Koukos G, Flier JS, Maratos-Flier E. Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. *Cell Metab* 2007; **5**: 426-437 [PMID: 17550778 DOI: 10.1016/j.cmet.2007.05.002]

102 **Giannini C**, Feldstein AE, Santoro N, Kim G, Kursawe R, Pierpont B, Caprio S. Circulating levels of FGF-21 in obese youth: associations with liver fat content and markers of liver damage. *J Clin Endocrinol Metab* 2013; **98**: 2993-3000 [PMID: 23626003 DOI: 10.1210/jc.2013-1250]

103 **Planavila A**, Redondo I, Hondares E, Vinciguerra M, Munts C, Iglesias R, Gabrielli LA, Sitges M, Giralt M, van Bilsen M, Villarroya F. Fibroblast growth factor 21 protects against cardiac hypertrophy in mice. *Nat Commun* 2013; **4**: 2019 [PMID: 23771152]

104 **Valenti L**, Alisi A, Galmozzi E, Bartuli A, Del Menico B, Alterio A, Dongiovanni P, Fargion S, Nobili V. I148M patatin-like phospholipase domain-containing 3 gene variant and severity of pediatric nonalcoholic fatty liver disease. *Hepatology* 2010; **52**: 1274-1280 [PMID: 20648474 DOI: 10.1002/hep.23823]

105 **Valenti L**, Motta BM, Alisi A, Sartorelli R, Buonaiuto G, Dongiovanni P, Rametta R, Pelusi S, Fargion S, Nobili V. LPIN1 rs13412852 polymorphism in pediatric nonalcoholic fatty liver disease. *J Pediatr Gastroenterol Nutr* 2012; **54**: 588-593 [PMID: 22157924]

106 **Rossi F**, Bellini G, Alisi A, Alterio A, Maione S, Perrone L, Locatelli F, Miraglia del Giudice E, Nobili V. Cannabinoid receptor type 2 functional variant influences liver damage in children with non-alcoholic fatty liver disease. *PLoS One* 2012; **7**: e42259 [PMID: 22927922 DOI: 10.1371/journal.pone.0042259]

107 **Lin YC**, Chang PF, Chang MH, Ni YH. A common variant in the peroxisome proliferator-activated receptor-γ coactivator-1α gene is associated with nonalcoholic fatty liver disease in obese children. *Am J Clin Nutr* 2013; **97**: 326-331 [PMID: 23269818 DOI: 10.3945/ajcn.112.046417]

108 **Adams LA**, White SW, Marsh JA, Lye SJ, Connor KL, Maganga R, Ayonrinde OT, Olynyk JK, Mori TA, Beilin LJ, Palmer LJ, Hamdorf JM, Pennell CE. Association between liver-specific gene polymorphisms and their expression levels with nonalcoholic fatty liver disease. *Hepatology* 2013; **57**: 590-600 [PMID: 23213074 DOI: 10.1002/hep.26184]

109 **Santoro N**, Zhang CK, Zhao H, Pakstis AJ, Kim G, Kursawe R, Dykas DJ, Bale AE, Giannini C, Pierpont B, Shaw MM, Groop L, Caprio S. Variant in the glucokinase regulatory protein (GCKR) gene is associated with fatty liver in obese children and adolescents. *Hepatology* 2012; **55**: 781-789 [PMID: 22105854 DOI: 10.1002/hep.24806]

110 **Al-Serri A**, Anstee QM, Valenti L, Nobili V, Leathart JB, Dongiovanni P, Patch J, Fracanzani A, Fargion S, Day CP, Daly AK. The SOD2 C47T polymorphism influences NAFLD fibrosis severity: evidence from case-control and intra-familial allele association studies. *J Hepatol* 2012; **56**: 448-454 [PMID: 21756849 DOI: 10.1016/j.jhep.2011.05.029]

111 **Romeo S**, Sentinelli F, Cambuli VM, Incani M, Congiu T, Matta V, Pilia S, Huang-Doran I, Cossu E, Loche S, Baroni MG. The 148M allele of the PNPLA3 gene is associated with indices of liver damage early in life. *J Hepatol* 2010; **53**: 335-338 [PMID: 20546964 DOI: 10.1016/j.jhep.2010.02.034]

112 **Santoro N**, Kursawe R, D'Adamo E, Dykas DJ, Zhang CK, Bale AE, Calí AM, Narayan D, Shaw MM, Pierpont B, Savoye M, Lartaud D, Eldrich S, Cushman SW, Zhao H, Shulman GI, Caprio S. A common variant in the patatin-like phospholipase 3 gene (PNPLA3) is associated with fatty liver disease in obese children and adolescents. *Hepatology* 2010; **52**: 1281-1290 [PMID: 20803499 DOI: 10.1002/hep.23832]

113 **Sookoian S**, Pirola CJ. Meta-analysis of the influence of I148M variant of patatin-like phospholipase domain containing 3 gene (PNPLA3) on the susceptibility and histological severity of nonalcoholic fatty liver disease. *Hepatology* 2011; **53**: 1883-1894 [PMID: 21381068 DOI: 10.1002/hep.24283]

114 **Valenti L**, Alisi A, Nobili V. I148M PNPLA3 variant and progressive liver disease: a new paradigm in hepatology. *Hepatology* 2012; **56**: 1883-1889 [PMID: 23012712 DOI: 10.1002/hep.25634]

115 **Speliotes EK**, Butler JL, Palmer CD, Voight BF, Hirschhorn JN. PNPLA3 variants specifically confer increased risk for histologic nonalcoholic fatty liver disease but not metabolic disease. *Hepatology* 2010; **52**: 904-912 [PMID: 20648472 DOI: 10.1002/hep.23768]

116 **Lin YC**, Chang PF, Hu FC, Yang WS, Chang MH, Ni YH. A common variant in the PNPLA3 gene is a risk factor for non-alcoholic fatty liver disease in obese Taiwanese children. *J Pediatr* 2011; **158**: 740-744 [PMID: 21168155 DOI: 10.1016/j.jpeds.2010.11.016]

117 **Goran MI**, Walker R, Le KA, Mahurkar S, Vikman S, Davis JN, Spruijt-Metz D, Weigensberg MJ, Allayee H. Effects of PNPLA3 on liver fat and metabolic profile in Hispanic children and adolescents. *Diabetes* 2010; **59**: 3127-3130 [PMID: 20852027 DOI: 10.2337/db10-0554]

118 **Woo KS**, Chook P, Yu CW, Sung RY, Qiao M, Leung SS, Lam CW, Metreweli C, Celermajer DS. Effects of diet and exercise on obesity-related vascular dysfunction in children. *Circulation* 2004; **109**: 1981-1986 [PMID: 15066949 DOI: 10.1161/01.CIR.0000126599.47470.BE]

119 **Wunsch R**, de Sousa G, Toschke AM, Reinehr T. Intima-media thickness in obese children before and after weight loss. *Pediatrics* 2006; **118**: 2334-2340 [PMID: 17142516 DOI: 10.1542/peds.2006-0302]

120 **Pahkala K**, Hietalampi H, Laitinen TT, Viikari JS, Rönnemaa T, Niinikoski H, Lagström H, Talvia S, Jula A, Heinonen OJ, Juonala M, Simell O, Raitakari OT. Ideal cardiovascular health in adolescence: effect of lifestyle intervention and association with vascular intima-media thickness and elasticity (the Special Turku Coronary Risk Factor Intervention Project for Children [STRIP] study). *Circulation* 2013; **127**: 2088-2096 [PMID: 23613255]

121 **Reinehr T**, Andler W. Changes in the atherogenic risk factor profile according to degree of weight loss. *Arch Dis Child* 2004; **89**: 419-422 [PMID: 15102630 DOI: 10.1136/adc.2003.028803]

122 **Shai I**, Spence JD, Schwarzfuchs D, Henkin Y, Parraga G, Rudich A, Fenster A, Mallett C, Liel-Cohen N, Tirosh A, Bolotin A, Thiery J, Fiedler GM, Blüher M, Stumvoll M, Stampfer MJ. Dietary intervention to reverse carotid atherosclerosis. *Circulation* 2010; **121**: 1200-1208 [PMID: 20194883]

123 **Wildman RP**, Schott LL, Brockwell S, Kuller LH, Sutton-Tyrrell K. A dietary and exercise intervention slows meno-

pause-associated progression of subclinical atherosclerosis as measured by intima-media thickness of the carotid arteries. *J Am Coll Cardiol* 2004; **44**: 579-585 [PMID: 15358024 DOI: 10.1016/j.jacc.2004.03.078]

124 **Pacifico L**, Arca M, Anania C, Cantisani V, Di Martino M, Chiesa C. Arterial function and structure after a 1-year lifestyle intervention in children with nonalcoholic fatty liver disease. *Nutr Metab Cardiovasc Dis* 2013; **23**: 1010-1016 [PMID: 23018041 DOI: 10.1016/j.numecd.2012]

125 **Karason K**, Wikstrand J, Sjöström L, Wendelhag I. Weight loss and progression of early atherosclerosis in the carotid artery: a four-year controlled study of obese subjects. *Int J Obes Relat Metab Disord* 1999; **23**: 948-956 [PMID: 10490801]

126 **Anderssen SA**, Hjelstuen AK, Hjermann I, Bjerkan K, Holme I. Fluvastatin and lifestyle modification for reduction of carotid intima-media thickness and left ventricular mass progression in drug-treated hypertensives. *Atherosclerosis* 2005; **178**: 387-397 [PMID: 15694949 DOI: 10.1016/j.atherosclerosis.2004]

127 **Koskinen J**, Magnussen CG, Taittonen L, Räsänen L, Mikkilä V, Laitinen T, Rönnemaa T, Kähönen M, Viikari JS, Raitakari OT, Juonala M. Arterial structure and function after recovery from the metabolic syndrome: the cardiovascular risk in Young Finns Study. *Circulation* 2010; **121**: 392-400 [PMID: 20065161]

128 **Musso G**, Cassader M, Rosina F, Gambino R. Impact of current treatments on liver disease, glucose metabolism and cardiovascular risk in non-alcoholic fatty liver disease (NAFLD): a systematic review and meta-analysis of randomised trials. *Diabetologia* 2012; **55**: 885-904 [PMID: 22278337 DOI: 10.1007/s00125-011-2446-4]

P- Reviewers: Atanasov G, Gong NQ, Najimi M
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI:10.3748/wjg.v20.i27.9072

World J Gastroenterol 2014 July 21; 20(27): 9072-9089
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Non-alcoholic fatty liver disease and type 2 diabetes mellitus: The liver disease of our age?

Gábor Firneisz

Gábor Firneisz, 2nd Department of Internal Medicine, Semmelweis University, H-1088 Budapest, Hungary
Author contributions: Firneisz G contributed solely to this manuscript.
Correspondence to: Gábor Firneisz, MD, PhD, 2nd Department of Internal Medicine, Semmelweis University, Szentkiralyi St 46, H-1088 Budapest, Hungary. firneisz.gabor@med.semmelweis-univ.hu
Telephone: +36-1-2100278-5520 Fax: +36-1-2661007
Received: November 14, 2013 Revised: March 20, 2014
Accepted: May 12, 2014
Published online: July 21, 2014

Abstract

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease that might affect up to one-third of the adult population in industrialised countries. NAFLD incorporates histologically and clinically different non-alcoholic entities; fatty liver (NAFL, steatosis hepatis) and steatohepatitis (NASH-characterised by hepatocyte ballooning and lobular inflammation ± fibrosis) might progress to cirrhosis and rarely to hepatocellular cancer. NAFL increasingly affects children (paediatric prevalence is 4.2%-9.6%). Type 2 diabetes mellitus (T2DM), insulin resistance (IR), obesity, metabolic syndrome and NAFLD are particularly closely related. Increased hepatic lipid storage is an early abnormality in insulin resistant women with a history of gestational diabetes mellitus. The accumulation of triacylglycerols in hepatocytes is predominantly derived from the plasma non-esterified fatty acid pool supplied largely by the adipose tissue. A few NAFLD susceptibility gene variants are associated with progressive liver disease, IR, T2DM and a higher risk for hepatocellular carcinoma. Although not approved, pharmacological approaches might be considered in NASH patients.

Key words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Liver cirrhosis; Hepatocellular cancer; Dysfunctional adipose tissue; Type 2 diabetes mellitus; Insulin resistance; Obesity; Genetics; Therapy

Core tip: In this review article, non-alcoholic fatty liver disease (NAFLD) spectrum disease is discussed in detail. The epidemiology of NAFLD/nonalcoholic steatohepatitis and the relationship of NAFLD to different forms of diabetes mellitus including type 2 diabetes mellitus and gestational diabetes mellitus are reviewed. Attention is paid to the main biochemical events, to dysfunctional adipose tissue and visceral adiposity associated with NAFLD and insulin resistance, to mitochondrial dysfunction and to the role of the entero-insular axis in NAFLD. Genetics and potential pharmacological approaches are discussed.

Firneisz G. Non-alcoholic fatty liver disease and type 2 diabetes mellitus: The liver disease of our age? *World J Gastroenterol* 2014; 20(27): 9072-9089 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9072.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9072

HISTORY

The work regarding non-alcoholic fatty liver disease (NAFLD) was launched only three decades ago, when Ludwig *et al*[1] described an "unnamed" and "poorly understood" liver disease they named non-alcoholic steatohepatitis (NASH) in 20 patients that histologically reminded authors of alcoholic hepatitis with a potential of progression to cirrhosis. Although 20 patients with NAFLD today could likely be recruited within one day in a lobby of a hotel, their observation that NASH is an obesity-associated disease largely accompanied by dia-

betes mellitus presenting with hepatomegaly and mild abnormalities of liver tests still accurately describes the most common clinical findings. There are a number of confounding factors in determining the true prevalence and incidence of NAFLD.

EPIDEMIOLOGY OF NAFLD/NASH BASED ON HISTOLOGY

NAFLD incorporates histologically and clinically different non-alcoholic entities as fatty liver (NAFL, steatosis hepatis) and steatohepatitis (NASH) with or without fibrosis that might progress to liver cirrhosis and in a few cases to hepatocellular cancer[2,3].

The gold standard methodologies are difficult to apply in a general population based study. Histological assessment had an exclusive role for decades as the only method for grading hepatic steatosis; however this method has been recently challenged and not only due to the difficulties in everyday use for diagnostic purposes in this spectrum of diseases. Hepatic steatosis is defined as intrahepatic fat content above 5.5%[4,5] or when more than 5% of the hepatocytes contain typically macrovesicular fat on the histology. Steatosis might be graded as-S1, mild (up to 10% of hepatocytes); S2, moderate (10% to 30% of hepatocytes); S3, severe (more than 30% of hepatocytes)-according to the proportion of the cells with macrovesicular changes in the liver cells containing fat.

Under routine clinical circumstances, liver biopsy is typically indicated when liver tests (LTs) are repeatedly and chronically elevated (*e.g.*, for six months) and are of unexplained origin. A few biopsy-based studies report the NASH prevalence in the general population. Williams *et al*[6] recruited 400 outpatients aged 18 to 70 years and performed 134 liver biopsies when the screening abdominal ultrasound suggested hepatic steatosis. Although ultrasound pre-screening theoretically might implicate a selection bias, the reported prevalence of NAFLD was 46% in this United States cohort. The recalculated NAFLD prevalence-taking into account those who refused the biopsy and those with normal liver histology-is 40% based on the biopsy findings. NASH was diagnosed in 12.2% of the entire United States cohort and 29.9% of the NAFLD patients (Table 1). Differences were found according to ethnic origin, with the highest risk for NAFLD and NASH presented in patients of Hispanic origin (> Caucasian > African-American) and according to the presence of diabetes mellitus (NAFLD prevalence, 74%, NASH prevalence, 22.2%); the NAFLD patients were more likely to be male (58.9%) and of older age, to have a higher BMI and to present with hypertension.

We might conclude that the prevalence of NAFLD and NASH is highly dependent on the structure of the study population due to the significant differences in prevalence among different sub-populations. The differences in the prevalence and clinical-pathological presentation are influenced by gender as follows: the males among NAFLD patients are more prone to present with elevated LTs, histologically determined NASH, hepatic fibrosis and higher overall mortality according to the majority of studies[7-9].

The NAFLD spectrum diseases do not differ from the demographic trends observed in metabolic diseases including type 2 diabetes mellitus that affect increasingly younger generations decade by decade[10]. The prevalence of NAFL in Poland and NAFLD in the United States was 4.2% and 9.6% based on histopathology at autopsy in pediatric populations, respectively[11,12]. Excess bodyweight was found in 55.6% of the children with NAFL in the European study, and the highest rate of NAFLD was observed in obese United States children (38%); the latter study found a difference in the NAFLD prevalence among different sub-populations (ethnic origin) in children[11,12].

EPIDEMIOLOGY OF NAFLD BASED ON PROTON MAGNETIC RESONANCE SPECTROSCOPY

Grading hepatic steatosis by histological examination potentially holds other biases, including sampling and observation biases. The amount of triglyceride accumulation in the liver could be assumed to be too low to allow the formation of macrovesicles and might not be assessed at histology. Proton magnetic resonance spectroscopy (^{1}HMRS) has appropriate sensitivity for the quantification of the intrahepatic lipid content and correlates better with the biochemical analysis results of liver specimens even if such a small lipid accumulation is in question[13,14]. Compared to traditional histology, ^{1}HMRS based steatosis (intrahepatocellular lipid content, IHCL) grading is based on a much larger mass of hepatic tissue that is investigated without the risks of liver biopsy (27 g *vs* 100 mg), providing additional advantages over the invasive method[13,14].

The NAFLD prevalence from the studies using ^{1}HMRS based IHCL measurements are indicated in Table 1. The prevalence of fatty liver disease in a population-based study in the United States using a ^{1}HMRS-based measurement for the determination of the intrahepatic triglyceride content (HTGC) was 34%, and in over 90% of the 2287 enrolled individuals, it was because of non-alcoholic causes[4]. The results of this multi-ethnic study demonstrated differences in the prevalence of hepatic steatosis among the different ethnic groups studied, with a higher prevalence in Hispanic patients, explained by the higher prevalence of obesity and insulin resistance. The authors concluded that ethnic differences in the prevalence of hepatic steatosis in the study reflected those observed previously for NAFLD-related cirrhosis (Hispanics > whites > blacks). The finding that the majority of subjects (79%) with NAFLD had normal levels of serum alanine aminotransferase should be taken into

Table 1 Prevalence of non-alcoholic fatty liver disease, non-alcoholic fatty liver and non-alcoholic steatohepatitis

NAFLD/NAFL prevalence	NASH prevalence	Population studied	Population size	Method of diagnosis	Remark	Ref.
46 (40)% NAFLD in the entire US cohort	12.2% in the US entire cohort 29.9% in patients with NAFLD	18-70 yr aged US cohort	328	Liver biopsy (in 134 ultrasound pre-screend patients)	Greatest risk for both NAFLD and NASH in Hispanics and with diabetes	[6]
NAFL: 49.3%-mild, moderate, and severe NAFL in 38.9%, 9.0% and 1.4% donor candidates respectively (mild steatosis was defined as fatty changes in 5%-30% of hepatocytes, moderate steatosis in 30% to 60% of hepatocytes, and severe steatosis in > 60% of hepatocytes without significant inflammation on liver histology)	2.2% (Asian population)	Korean living liver donor candidates	589	589 US guided liver biopsy		[126]
NAFL: 4.2% in pediatric European population	1% (pediatric population)	European children (6 mo-18 yr old)	342 medicolegal autopsy reports/265 children died from trauma	Histopathology at autopsy (and typical macroscopic imaging)	Excess body weight was observed in 55.6% of children with NAFL	[11]
NAFLD 9.6% in pediatric US population fatty liver was defined as > or = 5% of hepatocytes containing macrovesicular fat	NR	United States children (2-19 yr old)	742 children (2-19 yr old) who had autopsy (form 1993 to 2003)	Histopathology at autopsy	Different prevalence according to subpopulation (Asians: 10.2%; Black: 1.5%; Hispanic: 11.8%; White: 8.6%). The highest rate of NAFLD was seen in obese children (38%)	[12]
NAFLD 31% in adult Urban US population	NR	Large, ethnically diverse, probability-based adult population sample from Dallas, Texas, United States -participants in the Dallas Heart Study	2349	^{1}H-MRS of the liver to quantify HTGC	79% of patients with hepatic steatosis had normal levels of serum alanine aminotransferase. Different prevalence of hepatic steatosis in different sub-populations: 45% in Hispanics, 33% in Whites and 24% in Blacks	[4]

Based on histological examination or proton magnetic resonance spectroscopy (^{1}H-MRS) measurement in Hallmark Studies. HTGC: Hepatic triglyceride content; NAFLD: Non-alcoholic fatty liver disease; NAFL: Non-alcoholic fatty liver; NASH: Non-alcoholic steatohepatitis; NR: Not reported.

account when evaluating population-based studies using score systems that incorporate LT in the diagnosis of NAFLD[4].

In a recent study, biochemically measured hepatic triglyceride levels correlated significantly with the hepatic lipid level (IHCL) measured with ^{1}HMRS. The histologic and ^{1}HMRS grading of fatty liver was in agreement in the majority (65%) of these C-virus infected patients. In contrast, no linear correlation between the biochemically determined liver triglyceride content and histological examination was found[15].

ULTRASOUND BASED NAFLD PREVALENCE

The diagnostic plethora of NAFLD is further complicated by many studies using ultrasonography data to identify patients with NAFLD, although ultrasonography might not be regarded as accurate radiologic modality as the ^{1}HMRS in measuring the intrahepatic lipid level. The third National Health and Nutrition Examination Survey (NAHNES) assessed the prevalence of NAFLD from 1988 to 1994 in the United States based on the ultrasonography data of 12454 adults. They estimated that 28.8 million adults might be diagnosed with NAFLD in the United States; the corresponding prevalence is 19%[16]. The data obtained from the NAHNES study confirmed that NAFLD occurs with a higher prevalence in Mexican-Americans compared to non-Hispanic whites and non-Hispanic blacks. NAFLD was independently associated with insulin resistance and diabetes; and, among people without diabetes, with dyslipidaemia and obesity. The study confirmed that NAFLD is more common in males[16]. The NAFLD prevalence in the Italian Dionysos project in adults with and without suspected liver dis-

ease was 25% and 20%, respectively using the US based method for identification[17]. The prevalence of NAFLD in Japan increased to 2.4-fold from the 12.6% prevalence found in 1989 to the 30.3% prevalence observed in 1998[18]. A lower prevalence was reported in India, using ultrasonography for the identification of NAFLD: the prevalence of NAFLD was 18.9% in adults above 20 years of age, with a higher prevalence of NAFLD in males than females (24.6% *vs* 13.6%)[19].

A number of studies report the aminotransferase-based approach to diagnose; due to the observation that more than 75% of the patients with steatosis might have normal LT values, these studies are not discussed in detail here[4].

ASSESSMENT OF FIBROSIS AND STEATOHEPATITIS

In most patients with NAFLD, a non-invasive score proposed by Angulo *et al*[20] could be applied to assess liver fibrosis because advanced fibrosis could be diagnosed with accuracy in 90% of the cases. The authors analysed the clinical and liver biopsy data of more than 700 patients to construct this simple scoring system. Transient elastography (Fibroscan), which is a radiological modality used with high accuracy to non-invasively assess the degree of fibrosis based on the measurement of liver stiffness[21,22], might detect even low-grade steatosis because of a recently described novel ultrasonic controlled attenuation parameter of the machine[23].

Although there are promising biomarkers such as cytokeratin-18 fragments for NASH to be potentially applied as diagnostic tools in the future[24], the accurate diagnosis of NASH in some cases might require a liver biopsy. A biopsy is typically indicated in the cases in which the liver tests (LTs) are repeatedly elevated in a chronic manner (*e.g.*, for six months) and are of unexplained origin and when the patient must be carefully investigated for alternative diagnoses (*e.g.*, to measure assess hepatic iron concentration in a C282Y HFE heterozygote individual; when the case is suggestive of autoimmune liver disease; rarely, when the diagnosis of Wilson disease may not be established without quantitation of the liver copper content; in other storage diseases; or in rare distinct forms of liver disease)[25-29].

WHY IS NAFLD WORRISOME?

Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) and NAFLD are particularly closely related. This relationship among T2DM, insulin resistance (IR) and NAFLD is expected because insulin is subsequently delivered directly to the portal vein after secretion, taking the same route as the absorbed glucose, and the liver eliminates a large portion of portal insulin at the first pass.

Obesity in NAFLD is associated with dysfunctional adipose tissue, and lipotoxicity promotes insulin resistance and pancreatic β-cell dysfunction.

The prevalence of ultrasonographic NAFLD was 69.4% in 180 patients with T2DM[30]. NAFLD was associated with obesity (abdominal), hypertriglyceridemia and high-normal ALT levels. The authors concluded that the progression of NAFLD is independent of the diabetes progression[30].

The ultrasonography results of 204 patients with T2DM showed fatty infiltration in 62.2% of the patients; NAFLD was confirmed by liver biopsy with subsequent histology in 87% of the patients, indicating a 54.11% histologically confirmed prevalence in T2DM. Steatohepatitis and fibrosis were found in 38.9% and 23.2%, respectively, of Indian patients with T2DM[31].

Leite *et al*[32] found a 78% NASH prevalence at the histological examination in nearly 100 patients with T2DM and US evidence of NAFLD. The presence of high triglyceride, low HDL-cholesterol and increased ALT levels were independently associated with a higher risk of histologically confirmed NASH. The prevalence of advanced fibrosis (≥ stage 2) was found in 38% and 55% of the patients depending on the pathologist who conducted the histological examination. The presence of NASH was independently correlated with high serum γGT levels, older age and male gender[32].

Gestational diabetes mellitus

Recent novel findings have emerged to confirm the relationship between diabetes mellitus and NAFLD: Women with a history of gestational diabetes mellitus (GDM) have an increased risk of developing T2DM decades later. Prikoszovich *et al*[33] recruited women with a history of GDM (pGDM) four to five years after delivery and assessed the glucose tolerance and oral glucose insulin sensitivity to measure the whole-body insulin sensitivity during a 75 g CH OGTT. The lipid storage in the muscle, liver and flux through the ATP synthase were measured using $^{1}H/^{31}P$ magnetic resonance spectroscopy. In a comparison with women without any risk factor for T2DM, the hepatic content of lipids (HCL) was doubled in the insulin resistant pGDM women, despite they had normal glucose tolerance. HCL correlated positively with the body fat mass and inversely with insulin sensitivity. The authors concluded that increased hepatic lipid storage is an early and predominant abnormality in insulin resistant women with a history of GDM.

The fatty liver index (FLI) -measured using ^{1}HMRS- in women with previous GDM predicted further metabolic deterioration and subjects with the highest FLI values showed significant alterations in FFA kinetics with a higher risk to develop T2DM in the future[34]. The results of these studies should be taken into consideration when the role of NAFLD in determining the hepatic and whole body insulin sensitivity is under scrutiny in glucose tolerant individuals with insulin resistance.

In 2013, Brumbaugh *et al*[35] assessed the intrahepatic lipids in the neonatal offspring of obese women with gestational diabetes. The neonates born to obese women

with GDM underwent MRS for intrahepatic lipid content determination at 1-3 wk of age and demonstrated a mean 68% increase in the IHCL compared with infants born to normal-weight mothers. The intrahepatic fat deposition in the neonates positively correlated with the maternal pre-pregnancy BMI and not with subcutaneous adiposity.

"Metabolic syndrome"

Although many authors agree that the "metabolic syndrome" is a cluster of risk factors, but whether it may correctly be considered a syndrome is strongly questioned[36]. Provided the term "metabolic syndrome" is accepted, insulin resistance should be at the core of the "syndrome", and many authors agree that NAFLD that is generally asymptomatic is frequently associated with obesity, type 2 diabetes and the "metabolic syndrome"[17]. Although a statement was published for synchronising the various definitions for metabolic syndrome[37], our experience confirmed that a large proportion of patients with NAFLD did not fulfill the "metabolic syndrome" criteria (49%-46% depending on the NCEP in ATP-Ⅲ[38] or the AHA and IDF joint criteria[37] were applied). The high proportion of NAFLD patients without "metabolic syndrome" was observed despite that the HOMA2-IR values of the NAFLD patients were even higher in this study population than those with T2DM, provided that the NAFLD patients were excluded from the latter study group. None of the definitions of "metabolic syndrome" could be appraised as a consensus due to that the significant proportion of insulin-resistant NAFLD patients are excluded by these criteria[39].

BIOCHEMISTRY

The major biochemical event in NAFLD is the accumulation of triacylglycerols (TAG) in the hepatocytes. Because of the strong associations described above and to understand the relation of NAFLD to whole-body metabolic status, Donnelly *et al*[40] conducted a study using gas chromatography/mass spectrometry. Hepatic TAG might accumulate from different sources in the hepatocytes as follows: TAG as a nutrient after absorption from the intestine are delivered *via* chylomicrons to the liver, where they might subsequently be secreted as lipoproteins. Hepatic TAG synthesis is possible, and this process requires fatty acids and glycerol in the liver The required fatty acids might be from the plasma non-esterified fatty acid pool (NEFA), and they might be produced in the liver as *de novo* hepatic lipogenesis. It has been reported that approximately 60% of the TAG accumulated in the liver is derived from the plasma NEFA pool, even in the fed state, and that adipose tissue is the largest contributor to the fatty acid content of the plasma NEFA pool (80% in the fasted state). One-quarter of the TAG accumulation is derived from hepatic *de novo* lipogenesis that is elevated in the fasting state and demonstrates no diurnal variation, whereas approximately 15% is derived from the dietary intake. The situation is even worse when insulin resistance - a hallmark of metabolic syndrome - is also present because of the lack of (insulin induced) down-regulation of the hormone sensitive lipase that eventually results in enhanced lipolysis and an increased efflux of free fatty acids to the plasma NEFA pool from the adipocytes. Hyperglycaemia (and hyperinsulinaemia) induces SREBP-1c and ChREBP in the liver, and these transcription factors subsequently activate genes that are required for lipogenesis, eventually resulting in increased hepatic *de novo* lipogenesis. Hepatic *de novo* lipogenesis is increased in insulin-resistant states and in NAFLD[41]. Beta-oxidation of fatty acids is increased in patients with NASH; the oxidation might not overcome the increased hepatic TAG production, and the increased NEFA oxidation might result in increased oxidative stress, enhancing the transition of NAFL to NASH[42,43]. This (patho)biochemical path provides the reason that the association is remarkably strong between fatty liver and obesity-related insulin resistance[40,44].

NAFLD CONCURRENTLY WITH VISCERAL AND SUBCUTANEOUS ADIPOSITY AND INSULIN RESISTANCE

Is there accurate morphological evidence to support this biochemistry-driven hypothesis that the intrahepatic lipid content is hand-in-hand with different adipose tissue deposits, particularly the visceral adipose tissue accumulation that is strongly associated in impaired glucose metabolism?

Bosy-Westphal *et al*[45] assessed the fat volume including the visceral fat volumes (VAT), the pericardial adipose tissue (PAT, a well-known marker of visceral adiposity) and the abdominal subcutaneous adipose tissue using MRI and compared the results to the IHCL quantified by the highly sensitive ^{1}H-MRS method in thirty overweight, not yet diabetic women. The participating individuals were restricted to a low calorie diet for three months; at baseline, the visceral adipose tissue volume and PAT correlated with the IHCL as well as with the insulin resistance measured with the euglycaemic hyperinsulinaemic clamp and the homeostatic model assessment (HOMA)-IR. The strength of the relationship between the visceral fat volume and IHCL is shown by the finding that the reductions in IHCL induced by the dietary intervention and loss of body weight were only correlated with the decrease in VAT. The exceptional role of NAFLD in determining insulin resistance is confirmed by the improvements in HOMA-IR and HOMA2-%B after three months of diet and weight loss that were only related to the decrease in IHCL[45].

The interpretation of HOMA-IR has been recently challenged. Traditionally, HOMA-IR reflects the degree of insulin resistance; however, these estimates based on the fasting plasma insulin and glucose concentrations[46] do not take into account whether the secretion of insulin by the pancreatic β cells is altered or if an alteration is

in the insulin removal (clearance). The differentiation of total body insulin resistance, peripheral insulin resistance and hepatic insulin resistance merits research attention. A recent novel interpretation of HOMA-IR suggests that it is not a precise estimate of peripheral insulin action rather it might reflect the ability of insulin to suppress hepatic glucose production in the fasting state[47]. The clamp technique used in the previously mentioned study measures the peripheral insulin action in non-diabetic individuals resulting from hyperinsulinaemia during the measurement that is high enough to inhibit the hepatic glucose production completely[48]. This concept should be validated in studies using an insulin concentration that is lower than that regularly used during an euglycaemic-hyperinsulinaemic clamp measurement to avoid the absolute inhibition of hepatic glucose production[47]. Provided that this hypothesis regarding HOMA-IR is accurate, the ^{1}H-MRS based follow-up observation that improvements in HOMA-IR and HOMA2-%B after dietary intervention were only related to the decrease in the IHCL indicates that a decrease in the intrahepatocellular lipid content increases the ability of insulin to suppress the hepatic glucose production in the fasting state, which is a major determinant of fasting plasma glucose levels[45].

In addition the contribution of the subcutaneous adipose tissue (SAT) to the whole-body adipose tissue dysfunction has been also recently confronted against the broadly accepted "innocent bystander to VAT" concept. The results of an *in vivo* human study that functionally assessed SAT in patients with histology-confirmed NASH provided evidence that the abdominal SAT in NASH patients is highly insulin resistant and required > 6 × more insulin to the gain similar degree of glycerol release suppression (referring to impaired suppression of lipolysis in SAT by insulin) than in healthy subjects. Authors therefore suggested that abdominal SAT is dysfunctional in NASH and plays a profound role in NASH development and lipotoxicity[49].

LIPOTOXICITY

Free fatty acids (FFA) are directly hepatotoxic, and FFA levels are elevated in patients with NASH and correlate with disease severity[50]. Patients with severe fibrosis shown by liver biopsy had significantly greater serum concentration of free fatty acids than did the patients without severe fibrosis[50]. Saturated FFAs (*e.g.*, palmitate) are apparently more hepatotoxic than unsaturated (mono-unsaturated) FFAs (*e.g.*, palmitoleate); palmitoleate (known as a lipokine) was demonstrated to suppress hepatic steatosis[51,52]. Unsaturated fatty acids do not induce endoplasmic reticulum (ER) stress or apoptosis and are able to rescue the palmitate-induced ER stress and apoptosis in liver cells. It has been proposed that the difference in toxicity between saturated and unsaturated fatty acids is that unsaturated FFAs are more easily esterified into neutral triglycerides[53,54].

The impairment of liver cellular capacity in FFA utilisation, incorporation to TAGs and export contributes to the development of NASH, and hepatic injury is further accentuated by pathological FA oxidation and altered cell membrane composition[55]. Lipotoxicity induces hepatocellular apoptosis; Kupffer cell activation; impaired insulin signaling and hepatic insulin resistance; and hepatic stellate (Ito) cell activation with subsequent fibrosis. These pathological processes might eventually lead to cirrhosis[55].

DIACYLGLYCEROL ACYLTRANSFERASE 2: DISSOCIATION OF STEATOSIS AND INFLAMMATION-FIBROSIS

The hepatotoxicity of saturated FFAs is further supported by the genetic deletion of diacylglycerol acyltransferase 2 (DGAT2) in mice models as follows: in parallel with the decreased TAG synthesis due to the the increased oxidative stress from the lack of intracellular FFA esterification, hepatocellular apoptosis with subsequent fibrosis occurs, resulting in the dissociation of hepatic steatosis and hepatic fibrosis in the NASH model[56,57]. Overexpression of DGAT2 in the experimental model causes hepatic steatosis without concomitant liver fibrosis or insulin resistance, providing evidence for the different roles that toxic saturated FFAs and TAGs have in the development of NAFLD[57,58].

The pathological effect of lipotoxicity is not limited to the liver cells, and it might affect the pancreatic β-cells, contributing to the β-cell dysfunction that is frequently observed in T2DM[59]. This pathology that affects both the liver and the pancreatic β-cells is highly important in determining the plasma glucose levels.

Lipotoxicity in the pancreatic β-cells

The intracellular signaling pathways altered because of lipotoxicity should partially overlap in the liver cells and in the pancreatic β-cells (Figure 1).

Saturated fatty acids, among other factors, induce endoplasmic reticulum stress that induce JNK activation *via* the IRE1α/ASK1, a signal that has been described in pancreatic β-cells as well as in liver cells[60,61]. Subsequently, the activation of the JNK pathway induces the "sensitiser" BH3 proteins (DP5, Bad, Bik), which bind to the anti-apoptotic Bcl-2 proteins such as Bcl-2 and Bcl-XL; these proteins designate the pro-apoptotic BH3-only proteins, BIM and PUMA ("activators"), eventually leading to their activation, which results in the apoptotic death of the pancreatic β and liver cells *via* Bak and Bax[61-63]. There is a substantial homology of this unsaturated FFA induced path and the path of cytokine induced β cell apoptosis. Palmitoleate (a mono-unsaturated fatty acid) could inhibit lipoapoptosis by blocking the endoplasmic reticulum stress-associated increases of the BH3-only proteins, Bim and PUMA, in hepatocytes[64]. The deteriorating β cell function, in combination with the increasing hepatic IR and the decreasing suppression of hepatic glucose output, leads to hyperglycaemia that eventually

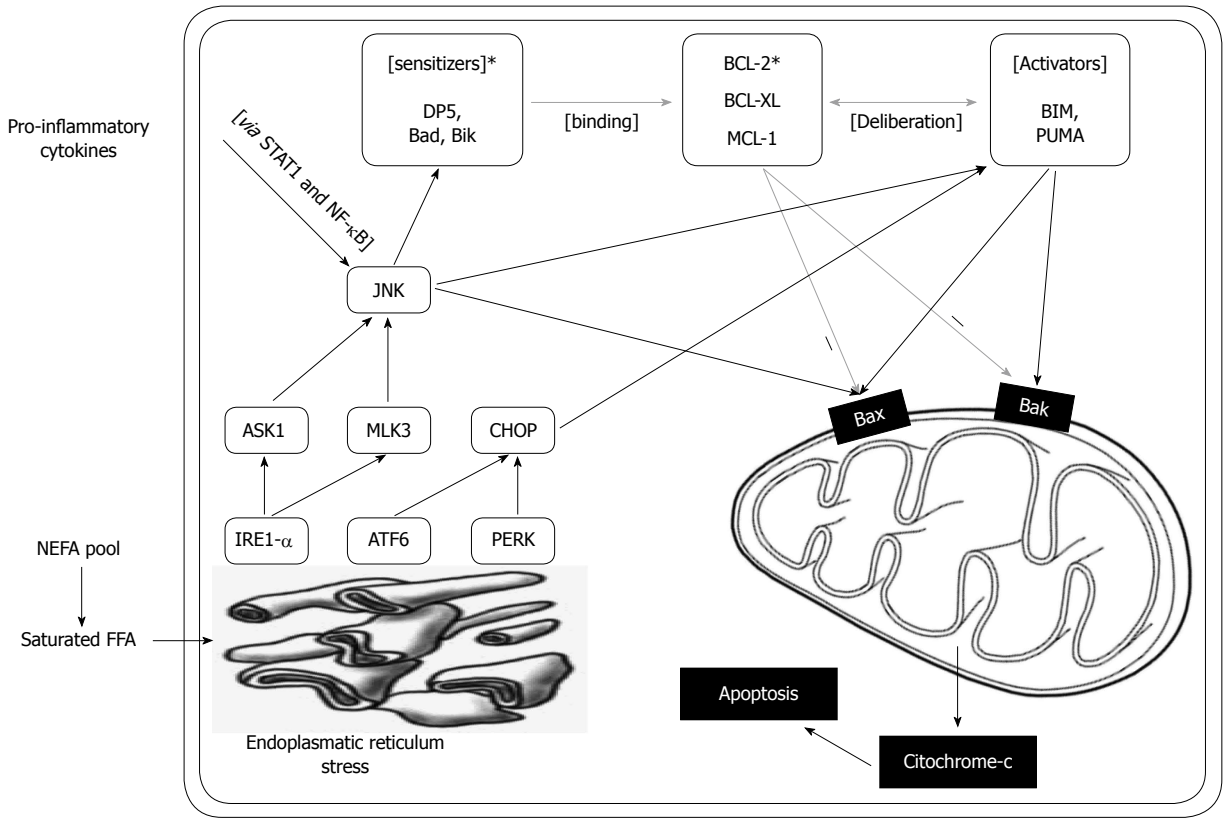


Figure 1 Endoplasmatic reticulum stress caused by saturated free fatty acids. Endoplasmatic reticulum stress caused by saturated free fatty acids, *via* three main mediators [inositol-requiring endoplasmic reticulum-to-nucleus signal kinase 1α (IRE1α), activating transcription factor 6 (ATF6) and RNA-dependent protein kinase (PRK)-like endoplasmic roticulum (ER) kinase (PERK)], results in the activation of c-Jun N-terminal kinase (JNK) and C/ CCAAT/enhancer binding protein (EBP) homologous protein. The sequence of BH3 protein activation based on a sensitizer and an activator group was described by Gurzov and Eizirik in β-cells[62,63]. The sensitizers bind the anti-apoptotic proteins Bcl-2, Bcl-XL and Mcl-1 and release the activators from this bond. JNK both mediates the induction of the sensitizer and the activator BH3 proteins and also activates Bax. Upregulation of Bcl-2 interacting mediator of cell death (BIM) and p53 upregulated modulator of apoptosis (PUMA) was also demonstrated in liver cells as a ruslt of free fatty acid (FFA) induction. Proinflammatory cytokines also activate JNK. ER sterss also results in CHOP actvation and subsequently the activation of the activator BH3 proteins. This complex signaling pathway might link the metabolic (saturated FFAs) stress and the effect of pro-inflammatory cytokines both in the pancreatic β-cell as well as in liver cells.

might directly (glucotoxicity) and by biochemical and metabolic consequences further promote this pathologic process. This cross-talk between the metabolic and cytokine induced pathways might facilitate the identification of novel drug targets (*e.g.*, DP5, Bim) that would inhibit the unsaturated FFA induced ER stress mediated apoptotic liver cell death and possess protective properties against cytokine induced pancreatic β cell death[60-63]. The cytokines, growth factors and inflammatory mediators that are important in NAFLD are summarised in Table 2.

MITOCHONDRIAL DYSFUNCTION

A defective hepatic mitochondrial respiratory chain (MRC) was described in NASH[65]. The mitochondrial dysfunction, as measured by the activity of the MRC complexes in liver tissue, was correlated with the serum TNF-alpha levels and with the degree of insulin resistance that were higher in NASH and with the BMI[65]. In addition to the mitochondrial dysfunction, Sanyal *et al*[66] described structural mitochondrial defects including the loss of the mitochondrial cristae and paracrystalline inclusions, the presence of linear crystalline inclusions and mitochondrial swelling in patients with NASH. Patients with T2DM of long duration might have decreased ATP production after fasting and after fructose administration. The mitochondrial dysfunction in NAFLD, from lipotoxicity, oxidative stress and as a result of inflammatory mediator effect, alters the hepatic energy metabolism, as recently reported by Koliaki and Roden[67].

ENTERO-INSULAR AXIS (DPP-4 AND INCRETINS) IN NAFLD

A number of studies have assessed both the glucagon-like peptide-1 (GLP-1) and glucose dependent insulinotropic peptide induced insulin secretion in T2DM patients and concluded that the response to the incretin hormone stimuli might be compromised as an early phenomenon in T2DM[68-71]. In the carriers of the transcription factor 7-like 2 gene polymorphism (TCF7L2-rs7903146, a widely known risk polymorphism in T2DM development) the GLP-1 induced insulin secretion, and not the GLP-1 secretion, is impaired[72]. Similar findings were presented for a common genetic variant in the

Table 2 Adipokine hormones, cytokines, growth factors and other mediators that play important role in non-alcoholic fatty liver disease pathology

Mediator/pathway	Observation /proposed mechanism	Ref.
Adiponectin	Shown to have anti-inflammatory and antifibrotic activity, serum levels were found to be decreased in NAFLD and NASH patients. Plasma adiponectin in NAFLD is related to hepatic insulin resistance and hepatic lipid content - not to liver disease severity	[127]
Ghrelin	Serum levels were found to be diminished in NAFLD and NASH patients - no correlation with histological grade. Ghrelin administration attenuated oxidative stress, inflammation and apoptosis in high fat diet induced NAFLD animal model	[128,129]
Leptin	Leptin levels are generally known to be higher in the sera of NASH patients, except for a subgroup; serum levels were shown to negatively correlate with AST/ALT levels. The livers of leptin-deficient mice were found to be unusually sensitive to LPS-induced injury. Recombinant leptin therapy was in clinical trial in patients with NASH and low leptin levels - no results were posted	[128] and ClinicalTrials.gov Identifier: NCT00596934
Resistin	Serum levels were shown to be significantly higher in patients with NAFLD and NASH	[130]
Small bowel bacterial overgrowth (SIBO)	Increased gut permeability and tight junction alterations in NAFLD. Higher prevalence of small intestinal bacterial overgrowth in NAFLD patients - correlated with the severity of liver steatosis	[131]
Toll-like receptor-4 (TLR4)	Both the TLR4 (endotoxin-receptor) protein and RNA levels were found to be elevated in liver in NASH	[132]
Nuclear factor-κB (NF-κB)	Increased activation of NF-κB was found in NASH	[132]
Tumor necrosis factor-α (TNF-α)	Key mediator of inflammation, serum levels are elevated in NASH. TNF-α expression in adipose tissue is upregulated in several models of obesity. In patients, TNF-α levels were shown to be higher in obese than in lean individuals, and were correlated with insulin resistance	[133]
Interleukin-6 (IL-6)	Increased plasma levels and hepatic expression was described in NASH patients. Increased hepatic IL-6 production may play an important role in NASH, insulin resistance and diabetes development	[134]
Transforming growth factor-β (TGF-β)	A key growth factor and a major inductor of hepatic stellate cell activation and therefore hepatic fibrosis, TGF-β signaling in hepatocytes may contribute to hepatocyte death and lipid accumulation *via* Smad signaling and ROS production	[135]
Th17 cells and IL-17	In the livers of mice on a high fat diet and also NASH patients an increased number of hepatic Th17 cells could be detected. In mice Il-17 neutralization ameliorated LPS induced liver injury Interleukin-17 exacerbates hepatic steatosis and inflammation in non-alcoholic fatty liver disease	[136]
Notch-mTOR pathway	Liver-specific ablation of Notch signaling, or its acute inhibition with a decoy Notch1 receptor, prevents hepatosteatosis by blocking mTor complex 1 (mTorc1) activity. Notch gain of function induces NAFL through constitutive activation of mTorc1	[137]
Mastocyte chymase	This enzyme is important in the convertion of angiotenzin-Ⅰ to angiotenzin-Ⅱ and the activation of matrix metalloproteinase-9, which both are involved in the development of liver fibrosis. Chymase inhibitor prevents the nonalcoholic steatohepatitis in a hamster model	[138]
Galectin 3	Galectin 3 is a β-galactoside-binding lectin with a multiple functions. It is also a receptor of advanced lipoxidation endproducts and plays important role in inflammation, fibrosis and carcinogenesis. Its role is suspected in NASH. Regression of fibrosis by galectin inhibitors in thioacetamide-induced liver disease animal model. Phase 1 Study with a Galectin inhibitor GR-MD-02 in patients with NASH and advanced fibrosis	[139,140], ClinicalTrials.gov Identifier: NCT01899859
Fibroblast geowth factor -19 (FGF19)	Both the intestinal FGF19 production and the hepatic response is impaired in NAFLD patients. A decrease in fasting FGF19 levels is associated with the development of non-alcoholic fatty liver disease in obese adolescents	[141]

A few of these molecules are therapeutic targets (*e.g.*, galectin 3) in early phase clinical trials. NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

WFS1 gene, and carriers of the polymorphism had impaired GLP-1-induced insulin secretion independently of insulin sensitivity[73]. Variants of the KCNQ1 (rs151290, rs2237892 and rs2237895) gene altered the endogenous GLP-1 secretion[74,75].

Despite these well-designed, elegant studies that assessed the role of the entero-insular axis in T2DM and its association with the risk gene polymorphisms in T2DM, there is limited data regarding the role of the entero-insular axis in NAFLD. Recently, β cell connectedness has been reported to influence the incretin-induced insulin secretion in human islets, and lipotoxicity was demonstrated to be able to disrupt this incretin-regulated human β cell connectivity that might result in the loss of the coordinated islet response to metabolic stimuli[76].

Increased serum DPP-4 activity (the soluble form of the enzyme in human sera) was described in patients with NAFLD in comparison to healthy controls and T2DM patients, provided that T2DM patients who presented with clinically obvious (with an US and biochemical based diagnosis) NAFLD were excluded from the study group[39]. We found a positive correlation among the γGT, ALAT and serum DPP-4 activities in NAFLD that supports the finding that the excess DPP-4 found in the serum of NAFLD patients is of hepatic origin. We concluded that it is the presence of (fatty) liver disease that primarily influenced the serum DPP-4 enzymatic activity and not hyperglycaemia alone[39]. Subsequently,

higher hepatic expression of DPP-4 at the mRNA level was described in NAFLD patients, and high glucose concentrations increased the DPP-4 expression in the HepG2 cells, in contrast to the insulin and FFAs that did not alter the DPP-4 expression[77]. The correlation among the serum levels of soluble CD26/DPP-4 activity and other liver disease biomarkers was also confirmed in Asian patients[78-80]. A positive correlation between the serum DPP-4 activity and insulin resistance (HOMA2-IR) in NAFLD was also found that is not surprising provided that the serum DPP-4 activity is considered as a novel liver disease biomarker. The existence of such a correlation further supported the recent suggestion that the soluble form of DPP-4 is a novel adipokine hormone that could induce insulin resistance without the presence of incretin hormones in an experimental system[81]. In a meta-analysis, Fadini *et al*[82] found that the DPP-4 activity was increased in T2D and was not lowered by glycemic control; the study confirmed that hyperglycaemia was not a direct determinant of DPP-4 activity and was lower in metformin-treated patients. A significant role of DPP-4 in hepatic glucose metabolism is supported by the study of Edgerton *et al*[83], who demonstrated that during DPP-4 inhibitor therapy (vildagliptin) and GLP-1 co-treatment, the net hepatic glucose uptake was three-fold greater in the DPP-4 inhibitor treated group than in the control group that was treated with portal vein GLP-1 infusion and not with the DPP-4 inhibitor; this effect was greater than that predicted by the change in insulin. This finding indicates that the DPP-4 inhibitor was able to increase the hepatic glucose disposal beyond the effects of GLP-1 on insulin and glucagon secretion.

The effect of DPP-4 inhibitor therapy on liver fibrosis should be assessed, particularly because the fibroblast activation protein (FAP), which is a duplicate molecule of DPP-4 (FAP-DPP-4 shows 88% homology at the cDNA level), is present at the tissue remodelling interface on hepatic stellate cells (HSCs, ITO cells) that primarily produce the accumulating extracellular matrix proteins (including collagens) in chronic liver diseases, eventually leading to fibrosis and cirrhosis of the liver[84].

DPP-4 INHIBITORS AND GLP-1 MIMETICS IN NAFLD TREATMENT

DPP-4 inhibitors were reported to improve hepatic steatosis and adipose tissue inflammation in mice[85,86]. Human treatments are also documented, in which the DPP-4 inhibitor, sitagliptin, was able to provide benefit for a refractory case of NAFLD[87]. Sitagliptin improved hepatocyte ballooning in a diabetic patient with NASH[88]. The available data on the use of GLP-1 mimetics is limited; the following effects were reported in animal models: improvement of the FA beta-oxidation; a decrease in the liver disease biomarker ALAT and hepatic TAGs; reduced ER-stress related hepatocyte cell death; and enhanced beneficial macroautophagy[89-92]. The GLP-1 derived nonpeptide (by the cleavage of the neutral endopeptidase), GLP-1 (28-36) amide, was shown to improve glucose disposal and decrease hepatic steatosis in high fat diet mice. The GLP-1 (28-36) amide suppressed the hepatic gluconeogenesis and improved the pyruvate tolerance in this model[93]. The large randomised controlled trials (RCTs) that would also assess safety issues of the DPP-4 inhibitors and the GLP-1 mimetics are missing in NAFL patients, as well as in NASH patients, in whom the indication for (an auxiliary) drug treatment (in addition to diet, weight loss and exercise) might be more obvious.

OTHER THERAPIES

There is consensus that the most effective treatment of NAFL in the overwhelming majority of cases is lifestyle change, including a supervised diet, exercise and weight loss. However doctors are not always successful in having patients reach this goal, and the potential pharmacological approaches that should be considered, predominantly in NASH patients, are briefly reviewed.

Metformin

Metformin is experiencing a booming renaissance in the treatment of T2DM; however, this drug has no significant effect on liver histology, probably in part because of the limited anti-steatogenic effect and failure to increase the adiponectin levels[94]. The guidelines do not recommend this biguanide specifically for the treatment of NASH (AASLD: Strength 1/Evidence: A)[26,95].

Thiazolidinediones

In contrast to metformin, thiazolidinediones (TZDs) are experiencing difficulty as pharmacological agents in human medicine. In certain countries there are no remaining agents from this drug class (*e.g.*, the French Agency for the Safety of Health Products requested a pharmaceutical company to suspend the use of pioglitazone containing products for the treatment of type 2 diabetes in France in 2011 because of an increased risk of urinary bladder cancer). EASL and AASLD discussed pioglitazone, outlining that, from the hepatologist point of view, glitazones consistently provided benefit for patients with NASH and could be used to treat biopsy proven steatohepatitis (AASLD: Strength: 1, Evidence: B)[26,95]. The associations emphasise that the long-term safety of pioglitazone in NASH is not established, and it is likely that no doctor is treating NASH isolated from other safety issues[26].

Vitamin E

The nature of the dilemma with vitamin E is somewhat similar to that with the TZDs. A meta-analyses (of over 100000 participants) reported increased all-cause mortality with a dose of vitamin E $\geqslant$ 400 IU/d, and one meta-analysis found a statistically significant relationship between vitamin E dosage and all-cause mortality in a dose-response analysis[96,97]. Although others have ques-

tioned the results obtained from these meta-analyses[98,99], recently vitamin E (400 IU/d) was shown to significantly increase the risk of prostate cancer among healthy men[100]. According to the AASLD recommendation, this vitamin E dose should be doubled (800 IU/d) to improve liver histology in non-diabetic adults with biopsy proven NASH (Strength: 1, Quality: B), and it is not recommended in NASH patients with T2DM (Strength: 1, Quality: C)[26]. Histological improvement in NASH in this context should be interpreted for all lesions, except for improvement in fibrosis, and vitamin E is not recommended for the treatment of NAFLD-cirrhosis or cryptogenic cirrhosis[26,95].

Ursodeoxycholic acid-nor-ursodeoxycholic acid

The AASLD does not recommend ursodeoxycholic acid (UDCA) A for the treatment of NAFLD and NASH (Strength 1, Quality: B), and long term, high dose UDCA increased the rate of serious adverse events (the development of cirrhosis, varices, cholangiocarcinoma, liver transplantation or death) in patients with primary sclerosing cholangitis[26,101]. The pharmacological properties of norUrsodeoxycholic acid (nor-UDCA) might be more attractive and might later be studied in patients with NASH[102].

Farnesoid X receptor agonists

The bile acids secreted upon feeding undergo enterohepatic circulation and serve as endogenous ligands to a class of nuclear hormone receptors that function as ligand-activated transcription factors. Farnesoid X receptor (FXR) belongs to this class and serves as a receptor for hydrophobic bile acids. In T2DM, the bile acid composition is altered. The bile acid taurochenodeoxycholic acid increases insulin release *via* the FXR dependent inhibition of the KATP channels, and FXR has been described to improve insulin sensitivity and glucose uptake in adipose tissue, liver and skeletal muscle by regulating the genes that are important in metabolic control[103,104]. The FXR agonist WAY-362450 was able to decrease inflammation and fibrosis in a murine model of NASH[105]. The following results of a 6-wk double-blind, randomised, placebo-controlled clinical phase Ⅱ trial of a semi-synthetic bile acid derivative, obeticholic acid (OCA), in patients with T2DM and presumed NAFLD has recently been published: administration of 25 or 50 mg of OCA for six weeks increased insulin sensitivity and reduced the markers of liver inflammation and fibrosis. A longer trial is ongoing with OCA in biopsy-proven NASH patients (Clinical trials identifier: NCT01265498)[106].

Despite the primary enthusiasm regarding the FXR agonists, the controversy might not be ignored due to the recent finding that under high fat diet conditions, FXR knockout (FXR-KO) mice benefited from the receptor deficiency, and FXR-KO protected against the HFD-induced impairment of fasting plasma glucose levels and glucose tolerance[107]. In parallel with the clinical trials, a better understanding of bile acid enterohepatic circulation and more research on the FXR-dependent and independent signaling pathways are warranted.

Pre and probiotics

The intestinal microbiome has an increasing role in the understanding of T2DM pathology. In a recent study, 20 patients with histology-proven NASH were randomised to receive probiotics or usual care for six months. The probiotic treatment decreased the ASAT, and the decrease in IHCL was confirmed using ^{1}H-MRS[108]. Other studies have suggested a potential role for prebiotic fibres (non-digestible carbohydrates modulating the human microbiome) in NAFLD treatment, and larger RCTs might provide conclusive evidence[109].

Omega 3 polyunsaturated FA

Di Minno *et al*[110] recently summarised the potential of omega-3 fatty acids for the treatment of NAFLD reported in seven human trials, the largest of which was a 6-mo follow-up RCT of 144 patients with NAFLD; however, the authors concluded that well-designed RCTs of adequate size and duration, with histological endpoints, are needed to assess the long-term safety and efficacy of such treatment.

Others

The phase 1 study to evaluate GR-MD-02, a Galectin-3 inhibitor in patients with NASH and advanced fibrosis (NCT01899859) could on one day be an excellent example of how a long term molecular research[139,140], may potentially pay out in benefiting certain patients. Galectin-3 protein (binding to terminal galactose residues in glycoproteins) is implicated in the pathogenesis of liver fibrosis and the results with compounds inhibiting the protein suggest a potential role of these drugs in human liver fibrosis and even in cirrhosis in the NAFLD spectrum as well (Table 2). Hypothetical approaches, including the development of a peripheral cannabinoid 1 receptor agonist without psychiatric side effects or the supplementation of vitamin D3 to overcome vitamin D3 deficiency that is highly prevalent in NAFLD[92,111,112], might have an effect on future therapies; however, they have yet to be tested and even limited evidence from concept studies is missing. There are ongoing randomised, controlled studies with potentially promising compounds such as resveratrol (500 mg three times daily for six months *vs* placebo-NCT01464801) in obese patients with NAFLD/NASH; however, conclusions from these studies would be premature.

Surgical interventions

Surgical interventions including Roux-en-Y gastric bypass might have beneficial effects on NAFLD from calorie intake reduction that could increase hepatic insulin sensitivity and augment postprandial GLP-1 secretion with a subsequently improved β cell function[26,113].

Table 3 Single nucleotide polymorphisms associated with non-alcoholic fatty liver disease in genome wide association studies

Region	SNPs	Reported gene(s)	Mapped gene	OR	Risk allele frequency in controls	*P* value	Gene product function	Context	Initial sample size	Replication sample size	Platform (SNPs passing QC)	Ref.
22q13.3	rs738409 (I148M), rs2896019-G rs738491, rs3761472, rs2143571 rs6006473, rs5764455, rs6006611	*PNPLA3* *SAMM50* *PARVB*	*PNPLA3* *SAMM50* *PARVB*	2.02	0.450	2×10^{-20}	Adiponutrin (PNPLA3)–nutritionally regulated lysophosphatidic acyltransferase: expressed in liver and adipose tissue. High CH diet increases expression. Has TAG hydrolase and DG transacylase activity. Strong predictor of steatosis, inflammation and fibrosis. Dysfunctional PNPLA3 promotes accumulation of lipotoxic substrates. I148M–association with HCC in severely obese individuals	Intron	392 Japanese cases, 934 Japanese controls	172 Japanese cases, 1012 Japanese control	Illumina [261540]	[119,120]
1p35	rs6691847-C	*PTPRU*	*PTPRU - MATN1*	1.32	0.770	7×10^{-6}	Member of the protein tyrosine phosphatase (PTP) family. PTPs are signaling molecules that regulate cell growth, differentiation, mitotic cycle, and oncogenic transformation					
4q13.3	rs222054-C	*GC*	*LDHAL6EP - GC*	2.54	0.301	1×10^{-6}	Albumin gene family. Multifunctional protein found in plasma, ascitic fluid, cerebrospinal fluid and on the surface of many cell types. It binds to vitamin D and its plasma metabolites and transports them to target tissues		126 European adolescent cases - 802 European adolescent controls	NR	Illumina [2078805] (imputed)	[142]
16q23	rs11864146-A	*SLC38, A8*	*SLC38A8*	3.14	0.100	2×10^{-6}	Amino acid transport: Putative sodium-coupled neutral amino acid transporter 8	Intron				
13q14.1	rs7324845-A	*LCP1*	*LCP1*	3.29	0.096	3×10^{-6}	L-plastin, an actin binding protein expressed in hemopoetic cell lineages. It is expressed by many solid tumor types of non-hemopoetic origin, suggesting its role in tumorigenesis	Intron				
1p21	rs12743824-C	*LPPR4*	*LPPR4 - PALMD*	2.30	0.441	5×10^{-6}	LPPR4: a lipid phosphate phosphatase, catalyzes the dephosphorylation of a number of bioactive lipid mediators, found to be important for axonal outgrowth; PALMD: cytosolic isoform of paralemmin-1, a lipid raft-associated protein implicated in cell shape control	NR				PALMD gene product - [143]
7p14	rs343064-A	Intergenic	*TBX20-HERPUD2*	1.31	0.400	3×10^{-8}	TBX20: transcription factor; HERPUD2: not reported		236 non-Hispanic Caucasian women	NR	Illumina [324623]	SNPs of other genes were also associated with qulatitative traits (*e.g.* NAFLD activity score and FDFT1 - rs2645424) as discussed in the text [123]
2q31	rs1529093-A	Intergenic	*RPL29P8 - KRT8P40*	4.13	0.410	2×10^{-6}	RPL29P8, KRT8P40: pseudogenes			NR	Illumina [324623]	
4p15.2	rs959903-A	23231	*SEL1L3*	3.81	0.290	7×10^{-6}		Intron		NR	Illumina [324623]	

Not all GWAS studies were replicated. Risk gene variants with OR higher than 1.31 are indicated in the table. Based on the National Human Genome Research. SNPs: Single nucleotide polymorphisms; NR: Not reported.

GENETICS

Familial clustering and prevalence differences according to ethnic origin

Although screening of family members is not recommended in NAFLD[26] based on a retrospective review of 90 cases, the authors concluded that familial clustering is common, and 18% of NASH patients had a first degree relative with a similar phenotype[114]. Schwimmer *et al*[115] found that NAFLD was more common in siblings (59%) and parents (78%), using ^{1}H-MRS based diagnostics of children with biopsy proven NAFLD in a familial aggregation study. After adjustment for age, sex, race and BMI, the study concluded that familial factors are a major determinant in NAFLD. Gene-environment interactions might have a role in the data *e.g.* the condition that family members are living in a common household might indicate common environmental risk factors (type of oil used in the diet *etc.*). The role of genetic risk factors is supported by the differences based on ethnic origin that were observed in the NAFLD prevalence in multi-ethnic cohorts (see the Prevalence chapter and Table 1), with a higher prevalence in Hispanics and a lower prevalence in African Americans compared to non-Hispanic whites[4,16].

Genome wide association studies

Five genome wide association scans (GWAS) are reported for NAFLD in the GWAS catalogue. Although these studies did not include case numbers that are typically employed in other GWA studies including T2DM, they provide remarkable evidence for genetic factors predisposing to or protecting from NAFLD. The risk polymorphisms with the highest ORs are summarised in Table 3, from the aspect of the NAFLD binary outcomes. Discussion of all the candidate genes is beyond the scope of this review.

PATATIN-LIKE PHOSPHOLIPASE DOMAIN CONTAINING 3 GENE (PNPLA3-ADIPONUTRIN) IN NAFLD SPECTRUM DISEASES -BINARY TRAITS

We should outline the single nucleotide polymorphism in the patatin-like phospholipase domain containing a 3-gene (PNPLA3) that is the most studied genetic risk variant in NAFLD. Adiponutrin is a nutritionally regulated lysophosphatidic-acyltransferase possessing the TAG hydrolase and DG transacylase activity. This gene is expressed in liver and adipose tissues, and a high carbohydrate diet increases the gene expression at the mRNA level in the liver. Dysfunctional PNPLA3 promotes the accumulation of lipotoxic substrates[116,117]. After the description that in carriers of the rs738409 C/G polymorphism of the PNPLA3 the hepatic lipid content was more than two-fold higher, PNPLA3 was subsequently confirmed in a Japanese GWAS as an NAFLD binary trait candidate gene[118,119] (Table 3). Polymorphisms in the SAMM50 and PARVB genes were associated with the development and progression of NAFLD in this Japanese GWA study[119].

The quality trait data demonstrate the following findings: PNPLA3 I148M (rs738409) is a genetic marker of progressive liver disease that is characterised with steatosis, inflammation and fibrosis; carriers are more insulin resistant and more susceptible to T2DM[120,121]; and, in a 15-year-follow-up, there is an association of this genetic variant and hepatocellular carcinoma incidence in severely obese individuals, with a hazard ratio of HCC of 5.9x for each PNPLA3 148M allele carried (reaching the HR- 16x in the PNPLA3 148M homozygotes even after adjustment for age, gender, BMI, type 2 diabetes status and ALAT)[121,122].

OTHER CANDIDATE GENES (SQUALENE SYNTHASE AND COLLAGEN XIII A1)-QUALITATIVE TRAITS

In the cases in which additional candidate genes were identified, qualitative traits were also assessed in GWAS. The NAFLD activity score was associated with the rs2645424 polymorphism of farnesyl diphosphate farnesyl transferase 1 (*FDFT1*), the degree of fibrosis was associated with the rs343062 SNP, and the lobular inflammation was associated with the rs1227756 polymorphism of the Collagen 13 A1 gene[123].

FDFT1 is a membrane-associated enzyme located at a branch point in the mevalonate pathway. The encoded protein is the first specific enzyme in cholesterol biosynthesis, catalysing the dimerisation of two molecules of farnesyl diphosphate in a two-step reaction to form squalene. In addition to the linkage of FDFT1 to the NAFLD activity score, a coding variant in the FDFT1 gene influences the plasma cholesterol levels, likely *via* alteration of the intracellular production of cholesterol[123,124].

Collagen XIII is one of the nonfibrillar collagens and belongs to the transmembrane collagens, and a number of alternatively spliced transcript variants have been described; integrins mediate the cell adhesion to the type XIII collagen[125]. There is no validation of these associations in larger studies.

In summary, we may conclude that genetic and molecular research might lead to the identification of additional risk and protective gene variants and this together with deeper understanding of gene-environment interactions might provide better insight into the molecular pathology and identification of molecular targets in NAFLD, which is the most common chronic liver disease affecting up to one-third of the adult population in industrialised countries. In addition, little is known regarding the long-term effect of the increasing NAFLD prevalence in paediatric populations.

ACKNOWLEDGEMENTS

EFSD New Horizons grant facilitated the construction of this manuscript. I am grateful to my colleagues, espe-

cially Prof. A. Somogyi, Dr. G. Lengyel, Prof. K. Rácz and Prof. Zs Tulassay, who made it possible to conduct research on this topic in the our institution. I am also grateful to my PhD-fellow, Dr. A. Zóka and my co-worker Dr. Z. Kukor for their comments.

REFERENCES

1 **Ludwig J**, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980; **55**: 434-438 [PMID: 7382552]

2 **Angulo P**. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231 [PMID: 11961152 DOI: 10.1056/NEJMra011775]

3 **Smith BW**, Adams LA. Non-alcoholic fatty liver disease. *Crit Rev Clin Lab Sci* 2011; **48**: 97-113 [PMID: 21875310 DOI: 10.3109/10408363.2011.596521]

4 **Browning JD**, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004; **40**: 1387-1395 [PMID: 15565570 DOI: 10.1002/hep.20466]

5 **Roden M**. Mechanisms of Disease: hepatic steatosis in type 2 diabetes--pathogenesis and clinical relevance. *Nat Clin Pract Endocrinol Metab* 2006; **2**: 335-348 [PMID: 16932311 DOI: 10.1038/ncpendmet0190]

6 **Williams CD**, Stengel J, Asike MI, Torres DM, Shaw J, Contreras M, Landt CL, Harrison SA. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. *Gastroenterology* 2011; **140**: 124-131 [PMID: 20858492 DOI: 10.1053/j.gastro.2010.09.038]

7 **Ong JP**, Pitts A, Younossi ZM. Increased overall mortality and liver-related mortality in non-alcoholic fatty liver disease. *J Hepatol* 2008; **49**: 608-612 [PMID: 18682312 DOI: 10.1016/j.jhep.2008.06.018]

8 **Kallwitz ER**, Kumar M, Aggarwal R, Berger R, Layden-Almer J, Gupta N, Cotler SJ. Ethnicity and nonalcoholic fatty liver disease in an obesity clinic: the impact of triglycerides. *Dig Dis Sci* 2008; **53**: 1358-1363 [PMID: 18347982 DOI: 10.1007/s10620-008-0234-x]

9 **Papatheodoridis GV**, Goulis J, Christodoulou D, Manolakopoulos S, Raptopoulou M, Andrioti E, Alexandropoulos N, Savvidou S, Papachristou A, Zervou E, Seferiadis K, Kousidou P, Vogiatzakis E, Tsianos E. High prevalence of elevated liver enzymes in blood donors: associations with male gender and central adiposity. *Eur J Gastroenterol Hepatol* 2007; **19**: 281-287 [PMID: 17353691 DOI: 10.1097/MEG.0b013e328011438b]

10 **Dabelea D**, Pihoker C, Talton JW, D'Agostino RB, Fujimoto W, Klingensmith GJ, Lawrence JM, Linder B, Marcovina SM, Mayer-Davis EJ, Imperatore G, Dolan LM. Etiological approach to characterization of diabetes type: the SEARCH for Diabetes in Youth Study. *Diabetes Care* 2011; **34**: 1628-1633 [PMID: 21636800 DOI: 10.2337/dc10-2324]

11 **Rorat M**, Jurek T, Kuchar E, Szenborn L, Golema W, Halon A. Liver steatosis in Polish children assessed by medicolegal autopsies. *World J Pediatr* 2013; **9**: 68-72 [PMID: 23275099 DOI: 10.1007/s12519-012-0387-8]

12 **Schwimmer JB**, Deutsch R, Kahen T, Lavine JE, Stanley C, Behling C. Prevalence of fatty liver in children and adolescents. *Pediatrics* 2006; **118**: 1388-1393 [PMID: 17015527 DOI: 10.1542/peds.2006-1212]

13 **Schwenzer NF**, Machann J, Martirosian P, Stefan N, Schraml C, Fritsche A, Claussen CD, Schick F. Quantification of pancreatic lipomatosis and liver steatosis by MRI: comparison of in/opposed-phase and spectral-spatial excitation techniques. *Invest Radiol* 2008; **43**: 330-337 [PMID: 18424954 DOI: 10.1097/RLI.0b013e31816a88c6]

14 **Szczepaniak LS**, Babcock EE, Schick F, Dobbins RL, Garg A, Burns DK, McGarry JD, Stein DT. Measurement of intracellular triglyceride stores by H spectroscopy: validation in vivo. *Am J Physiol* 1999; **276**: E977-E989 [PMID: 10329993]

15 **Krssák M**, Hofer H, Wrba F, Meyerspeer M, Brehm A, Lohninger A, Steindl-Munda P, Moser E, Ferenci P, Roden M. Non-invasive assessment of hepatic fat accumulation in chronic hepatitis C by 1H magnetic resonance spectroscopy. *Eur J Radiol* 2010; **74**: e60-e66 [PMID: 19406596 DOI: 10.1016/j.ejrad.2009.03.062]

16 **Lazo M**, Hernaez R, Eberhardt MS, Bonekamp S, Kamel I, Guallar E, Koteish A, Brancati FL, Clark JM. Prevalence of nonalcoholic fatty liver disease in the United States: the Third National Health and Nutrition Examination Survey, 1988-1994. *Am J Epidemiol* 2013; **178**: 38-45 [PMID: 23703888 DOI: 10.1093/aje/kws448]

17 **Bedogni G**, Miglioli L, Masutti F, Tiribelli C, Marchesini G, Bellentani S. Prevalence of and risk factors for nonalcoholic fatty liver disease: the Dionysos nutrition and liver study. *Hepatology* 2005; **42**: 44-52 [PMID: 15895401 DOI: 10.1002/hep.20734]

18 **Kojima S**, Watanabe N, Numata M, Ogawa T, Matsuzaki S. Increase in the prevalence of fatty liver in Japan over the past 12 years: analysis of clinical background. *J Gastroenterol* 2003; **38**: 954-961 [PMID: 14614602 DOI: 10.1007/s00535-003-1178-8]

19 **Amarapurkar D**, Kamani P, Patel N, Gupte P, Kumar P, Agal S, Baijal R, Lala S, Chaudhary D, Deshpande A. Prevalence of non-alcoholic fatty liver disease: population based study. *Ann Hepatol* 2007; **6**: 161-163 [PMID: 17786142]

20 **Angulo P**, Hui JM, Marchesini G, Bugianesi E, George J, Farrell GC, Enders F, Saksena S, Burt AD, Bida JP, Lindor K, Sanderson SO, Lenzi M, Adams LA, Kench J, Therneau TM, Day CP. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology* 2007; **45**: 846-854 [PMID: 17393509 DOI: 10.1002/hep.21496]

21 **Musso G**, Gambino R, Cassader M, Pagano G. Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. *Ann Med* 2011; **43**: 617-649 [PMID: 21039302 DOI: 10.3109/07853890.2010.518623]

22 **Myers RP**, Pomier-Layrargues G, Kirsch R, Pollett A, Duarte-Rojo A, Wong D, Beaton M, Levstik M, Crotty P, Elkashab M. Feasibility and diagnostic performance of the FibroScan XL probe for liver stiffness measurement in overweight and obese patients. *Hepatology* 2012; **55**: 199-208 [PMID: 21898479 DOI: 10.1002/hep.24624]

23 **Wong GL**. Transient elastography: Kill two birds with one stone? *World J Hepatol* 2013; **5**: 264-274 [PMID: 23717737 DOI: 10.4254/wjh.v5.i5.264]

24 **Feldstein AE**, Wieckowska A, Lopez AR, Liu YC, Zein NN, McCullough AJ. Cytokeratin-18 fragment levels as noninvasive biomarkers for nonalcoholic steatohepatitis: a multicenter validation study. *Hepatology* 2009; **50**: 1072-1078 [PMID: 19585618 DOI: 10.1002/hep.23050]

25 **Musso G**, Gambino R, Bo S, Uberti B, Biroli G, Pagano G, Cassader M. Should nonalcoholic fatty liver disease be included in the definition of metabolic syndrome? A cross-sectional comparison with Adult Treatment Panel III criteria in nonobese nondiabetic subjects. *Diabetes Care* 2008; **31**: 562-568 [PMID: 18056890 DOI: 10.2337/dc07-1526]

26 **Chalasani N**, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, Charlton M, Sanyal AJ. The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* 2012; **55**: 2005-2023 [PMID: 22488764 DOI: 10.1002/hep.25762]

27 **Ferenci P**, Steindl-Munda P, Vogel W, Jessner W, Gschwantler M, Stauber R, Datz C, Hackl F, Wrba F, Bauer P, Lorenz O. Diagnostic value of quantitative hepatic copper determination in patients with Wilson's Disease. *Clin Gastroenterol Hepatol* 2005; **3**: 811-818 [PMID: 16234011]

28 **Pietrangelo A**, Schilsky M. Metal storage disorders. Forward. *Semin Liver Dis* 2011; **31**: 231-232 [PMID: 21901652 DOI: 10.1055/s-0031-1286053]

29 **Fink S**, Schilsky ML. Inherited metabolic disease of the liver. *Curr Opin Gastroenterol* 2007; **23**: 237-243 [PMID: 17414838 DOI: 10.1097/MOG.0b013e3280ef68e4]

30 **Leite NC**, Salles GF, Araujo AL, Villela-Nogueira CA, Cardoso CR. Prevalence and associated factors of non-alcoholic fatty liver disease in patients with type-2 diabetes mellitus. *Liver Int* 2009; **29**: 113-119 [PMID: 18384521 DOI: 10.1111/j.1478-3231.2008.01718.x]

31 **Prashanth M**, Ganesh HK, Vima MV, John M, Bandgar T, Joshi SR, Shah SR, Rathi PM, Joshi AS, Thakkar H, Menon PS, Shah NS. Prevalence of nonalcoholic fatty liver disease in patients with type 2 diabetes mellitus. *J Assoc Physicians India* 2009; **57**: 205-210 [PMID: 19588648]

32 **Leite NC**, Villela-Nogueira CA, Pannain VL, Bottino AC, Rezende GF, Cardoso CR, Salles GF. Histopathological stages of nonalcoholic fatty liver disease in type 2 diabetes: prevalences and correlated factors. *Liver Int* 2011; **31**: 700-706 [PMID: 21457442 DOI: 10.1111/j.1478-3231.2011.02482.x]

33 **Prikoszovich T**, Winzer C, Schmid AI, Szendroedi J, Chmelik M, Pacini G, Krssák M, Moser E, Funahashi T, Waldhäusl W, Kautzky-Willer A, Roden M. Body and liver fat mass rather than muscle mitochondrial function determine glucose metabolism in women with a history of gestational diabetes mellitus. *Diabetes Care* 2011; **34**: 430-436 [PMID: 20978097 DOI: 10.2337/dc10-1002]

34 **Bozkurt L**, Göbl CS, Tura A, Chmelik M, Prikoszovich T, Kosi L, Wagner O, Roden M, Pacini G, Gastaldelli A, Kautzky-Willer A. Fatty liver index predicts further metabolic deteriorations in women with previous gestational diabetes. *PLoS One* 2012; **7**: e32710 [PMID: 22393439 DOI: 10.1371/journal.pone.0032710]

35 **Brumbaugh DE**, Tearse P, Cree-Green M, Fenton LZ, Brown M, Scherzinger A, Reynolds R, Alston M, Hoffman C, Pan Z, Friedman JE, Barbour LA. Intrahepatic fat is increased in the neonatal offspring of obese women with gestational diabetes. *J Pediatr* 2013; **162**: 930-936.e1 [PMID: 23260099 DOI: 10.1016/j.jpeds.2012.11.017]

36 **Borch-Johnsen K**, Wareham N. The rise and fall of the metabolic syndrome. *Diabetologia* 2010; **53**: 597-599 [PMID: 20084362 DOI: 10.1007/s00125-010-1659-2]

37 **Alberti KG**, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WP, Loria CM, Smith SC. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation* 2009; **120**: 1640-1645 [PMID: 19805654 DOI: 10.1161/CIRCULATIONAHA.109.192644]

38 **Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults**. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA* 2001; **285**: 2486-2497 [PMID: 11368702]

39 **Firneisz G**, Varga T, Lengyel G, Fehér J, Ghyczy D, Wichmann B, Selmeci L, Tulassay Z, Rácz K, Somogyi A. Serum dipeptidyl peptidase-4 activity in insulin resistant patients with non-alcoholic fatty liver disease: a novel liver disease biomarker. *PLoS One* 2010; **5**: e12226 [PMID: 20805868 DOI: 10.1371/journal.pone.0012226]

40 **Donnelly KL**, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest* 2005; **115**: 1343-1351 [PMID: 15864352 DOI: 10.1172/JCI23621]

41 **Schwarz JM**, Linfoot P, Dare D, Aghajanian K. Hepatic de novo lipogenesis in normoinsulinemic and hyperinsulinemic subjects consuming high-fat, low-carbohydrate and low-fat, high-carbohydrate isoenergetic diets. *Am J Clin Nutr* 2003; **77**: 43-50 [PMID: 12499321]

42 **Bugianesi E**, Leone N, Vanni E, Marchesini G, Brunello F, Carucci P, Musso A, De Paolis P, Capussotti L, Salizzoni M, Rizzetto M. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. *Gastroenterology* 2002; **123**: 134-140 [PMID: 12105842]

43 **Bugianesi E**, Moscatiello S, Ciaravella MF, Marchesini G. Insulin resistance in nonalcoholic fatty liver disease. *Curr Pharm Des* 2010; **16**: 1941-1951 [PMID: 20370677]

44 **Tamura S**, Shimomura I. Contribution of adipose tissue and de novo lipogenesis to nonalcoholic fatty liver disease. *J Clin Invest* 2005; **115**: 1139-1142 [PMID: 15864343 DOI: 10.1172/JCI24930]

45 **Bosy-Westphal A**, Kossel E, Goele K, Blöcker T, Lagerpusch M, Later W, Heller M, Glüer CC, Müller MJ. Association of pericardial fat with liver fat and insulin sensitivity after diet-induced weight loss in overweight women. *Obesity* (Silver Spring) 2010; **18**: 2111-2117 [PMID: 20224561 DOI: 10.1038/oby.2010.49]

46 **Wallace TM**, Levy JC, Matthews DR. Use and abuse of HOMA modeling. *Diabetes Care* 2004; **27**: 1487-1495 [PMID: 15161807]

47 **Reaven GM**. What do we learn from measurements of HOMA-IR? *Diabetologia* 2013; **56**: 1867-1868 [PMID: 23722624 DOI: 10.1007/s00125-013-2948-3]

48 **Pisprasert V**, Ingram KH, Lopez-Davila MF, Munoz AJ, Garvey WT. Limitations in the use of indices using glucose and insulin levels to predict insulin sensitivity: impact of race and gender and superiority of the indices derived from oral glucose tolerance test in African Americans. *Diabetes Care* 2013; **36**: 845-853 [PMID: 23223406 DOI: 10.2337/dc12-0840]

49 **Armstrong MJ**, Hazlehurst JM, Hull D, Guo K, Borrows S, Yu J, Gough SC, Newsome PN, Tomlinson JW. Abdominal subcutaneous adipose tissue insulin resistance and lipolysis in patients with non-alcoholic steatohepatitis. *Diabetes Obes Metab* 2014; **16**: 651-660 [PMID: 24962805 DOI: 10.1111/dom.12272]

50 **Nehra V**, Angulo P, Buchman AL, Lindor KD. Nutritional and metabolic considerations in the etiology of nonalcoholic steatohepatitis. *Dig Dis Sci* 2001; **46**: 2347-2352 [PMID: 11713934]

51 **Wei Y**, Wang D, Topczewski F, Pagliassotti MJ. Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. *Am J Physiol Endocrinol Metab* 2006; **291**: E275-E281 [PMID: 16492686 DOI: 10.1152/ajpendo.00644.2005]

52 **Cao H**, Gerhold K, Mayers JR, Wiest MM, Watkins SM, Hotamisligil GS. Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism. *Cell* 2008; **134**: 933-944 [PMID: 18805087 DOI: 10.1016/j.cell.2008.07.048]

53 **Li ZZ**, Berk M, McIntyre TM, Feldstein AE. Hepatic lipid partitioning and liver damage in nonalcoholic fatty liver disease: role of stearoyl-CoA desaturase. *J Biol Chem* 2009; **284**: 5637-5644 [PMID: 19119140 DOI: 10.1074/jbc.M807616200]

54 **Listenberger LL**, Han X, Lewis SE, Cases S, Farese RV, Ory DS, Schaffer JE. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc Natl Acad Sci USA* 2003; **100**: 3077-3082 [PMID: 12629214 DOI: 10.1073/

pnas.0630588100]
55 **Trauner M**, Arrese M, Wagner M. Fatty liver and lipotoxicity. *Biochim Biophys Acta* 2010; **1801**: 299-310 [PMID: 19857603 DOI: 10.1016/j.bbalip.2009.10.007]
56 **Yamaguchi K**, Yang L, McCall S, Huang J, Yu XX, Pandey SK, Bhanot S, Monia BP, Li YX, Diehl AM. Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. *Hepatology* 2007; **45**: 1366-1374 [PMID: 17476695 DOI: 10.1002/hep.21655]
57 **Monetti M**, Levin MC, Watt MJ, Sajan MP, Marmor S, Hubbard BK, Stevens RD, Bain JR, Newgard CB, Farese RV, Hevener AL, Farese RV. Dissociation of hepatic steatosis and insulin resistance in mice overexpressing DGAT in the liver. *Cell Metab* 2007; **6**: 69-78 [PMID: 17618857 DOI: 10.1016/j.cmet.2007.05.005]
58 **Ibrahim SH**, Kohli R, Gores GJ. Mechanisms of lipotoxicity in NAFLD and clinical implications. *J Pediatr Gastroenterol Nutr* 2011; **53**: 131-140 [PMID: 21629127 DOI: 10.1097/MPG.0b013e31822578db]
59 **Marchetti P**, Bugliani M, Boggi U, Masini M, Marselli L. The pancreatic beta cells in human type 2 diabetes. *Adv Exp Med Biol* 2012; **771**: 288-309 [PMID: 23393686]
60 **Miani M**, Barthson J, Colli ML, Brozzi F, Cnop M, Eizirik DL. Endoplasmic reticulum stress sensitizes pancreatic beta cells to interleukin-1β-induced apoptosis via Bim/A1 imbalance. *Cell Death Dis* 2013; **4**: e701 [PMID: 23828564 DOI: 10.1038/cddis.2013.236]
61 **Ibrahim SH**, Gores GJ. Who pulls the trigger: JNK activation in liver lipotoxicity? *J Hepatol* 2012; **56**: 17-19 [PMID: 21703172 DOI: 10.1016/j.jhep.2011.04.017]
62 **Eizirik DL**, Colli ML, Ortis F. The role of inflammation in insulitis and beta-cell loss in type 1 diabetes. *Nat Rev Endocrinol* 2009; **5**: 219-226 [PMID: 19352320 DOI: 10.1038/nrendo.2009.21]
63 **Gurzov EN**, Eizirik DL. Bcl-2 proteins in diabetes: mitochondrial pathways of β-cell death and dysfunction. *Trends Cell Biol* 2011; **21**: 424-431 [PMID: 21481590 DOI: 10.1016/j.tcb.2011.03.001]
64 **Akazawa Y**, Cazanave S, Mott JL, Elmi N, Bronk SF, Kohno S, Charlton MR, Gores GJ. Palmitoleate attenuates palmitate-induced Bim and PUMA up-regulation and hepatocyte lipoapoptosis. *J Hepatol* 2010; **52**: 586-593 [PMID: 20206402 DOI: 10.1016/j.jhep.2010.01.003]
65 **Pérez-Carreras M**, Del Hoyo P, Martín MA, Rubio JC, Martín A, Castellano G, Colina F, Arenas J, Solis-Herruzo JA. Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology* 2003; **38**: 999-1007 [PMID: 14512887 DOI: 10.1053/jhep.2003.50398]
66 **Sanyal AJ**, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, Luketic VA, Shiffman ML, Clore JN. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 2001; **120**: 1183-1192 [PMID: 11266382 DOI: 10.1053/gast.2001.23256]
67 **Koliaki C**, Roden M. Hepatic energy metabolism in human diabetes mellitus, obesity and non-alcoholic fatty liver disease. *Mol Cell Endocrinol* 2013; **379**: 35-42 [PMID: 23770462 DOI: 10.1016/j.mce.2013.06.002]
68 **Kjems LL**, Holst JJ, Vølund A, Madsbad S. The influence of GLP-1 on glucose-stimulated insulin secretion: effects on beta-cell sensitivity in type 2 and nondiabetic subjects. *Diabetes* 2003; **52**: 380-386 [PMID: 12540611]
69 **Højberg PV**, Vilsbøll T, Rabøl R, Knop FK, Bache M, Krarup T, Holst JJ, Madsbad S. Four weeks of near-normalisation of blood glucose improves the insulin response to glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide in patients with type 2 diabetes. *Diabetologia* 2009; **52**: 199-207 [PMID: 19037628 DOI: 10.1007/s00125-008-1195-5]
70 **Holst JJ**, Knop FK, Vilsbøll T, Krarup T, Madsbad S. Loss of incretin effect is a specific, important, and early characteristic of type 2 diabetes. *Diabetes Care* 2011; **34** Suppl 2: S251-S257 [PMID: 21525464 DOI: 10.2337/dc11-s227]
71 **Knop FK**, Aaboe K, Vilsbøll T, Vølund A, Holst JJ, Krarup T, Madsbad S. Impaired incretin effect and fasting hyperglucagonaemia characterizing type 2 diabetic subjects are early signs of dysmetabolism in obesity. *Diabetes Obes Metab* 2012; **14**: 500-510 [PMID: 22171657 DOI: 10.1111/j.1463-1326.2011.01549.x]
72 **Schäfer SA**, Tschritter O, Machicao F, Thamer C, Stefan N, Gallwitz B, Holst JJ, Dekker JM, 't Hart LM, Nijpels G, van Haeften TW, Häring HU, Fritsche A. Impaired glucagon-like peptide-1-induced insulin secretion in carriers of transcription factor 7-like 2 (TCF7L2) gene polymorphisms. *Diabetologia* 2007; **50**: 2443-2450 [PMID: 17661009 DOI: 10.1007/s00125-007-0753-6]
73 **Schäfer SA**, Müssig K, Staiger H, Machicao F, Stefan N, Gallwitz B, Häring HU, Fritsche A. A common genetic variant in WFS1 determines impaired glucagon-like peptide-1-induced insulin secretion. *Diabetologia* 2009; **52**: 1075-1082 [PMID: 19330314 DOI: 10.1007/s00125-009-1344-5]
74 **Smushkin G**, Sathananthan M, Sathananthan A, Dalla Man C, Micheletto F, Zinsmeister AR, Cobelli C, Vella A. Diabetes-associated common genetic variation and its association with GLP-1 concentrations and response to exogenous GLP-1. *Diabetes* 2012; **61**: 1082-1089 [PMID: 22461567 DOI: 10.2337/db11-1732]
75 **Müssig K**, Staiger H, Machicao F, Kirchhoff K, Guthoff M, Schäfer SA, Kantartzis K, Silbernagel G, Stefan N, Holst JJ, Gallwitz B, Häring HU, Fritsche A. Association of type 2 diabetes candidate polymorphisms in KCNQ1 with incretin and insulin secretion. *Diabetes* 2009; **58**: 1715-1720 [PMID: 19366866 DOI: 10.2337/db08-1589]
76 **Hodson DJ**, Mitchell RK, Bellomo EA, Sun G, Vinet L, Meda P, Li D, Li WH, Bugliani M, Marchetti P, Bosco D, Piemonti L, Johnson P, Hughes SJ, Rutter GA. Lipotoxicity disrupts incretin-regulated human β cell connectivity. *J Clin Invest* 2013; **123**: 4182-4194 [PMID: 24018562 DOI: 10.1172/JCI68459]
77 **Miyazaki M**, Kato M, Tanaka K, Tanaka M, Kohjima M, Nakamura K, Enjoji M, Nakamuta M, Kotoh K, Takayanagi R. Increased hepatic expression of dipeptidyl peptidase-4 in non-alcoholic fatty liver disease and its association with insulin resistance and glucose metabolism. *Mol Med Rep* 2012; **5**: 729-733 [PMID: 22179204 DOI: 10.3892/mmr.2011.707]
78 **Aso Y**, Ozeki N, Terasawa T, Naruse R, Hara K, Suetsugu M, Takebayashi K, Shibazaki M, Haruki K, Morita K, Inukai T. Serum level of soluble CD26/dipeptidyl peptidase-4 (DPP-4) predicts the response to sitagliptin, a DPP-4 inhibitor, in patients with type 2 diabetes controlled inadequately by metformin and/or sulfonylurea. *Transl Res* 2012; **159**: 25-31 [PMID: 22153807 DOI: 10.1016/j.trsl.2011.09.005]
79 **Firneisz G**, Somogyi A. Serum level of soluble CD26/dieptidyl peptidase-4 (DPP-4) activity correlates with other liver disease biomarkers both in Asian and European patients. *Transl Res* 2012; **160**: 95-96 [PMID: 22683411 DOI: 10.1016/j.trsl.2012.01.016]
80 **Aso Y**. Re: Serum level of soluble CD26/dipeptidyl peptidase-4 (DPP-4). *Transl Res* 2012; **160**: 164 [PMID: 22704734 DOI: 10.1016/j.trsl.2012.01.017]
81 **Lamers D**, Famulla S, Wronkowitz N, Hartwig S, Lehr S, Ouwens DM, Eckardt K, Kaufman JM, Ryden M, Müller S, Hanisch FG, Ruige J, Arner P, Sell H, Eckel J. Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome. *Diabetes* 2011; **60**: 1917-1925 [PMID: 21593202 DOI: 10.2337/db10-1707]
82 **Fadini GP**, Albiero M, Menegazzo L, de Kreutzenberg SV, Avogaro A. The increased dipeptidyl peptidase-4 activity is not counteracted by optimized glucose control in type 2

diabetes, but is lower in metformin-treated patients. *Diabetes Obes Metab* 2012; **14**: 518-522 [PMID: 22171692 DOI: 10.1111/j.1463-1326.2011.01550.x]

83 **Edgerton DS**, Johnson KM, Neal DW, Scott M, Hobbs CH, Zhang X, Duttaroy A, Cherrington AD. Inhibition of dipeptidyl peptidase-4 by vildagliptin during glucagon-like Peptide 1 infusion increases liver glucose uptake in the conscious dog. *Diabetes* 2009; **58**: 243-249 [PMID: 18840785 DOI: 10.2337/db08-0515]

84 **Levy MT**, McCaughan GW, Abbott CA, Park JE, Cunningham AM, Müller E, Rettig WJ, Gorrell MD. Fibroblast activation protein: a cell surface dipeptidyl peptidase and gelatinase expressed by stellate cells at the tissue remodelling interface in human cirrhosis. *Hepatology* 1999; **29**: 1768-1778 [PMID: 10347120 DOI: 10.1002/hep.510290631]

85 **Shirakawa J**, Fujii H, Ohnuma K, Sato K, Ito Y, Kaji M, Sakamoto E, Koganei M, Sasaki H, Nagashima Y, Amo K, Aoki K, Morimoto C, Takeda E, Terauchi Y. Diet-induced adipose tissue inflammation and liver steatosis are prevented by DPP-4 inhibition in diabetic mice. *Diabetes* 2011; **60**: 1246-1257 [PMID: 21330637 DOI: 10.2337/db10-1338]

86 **Kern M**, Klöting N, Niessen HG, Thomas L, Stiller D, Mark M, Klein T, Blüher M. Linagliptin improves insulin sensitivity and hepatic steatosis in diet-induced obesity. *PLoS One* 2012; **7**: e38744 [PMID: 22761701 DOI: 10.1371/journal.pone.0038744]

87 **Itou M**, Kawaguchi T, Taniguchi E, Oriishi T, Sata M. Dipeptidyl Peptidase IV Inhibitor Improves Insulin Resistance and Steatosis in a Refractory Nonalcoholic Fatty Liver Disease Patient: A Case Report. *Case Rep Gastroenterol* 2012; **6**: 538-544 [PMID: 22949894 DOI: 10.1159/000341510]

88 **Yilmaz Y**, Yonal O, Deyneli O, Celikel CA, Kalayci C, Duman DG. Effects of sitagliptin in diabetic patients with nonalcoholic steatohepatitis. *Acta Gastroenterol Belg* 2012; **75**: 240-244 [PMID: 22870790]

89 **Mells JE**, Fu PP, Sharma S, Olson D, Cheng L, Handy JA, Saxena NK, Sorescu D, Anania FA. Glp-1 analog, liraglutide, ameliorates hepatic steatosis and cardiac hypertrophy in C57BL/6J mice fed a Western diet. *Am J Physiol Gastrointest Liver Physiol* 2012; **302**: G225-G235 [PMID: 22038829 DOI: 10.1152/ajpgi.00274.2011]

90 **Trevaskis JL**, Griffin PS, Wittmer C, Neuschwander-Tetri BA, Brunt EM, Dolman CS, Erickson MR, Napora J, Parkes DG, Roth JD. Glucagon-like peptide-1 receptor agonism improves metabolic, biochemical, and histopathological indices of nonalcoholic steatohepatitis in mice. *Am J Physiol Gastrointest Liver Physiol* 2012; **302**: G762-G772 [PMID: 22268099 DOI: 10.1152/ajpgi.00476.2011]

91 **Sharma S**, Mells JE, Fu PP, Saxena NK, Anania FA. GLP-1 analogs reduce hepatocyte steatosis and improve survival by enhancing the unfolded protein response and promoting macroautophagy. *PLoS One* 2011; **6**: e25269 [PMID: 21957486 DOI: 10.1371/journal.pone.0025269]

92 **Schuppan D**, Schattenberg JM. Non-alcoholic steatohepatitis: pathogenesis and novel therapeutic approaches. *J Gastroenterol Hepatol* 2013; **28** Suppl 1: 68-76 [PMID: 23855299 DOI: 10.1111/jgh.12212]

93 **Ip W**, Shao W, Chiang YT, Jin T. GLP-1-derived nonapeptide GLP-1(28-36)amide represses hepatic gluconeogenic gene expression and improves pyruvate tolerance in high-fat diet-fed mice. *Am J Physiol Endocrinol Metab* 2013; **305**: E1348-E1358 [PMID: 24085036 DOI: 10.1152/ajpendo.00376.2013]

94 **Tiikkainen M**, Häkkinen AM, Korsheninnikova E, Nyman T, Mäkimattila S, Yki-Järvinen H. Effects of rosiglitazone and metformin on liver fat content, hepatic insulin resistance, insulin clearance, and gene expression in adipose tissue in patients with type 2 diabetes. *Diabetes* 2004; **53**: 2169-2176 [PMID: 15277403]

95 **Ratziu V**, Bellentani S, Cortez-Pinto H, Day C, Marchesini G. A position statement on NAFLD/NASH based on the EASL 2009 special conference. *J Hepatol* 2010; **53**: 372-384 [PMID: 20494470 DOI: 10.1016/j.jhep.2010.04.008]

96 **Miller ER**, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann Intern Med* 2005; **142**: 37-46 [PMID: 15537682]

97 **Bjelakovic G**, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. *JAMA* 2007; **297**: 842-857 [PMID: 17327526 DOI: 10.1001/jama.297.8.842]

98 **Berry D**, Wathen JK, Newell M. Bayesian model averaging in meta-analysis: vitamin E supplementation and mortality. *Clin Trials* 2009; **6**: 28-41 [PMID: 19254931 DOI: 10.1177/1740774508101279]

99 **Gerss J**, Köpcke W. The questionable association of vitamin E supplementation and mortality--inconsistent results of different meta-analytic approaches. *Cell Mol Biol* (Noisy-le-grand) 2009; **55** Suppl: OL1111-OL1120 [PMID: 19267994]

100 **Klein EA**, Thompson IM, Tangen CM, Crowley JJ, Lucia MS, Goodman PJ, Minasian LM, Ford LG, Parnes HL, Gaziano JM, Karp DD, Lieber MM, Walther PJ, Klotz L, Parsons JK, Chin JL, Darke AK, Lippman SM, Goodman GE, Meyskens FL, Baker LH. Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA* 2011; **306**: 1549-1556 [PMID: 21990298 DOI: 10.1001/jama.2011.1437]

101 **Lindor KD**, Kowdley KV, Luketic VA, Harrison ME, McCashland T, Befeler AS, Harnois D, Jorgensen R, Petz J, Keach J, Mooney J, Sargeant C, Braaten J, Bernard T, King D, Miceli E, Schmoll J, Hoskin T, Thapa P, Enders F. High-dose ursodeoxycholic acid for the treatment of primary sclerosing cholangitis. *Hepatology* 2009; **50**: 808-814 [PMID: 19585548 DOI: 10.1002/hep.23082]

102 **Beraza N**, Ofner-Ziegenfuss L, Ehedego H, Boekschoten M, Bischoff SC, Mueller M, Trauner M, Trautwein C. Norursodeoxycholic acid reverses hepatocyte-specific nemo-dependent steatohepatitis. *Gut* 2011; **60**: 387-396 [PMID: 21115542 DOI: 10.1136/gut.2010.223834]

103 **Düfer M**, Hörth K, Wagner R, Schittenhelm B, Prowald S, Wagner TF, Oberwinkler J, Lukowski R, Gonzalez FJ, Krippeit-Drews P, Drews G. Bile acids acutely stimulate insulin secretion of mouse β-cells via farnesoid X receptor activation and K(ATP) channel inhibition. *Diabetes* 2012; **61**: 1479-1489 [PMID: 22492528 DOI: 10.2337/db11-0815]

104 **Adorini L**, Pruzanski M, Shapiro D. Farnesoid X receptor targeting to treat nonalcoholic steatohepatitis. *Drug Discov Today* 2012; **17**: 988-997 [PMID: 22652341 DOI: 10.1016/j.drudis.2012.05.012]

105 **Zhang S**, Wang J, Liu Q, Harnish DC. Farnesoid X receptor agonist WAY-362450 attenuates liver inflammation and fibrosis in murine model of non-alcoholic steatohepatitis. *J Hepatol* 2009; **51**: 380-388 [PMID: 19501927 DOI: 10.1016/j.jhep.2009.03.025]

106 **Mudaliar S**, Henry RR, Sanyal AJ, Morrow L, Marschall HU, Kipnes M, Adorini L, Sciacca CI, Clopton P, Castelloe E, Dillon P, Pruzanski M, Shapiro D. Efficacy and safety of the farnesoid X receptor agonist obeticholic acid in patients with type 2 diabetes and nonalcoholic fatty liver disease. *Gastroenterology* 2013; **145**: 574-582.e1 [PMID: 23727264 DOI: 10.1053/j.gastro.2013.05.042]

107 Abstracts of the 49th EASD (European Association for the Study of Diabetes) Annual Meeting. September 23-27, 2013. Barcelona, Spain. *Diabetologia* 2013; **56** Suppl 1: 1-566 [PMID: 23949610 DOI: 10.1007/s00125-013-3012-z]

108 **Wong VW**, Won GL, Chim AM, Chu WC, Yeung DK, Li KC, Chan HL. Treatment of nonalcoholic steatohepatitis with probiotics. A proof-of-concept study. *Ann Hepatol* 2013; **12**: 256-262 [PMID: 23396737]

109 **Parnell JA**, Raman M, Rioux KP, Reimer RA. The potential role of prebiotic fibre for treatment and management of non-alcoholic fatty liver disease and associated obesity and insulin resistance. *Liver Int* 2012; **32**: 701-711 [PMID: 22221818 DOI: 10.1111/j.1478-3231.2011.02730.x]

110 **Di Minno MN**, Russolillo A, Lupoli R, Ambrosino P, Di Minno A, Tarantino G. Omega-3 fatty acids for the treatment of non-alcoholic fatty liver disease. *World J Gastroenterol* 2012; **18**: 5839-5847 [PMID: 23139599 DOI: 10.3748/wjg.v18.i41.5839]

111 **Targher G**, Scorletti E, Mantovani A, Byrne CD. Nonalcoholic fatty liver disease and reduced serum vitamin D(3) levels. *Metab Syndr Relat Disord* 2013; **11**: 217-228 [PMID: 23745619 DOI: 10.1089/met.2013.0044]

112 **Eliades M**, Spyrou E, Agrawal N, Lazo M, Brancati FL, Potter JJ, Koteish AA, Clark JM, Guallar E, Hernaez R. Meta-analysis: vitamin D and non-alcoholic fatty liver disease. *Aliment Pharmacol Ther* 2013; **38**: 246-254 [PMID: 23786213 DOI: 10.1111/apt.12377]

113 **Dirksen C**, Jørgensen NB, Bojsen-Møller KN, Jacobsen SH, Hansen DL, Worm D, Holst JJ, Madsbad S. Mechanisms of improved glycaemic control after Roux-en-Y gastric bypass. *Diabetologia* 2012; **55**: 1890-1901 [PMID: 22538359 DOI: 10.1007/s00125-012-2556-7]

114 **Willner IR**, Waters B, Patil SR, Reuben A, Morelli J, Riely CA. Ninety patients with nonalcoholic steatohepatitis: insulin resistance, familial tendency, and severity of disease. *Am J Gastroenterol* 2001; **96**: 2957-2961 [PMID: 11693332 DOI: 10.1111/j.1572-0241.2001.04667.x]

115 **Schwimmer JB**, Celedon MA, Lavine JE, Salem R, Campbell N, Schork NJ, Shiehmorteza M, Yokoo T, Chavez A, Middleton MS, Sirlin CB. Heritability of nonalcoholic fatty liver disease. *Gastroenterology* 2009; **136**: 1585-1592 [PMID: 19208353 DOI: 10.1053/j.gastro.2009.01.050]

116 **Dubuquoy C**, Robichon C, Lasnier F, Langlois C, Dugail I, Foufelle F, Girard J, Burnol AF, Postic C, Moldes M. Distinct regulation of adiponutrin/PNPLA3 gene expression by the transcription factors ChREBP and SREBP1c in mouse and human hepatocytes. *J Hepatol* 2011; **55**: 145-153 [PMID: 21145868 DOI: 10.1016/j.jhep.2010.10.024]

117 **Li JZ**, Huang Y, Karaman R, Ivanova PT, Brown HA, Roddy T, Castro-Perez J, Cohen JC, Hobbs HH. Chronic overexpression of PNPLA3I148M in mouse liver causes hepatic steatosis. *J Clin Invest* 2012; **122**: 4130-4144 [PMID: 23023705 DOI: 10.1172/JCI65179]

118 **Romeo S**, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, Boerwinkle E, Cohen JC, Hobbs HH. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet* 2008; **40**: 1461-1465 [PMID: 18820647 DOI: 10.1038/ng.257]

119 **Kitamoto T**, Kitamoto A, Yoneda M, Hyogo H, Ochi H, Nakamura T, Teranishi H, Mizusawa S, Ueno T, Chayama K, Nakajima A, Nakao K, Sekine A, Hotta K. Genome-wide scan revealed that polymorphisms in the PNPLA3, SAMM50, and PARVB genes are associated with development and progression of nonalcoholic fatty liver disease in Japan. *Hum Genet* 2013; **132**: 783-792 [PMID: 23535911 DOI: 10.1007/s00439-013-1294-3]

120 **Dubuquoy C**, Burnol AF, Moldes M. PNPLA3, a genetic marker of progressive liver disease, still hiding its metabolic function? *Clin Res Hepatol Gastroenterol* 2013; **37**: 30-35 [PMID: 22884299 DOI: 10.1016/j.clinre.2012.06.014]

121 **Palmer CN**, Maglio C, Pirazzi C, Burza MA, Adiels M, Burch L, Donnelly LA, Colhoun H, Doney AS, Dillon JF, Pearson ER, McCarthy M, Hattersley AT, Frayling T, Morris AD, Peltonen M, Svensson PA, Jacobson P, Borén J, Sjöström L, Carlsson LM, Romeo S. Paradoxical lower serum triglyceride levels and higher type 2 diabetes mellitus susceptibility in obese individuals with the PNPLA3 148M variant. *PLoS One* 2012; **7**: e39362 [PMID: 22724004 DOI: 10.1371/journal.pone.0039362]

122 **Burza MA**, Pirazzi C, Maglio C, Sjöholm K, Mancina RM, Svensson PA, Jacobson P, Adiels M, Baroni MG, Borén J, Ginanni Corradini S, Montalcini T, Sjöström L, Carlsson LM, Romeo S. PNPLA3 I148M (rs738409) genetic variant is associated with hepatocellular carcinoma in obese individuals. *Dig Liver Dis* 2012; **44**: 1037-1041 [PMID: 22704398 DOI: 10.1016/j.dld.2012.05.006]

123 **Chalasani N**, Guo X, Loomba R, Goodarzi MO, Haritunians T, Kwon S, Cui J, Taylor KD, Wilson L, Cummings OW, Chen YD, Rotter JI, Nonalcoholic Steatohepatitis Clinical Research Network. Genome-wide association study identifies variants associated with histologic features of nonalcoholic Fatty liver disease. *Gastroenterology* 2010; **139**: 1567-1576, 1576.e1-6 [PMID: 20708005 DOI: 10.1053/j.gastro.2010.07.057]

124 **Do R**, Paré G, Montpetit A, Hudson TJ, Gaudet D, Engert JC. K45R variant of squalene synthase increases total cholesterol levels in two study samples from a French Canadian population. *Hum Mutat* 2008; **29**: 689-694 [PMID: 18350552 DOI: 10.1002/humu.20702]

125 **Nykvist P**, Tu H, Ivaska J, Käpylä J, Pihlajaniemi T, Heino J. Distinct recognition of collagen subtypes by alpha(1)beta(1) and alpha(2)beta(1) integrins. Alpha(1)beta(1) mediates cell adhesion to type XIII collagen. *J Biol Chem* 2000; **275**: 8255-8261 [PMID: 10713152]

126 **Lee JY**, Kim KM, Lee SG, Yu E, Lim YS, Lee HC, Chung YH, Lee YS, Suh DJ. Prevalence and risk factors of non-alcoholic fatty liver disease in potential living liver donors in Korea: a review of 589 consecutive liver biopsies in a single center. *J Hepatol* 2007; **47**: 239-244 [PMID: 17400323]

127 **Bugianesi E**, Pagotto U, Manini R, Vanni E, Gastaldelli A, de Iasio R, Gentilcore E, Natale S, Cassader M, Rizzetto M, Pasquali R, Marchesini G. Plasma adiponectin in non-alcoholic fatty liver is related to hepatic insulin resistance and hepatic fat content, not to liver disease severity. *J Clin Endocrinol Metab* 2005; **90**: 3498-3504 [PMID: 15797948 DOI: 10.1210/jc.2004-2240]

128 **Yalniz M**, Bahcecioglu IH, Ataseven H, Ustundag B, Ilhan F, Poyrazoglu OK, Erensoy A. Serum adipokine and ghrelin levels in nonalcoholic steatohepatitis. *Mediators Inflamm* 2006; **2006**: 34295 [PMID: 17392582 DOI: 10.1155/MI/2006/34295]

129 **Li Y**, Hai J, Li L, Chen X, Peng H, Cao M, Zhang Q. Administration of ghrelin improves inflammation, oxidative stress, and apoptosis during and after non-alcoholic fatty liver disease development. *Endocrine* 2013; **43**: 376-386 [PMID: 22843123 DOI: 10.1007/s12020-012-9761-5]

130 **Senateş E**, Colak Y, Yeşil A, Coşkunpinar E, Sahin O, Kahraman OT, Erkalma Şenateş B, Tuncer I. Circulating resistin is elevated in patients with non-alcoholic fatty liver disease and is associated with steatosis, portal inflammation, insulin resistance and nonalcoholic steatohepatitis scores. *Minerva Med* 2012; **103**: 369-376 [PMID: 23042372]

131 **Miele L**, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, Mascianà R, Forgione A, Gabrieli ML, Perotti G, Vecchio FM, Rapaccini G, Gasbarrini G, Day CP, Grieco A. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. *Hepatology* 2009; **49**: 1877-1887 [PMID: 19291785 DOI: 10.1002/hep.22848]

132 **Singh R**, Bullard J, Kalra M, Assefa S, Kaul AK, Vonfeldt K, Strom SC, Conrad RS, Sharp HL, Kaul R. Status of bacterial colonization, Toll-like receptor expression and nuclear factor-kappa B activation in normal and diseased human livers. *Clin Immunol* 2011; **138**: 41-49 [PMID: 20940109 DOI: 10.1016/j.clim.2010.09.006]

133 **Braunersreuther V**, Viviani GL, Mach F, Montecucco F. Role of cytokines and chemokines in non-alcoholic fatty liver disease. *World J Gastroenterol* 2012; **18**: 727-735 [PMID: 22371632 DOI: 10.3748/wjg.v18.i8.727]

134 **Wieckowska A**, Papouchado BG, Li Z, Lopez R, Zein NN, Feldstein AE. Increased hepatic and circulating interleukin-6 levels in human nonalcoholic steatohepatitis. *Am J*

Gastroenterol 2008; **103**: 1372-1379 [PMID: 18510618 DOI: 10.1111/j.1572-0241.2007.01774.x]

135 **Yang L**, Roh YS, Song J, Zhang B, Liu C, Loomba R, Seki E. Transforming growth factor beta signaling in hepatocytes participates in steatohepatitis through regulation of cell death and lipid metabolism in mice. *Hepatology* 2014; **59**: 483-495 [PMID: 23996730 DOI: 10.1002/hep.26698]

136 **Tang Y**, Bian Z, Zhao L, Liu Y, Liang S, Wang Q, Han X, Peng Y, Chen X, Shen L, Qiu D, Li Z, Ma X. Interleukin-17 exacerbates hepatic steatosis and inflammation in non-alcoholic fatty liver disease. *Clin Exp Immunol* 2011; **166**: 281-290 [PMID: 21985374 DOI: 10.1111/j.1365-2249.2011.04471.x]

137 **Pajvani UB**, Qiang L, Kangsamaksin T, Kitajewski J, Ginsberg HN, Accili D. Inhibition of Notch uncouples Akt activation from hepatic lipid accumulation by decreasing mTorc1 stability. *Nat Med* 2013; **19**: 1054-1060 [PMID: 23832089 DOI: 10.1038/nm.3259]

138 **Tashiro K**, Takai S, Jin D, Yamamoto H, Komeda K, Hayashi M, Tanaka K, Tanigawa N, Miyazaki M. Chymase inhibitor prevents the nonalcoholic steatohepatitis in hamsters fed a methionine- and choline-deficient diet. *Hepatol Res* 2010; **40**: 514-523 [PMID: 20374300 DOI: 10.1111/j.1872-034X.2010.00627.x]

139 **Iacobini C**, Menini S, Ricci C, Blasetti Fantauzzi C, Scipioni A, Salvi L, Cordone S, Delucchi F, Serino M, Federici M, Pricci F, Pugliese G. Galectin-3 ablation protects mice from diet-induced NASH: a major scavenging role for galectin-3 in liver. *J Hepatol* 2011; **54**: 975-983 [PMID: 21145823 DOI: 10.1016/j.jhep.2010.09.020]

140 **Traber PG**, Chou H, Zomer E, Hong F, Klyosov A, Fiel MI, Friedman SL. Regression of fibrosis and reversal of cirrhosis in rats by galectin inhibitors in thioacetamide-induced liver disease. *PLoS One* 2013; **8**: e75361 [PMID: 24130706 DOI: 10.1371/journal.pone.0075361]

141 **Wojcik M**, Janus D, Dolezal-Oltarzewska K, Kalicka-Kasperczyk A, Poplawska K, Drozdz D, Sztefko K, Starzyk JB. A decrease in fasting FGF19 levels is associated with the development of non-alcoholic fatty liver disease in obese adolescents. *J Pediatr Endocrinol Metab* 2012; **25**: 1089-1093 [PMID: 23329754 DOI: 10.1515/jpem-2012-0253]

142 **Adams LA**, White SW, Marsh JA, Lye SJ, Connor KL, Maganga R, Ayonrinde OT, Olynyk JK, Mori TA, Beilin LJ, Palmer LJ, Hamdorf JM, Pennell CE. Association between liver-specific gene polymorphisms and their expression levels with nonalcoholic fatty liver disease. *Hepatology* 2013; **57**: 590-600 [PMID: 23213074 DOI: 10.1002/hep.26184]

143 **Hu B**, Petrasch-Parwez E, Laue MM, Kilimann MW. Molecular characterization and immunohistochemical localization of palmdelphin, a cytosolic isoform of the paralemmin protein family implicated in membrane dynamics. *Eur J Cell Biol* 2005; **84**: 853-866 [PMID: 16323283 DOI: 10.1016/j.ejcb.2005.07.002]

P- Reviewers: Fontana L, Rocha R **S- Editor**: Gou SX
L- Editor: A **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9090

World J Gastroenterol 2014 July 21; 20(27): 9090-9097
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (12): Nonalcoholic fatty liver disease

Potential roles of glucagon-like peptide-1-based therapies in treating non-alcoholic fatty liver disease

Ye Liu, Rui Wei, Tian-Pei Hong

Ye Liu, Rui Wei, Tian-Pei Hong, Department of Endocrinology and Metabolism, Peking University Third Hospital, Beijing 100191, China
Author contributions: Liu Y collected the data and drafted the article; Wei R revised the article; Hong TP designed the work and supervised the data collection and writing of the article.
Supported by Chinese National 973 Program No. 2012CB517502; and the Research Fund for the Doctoral Program of Higher Education of China No. 20120001120069
Correspondence to: Tian-Pei Hong, MD, PhD, Chief, Department of Endocrinology and Metabolism, Peking University Third Hospital, 49 North Garden Road, Haidian District, Beijing 100191, China. tpho66@bjmu.edu.cn
Telephone: +86-10-82265515 Fax: +86-10-62017700
Received: December 30, 2013 Revised: March 30, 2014
Accepted: April 30, 2014
Published online: July 21, 2014

Abstract

Glucagon-like peptide-1 (GLP-1)-based therapies have demonstrated efficacy and safety in treating type 2 diabetes, which shares a similar pathophysiological mechanism with non-alcoholic fatty liver disease (NAFLD). Recent studies showed that glucose-induced GLP-1 secretion was decreased in patients with NAFLD and that the level of dipeptidyl peptidase-4, which inactivates intact GLP-1, was upregulated. Moreover, the expression of the GLP-1 receptor was downregulated in livers from patients with NAFLD, indicating an association of defective GLP-1 signalling with NAFLD. Notably, GLP-1-based therapies are reported to be effective in improving hepatic endpoints in patients with NAFLD, such as reducing hepatic fat content, hepatic steatosis and plasma transaminase levels, and preventing fibrosis. GLP-1-based therapies are beneficial for body weight control and glycaemic normalisation, which are important for the management of NAFLD. Moreover, clinical and preclinical studies showed that GLP-1-based agents might directly exert their actions on the liver through activation of functional GLP-1 receptors in hepatocytes. The possible mechanisms involve regulating gene expression that is associated with insulin resistance and lipid metabolism, and suppressing oxidative stress in the liver cells, thus preventing the development and progression of NAFLD. Based on these promising data, large-scale randomised controlled trials are warranted to assess the efficacy and safety of GLP-1-based therapies in treating NAFLD.

Key words: Non-alcoholic fatty liver disease; Glucagon-like peptide-1; Dipeptidyl peptidase-4; Insulin resistance; Oxidative stress; Lipid metabolism

Core tip: Recently, an association of defective glucagon-like peptide-1 (GLP-1) signalling with non-alcoholic fatty liver disease (NAFLD) has been documented. GLP-1-based therapies, which are well accepted in treating diabetes, are effective in improving hepatic endpoints in NAFLD. In addition to the benefits in controlling metabolic disorders, GLP-1-based agents may directly exert actions on the liver through activation of GLP-1 receptors in hepatocytes, resulting in the regulation of gene expression associated with insulin resistance and lipid metabolism, and the suppression of oxidative stress in liver cells. Therefore, GLP-1-based therapies may have potential roles in treating NAFLD.

Liu Y, Wei R, Hong TP. Potential roles of glucagon-like peptide-1-based therapies in treating non-alcoholic fatty liver disease. *World J Gastroenterol* 2014; 20(27): 9090-9097 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9090.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9090

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) encompasses

a spectrum of diseases ranging from simple hepatic steatosis through steatohepatitis (NASH) to increasing fibrosis and eventual cirrhosis. NAFLD used to be considered the leading cause of chronic liver damage in Western countries. With the spread of a Westernised lifestyle, NAFLD is exhibiting an increasingly universal distribution[1,2]. NAFLD is strongly associated with obesity, type 2 diabetes (T2DM), dyslipidemia and cardiovascular disease[3]. Although the cornerstone of NAFLD therapy remains lifestyle intervention, many patients have difficulty maintaining lifestyle changes and achieving the treatment goals.

As novel anti-diabetic treatment options, glucagon-like peptide-1 (GLP-1)-based agents have been shown to improve the hepatic parameters in T2DM patients with NAFLD. GLP-1 is an incretin hormone secreted from intestinal L cells in response to nutrient ingestion, and has various bioactivities including enhancing glucose-dependent insulin secretion from pancreatic β-cells, promoting β-cell survival, inhibiting glucagon secretion from α-cell, slowing gastric empty and controlling food intake. These effects help to maintain a euglycaemic level and are beneficial for weight control. However, under physiological conditions, the intact active GLP-1 is quickly degraded by dipeptidyl peptidase-4 (DPP-4) into the inactive GLP-1. Therefore, GLP-1 receptor (GLP-1R) agonists, which are resistant to DPP-4, or DPP-4 inhibitors, which protect the endogenous GLP-1 from degradation, were developed by pharmaceutical enterprises. These GLP-1-based therapies have been widely accepted in the treatment of T2DM in recent years. It has been proven that GLP-1-based therapies are beneficial for body weight control, improve insulin sensitivity and dyslipidemia, and prevent oxidative stress in patients with T2DM[4-7]. Apart from these well-documented effects that are helpful for the metabolic disorders in NAFLD, recent studies have shown that GLP-1 agents may also have direct effects on hepatocytes.

In this article, we review the data from both clinical and preclinical studies that investigate the relationship between GLP-1 and NAFLD. The possible mechanisms, especially the direct effects of GLP-1-based therapies on hepatocytes, are also discussed.

ASSOCIATION OF GLP-1 SIGNALING WITH NAFLD

Observations from bariatric surgery

Bariatric surgery is recommended as a treatment option for adults with morbid obesity or obese patients with T2DM. The benefits of weight loss and lowering of blood glucose have been well documented in patients undergoing the procedure. Additionally, clinical studies and systemic reviews have reported that the procedure also improves hepatic histology in most obese patients with NAFLD or NASH, reversing steatosis, inflammation and even fibrosis in the liver[8,9]. Although weight loss is one of the main factors associated with the improvement of liver histology in individuals with NAFLD, several other factors that are independent of weight loss may also contribute to this effect. For example, one important factor is an elevated serum level of GLP-1 resulting from the altered secretion pattern of this gut hormone after re-routing the flow of chyme, whereby food more quickly reaches the distal ileum and stimulates the secretion of GLP-1 from the L cells located there. Nevertheless, whether NAFLD improvement is associated with the elevation of GLP-1 level in patients undergoing bariatric surgery remains to be clarified.

Evidence from NAFLD patients and animal models

GLP-1 levels and DPP-4 expression in NAFLD: Bernsmeier *et al*[10] evaluated the plasma level of active GLP-1 in patients with NAFLD or NASH and healthy controls with a standardised oral glucose tolerance test. The glucose-induced GLP-1 secretion was dramatically decreased in patients with NAFLD or NASH compared to the controls, suggesting a deficiency of GLP-1 signalling in NAFLD. McDonald *et al*[11] found that the fasting GLP-1 level was not significantly different between the high fat diet (HFD)-treated rats and the control animals throughout the development of NAFLD, although there was an age-related decline. Unfortunately, the ELISA kit that this previous study used for measuring plasma GLP-1 level had a 100% cross-reaction of active GLP-1 and inactive GLP-1 and could not therefore identify the level of active GLP-1 from that of total GLP-1. On the other hand, DPP-4, which inactivates intact GLP-1, has been shown to be upregulated in the livers of patients with NAFLD. In a recent report, hepatic DPP-4 mRNA expression level in liver biopsy samples was significantly higher in patients with NAFLD compared to healthy subjects[12]. Moreover, serum DPP-4 activity and the intensity of DPP-4 expression in the human liver were correlated with markers of liver damage, histopathological grade and insulin resistance in patients with NAFLD[13,14]. These results suggest that excessive GLP-1 inactivation resulting from DPP-4 overactivity may have a role in the development of NAFLD.

Expression of GLP-1R in NAFLD: The GLP-1R has been detected in human liver biopsy samples, primary human hepatocytes and human hepatoma cell lines at both the mRNA and protein level[15,16], which raises the possibility that GLP-1 may exert a direct effect on hepatocytes *via* functional GLP-1R. Hepatic GLP-1R expression level in patients with NASH was significantly lower than that in healthy controls. A similar phenomenon was also found in animal models of NASH. In HFD-induced NASH rats, GLP-1R mRNA expression was significantly decreased in the liver after one month of HFD exposure, indicating an association of downregulated GLP-1R expression with NASH[15].

Based on the aforementioned human data and animal studies, defective GLP-1 signalling may play a role in the development and progression of NAFLD. GLP-1-based

agents have been well accepted as safe and efficacious options in the treatment of T2DM, which shares similar pathophysiological mechanisms with NAFLD. Currently, increasing amount of data from both clinical and animal studies suggest that GLP-1-based agents are also beneficial for NAFLD.

BENEFICIAL EFFECTS OF GLP-1-BASED THERAPIES ON NAFLD

Suggestions from small clinical studies

In one case report, a 59-year-old male T2DM patient was treated with exenatide, a GLP-1R agonist, adding to metformin monotherapy. His liver fat content declined from 15.8% to 4.3% as measured by magnetic resonance spectroscopy (MRS) following a 44-wk treatment. This dramatic decrease in liver fat content was accompanied by a significant improvement of liver enzymes, in particular alanine transaminase (ALT), the most important marker of liver steatosis[17].

Cuthbertson *et al*[18] investigated the impact of GLP-1R agonist therapy on intrahepatic lipid in obese T2DM patients with hepatic steatosis in a prospective clinical study. Twenty-five patients were given 6 mo of treatment with GLP-1R agonist (exenatide in 19, and liraglutide in 6 subjects). After 6 mo of treatment with the GLP-1R agonists, the relative reduction from baseline in intrahepatic lipid, as quantified by MRS, was 42%. This reduction was significantly correlated with a relative reduction in haemoglobin A1c (HbA1c) but not with that in total body weight.

Thiazolidinediones (TZDs) are considered an effective option in the treatment of NAFLD[19]. In a small randomised controlled trial, Sathyanarayana *et al*[20] compared the effects of exenatide and pioglitazone combination therapy with pioglitazone alone on hepatic fat content in T2DM patients who were already on diet control and/or metformin therapy. The exenatide and pioglitazone combination therapy was associated with a significantly greater decrease in hepatic fat content as evaluated by MRS when compared to pioglitazone monotherapy, despite a lack of a significant change in body weight. Reduction in plasma levels of ALT and triglycerides (TG) was also significantly greater following the combination therapy with pioglitazone and exenatide. Moreover, the fasting plasma level of fibroblast growth factor-21, an independent predictor of NAFLD, was significantly decreased in the exenatide and pioglitazone combination therapy and was unchanged in the pioglitazone monotherapy[21]. These results indicate an additive effect of exenatide on the TZDs for improving hepatic parameters in T2DM patients with NAFLD.

Analogous to GLP-1R agonists, DPP-4 inhibitors also show potential benefit for NAFLD patients with T2DM. In a small clinical study, 30 NAFLD patients with T2DM were given sitagliptin, a DPP-4 inhibitor. After a 4-month treatment, not only glycaemic control but also serum ALT, aspartate aminotransferase (AST) and γ-glutamyl transpeptidase levels were significantly improved relative to baseline[22].

Evidence from prospective placebo-controlled trials

The Liraglutide Effect and Action in Diabetes (LEAD) program assessed the efficacy and safety of liraglutide therapy on liver parameters in comparison with placebo-controls in a meta-analysis performed using data from six 26-wk, phase-Ⅲ, and randomised controlled trials in patients with T2DM. In this program, 2241 (50.8%) patients had an abnormal elevation of plasma ALT levels at baseline. Liraglutide dose-dependently reduced the ALT levels in these patients, with similar adverse effects between the liraglutide and control groups. Additionally, in a sub-study of LEAD-2 where hepatic steatosis was measured by computerised tomography scan, once-daily subcutaneous injection of liraglutide (1.8 mg) showed a trend towards improving hepatic steatosis compared with placebo (liver-to-spleen attenuation ratio: 0.10 *vs* 0.00; $P = 0.07$). However, this difference became smaller after adjusting for reduction in body weight and HbA1c[6].

Evidence from animal studies

Zhang *et al*[23] investigated the effect of liraglutide on hepatic lipid accumulation in hypoadiponectinemia ApoE$^{-/-}$ mice fed on HFD. Hepatic steatosis in this animal model was improved histologically, and hepatic TG content, total cholesterol (TC) content and total lipid content were all dramatically reduced by an 8-wk liraglutide treatment. Similar results were found in a high fat and high fructose diet-induced NAFLD model in mice[24].

In agreement with the beneficial effects found in patients with T2DM, exendin-4 (also known as exenatide) therapy led to a reduction in the net weight gain, serum glucose level and insulin resistance in obese mice compared to their counterparts. Moreover, the increased liver weight, elevated hepatic TG content and hepatic steatosis were greatly attenuated by exendin-4 treatment[21,25]. Administration of AC3174, an exendin-4 analogue, to *ob/ob* mice ameliorated hepatic steatosis and fibrosis histologically in high trans-fat or high lard-fat diet models. Interestingly, this positive effect on the liver endpoints was at least partly body weight-independent. AC3174 treatment significantly reduced liver mass (-14.2%), liver lipid content (-12.9%), and plasma level of ALT and TG, whereas a calorie-restricted, weight-matched group displayed only modest nonsignificant reductions in liver mass (-9%) and liver lipid content (-5.1%) relative to the controls. Moreover, the improvement by AC3174 in liver and lipid parameters were abolished in GLP-1R knockout mice, suggesting the benefits resulted from the exendin-4 analogue was dependent on GLP-1R[26].

Shirakawa *et al*[27] found that the DPP-4 inhibitor sitagliptin monotherapy improved the diet-induced hepatic steatosis histologically, and significantly decreased the grade of steatosis and hepatic TC contents in both wild-type and pancreatic β-cell-specific glucokinase haploinsufficient (GCK$^{+/-}$) diabetic mice. In a DPP-4-deficient

rat model, the serum active GLP-1 level was chronically elevated. The DPP-4-deficient rats had markedly less hepatic fat and TG content and exported significantly less TG into the circulation than the wild-type animals. HFD-induced hepatic fat accumulation, blood lipid abnormality, and serum ALT and AST level elevation were much less in the DPP-4-deficient rats than those of the wild-type animals[28,29]. Similar results were also observed in a DPP-4-deficient mouse model[30].

Collectively, the results from both clinical data and animal studies suggest that GLP-1-based therapies may have beneficial effects in the treatment of NAFLD.

Safety issues of GLP-1-based therapies

On the other hand, the safety concerns of GLP-1-based therapies, especially the long-term safety, have been raised with their wide usage in the treatment of T2DM. Two major areas of interest are whether these therapies have an impact on pancreatitis and cancer. Elashoff *et al*[31] raised safety concerns in an analysis of case reports of pancreatitis, pancreatic cancer and other cancers in patients treated with exenatide or sitagliptin for T2DM. The data showed that the use of sitagliptin or exenatide increased the odds ratio for reported pancreatitis compared with 4 other anti-diabetic medications. Pancreatic cancer was more commonly reported among patients who took sitagliptin or exenatide compared with the other therapies. However, it is noteworthy that Elashoff's study was based on the United States Food and Drug Administration database containing spontaneous reports of adverse events, where over-reporting biases were highly likely to exist. Moreover, T2DM itself is associated with increased risk of acute pancreatitis and solid tumours, including pancreatic cancer[32,33]. Retrospective case control studies do not associate pancreatitis with GLP-1R agonists or DPP-4 inhibitors[34,35]. In line with the observations, two large-scale endpoint trials have recently shown that the DPP-4 inhibitors saxagliptin and alogliptin do not increase the risk of acute and chronic pancreatitis[36,37]. It has been reported that GLP-1R activation promoted C-cell hyperplasia and medullary thyroid cancer in rodents[38]. However, C cells within the monkey and human thyroid gland exhibit lower levels of GLP-1R expression. Long-term clinical studies of sufficient size and duration to permit conclusions regarding the association of GLP-1-based therapies with cancer have not yet been completed[35].

POTENTIAL MECHANISMS OF GLP-1-BASED THERAPIES IN AMELIORATING NAFLD

It has been well accepted that GLP-1-based therapies lead to weight control and glycaemic improvement, which are both important for the improvement of NAFLD. Moreover, recent reports have shown that GLP-1-based therapies also have direct effects on the liver (Figure 1).

Functional GLP-1R is expressed in hepatocytes

GLP-1R was detected in human liver biopsies at both mRNA and protein levels, although it was downregulated in patients with NASH[15]. Gupta *et al*[16] confirmed that GLP-1R was expressed in primary human hepatocytes and in two human hepatocellular carcinoma cell lines, HepG2 and HuH-7. Likewise, GLP-1R was also detectable in rodent hepatocytes[15,25]. Moreover, GLP-1R was present on the cell membrane of human hepatocytes and was internalised upon GLP-1 and exendin-4 stimulation[16]. Both GLP-1 and exendin-4 treatments resulted in a dramatic increase of cyclic adenosine monophosphate (cAMP) production in the hepatocytes. When the hepatocytes were pre-treated with the GLP-1R antagonist exendin (9-39), cAMP production that was induced by either GLP-1 or exendin-4 was significantly reduced to below basal levels[25]. These results indicate that functional GLP-1R is expressed in hepatocytes, and native GLP-1 or GLP-1R agonists may have a direct effect on hepatocytes via activation of GLP-1R.

Attenuation of insulin resistance in the liver by GLP-1-based therapies

Insulin resistance is an essential requirement and is believed to influence "the first hit" in the development of NAFLD. Recent studies have shown that this crucial process in the liver may be attenuated by GLP-1-based therapies.

In rodent NAFLD animal models and isolated primary rodent hepatocytes, either elevated endogenous GLP-1 level or liraglutide treatment was able to enhance activation of AMP-activated protein kinase (AMPK), a critical signal molecule involved in the regulation of hepatic insulin sensitivity[23,28]. GLP-1 overexpression increased insulin receptor substrate 1 (IRS-1) expression in the liver, promoted the hepatic activation of IRS-1 and protein kinase C (PKC) by insulin, and decreased hepatic glucose production (HGP) and hepatic fatty acid synthesis in the diabetic *ob/ob* mice[39]. In an HFD-induced insulin resistance model of ApoE$^{-/-}$ mice, hypoadiponectinemia induced by siRNA further led to a decrease in glucose infusion rate and insulin's ability to suppress HGP, accompanied by a reduction in the expression of glucose transporter-1 and elevation in the expression of phosphoenolpyruvate carboxykinase, which is a key enzyme of HGP. Liraglutide treatment completely or partially restored the deterioration in insulin resistance and the alteration of regulatory factors involved in hepatic insulin sensitivity[40]. Another GLP-1R agonist exendin-4 stimulated the phosphorylation of other key elements of the insulin signalling pathway, including phosphoinositide-dependent kinase-1 (PDK-1), protein kinase B (also called Akt) and PKC, in human hepatocellular cell lines. GLP-1R knockdown in the cells abolished the effects of exendin-4 on PDK-1 and PKC[16]. Exendin-4 treatment increased protein kinase A (PKA) activity and Akt phosphorylation in the hepatocytes isolated from NASH rats[15], indicating that the GLP-1R agonist attenuates in-

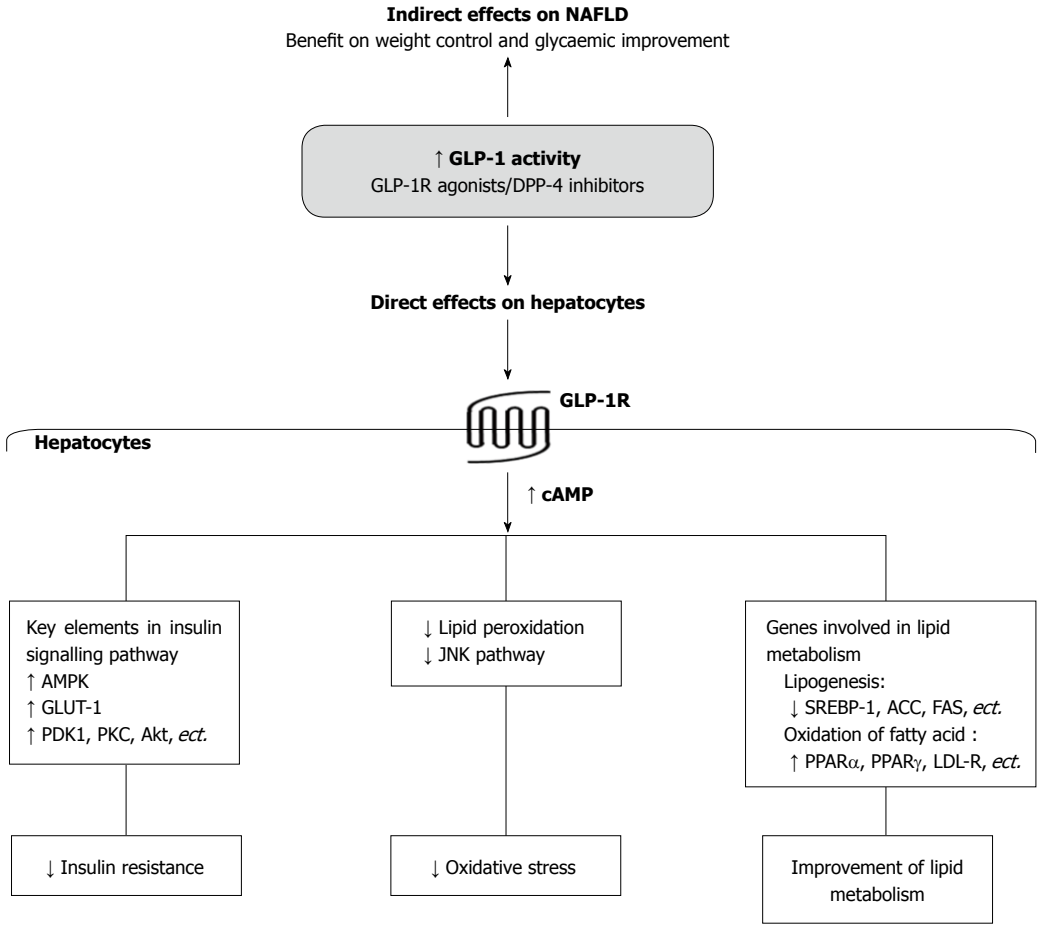


Figure 1 Potential mechanistic roles of Glucagon-like peptide-1 and Glucagon-like peptide-1-based therapies in non-alcoholic fatty liver disease. GLP-1: Glucagon-like peptide-1; GLP-1R: Glucagon-like peptide-1 receptor; DPP-4: Dipeptidyl peptidase-4; NAFLD: Non-alcoholic fatty liver disease; cAMP: Cyclic adenosine monophosphate; AMPK: AMP-activated protein kinase; PDK-1: Phosphoinositide-dependent kinase-1; PKC: Protein kinase C; Akt: Protein kinase B; GLUT-1: Glucose transporter-1; JNK: c-Jun N-terminal protein kinase; SREBP-1: Sterol regulatory element-binding protein 1; ACC: Acetyl-CoA carboxylase; FAS: Fatty acid synthase; PPAR: Peroxisome proliferator activator receptor; LDL-R: Low-density lipoprotein receptor.

sulin resistance in the liver of NASH animals.

Suppression of oxidative stress in the liver by GLP-1-based therapies

Exendin-4-treated *ob/ob* mice exhibited a significant decrease in hepatic level of thiobarbituric reactive substance, an important assessment of lipid peroxidation, compared with saline-treated group[25]. As a key mechanism involved in hepatic oxidative stress, c-Jun N-terminal protein kinase signalling pathway was depressed by treatment with either exenatide[15] or liraglutide[23]. These findings indicate that GLP-1R agonists attenuate liver cell oxidative stress, one of the essential steps in the progression of NAFLD or NASH.

Interestingly, liver cells seem to be able to synthesise and secrete GLP-1 as intestinal L cells. Nobili *et al*[41] evaluated hepatic progenitor cells (HPCs) in liver biopsies from 30 paediatric NAFLD subjects. They found that the HPC compartment expanded in the paediatric NAFLD individuals relative to healthy controls. GLP-1 expression in the HPCs of the paediatric NAFLD was upregulated, and GLP-1 expression level was associated with the degree of steatosis and NAFLD activity score. Therefore, the authors hypothesised that HPC activation was involved in the response of the liver to oxidative stress in the paediatric NAFLD subjects. GLP-1 may play a protective role in this process by inducing the resistance of HPCs to oxidative stress, favouring their proliferation and differentiation.

Regulation of lipid metabolism-related gene expression in the liver by GLP-1-based therapies

GLP-1-treated hepatocytes presented a significant increase in cAMP production and an alteration of gene expression profile, including increased expression of both peroxisome proliferator activator receptor α (PPAR-α) and acetyl-CoA oxidase, as well as decreased expression of stearoyl-CoA desaturase 1, sterol regulatory element-binding protein 1 (SREBP-1) and acetyl-CoA carboxylase (ACC)[25]. In *ob/ob* mice, overexpression of GLP-1 reduced hepatic expression of fatty acid synthase (FAS)[39]. These findings suggest that GLP-1 impairs hepatocyte lipogenesis and enhances β-oxidation of fatty acids. Moreover, pre-treatment with exendin (9-39) abolished the effects of GLP-1 on the expression of these genes, suggesting that GLP-1R is required in this regulatory

process[25].

Similarly, GLP-1R agonists are also effective in modulating the expression of these aforementioned genes. Liraglutide downregulated the expression of ACC and FAS in the liver of NAFLD mice[23] and restored hepatic transcripts of PPAR-α, low-density lipoprotein receptor and insulin-induced geng-2, which were downregulated by hypo-adiponectin in a NAFLD model[40]. Exenatide increased PPAR-γ expression and determined a PKA-dependent increase of PPAR-α activity in rats with NASH. The regulatory effect of exenatide on the expression of genes related to fatty acid β-oxidation was abolished by either PKA or AMPK inhibitors[15], indicating that the effects of the GLP-1R agonists on lipid metabolism are mediated *via* PKA and AMPK signalling pathway.

In mice fed on HFD, the DPP-4 inhibitor vildagliptin reduced hepatic expression of phosphomevalonate kinase and farnesyl diphosphate transferase 1, which were important for cholesterol synthesis[42]. In DPP-4-deficient mice, elevated GLP-1 level was associated with depressed SREBP-1 expression in hepatocytes[30].

Taken together, these findings indicate that GLP-1-based agents are able to regulate the expression of hepatic genes that are important for lipid metabolism.

Other effects of GLP-1-based therapies that may be beneficial for NAFLD

Liraglutide downregulated the expression of proinflammatory cytokines and transcription factors, including tumour necrosis factor α and nuclear factor κB, in the liver tissues of NAFLD[23]. In fat-loaded primary human hepatocytes, exendin-4 restored the change in endoplasmic reticulum stress markers, GPR78 and C/EBP homologous protein. Moreover, exendin-4 clearly promoted hepatocyte autophagy-associated events, as shown by enhanced production of both beclin-1 and LC3B-Ⅱ detected by western blot and increased autophagic vacuoles visualised by transmission electron microscopy. As a result, the survival of hepatocytes in the fat load was promoted, and apoptosis was inhibited by exendin-4 treatment. Similar observations were made in mouse liver lysates after mice were fed on a high-fat, high-fructose diet and treated with liraglutide[24].

CONCLUSION

NAFLD has been reported to be associated with defective GLP-1 signalling. GLP-1-based therapies were effective in improving hepatic endpoints in NAFLD, such as reducing hepatic fat content, hepatic steatosis and plasma transaminase, and preventing fibrosis. Apart from the benefits in controlling body weight and blood glucose, GLP-1-based agents may directly exert an action on the liver through activation of functional GLP-1R in hepatocytes. The possible mechanisms include regulating the expression of genes associated with insulin resistance and lipid metabolism, and suppressing oxidative stress in the liver cells, thus preventing the development and progression of NAFLD. With these promising findings, large-scale randomised controlled trials are warranted to investigate the efficacy and safety of GLP-1-based therapies in the treatment of patients with NAFLD.

REFERENCES

1 **Angulo P**. GI epidemiology: nonalcoholic fatty liver disease. *Aliment Pharmacol Ther* 2007; **25**: 883-889 [PMID: 17402991 DOI: 10.1111/j.1365-2036.2007.03246.x]

2 **Amarapurkar DN**, Hashimoto E, Lesmana LA, Sollano JD, Chen PJ, Goh KL. How common is non-alcoholic fatty liver disease in the Asia-Pacific region and are there local differences? *J Gastroenterol Hepatol* 2007; **22**: 788-793 [PMID: 17565631 DOI: 10.1111/j.1440-1746.2007.05042.x]

3 **Lomonaco R**, Sunny NE, Bril F, Cusi K. Nonalcoholic fatty liver disease: current issues and novel treatment approaches. *Drugs* 2013; **73**: 1-14 [PMID: 23329465 DOI: 10.1007/s40265-012-0004-0]

4 **Drucker DJ**, Sherman SI, Gorelick FS, Bergenstal RM, Sherwin RS, Buse JB. Incretin-based therapies for the treatment of type 2 diabetes: evaluation of the risks and benefits. *Diabetes Care* 2010; **33**: 428-433 [PMID: 20103558 DOI: 10.2337/dc09-1499]

5 **Azuma K**, Rádiková Z, Mancino J, Toledo FG, Thomas E, Kangani C, Dalla Man C, Cobelli C, Holst JJ, Deacon CF, He Y, Ligueros-Saylan M, Serra D, Foley JE, Kelley DE. Measurements of islet function and glucose metabolism with the dipeptidyl peptidase 4 inhibitor vildagliptin in patients with type 2 diabetes. *J Clin Endocrinol Metab* 2008; **93**: 459-464 [PMID: 18042650 DOI: 10.1210/jc.2007-1369]

6 **Armstrong MJ**, Houlihan DD, Rowe IA, Clausen WH, Elbrønd B, Gough SC, Tomlinson JW, Newsome PN. Safety and efficacy of liraglutide in patients with type 2 diabetes and elevated liver enzymes: individual patient data meta-analysis of the LEAD program. *Aliment Pharmacol Ther* 2013; **37**: 234-242 [PMID: 23163663 DOI: 10.1111/apt.12149]

7 **Ceriello A**, Esposito K, Testa R, Bonfigli AR, Marra M, Giugliano D. The possible protective role of glucagon-like peptide 1 on endothelium during the meal and evidence for an "endothelial resistance" to glucagon-like peptide 1 in diabetes. *Diabetes Care* 2011; **34**: 697-702 [PMID: 21273492 DOI: 10.2337/dc10-1949]

8 **Rabl C**, Campos GM. The impact of bariatric surgery on nonalcoholic steatohepatitis. *Semin Liver Dis* 2012; **32**: 80-91 [PMID: 22418890 DOI: 10.1055/s-0032-1306428]

9 **Hafeez S**, Ahmed MH. Bariatric surgery as potential treatment for nonalcoholic fatty liver disease: a future treatment by choice or by chance? *J Obes* 2013; **2013**: 839275 [PMID: 23431426 DOI: 10.1155/2013/839275]

10 **Bernsmeier C**, Meyer-Gerspach AC, Blaser LS, Jeker L, Steinert RE, Heim MH, Beglinger C. Glucose-induced glucagon-like Peptide 1 secretion is deficient in patients with non-alcoholic fatty liver disease. *PLoS One* 2014; **9**: e87488 [PMID: 24489924 DOI: 10.1371/journal.pone.0087488]

11 **McDonald SD**, Pesarchuk E, Don-Wauchope A, El Zimaity H, Holloway AC. Adverse metabolic effects of a hypercaloric, high-fat diet in rodents precede observable changes in body weight. *Nutr Res* 2011; **31**: 707-714 [PMID: 22024495 DOI: 10.1016/j.nutres.2011.08.009]

12 **Miyazaki M**, Kato M, Tanaka K, Tanaka M, Kohjima M, Nakamura K, Enjoji M, Nakamuta M, Kotoh K, Takayanagi R. Increased hepatic expression of dipeptidyl peptidase-4 in non-alcoholic fatty liver disease and its association with insulin resistance and glucose metabolism. *Mol Med Rep* 2012; **5**: 729-733 [PMID: 22179204 DOI: 10.3892/mmr.2011.707]

13 **Balaban YH**, Korkusuz P, Simsek H, Gokcan H, Gedikoglu G, Pinar A, Hascelik G, Asan E, Hamaloglu E, Tatar G. Dipeptidyl peptidase IV (DDP IV) in NASH patients. *Ann*

Hepatol 2007; **6**: 242-250 [PMID: 18007554]
14 **Firneisz G**, Varga T, Lengyel G, Fehér J, Ghyczy D, Wichmann B, Selmeci L, Tulassay Z, Rácz K, Somogyi A. Serum dipeptidyl peptidase-4 activity in insulin resistant patients with non-alcoholic fatty liver disease: a novel liver disease biomarker. *PLoS One* 2010; **5**: e12226 [PMID: 20805868 DOI: 10.1371/journal.pone.0012226]
15 **Svegliati-Baroni G**, Saccomanno S, Rychlicki C, Agostinelli L, De Minicis S, Candelaresi C, Faraci G, Pacetti D, Vivarelli M, Nicolini D, Garelli P, Casini A, Manco M, Mingrone G, Risaliti A, Frega GN, Benedetti A, Gastaldelli A. Glucagon-like peptide-1 receptor activation stimulates hepatic lipid oxidation and restores hepatic signalling alteration induced by a high-fat diet in nonalcoholic steatohepatitis. *Liver Int* 2011; **31**: 1285-1297 [PMID: 21745271 DOI: 10.1111/j.1478-3231.2011.02462.x]
16 **Gupta NA**, Mells J, Dunham RM, Grakoui A, Handy J, Saxena NK, Anania FA. Glucagon-like peptide-1 receptor is present on human hepatocytes and has a direct role in decreasing hepatic steatosis in vitro by modulating elements of the insulin signaling pathway. *Hepatology* 2010; **51**: 1584-1592 [PMID: 20225248 DOI: 10.1002/hep.23569]
17 **Tushuizen ME**, Bunck MC, Pouwels PJ, van Waesberghe JH, Diamant M, Heine RJ. Incretin mimetics as a novel therapeutic option for hepatic steatosis. *Liver Int* 2006; **26**: 1015-1017 [PMID: 16953843 DOI: 10.1111/j.1478-3231.2006.01315.x]
18 **Cuthbertson DJ**, Irwin A, Gardner CJ, Daousi C, Purewal T, Furlong N, Goenka N, Thomas EL, Adams VL, Pushpakom SP, Pirmohamed M, Kemp GJ. Improved glycaemia correlates with liver fat reduction in obese, type 2 diabetes, patients given glucagon-like peptide-1 (GLP-1) receptor agonists. *PLoS One* 2012; **7**: e50117 [PMID: 23236362 DOI: 10.1371/journal.pone.0050117]
19 **Boettcher E**, Csako G, Pucino F, Wesley R, Loomba R. Meta-analysis: pioglitazone improves liver histology and fibrosis in patients with non-alcoholic steatohepatitis. *Aliment Pharmacol Ther* 2012; **35**: 66-75 [PMID: 22050199 DOI: 10.1111/j.1365-2036.2011.04912.x]
20 **Sathyanarayana P**, Jogi M, Muthupillai R, Krishnamurthy R, Samson SL, Bajaj M. Effects of combined exenatide and pioglitazone therapy on hepatic fat content in type 2 diabetes. *Obesity* (Silver Spring) 2011; **19**: 2310-2315 [PMID: 21660077 DOI: 10.1038/oby.2011.152]
21 **Samson SL**, Sathyanarayana P, Jogi M, Gonzalez EV, Gutierrez A, Krishnamurthy R, Muthupillai R, Chan L, Bajaj M. Exenatide decreases hepatic fibroblast growth factor 21 resistance in non-alcoholic fatty liver disease in a mouse model of obesity and in a randomised controlled trial. *Diabetologia* 2011; **54**: 3093-3100 [PMID: 21956711 DOI: 10.1007/s00125-011-2317-z]
22 **Iwasaki T**, Yoneda M, Inamori M, Shirakawa J, Higurashi T, Maeda S, Terauchi Y, Nakajima A. Sitagliptin as a novel treatment agent for non-alcoholic Fatty liver disease patients with type 2 diabetes mellitus. *Hepatogastroenterology* 2011; **58**: 2103-2105 [PMID: 22024083 DOI: 10.5754/hge11263]
23 **Zhang L**, Yang M, Ren H, Hu H, Boden G, Li L, Yang G. GLP-1 analogue prevents NAFLD in ApoE KO mice with diet and Acrp30 knockdown by inhibiting c-JNK. *Liver Int* 2013; **33**: 794-804 [PMID: 23432843 DOI: 10.1111/liv.12120]
24 **Sharma S**, Mells JE, Fu PP, Saxena NK, Anania FA. GLP-1 analogs reduce hepatocyte steatosis and improve survival by enhancing the unfolded protein response and promoting macroautophagy. *PLoS One* 2011; **6**: e25269 [PMID: 21957486 DOI: 10.1371/journal.pone.0025269]
25 **Ding X**, Saxena NK, Lin S, Gupta NA, Anania FA. Exendin-4, a glucagon-like protein-1 (GLP-1) receptor agonist, reverses hepatic steatosis in ob/ob mice. *Hepatology* 2006; **43**: 173-181 [PMID: 16374859 DOI: 10.1002/hep.21006]
26 **Trevaskis JL**, Griffin PS, Wittmer C, Neuschwander-Tetri BA, Brunt EM, Dolman CS, Erickson MR, Napora J, Parkes DG, Roth JD. Glucagon-like peptide-1 receptor agonism improves metabolic, biochemical, and histopathological indices of nonalcoholic steatohepatitis in mice. *Am J Physiol Gastrointest Liver Physiol* 2012; **302**: G762-G772 [PMID: 22268099 DOI: 10.1152/ajpgi.00476.2011]
27 **Shirakawa J**, Fujii H, Ohnuma K, Sato K, Ito Y, Kaji M, Sakamoto E, Koganei M, Sasaki H, Nagashima Y, Amo K, Aoki K, Morimoto C, Takeda E, Terauchi Y. Diet-induced adipose tissue inflammation and liver steatosis are prevented by DPP-4 inhibition in diabetic mice. *Diabetes* 2011; **60**: 1246-1257 [PMID: 21330637 DOI: 10.2337/db10-1338]
28 **Ben-Shlomo S**, Zvibel I, Shnell M, Shlomai A, Chepurko E, Halpern Z, Barzilai N, Oren R, Fishman S. Glucagon-like peptide-1 reduces hepatic lipogenesis via activation of AMP-activated protein kinase. *J Hepatol* 2011; **54**: 1214-1223 [PMID: 21145820 DOI: 10.1016/j.jhep.2010.09.032]
29 **Ben-Shlomo S**, Zvibel I, Rabinowich L, Goldiner I, Shlomai A, Santo EM, Halpern Z, Oren R, Fishman S. Dipeptidyl peptidase 4-deficient rats have improved bile secretory function in high fat diet-induced steatosis. *Dig Dis Sci* 2013; **58**: 172-178 [PMID: 22918684 DOI: 10.1007/s10620-012-2353-7]
30 **Conarello SL**, Li Z, Ronan J, Roy RS, Zhu L, Jiang G, Liu F, Woods J, Zycband E, Moller DE, Thornberry NA, Zhang BB. Mice lacking dipeptidyl peptidase IV are protected against obesity and insulin resistance. *Proc Natl Acad Sci USA* 2003; **100**: 6825-6830 [PMID: 12748388 DOI: 10.1073/pnas.0631828100]
31 **Elashoff M**, Matveyenko AV, Gier B, Elashoff R, Butler PC. Pancreatitis, pancreatic, and thyroid cancer with glucagon-like peptide-1-based therapies. *Gastroenterology* 2011; **141**: 150-156 [PMID: 21334333 DOI: 10.1053/j.gastro.2011.02.018]
32 **Girman CJ**, Kou TD, Cai B, Alexander CM, O'Neill EA, Williams-Herman DE, Katz L. Patients with type 2 diabetes mellitus have higher risk for acute pancreatitis compared with those without diabetes. *Diabetes Obes Metab* 2010; **12**: 766-771 [PMID: 20649628 DOI: 10.1111/j.1463-1326.2010.01231.x]
33 **Seshasai SR**, Kaptoge S, Thompson A, Di Angelantonio E, Gao P, Sarwar N, Whincup PH, Mukamal KJ, Gillum RF, Holme I, Njølstad I, Fletcher A, Nilsson P, Lewington S, Collins R, Gudnason V, Thompson SG, Sattar N, Selvin E, Hu FB, Danesh J. Diabetes mellitus, fasting glucose, and risk of cause-specific death. *N Engl J Med* 2011; **364**: 829-841 [PMID: 21366474 DOI: 10.1056/NEJMoa1008862]
34 **Spranger J**, Gundert-Remy U, Stammschulte T. GLP-1-based therapies: the dilemma of uncertainty. *Gastroenterology* 2011; **141**: 20-23 [PMID: 21723985 DOI: 10.1053/j.gastro.2011.05.019]
35 **Drucker DJ**, Sherman SI, Bergenstal RM, Buse JB. The safety of incretin-based therapies--review of the scientific evidence. *J Clin Endocrinol Metab* 2011; **96**: 2027-2031 [PMID: 21734003 DOI: 10.1210/jc.2011-0599]
36 **Scirica BM**, Bhatt DL, Braunwald E, Steg PG, Davidson J, Hirshberg B, Ohman P, Frederich R, Wiviott SD, Hoffman EB, Cavender MA, Udell JA, Desai NR, Mosenzon O, McGuire DK, Ray KK, Leiter LA, Raz I. Saxagliptin and cardiovascular outcomes in patients with type 2 diabetes mellitus. *N Engl J Med* 2013; **369**: 1317-1326 [PMID: 23992601 DOI: 10.1056/NEJMoa1307684]
37 **White WB**, Cannon CP, Heller SR, Nissen SE, Bergenstal RM, Bakris GL, Perez AT, Fleck PR, Mehta CR, Kupfer S, Wilson C, Cushman WC, Zannad F. Alogliptin after acute coronary syndrome in patients with type 2 diabetes. *N Engl J Med* 2013; **369**: 1327-1335 [PMID: 23992602 DOI: 10.1056/NEJMoa1305889]
38 **Bjerre Knudsen L**, Madsen LW, Andersen S, Almholt K, de Boer AS, Drucker DJ, Gotfredsen C, Egerod FL, Hegelund AC, Jacobsen H, Jacobsen SD, Moses AC, Mølck AM, Nielsen HS, Nowak J, Solberg H, Thi TD, Zdravkovic M, Moerch U. Glucagon-like Peptide-1 receptor agonists activate rodent thyroid C-cells causing calcitonin release and C-cell proliferation. *Endocrinology* 2010; **151**: 1473-1486 [PMID: 20203154 DOI: 10.1210/en.2009-1272]
39 **Lee YS**, Shin S, Shigihara T, Hahm E, Liu MJ, Han J, Yoon

JW, Jun HS. Glucagon-like peptide-1 gene therapy in obese diabetic mice results in long-term cure of diabetes by improving insulin sensitivity and reducing hepatic gluconeogenesis. *Diabetes* 2007; **56**: 1671-1679 [PMID: 17369525 DOI: 10.2337/db06-1182]

40 **Li L**, Miao Z, Liu R, Yang M, Liu H, Yang G. Liraglutide prevents hypoadiponectinemia-induced insulin resistance and alterations of gene expression involved in glucose and lipid metabolism. *Mol Med* 2011; **17**: 1168-1178 [PMID: 21785811 DOI: 10.2119/molmed.2011.00051]

41 **Nobili V**, Carpino G, Alisi A, Franchitto A, Alpini G, De Vito R, Onori P, Alvaro D, Gaudio E. Hepatic progenitor cells activation, fibrosis, and adipokines production in pediatric nonalcoholic fatty liver disease. *Hepatology* 2012; **56**: 2142-2153 [PMID: 22467277 DOI: 10.1002/hep.25742]

42 **Flock G**, Baggio LL, Longuet C, Drucker DJ. Incretin receptors for glucagon-like peptide 1 and glucose-dependent insulinotropic polypeptide are essential for the sustained metabolic actions of vildagliptin in mice. *Diabetes* 2007; **56**: 3006-3013 [PMID: 17717280 DOI: 10.2337/db07-0697]

P- Reviewers: Conti B, Silva LD, Vairappan B, Zhu L
S- Editor: Qi Y **L- Editor**: A **E- Editor**: Ma S

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9098

World J Gastroenterol 2014 July 21; 20(27): 9098-9105
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Bone marrow derived stem cells for the treatment of end-stage liver disease

Cristina Margini, Ranka Vukotic, Lucia Brodosi, Mauro Bernardi, Pietro Andreone

Cristina Margini, Ranka Vukotic, Lucia Brodosi, Mauro Bernardi, Pietro Andreone, Dipartimento di Scienze mediche e Chirurgiche, Unità Operativa di Semeiotica Medica, University of Bologna, 40138 Bologna, Italy
Author contributions: Margini C conceived of and designed the study, performed the literature search and critical analyses, interpreted and contextualized the available data, drafted the manuscript and revised it for scientific content; Vukotic R contributed to the interpretation of the collected data, drafted the article and revised the manuscript for scientific content; Brodosi L contributed to revising the paper for scientific content; Bernardi M revised the draft for scientific content; Andreone P conceived of and designed the study, contributed to the interpretation of the data and to the revision of the draft for scientific content, and is the guarantor of this review article; all authors approved the final version of the manuscript.
Correspondence to: Pietro Andreone, MD, Professor of Internal Medicine, Dipartimento di Scienze mediche e Chirurgiche, Unità Operativa di Semeiotica Medica, University of Bologna, Padiglione 11, Via Massarenti 9, 40138 Bologna, Italy. pietro.andreone@unibo.it
Telephone: +39-51-6364107 Fax: +39-51-345806
Received: October 29, 2013 Revised: February 27, 2014
Accepted: April 15, 2014
Published online: July 21, 2014

Abstract

End-stage disease due to liver cirrhosis is an important cause of death worldwide. Cirrhosis results from progressive, extensive fibrosis and impaired hepatocyte regeneration. The only curative treatment is liver transplantation, but due to the several limitations of this procedure, the interest in alternative therapeutic strategies is increasing. In particular, the potential of bone marrow stem cell (BMSC) therapy in cirrhosis has been explored in different trials. In this article, we evaluate the results of 18 prospective clinical trials, and we provide a descriptive overview of recent advances in the research on hepatic regenerative medicine. The main message from the currently available data in the literature is that BMSC therapy is extremely promising in the context of liver cirrhosis. However, its application should be further explored in randomized, controlled trials with large cohorts and long follow-ups.

Key words: Liver cirrhosis; Liver regeneration; Hematopoietic stem cells; Mesenchymal stem cells; End stage liver disease

Core tip: In recent years, the role of bone marrow stem cells (BMSCs) in liver regeneration has been explored in various clinical trials. Because these trials were very diverse, we conducted a descriptive overview to understand the effects of BMSC transplantation on liver histology and morphology, on laboratory parameters and prognostic scores, and finally, on clinical manifestations and quality of life. This overview suggests that the efficacy of BMSC therapy might be temporary, and therefore, repeated cycles of BMSCs could be useful to achieve a sustained benefit.

Margini C, Vukotic R, Brodosi L, Bernardi M, Andreone P. Bone marrow derived stem cells for the treatment of end-stage liver disease. *World J Gastroenterol* 2014; 20(27): 9098-9105 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9098.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9098

INTRODUCTION

End-stage disease due to liver cirrhosis is an important cause of death worldwide[1-3]. Currently, the only effective treatment is liver transplantation, but because of the lack of organ donors, surgical complications, risk of rejection and high costs[4,5], the pressure on finding new treatment strategies is increasing[6]. When a successful etiologic approach is unavailable or has failed, progressive, extensive fibrosis[7,8] with concurrently impaired

hepatocyte regeneration[9,10] leads to irreversible cirrhosis[11,12]. Therefore, the development of new techniques to stimulate liver regeneration and reduce the scarring process is urgently needed. In this respect, there is great interest in the potential of BMSC therapy to promote liver regeneration through the use of unsorted mononuclear stem cells (MNCs), hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). This review article summarizes the results of the main prospective clinical trials on BMSC transplantation in patients with cirrhosis, providing a descriptive overview of recent advances in the research on hepatic regenerative medicine.

Liver fibrosis

Cirrhosis is the common final outcome of chronic liver disease, leading to portal hypertension and end-stage liver disease (ESLD), and it is mainly caused by alcohol abuse and viral infections. The prevalence is estimated at 0.15% in the United States[6], and liver cirrhosis-related deaths constitute 1.8% of all deaths in Europe[13].

The main pathogenetic mechanism that leads to the subversion of liver architecture is an aberrant healing process referred to as fibrogenesis, which can be triggered by various factors such as viruses, alcohol abuse, steatohepatitis, autoantibodies, oxidative stress, and others. The common pathway leading to the deposition of extracellular matrix (ECM) is the activation of myofibroblasts. Myofibroblasts originate from different sources such as hepatic stem cells, portal fibroblasts and bone marrow (BM) derived fibrocytes (haematopoietic lineage) and mesenchymal cells. These cells can also arise from epithelial cells via epithelial to mesenchymal transition[8,14]. In the first phase of hepatic injury, the production of ECM is counterbalanced by the action of proteolytic enzymes such as matrix metalloproteinases (MMPs). With persistent damage, this equilibrium is compromised, and the presence of tissue inhibitors of MMPs abnormally enhances the deposition of ECM and ultimately leads to the alteration of normal liver structure[15].

Liver regeneration

The processes and the pathways responsible for liver regeneration are not yet completely understood. Under physiological circumstances, the ability of hepatocytes to re-enter the cell cycle enables liver regeneration and repair through compensatory hyperplasia and hypertrophy[16-18]. In the case of chronic liver injury, this ability is compromised, and liver regeneration is carried out by liver progenitor cells (LPCs). Progenitor-dependent regeneration takes place if hepatocytes are severely damaged and unable to regenerate efficiently, as occurs in cirrhosis. This hypothesis was first suggested by the finding that the LPC concentration in patients with chronic liver disease is greatly increased[19].

These cells were initially identified in animal models and called "oval cells". It has been demonstrated that they are a bi-potential progenitor for hepatocytes and biliary cells[20]. Highly conserved intracellular pathways are responsible for oval cell differentiation. In particular, Wnt signalling is involved in LPC proliferation, while *Notch* signalling is involved in biliary differentiation[21]. The human equivalent of oval cells have been detected in the canal of Hering, and according to the so called "streaming liver hypothesis", these cells migrate to the central vein and progressively differentiate into hepatocytes[22,23]. Conversely, Kuwahara *et al*[24] have suggested that LPCs can be located in four different cell niches: canal of Hering, intralobular bile ducts, periductular mononuclear cells and peribiliary hepatocytes. The space of Disse has also been reported to be a potential niche for LPCs[25].

The origin of LPCs is controversial; they might be *in situ* cells, descendants of the foetal ductal plate[26], or derive from BMSCs, as first described by Petersen *et al*[27]. Furthermore, it has also been hypothesized that they might arise from MSCs *via* the mesenchymal to epithelial transition[28].

Using Y chromosome tracking in rodents[29] and humans[30], it has also been reported that BMSCs may contribute to hepatocyte differentiation independently of mature hepatocytes and LPCs. This limited evidence highlights the fact that liver regeneration processes are not yet fully understood, and only with a better understanding of these molecular and cellular mechanisms will it be possible to develop a targeted therapy for liver fibrosis.

STEM CELL THERAPY

In recent years, unsorted MNCs, HSCs and MSCs have been employed in research focused on liver regeneration[31,32].

HSCs are traceable using CD34 and CD133 markers. The latter is believed to represent a subpopulation of the $CD34^+$ cells that have a higher differentiation potential[33]. HSCs can be obtained by BM aspiration or from peripheral collection through leukapheresis after granulocyte-colony stimulating factor (G-CSF) administration, whereas MNCs and MSCs can be harvested mainly by BM aspiration, which requires an invasive procedure. G-CSF has been used in liver regeneration because of its ability to increase the number of circulating BMSCs and to promote repair in the cirrhotic liver[34]. As suggested by Jin *et al*[35], G-CSF may also enhance MNC homing to the liver.

The feasibility and safety of mobilizing BM derived cells following G-CSF administration was demonstrated by Gaia *et al*[36] in eight patients with ESLD. Additionally, this study reported improved model for end-stage liver disease (MELD) scores and did not find any development of hepatocellular carcinoma or increase in alpha-fetoprotein up to eight months after G-CSF administration. A favourable effect of G-CSF administration on survival and clinical parameters in patients with liver failure has also been reported in other studies[37]. Lorenzini *et al*[38] demonstrated the safety of BMSC mobilization

and collection through leukapheresis in patients with cirrhosis, even though no improvement of liver function tests occurred. G-CSF administration can be also associated with the risk of spleen enlargement[39] or even rupture, as reported by Falzetti *et al*[40] in a healthy donor.

Other cells that have been utilized in hepatic regeneration research include foetal annex stem cells (cord blood and placenta) and embryonic stem cells[41,42]. The use of embryonic stem cells is limited to *in vitro* and animal studies because of difficulties in controlling their proliferative and differentiation potential. Another type of cell employed in animal experiments is induced pluripotent stem cells, which are embryonic-like stem cells derived from somatic cells through the expression of reprogramming factors[43].

Hypothesized mechanisms

Stem cell therapy may contribute to the improvement of liver function[44]. Although the mechanisms involved are not yet fully understood, some hypotheses have been proposed[45,46]. One hypothesis is that genomic plasticity, in response to the microenvironment, causes the trans-differentiation of stem cells into functional hepatocytes[47,48]. Another mechanism is presumably related to the cell fusion of BMSCs and hepatocytes[49,50]. Additionally, it has been proposed that stem cells may exert paracrine effects on endogenous hepatocytes to increase their ability to regenerate, through the release of proliferative cytokines and the production of matrix metalloproteinase-9[51] or by enhancing angiogenesis through the release of vascular endothelial growth factors[52]. A better understanding of the action of stem cells in the context of a fibrotic liver might allow more rational use of BMSC therapy in liver cirrhosis.

CLINICAL TRIALS

Many clinical trials have recently been published on BMSC therapy in cirrhotic patients. The results of the main prospective clinical trials are summarized in Table 1. These studies differ with respect to study design, inclusion/exclusion criteria, type and number of cells infused, route of delivery and end points. The stage of cirrhosis varied from Child Turcotte Pugh score (CTP) A to C. Although cells were harvested mainly by BM aspiration, in some studies, leukapheresis after G-CSF administration was performed. Different types of cells were infused, including MNCs, MSCs or HSCs. The number of injected cells varied from 10^6 to 10^9. The most commonly used routes of delivery were the hepatic artery or portal vein, but in some studies, peripheral vein and intrasplenic injection were also utilized.

Safety of BMSCs therapy

The majority of the clinical trials demonstrated the safety of the procedure. Couto *et al*[53] reported a case of artery dissection and a case of Tako-Tsubo syndrome after the injection of BMSCs through the hepatic artery, and Levicar *et al*[54] reported thrombocytopenia after leukapheresis. Finally, Mohamadnejad *et al*[55] reported a case of radiocontrast nephropathy that progressed to type 1 hepatorenal syndrome and caused the death of a patient. For this reason, the clinical trial was prematurely stopped, and BMSC therapy through the hepatic artery was not considered safe.

Effect of BMSCs therapy on liver histology and morphology

In a study by Kim *et al*[56], which enrolled ten patients, a significant increase in liver volume compared to baseline was documented using MRI six months after MNCs transplantation through a peripheral injection but this result has not been confirmed[57,58]. In that study, serial biopsies were performed. All biopsies at baseline showed low levels of LPC activation and differentiation. After BMSC therapy, a gradual increase in the LPC count occurred in all patients, with a peak three months after reinfusion. In contrast, no changes in the degree of stellate cell activation were observed[56].

Terai *et al*[44] demonstrated increased expression of proliferating cell nuclear antigen in liver biopsy tissue one month after peripheral MNC injection. Jang *et al*[59] performed a histological evaluation in eleven patients with alcohol-induced cirrhosis after two MSC transplantations through the hepatic artery. After five months, significant histological improvement according to the Laennec system[60] was observed, along with a significant decrease in the expression of transforming growth factor-beta1, type I -collagen and alpha-smooth muscle actin.

An interesting study by Couto *et al*[53] suggested that the hepatic retention of MNCs is fair. In that study, MNCs (MSCs and HSCs) were labelled with Tc99 and then injected through the hepatic artery in eight patients with CTP B and C. Remarkably, whole body scintigraphy at 3 and 24 h after injection showed a mean radiotracer retention of 41% and 32%, respectively. Few studies have evaluated liver histology and morphology, but the available data are consistent with histological improvement, increased LPC count and decreased expression of fibrosis markers after BMSC therapy.

Effect of BMSC therapy on laboratory parameters and prognostic scores

The efficacy of BMSC therapy in patients with cirrhosis was assessed using laboratory parameters such as International Normalized Ratio (INR), total bilirubin (TBil), creatinine (Cr), albumin (Alb) and/or prognostic scores (CTP and MELD). These results are summarized in Table 2.

Significant improvements in laboratory tests were reported at one month after BMSC infusion in one study[53], at three months in four studies[59,61-63], at six months in six studies[44,56,64-67] and after twelve months of follow up in one study[68]. In the remaining studies, the limited number of patients enrolled prevented statistical evaluation, but

Table 1 Prospective studies on bone marrow stem cells therapy in patients with cirrhosis

Ref.	Study design	No. of patients	Disease cause/stage	Cell/harvest	Infusion route/n cell infused
Park *et al*[69]	Case series	5	Mixed	MNCs (MSCs)	Hepatic artery
Cytotherapy, 2013			CTP B-C	BM aspiration	10^6-10^7/kg
Amin *et al*[64]	Case series	20	HCV	MSCs	Intrasplenic
Clin Transplant, 2013			CTP C	BM aspiration	10^7
Mohamadnejad *et al*[57]	Randomized	15	Mixed	MSCs/placebo	Peripheral vein
Liver Int, 2013	Vs untreated control	12 control	CTP A-C	BM aspiration	10^8
Jang *et al*[59]	Case series	12	Alcohol	MSCs	Hepatic artery
Liver Int, 2013			CTP A-B	BM aspiration	10^7
Salama *et al*[68]	Not-randomized	50	HCV	HSCs	Portal vein/hepatic artery
Stem Cell Res Ther, 2012	Vs untreated control	50 control	ESLD	Leukapheresis	
				G-CSF	10^9
El-Ansary *et al*[65]	Not-randomized	15	HCV	MSCs	Peripheral vein
Stem Cell Rev, 2012	Vs untreated control	10 control	CTP C	BM aspiration	10^6
Peng *et al*[61]	Not-randomized	53	HBV	MSCs	Hepatic artery
Hepatology, 2011	Vs untreated control	105 control	Mixed[1]	BM aspiration	NA
Couto *et al*[53]	Case series	8	Mixed	MNCs	Hepatic artery
Liver Int, 2010			CTP B-C	BM aspiration	10^9
Nikeghbalian *et al*[58]	Case series	6	Mixed	MNCs/HSCs	Portalvein
Arch Iran Med, 2011			CTP C	BM aspiration	10^6-10^9
Salama *et al*[66]	Randomized	90	HCV	HSCs	Portal vein
World J Gastroenterol, 2010	Vs untreated control	50 control	NA	BM aspiration	10^7
				G-CSF	
Kim *et al*[56]	Case series	10	HBV	MNCs	Peripheral vein
Cell Transplantation, 2010			MELD 7-13	BM aspiration	10^8/kg
Lyra *et al*[62]	Randomized	15	Mixed	MNCs	Hepatic artery
Eur J Gastroenterol Hepatol, 2009	Vs untreated control	15 control	CTP B-C	BM aspiration	10^8
Kharaziha *et al*[67]	Case series	8	Mixed	MSCs	Portal vein
Eur J Gastroenterol Hepatol, 2009			MELD > 10	BM aspiration	10^8
Pai *et al*[63]	Case series	9	alcohol	HSCs	Hepatic artery
AM J Gastroenterol, 2008			CTP B	Leukapheresis	10^8
				G-CSF	
Levicar *et al*[54]	Case series	5	Mixed	HSCs	Portalvein/
Cell Proliferat, 2008			CTP A-B	Leukapheresis	hepaticartery
				G-CSF	10^8
Mohamadnejad *et al*[55]	Case series	4	Mixed	HSCs	Hepatic artery
World J Gastroenterol, 2007			CTP B-C	BM aspiration	10^6-10^7
Lyra *et al*[70]	Case series	10	Mixed	MNCs	Hepatic artery
World J Gastroenterol, 2007			CTP B-C	BM aspiration	10^8
Terai *et al*[44]	Case series	9	Mixed	MNCs	Peripheral vein
Stem cells, 2006			CTP B-C	BM aspiration	10^9

[1]In this study the patients had cirrhosis or chronic hepatitis. BMSC: Bone marrow stem cell; CTP :Child Turcotte Pugh score; MNC: Mononuclear stem cell; HSC: Hematopoietic stem cell; MSC: Mesenchymal stem cells; NA: Not available; GI: Gastrointestinal; Pt: Patients.

some improvement in laboratory tests was reported[54,69,70].

A significant improvement in MELD and/or CTP score three months after BMSCs infusion in two studies[59,62], at six months in four studies[44,56,65,67] and at nine months in one study[61] has also been described.

However, the controlled randomized clinical trial performed by Mohamadnejad *et al*[57] did not show any significant difference in either INR or prognostic scores between treatment and control groups at three and twelve months after BMSCs infusion. The majority of the clinical trials showed a significant but time-limited improvement in laboratory parameters and prognostic scores, offering encouraging prospects for future trials.

Effect of BMSC therapy on clinical manifestations and quality of life

Many studies have evaluated the clinical manifestations associated with ESLD, including hepatic encephalopathy, lower limb oedema, hematemesis, ascites and jaundice. Seven studies reported an improvement in at least one of the previously mentioned clinical manifestations[44,56,63-66,68]. However, it should be noted that some clinical manifestations, such as ascites, might not accurately reflect efficacy. In fact, ascites can be over- or underestimated by physical examination and can be modified by pharmacological interventions other than BMSC therapy (*e.g.*, diuretics, albumin).

Health-related quality of life after BMSC therapy was the primary outcome in an interesting study performed by Salama *et al*[68]. One hundred patients were assigned to the treatment or control groups and completed the Short Form-36 health status evaluation. Self-reported physical and mental status significantly improved in the treatment group during the six months following BMSC reinfusion, while status significantly deteriorated in the control group; these data are also supported by another

Table 2 Studies that reported a modification of laboratory parameters and prognostic scores

Author	Results compared to	Laboratory parameters				Prognostic scores	
		INR	TBil	Alb	Cr	MELD	CTP
Amin *et al*[64]	Baseline	I	I	I	NR	NR	NR
Mohamadnejad *et al*[57]	Control group	NI	NR	NI	NR	NI	NI
Jang *et al*[59]	Baseline	I	NI	I	NI	I	I
Salama *et al*[68]	Baseline	NR	I	NR	NR	NR	NR
El-Ansary *et al*[65]	Control group	I[1]	I	I	NR	I	NR
Peng *et al*[61]	Control group	I	I	I	NR	I	NR
Couto *et al*[53]	Baseline	NR	I	I	NR	NR	NR
Salama *et al*[66]	Control group	NR	I	I	NR	NR	NR
Kim *et al*[56]	Baseline	I	NR	I	NR	NI	I
Lyra *et al*[62]	Baseline	NR	NR	I	NR	NI	I
Kharaziha *et al*[67]	Baseline	I	NI	NI	I	I	NR
Pai *et al*[63]	Baseline	NR	I	NI	NR	NR	NR
Terai *et al*[44]	Baseline	NR	NR	I	NR	NR	I

[1]In this study it was reported prothrombin concentration (PC) and not INR. I: Improved significantly; NI: Not improved; NR: Not reported; INR: International normalized ratio; MELD: Model for end-stage liver disease; CTP: Child turcotte pugh score.

recent study[56].

In addition, Salama *et al*[68] reported a significantly higher survival rate in the treatment group compared to the control group. An improved survival rate after BMSC therapy was also reported in other studies, but the data were not statistically significant[61,66]. In summary, clinical manifestations, health-related quality of life and survival rate have been reported to be improved after BMSCs therapy.

CONCLUSION

There are still many open questions concerning BMSC therapy for the treatment of liver cirrhosis. First, it is crucial to understand the homing processes of BMSCs to the liver and to elucidate the relationships that exist not only between BMSCs and hepatocytes (regeneration) but also between MSCs, myofibroblasts and stellate cells (fibrogenesis). It is essential to clarify the mechanisms by which different types of BMSCs act in the liver, as this would allow the tailoring of stem cell therapy to the specific patient. The hypothesis that BMSCs act through the delivery of specific substances (cytokines and growth factors), rather than through transdifferentiation or cell fusion, suggests that improvements in liver function might be temporary. This hypothesis is supported by the results of the majority of the clinical trials: the improvement in laboratory data and CTP and MELD scores did not persist longer than three-six months regardless of the type of BMSCs infused, the route of delivery or the aetiology of the disease. In addition, the histological evaluations support this hypothesis, as an increase in LPC count was documented, peaking three months after BMSCs infusion. These results suggest that repeated cycles of BMSC therapy could be useful to obtain a sustained benefit.

BMSC therapy, although promising, needs to be further evaluated in large randomized, controlled clinical trials with longer follow-ups because the characteristics of the study populations reported in the current literature do not allow analytic comparison between the studies. In particular, a crucial issue is the different types of stem cells used, and in this regard, it could be interesting to compare the effects of the different types of BMSCs (unsorted MNCs, MSCs, and HSCs) on objective liver function parameters.

REFERENCES

1 **Lim YS**, Kim WR. The global impact of hepatic fibrosis and end-stage liver disease. *Clin Liver Dis* 2008; **12**: 733-746, vii [PMID: 18984463 DOI: 10.1016/j.cld.2008.07.007]

2 **D'Amico G**, Garcia-Tsao G, Pagliaro L. Natural history and prognostic indicators of survival in cirrhosis: a systematic review of 118 studies. *J Hepatol* 2006; **44**: 217-231 [PMID: 16298014 DOI: 10.1016/j.jhep.2005.10.013]

3 **Asrani SK**, Kamath PS. Natural history of cirrhosis. *Curr Gastroenterol Rep* 2013; **15**: 308 [PMID: 23314828 DOI: 10.1007/11894-0.12-0308-y]

4 **Lucey MR**, Terrault N, Ojo L, Hay JE, Neuberger J, Blumberg E, Teperman LW. Long-term management of the successful adult liver transplant: 2012 practice guideline by the American Association for the Study of Liver Diseases and the American Society of Transplantation. *Liver Transpl* 2013; **19**: 3-26 [PMID: 23281277 DOI: 10.1002/lt.23566]

5 **Crespo G**, Mariño Z, Navasa M, Forns X. Viral hepatitis in liver transplantation. *Gastroenterology* 2012; **142**: 1373-1383. e1 [PMID: 22537446 DOI: 10.1053/j.gastro.2012.02.011]

6 **Schuppan D**, Afdhal NH. Liver cirrhosis. *Lancet* 2008; **371**: 838-851 [PMID: 18328931 DOI: 10.1016/S0140-6736(08)60383-9]

7 **Török NJ**. Recent advances in the pathogenesis and diagnosis of liver fibrosis. *J Gastroenterol* 2008; **43**: 315-321 [PMID: 18592147 DOI: 10.1007/s00535-008-2181-x]

8 **Bataller R**, Brenner DA. Liver fibrosis. *J Clin Invest* 2005; **115**: 209-218 [PMID: 15690074 DOI: 10.1172/JCI24282]

9 **Wiemann SU**, Satyanarayana A, Tsahuridu M, Tillmann HL, Zender L, Klempnauer J, Flemming P, Franco S, Blasco MA, Manns MP, Rudolph KL. Hepatocyte telomere shortening and senescence are general markers of human liver cirrhosis. *FASEB J* 2002; **16**: 935-942 [PMID: 12087054 DOI: 10.1096/fj.01-0977com]

10 **Mormone E**, George J, Nieto N. Molecular pathogenesis of hepatic fibrosis and current therapeutic approaches. *Chem Biol Interact* 2011; **193**: 225-231 [PMID: 21803030 DOI: 10.1016/j.cbi.2011.07.001]

11 **Pinzani M**, Rosselli M, Zuckermann M. Liver cirrhosis. *Best*

Pract Res Clin Gastroenterol 2011; **25**: 281-290 [PMID: 21497745]
12 **Pinzani M**, Macias-Barragan J. Update on the pathophysiology of liver fibrosis. *Expert Rev Gastroenterol Hepatol* 2010; **4**: 459-472 [PMID: 20678019]
13 **Blachier M**, Leleu H, Peck-Radosavljevic M, Valla DC, Roudot-Thoraval F. The burden of liver disease in Europe: a review of available epidemiological data. *J Hepatol* 2013; **58**: 593-608 [PMID: 23419824 DOI: 10.1016/j.jhep.2012.12.005]
14 **Liu X**, Xu J, Brenner DA, Kisseleva T. Reversibility of Liver Fibrosis and Inactivation of Fibrogenic Myofibroblasts. *Curr Pathobiol Rep* 2013; **1**: 209-214 [PMID: 24000319 DOI: 10.1007/s40139-013-0018-7]
15 **Lichtinghagen R**, Michels D, Haberkorn CI, Arndt B, Bahr M, Flemming P, Manns MP, Boeker KH. Matrix metalloproteinase (MMP)-2, MMP-7, and tissue inhibitor of metalloproteinase-1 are closely related to the fibroproliferative process in the liver during chronic hepatitis C. *J Hepatol* 2001; **34**: 239-247 [PMID: 11281552 DOI: 10.1016/S0168-8278(00)00037-4]
16 **Orstavik D**, Mjör IA. Usage test of four endodontic sealers in Macaca fascicularis monkeys. *Oral Surg Oral Med Oral Pathol* 1992; **73**: 337-344 [PMID: 1545966 DOI: 10.1038/nrm1489]
17 **Fausto N**, Campbell JS, Riehle KJ. Liver regeneration. *Hepatology* 2006; **43**: S45-S53 [PMID: 16447274 DOI: 10.1002/hep.20969]
18 **Miyaoka Y**, Miyajima A. To divide or not to divide: revisiting liver regeneration. *Cell Div* 2013; **8**: 8 [PMID: 23786799 DOI: 10.1186/1747-1028-8-8]
19 **Libbrecht L**, Roskams T. Hepatic progenitor cells in human liver diseases. *Semin Cell Dev Biol* 2002; **13**: 389-396 [PMID: 12468238 DOI: 10.1016/S1084952102001258]
20 **Dezső K**, Papp V, Bugyik E, Hegyesi H, Sáfrány G, Bödör C, Nagy P, Paku S. Structural analysis of oval-cell-mediated liver regeneration in rats. *Hepatology* 2012; **56**: 1457-1467 [PMID: 22419534 DOI: 10.1002/hep.25713]
21 **Spee B**, Carpino G, Schotanus BA, Katoonizadeh A, Vander Borght S, Gaudio E, Roskams T. Characterisation of the liver progenitor cell niche in liver diseases: potential involvement of Wnt and Notch signalling. *Gut* 2010; **59**: 247-257 [PMID: 19880964 DOI: 10.1136/gut.2009.188367]
22 **Fellous TG**, Islam S, Tadrous PJ, Elia G, Kocher HM, Bhattacharya S, Mears L, Turnbull DM, Taylor RW, Greaves LC, Chinnery PF, Taylor G, McDonald SA, Wright NA, Alison MR. Locating the stem cell niche and tracing hepatocyte lineages in human liver. *Hepatology* 2009; **49**: 1655-1663 [PMID: 19309719 DOI: 10.1002/hep.22791]
23 **Lin WR**, Lim SN, McDonald SA, Graham T, Wright VL, Peplow CL, Humphries A, Kocher HM, Wright NA, Dhillon AP, Alison MR. The histogenesis of regenerative nodules in human liver cirrhosis. *Hepatology* 2010; **51**: 1017-1026 [PMID: 20198634 DOI: 10.1002/hep.23483]
24 **Kuwahara R**, Kofman AV, Landis CS, Swenson ES, Barendswaard E, Theise ND. The hepatic stem cell niche: identification by label-retaining cell assay. *Hepatology* 2008; **47**: 1994-2002 [PMID: 18454509 DOI: 10.1002/hep.22218]
25 **Kordes C**, Häussinger D. Hepatic stem cell niches. *J Clin Invest* 2013; **123**: 1874-1880 [PMID: 23635785 DOI: 10.1172/JCI66027]
26 **Zhang L**, Theise N, Chua M, Reid LM. The stem cell niche of human livers: symmetry between development and regeneration. *Hepatology* 2008; **48**: 1598-1607 [PMID: 18972441 DOI: 10.1002/hep.22516]
27 **Petersen BE**, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP. Bone marrow as a potential source of hepatic oval cells. *Science* 1999; **284**: 1168-1170 [PMID: 10325227]
28 **Banas A**, Teratani T, Yamamoto Y, Tokuhara M, Takeshita F, Quinn G, Okochi H, Ochiya T. Adipose tissue-derived mesenchymal stem cells as a source of human hepatocytes. *Hepatology* 2007; **46**: 219-228 [PMID: 17596885 DOI: 10.1002/hep.21704]
29 **Mallet VO**, Mitchell C, Mezey E, Fabre M, Guidotti JE, Renia L, Coulombel L, Kahn A, Gilgenkrantz H. Bone marrow transplantation in mice leads to a minor population of hepatocytes that can be selectively amplified in vivo. *Hepatology* 2002; **35**: 799-804 [PMID: 11915025 DOI: 10.1053/jhep.2002.32530]
30 **Alison MR**, Poulsom R, Jeffery R, Dhillon AP, Quaglia A, Jacob J, Novelli M, Prentice G, Williamson J, Wright NA. Hepatocytes from non-hepatic adult stem cells. *Nature* 2000; **406**: 257 [PMID: 10917519 DOI: 10.1038/35018642]
31 **Lorenzini S**, Gitto S, Grandini E, Andreone P, Bernardi M. Stem cells for end stage liver disease: how far have we got? *World J Gastroenterol* 2008; **14**: 4593-4599 [PMID: 18698672 DOI: 10.3748/wjg.14.4593]
32 **Fausto N**. Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. *Hepatology* 2004; **39**: 1477-1487 [PMID: 15185286 DOI: 10.1002/hep.20214]
33 **de Wynter EA**, Buck D, Hart C, Heywood R, Coutinho LH, Clayton A, Rafferty JA, Burt D, Guenechea G, Bueren JA, Gagen D, Fairbairn LJ, Lord BI, Testa NG. CD34+AC133+ cells isolated from cord blood are highly enriched in long-term culture-initiating cells, NOD/SCID-repopulating cells and dendritic cell progenitors. *Stem Cells* 1998; **16**: 387-396 [PMID: 9831864 DOI: 10.1002/stem.160387]
34 **Mizunaga Y**, Terai S, Yamamoto N, Uchida K, Yamasaki T, Nishina H, Fujita Y, Shinoda K, Hamamoto Y, Sakaida I. Granulocyte colony-stimulating factor and interleukin-1β are important cytokines in repair of the cirrhotic liver after bone marrow cell infusion: comparison of humans and model mice. *Cell Transplant* 2012; **21**: 2363-2375 [PMID: 22507241 DOI: 10.3727/096368912X638856]
35 **Jin SZ**, Meng XW, Sun X, Han MZ, Liu BR, Wang XH, Sun LY, Huang Q, Zhao RB, Ban X, Yu HY, Yu HW. Granulocyte colony-stimulating factor enhances bone marrow mononuclear cell homing to the liver in a mouse model of acute hepatic injury. *Dig Dis Sci* 2010; **55**: 2805-2813 [PMID: 20130994 DOI: 10.1007/s10620-009-1117-5]
36 **Gaia S**, Smedile A, Omedè P, Olivero A, Sanavio F, Balzola F, Ottobrelli A, Abate ML, Marzano A, Rizzetto M, Tarella C. Feasibility and safety of G-CSF administration to induce bone marrow-derived cells mobilization in patients with end stage liver disease. *J Hepatol* 2006; **45**: 13-19 [PMID: 16635534 DOI: 10.1016/j.jhep.2006.02.018]
37 **Garg V**, Garg H, Khan A, Trehanpati N, Kumar A, Sharma BC, Sakhuja P, Sarin SK. Granulocyte colony-stimulating factor mobilizes CD34(+) cells and improves survival of patients with acute-on-chronic liver failure. *Gastroenterology* 2012; **142**: 505-512.e1 [PMID: 22119930 DOI: 10.1053/j.gastro.2011.11.027]
38 **Lorenzini S**, Isidori A, Catani L, Gramenzi A, Talarico S, Bonifazi F, Giudice V, Conte R, Baccarani M, Bernardi M, Forbes SJ, Lemoli RM, Andreone P. Stem cell mobilization and collection in patients with liver cirrhosis. *Aliment Pharmacol Ther* 2008; **27**: 932-939 [PMID: 18315586 DOI: 10.1111/j.1365-2036.2008.03670.x]
39 **Picardi M**, De Rosa G, Selleri C, Scarpato N, Soscia E, Martinelli V, Ciancia R, Rotoli B. Spleen enlargement following recombinant human granulocyte colony-stimulating factor administration for peripheral blood stem cell mobilization. *Haematologica* 2003; **88**: 794-800 [PMID: 12857559]
40 **Falzetti F**, Aversa F, Minelli O, Tabilio A. Spontaneous rupture of spleen during peripheral blood stem-cell mobilisation in a healthy donor. *Lancet* 1999; **353**: 555 [PMID: 10028986 DOI: 10.1016/S0140-6736(99)00268-8]
41 **Campard D**, Lysy PA, Najimi M, Sokal EM. Native umbilical cord matrix stem cells express hepatic markers and differentiate into hepatocyte-like cells. *Gastroenterology* 2008; **134**: 833-848 [PMID: 18243183 DOI: 10.1053/j.gastro.2007.12.024]
42 **Teramoto K**, Asahina K, Kumashiro Y, Kakinuma S, Chinzei R, Shimizu-Saito K, Tanaka Y, Teraoka H, Arii S. Hepato-

cyte differentiation from embryonic stem cells and umbilical cord blood cells. *J Hepatobiliary Pancreat Surg* 2005; **12**: 196-202 [PMID: 15995807 DOI: 10.1007/s00534-005-0980-5]
43 **Piscaglia AC**, Campanale M, Gasbarrini A, Gasbarrini G. Stem cell-based therapies for liver diseases: state of the art and new perspectives. *Stem Cells Int* 2010; **2010**: 259461 [PMID: 21048845 DOI: 10.4061/2010/259461]
44 **Terai S**, Ishikawa T, Omori K, Aoyama K, Marumoto Y, Urata Y, Yokoyama Y, Uchida K, Yamasaki T, Fujii Y, Okita K, Sakaida I. Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy. *Stem Cells* 2006; **24**: 2292-2298 [PMID: 16778155 DOI: 10.1634/stemcells.2005-0542]
45 **Kisseleva T**, Brenner DA. The phenotypic fate and functional role for bone marrow-derived stem cells in liver fibrosis. *J Hepatol* 2012; **56**: 965-972 [PMID: 22173163 DOI: 10.1016/j.jhep.2011.09.021]
46 **Lorenzini S**, Andreone P. Regenerative medicine and liver injury: what role for bone marrow derived stem cells? *Curr Stem Cell Res Ther* 2007; **2**: 83-88 [PMID: 18220893]
47 **Terai S**, Sakaida I, Yamamoto N, Omori K, Watanabe T, Ohata S, Katada T, Miyamoto K, Shinoda K, Nishina H, Okita K. An in vivo model for monitoring trans-differentiation of bone marrow cells into functional hepatocytes. *J Biochem* 2003; **134**: 551-558 [PMID: 14607982 DOI: 10.1093/jb/mvg173]
48 **Jang YY**, Collector MI, Baylin SB, Diehl AM, Sharkis SJ. Hematopoietic stem cells convert into liver cells within days without fusion. *Nat Cell Biol* 2004; **6**: 532-539 [PMID: 15133469 DOI: 10.1038/ncb1132]
49 **Wang X**, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, Grompe M. Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* 2003; **422**: 897-901 [PMID: 12665832 DOI: 10.1038/nature01531]
50 **Vassilopoulos G**, Wang PR, Russell DW. Transplanted bone marrow regenerates liver by cell fusion. *Nature* 2003; **422**: 901-904 [PMID: 12665833 DOI: 10.1038/nature01539]
51 **Sakaida I**, Terai S, Yamamoto N, Aoyama K, Ishikawa T, Nishina H, Okita K. Transplantation of bone marrow cells reduces CCl4-induced liver fibrosis in mice. *Hepatology* 2004; **40**: 1304-1311 [PMID: 15565662 DOI: 10.1002/hep.20452]
52 **Wang L**, Wang X, Wang L, Chiu JD, van de Ven G, Gaarde WA, Deleve LD. Hepatic vascular endothelial growth factor regulates recruitment of rat liver sinusoidal endothelial cell progenitor cells. *Gastroenterology* 2012; **143**: 1555-1563.e2 [PMID: 22902870 DOI: 10.1053/j.gastro.2012.08.008]
53 **Couto BG**, Goldenberg RC, da Fonseca LM, Thomas J, Gutfilen B, Resende CM, Azevedo F, Mercante DR, Torres AL, Coelho HS, Maiolino A, Alves AL, Dias JV, Moreira MC, Sampaio AL, Sousa MA, Kasai-Brunswick TH, Souza SA, Campos-de-Carvalho AC, Rezende GF. Bone marrow mononuclear cell therapy for patients with cirrhosis: a Phase 1 study. *Liver Int* 2011; **31**: 391-400 [PMID: 21281433 DOI: 10.1111/j.1478-3231.2010.02424.x]
54 **Levicar N**, Pai M, Habib NA, Tait P, Jiao LR, Marley SB, Davis J, Dazzi F, Smadja C, Jensen SL, Nicholls JP, Apperley JF, Gordon MY. Long-term clinical results of autologous infusion of mobilized adult bone marrow derived CD34+ cells in patients with chronic liver disease. *Cell Prolif* 2008; **41** Suppl 1: 115-125 [PMID: 18181952 DOI: 10.1111/j.1365-2184.2008.00491.x]
55 **Mohamadnejad M**, Namiri M, Bagheri M, Hashemi SM, Ghanaati H, Zare Mehrjardi N, Kazemi Ashtiani S, Malekzadeh R, Baharvand H. Phase 1 human trial of autologous bone marrow-hematopoietic stem cell transplantation in patients with decompensated cirrhosis. *World J Gastroenterol* 2007; **13**: 3359-3363 [PMID: 17659676]
56 **Kim JK**, Park YN, Kim JS, Park MS, Paik YH, Seok JY, Chung YE, Kim HO, Kim KS, Ahn SH, Kim do Y, Kim MJ, Lee KS, Chon CY, Kim SJ, Terai S, Sakaida I, Han KH. Autologous bone marrow infusion activates the progenitor cell compartment in patients with advanced liver cirrhosis. *Cell Transplant* 2010; **19**: 1237-1246 [PMID: 20525430 DOI: 10.3727/096368910X506863]
57 **Mohamadnejad M**, Alimoghaddam K, Bagheri M, Ashrafi M, Abdollahzadeh L, Akhlaghpoor S, Bashtar M, Ghavamzadeh A, Malekzadeh R. Randomized placebo-controlled trial of mesenchymal stem cell transplantation in decompensated cirrhosis. *Liver Int* 2013; **33**: 1490-1496 [PMID: 23763455 DOI: 10.1111/liv.12228]
58 **Nikeghbalian S**, Pournasr B, Aghdami N, Rasekhi A, Geramizadeh B, Hosseini Asl SM, Ramzi M, Kakaei F, Namiri M, Malekzadeh R, Vosough Dizaj A, Malek-Hosseini SA, Baharvand H. Autologous transplantation of bone marrow-derived mononuclear and CD133(+) cells in patients with decompensated cirrhosis. *Arch Iran Med* 2011; **14**: 12-17 [PMID: 21194255]
59 **Jang YO**, Kim YJ, Baik SK, Kim MY, Eom YW, Cho MY, Park HJ, Park SY, Kim BR, Kim JW, Soo Kim H, Kwon SO, Choi EH, Kim YM. Histological improvement following administration of autologous bone marrow-derived mesenchymal stem cells for alcoholic cirrhosis: a pilot study. *Liver Int* 2014; **34**: 33-41 [PMID: 23782511 DOI: 10.1111/liv.12218]
60 **Kim SU**, Oh HJ, Wanless IR, Lee S, Han KH, Park YN. The Laennec staging system for histological sub-classification of cirrhosis is useful for stratification of prognosis in patients with liver cirrhosis. *J Hepatol* 2012; **57**: 556-563 [PMID: 22617153 DOI: 10.1016/j.jhep.2012.04.029]
61 **Peng L**, Xie DY, Lin BL, Liu J, Zhu HP, Xie C, Zheng YB, Gao ZL. Autologous bone marrow mesenchymal stem cell transplantation in liver failure patients caused by hepatitis B: short-term and long-term outcomes. *Hepatology* 2011; **54**: 820-828 [PMID: 21608000 DOI: 10.1002/hep.24434]
62 **Lyra AC**, Soares MB, da Silva LF, Braga EL, Oliveira SA, Fortes MF, Silva AG, Brustolim D, Genser B, Dos Santos RR, Lyra LG. Infusion of autologous bone marrow mononuclear cells through hepatic artery results in a short-term improvement of liver function in patients with chronic liver disease: a pilot randomized controlled study. *Eur J Gastroenterol Hepatol* 2010; **22**: 33-42 [PMID: 19654548 DOI: 10.1097/MEG.0b013e32832eb69a]
63 **Pai M**, Zacharoulis D, Milicevic MN, Helmy S, Jiao LR, Levicar N, Tait P, Scott M, Marley SB, Jestice K, Glibetic M, Bansi D, Khan SA, Kyriakou D, Rountas C, Thillainayagam A, Nicholls JP, Jensen S, Apperley JF, Gordon MY, Habib NA. Autologous infusion of expanded mobilized adult bone marrow-derived CD34+ cells into patients with alcoholic liver cirrhosis. *Am J Gastroenterol* 2008; **103**: 1952-1958 [PMID: 18637092 DOI: 10.1111/j.1572-0241.2008.01993.x]
64 **Amin MA**, Sabry D, Rashed LA, Aref WM, el-Ghobary MA, Farhan MS, Fouad HA, Youssef YA. Short-term evaluation of autologous transplantation of bone marrow-derived mesenchymal stem cells in patients with cirrhosis: Egyptian study. *Clin Transplant* 2013; **27**: 607-612 [PMID: 23923970 DOI: 10.1111/ctr.12179]
65 **El-Ansary M**, Abdel-Aziz I, Mogawer S, Abdel-Hamid S, Hammam O, Teaema S, Wahdan M. Phase II trial: undifferentiated versus differentiated autologous mesenchymal stem cells transplantation in Egyptian patients with HCV induced liver cirrhosis. *Stem Cell Rev* 2012; **8**: 972-981 [PMID: 21989829 DOI: 10.1007/s12015-011-9322-y]
66 **Salama H**, Zekri AR, Bahnassy AA, Medhat E, Halim HA, Ahmed OS, Mohamed G, Al Alim SA, Sherif GM. Autologous CD34+ and CD133+ stem cells transplantation in patients with end stage liver disease. *World J Gastroenterol* 2010; **16**: 5297-5305 [PMID: 21072892 DOI: 10.3748/wjg.v16.i42.5297]
67 **Kharaziha P**, Hellström PM, Noorinayer B, Farzaneh F, Aghajani K, Jafari F, Telkabadi M, Atashi A, Honardoost M, Zali MR, Soleimani M. Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem

cell injection: a phase I-II clinical trial. *Eur J Gastroenterol Hepatol* 2009; **21**: 1199-1205 [PMID: 19455046 DOI: 10.1097/MEG.0b013e32832a1f6c]

68 **Salama H**, Zekri AR, Ahmed R, Medhat I, Abdallah ES, Darwish T, Ahmed OS, Bahnassy A. Assessment of health-related quality of life in patients receiving stem cell therapy for end-stage liver disease: an Egyptian study. *Stem Cell Res Ther* 2012; **3**: 49 [PMID: 23206927 DOI: 10.1186/scrt140]

69 **Park CH**, Bae SH, Kim HY, Kim JK, Jung ES, Chun HJ, Song MJ, Lee SE, Cho SG, Lee JW, Choi JY, Yoon SK, Han NI, Lee YS. A pilot study of autologous CD34-depleted bone marrow mononuclear cell transplantation via the hepatic artery in five patients with liver failure. *Cytotherapy* 2013; **15**: 1571-1579 [PMID: 23849977 DOI: 10.1016/j.jcyt.2013.05.013]

70 **Lyra AC**, Soares MB, da Silva LF, Fortes MF, Silva AG, Mota AC, Oliveira SA, Braga EL, de Carvalho WA, Genser B, dos Santos RR, Lyra LG. Feasibility and safety of autologous bone marrow mononuclear cell transplantation in patients with advanced chronic liver disease. *World J Gastroenterol* 2007; **13**: 1067-1073 [PMID: 17373741]

P- Reviewers: Kan L, Minana MD, Rameshwar P
S- Editor: Zhai HH **L- Editor**: A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9106

World J Gastroenterol 2014 July 21; 20(27): 9106-9115
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Mast cell deficiency exacerbates inflammatory bowel symptoms in interleukin-10-deficient mice

Hanying Zhang, Yansong Xue, Hui Wang, Yan Huang, Min Du, Qiyuan Yang, Mei-Jun Zhu

Hanying Zhang, Yansong Xue, Mei-Jun Zhu, School of Food Science, Washington State University, Pullman, WA 99164, United States
Hanying Zhang, Hui Wang, Yan Huang, Department of Animal Science, University of Wyoming, Laramie, WY 82071, United States
Min Du, Qiyuan Yang, Department of Animal Science, Washington State University, Pullman, WA 99164, United States
Author contributions: Zhang H performed research; Xue Y, Wang H, Huang Y and Yang Q contributed analytic tools; Zhang H and Zhu MJ analyzed data; Zhang H, Du M and Zhu MJ wrote the paper.
Supported by USDA-AFRI, No. 2009-65203-05716; NIH, No. 1R15HD073864; and NIH-INBRE, No. P20RR016474
Correspondence to: Dr. Mei-Jun Zhu, School of Food Science, Washington State University, Pullman, WA 99164, United States. meijun.zhu@wsu.edu
Telephone: +1-509-3354016 Fax: +1-509-3354815
Received: December 6, 2013 Revised: January 7, 2014
Accepted: March 5, 2014
Published online: July 21, 2014

Abstract

AIM: To test the role of mast cells in gut inflammation and colitis using interleukin (IL)-10-deficient mice as an experimental model.

METHODS: Mast cell-deficient ($Kit^{W\text{-}sh/W\text{-}sh}$) mice were crossbred with IL-10-deficient mice to obtain double knockout (DKO) mice. The growth, mucosal damage and colitis status of DKO mice were compared with their IL-10-deficient littermates.

RESULTS: DKO mice exhibited exacerbated colitis compared with their IL-10-deficient littermates, as shown by increased pathological score, higher myeloperoxidase content, enhanced Th1 type pro-inflammatory cytokines and inflammatory signaling, elevated oxidative stress, as well as pronounced goblet cell loss. In addition, deficiency in mast cells resulted in enhanced mucosal damage, increased gut permeability, and impaired epithelial tight junctions. Mast cell deficiency was also linked to systemic inflammation, as demonstrated by higher serum levels of tumor necrosis factor α and interferon γ in DKO mice than that in IL-10-deficient mice.

CONCLUSION: Mast cell deficiency in IL-10-deficient mice resulted in systematic and gut inflammation, impaired gut barrier function, and severer Th1-mediated colitis when compared to mice with only IL-10-deficiency. Inflammation and impaired gut epithelial barrier function likely form a vicious cycle to worsen colitis in the DKO mice.

Key words: Colitis; Interleukin-10; Inflammation; Inflammatory bowel disease; Mast cells; Mice

Core tip: Colitis is characterized by chronic inflammation and mast cells accumulate at the pathological sites, implicating their mediating roles, but the exact roles of mast cells in colitis remain poorly defined and controversial. In this study, the authors cross-bred mast cell-deficient mice with interleukin-10-deficient mice to investigate the role of mast cells in gut inflammation and the onset of colitis. Data show that mast cells have protective roles in the development of colitis by suppressing Th1 type immune response and inflammation, altering gut microbiota composition, improving gut epithelial barrier function, and reducing epithelial damage.

Zhang H, Xue Y, Wang H, Huang Y, Du M, Yang Q, Zhu MJ. Mast cell deficiency exacerbates inflammatory bowel symptoms in interleukin-10-deficient mice. *World J Gastroenterol* 2014; 20(27): 9106-9115 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9106.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9106

INTRODUCTION

Inflammatory bowel disease (IBD), primarily Crohn's disease and ulcerative colitis, is one of the most common gastrointestinal diseases. Our understanding of IBD etiology, however, is far from complete. Interleukin (IL)-10-deficient mice develop IBD spontaneously after 3 mo of age, which is a common model for studying the etiology of Crohn's disease. The onset of colitis in these mice is associated with enhanced $CD4^+$ Th1/Th17 mediated inflammatory responses[1].

Mast cells are generated from bone marrow derived hematopoietic progenitor cells that migrate into vascularized tissues, where they undergo final maturation[2]. The resident mast cells comprise about 2%-3% of mucosal cells in the healthy gut[3], but they can be recruited in large numbers in response to an array of stimuli. They regulate epithelial barrier function and inflammation through: (1) affecting the expression and distribution of tight junction proteins[4,5]; (2) regulating enteric nervous system by secretion of neurotransmitters[6]; and (3) recruiting and activating other immune cells by releasing cytokines[7].

The density of mast cells increases in the gastrointestinal tract of IBD patients, indicating the likely involvement of mast cells in the etiology of IBD[8-10]. It has been reported that mast cells potentiated inflammation in Dextran Sodium Sulfate (DSS) induced colitis, since mast cell deficiency dampened DSS-induced body weight loss and attenuated colonic hypersensitivity[11]. In stress-induced gut inflammation, mast cells mediated epithelial barrier dysfunction in rats[12,13], and mast cell-deficient mice had decreased basal jejunum permeability *ex vivo*[14]. However, mast cells have also been documented to have a protective role in colonic colitis; deletion of mast cells in IL-10-deficient mice resulted in enhanced mucosal epithelial permeability[15], while mast cell deficiency has no inhibitory role in helicobacter induced gut inflammatory response in IL-10-deficient mice[15]. These data indicate a complex nature of the role of mast cells in gut inflammation and IBD pathogenesis, which possibly depends on the genotype and physiological status of mice as well as environmental factors. Here in this study, we cross-bred mast cell-deficient mice with IL-10-deficient mice to investigate the role of mast cells in gut inflammation and the onset of colitis, and further explored underlying mechanisms.

MATERIALS AND METHODS

Animal care and experimental design

All animal procedures were approved by the University of Wyoming Animal Care and Use Committee. IL-10-deficient mice (B6.129P2-*IL-10*^{tm1Cgn}/J; stock #002251) and mast cell-deficient mice (STOCK *Kit*$^{W\text{-}sh}$/HNihrJaeBsmJ; stock #005051) were obtained from the Jackson Laboratory (Bar Harbor, ME). Both strains are on the C57/BL6 background. IL-10-deficient mice and mast cell-deficient mice were cross-bred for two generations to obtain mast cell heterozygous IL-10-deficient mice. At five weeks of age, mast cell heterozygous, IL-10-deficient female mice were fed either with a control diet (D12450B, 10% energy from fat, Research Diets Inc.) or a high energy diet (D12451, 45% energy from fat) for 3 mo and then bred with the same genotype male mice fed with the control diet. Offspring with both mast cell-deficient and IL-10-deficient (double deficient, for simplicity, we called double knockout, DKO) mice and only IL-10-deficient mice from the same litter were obtained and used for further studies. All mice were housed in sterile high-efficiency particulate air filter cages, with access to food and water *ad libitum*. However, for unknown reasons, very few or no viable neonatal DKO mice could be obtained from mothers fed the control diet. Therefore, only IL-10-deficient and DKO offspring from mothers fed the high energy diet were used for further studies. All mice were sacrificed at 10 wk of age.

Tissue collection

After euthanasia, the colonic tissue was dissected from surrounding tissue. A 5 mm section from the colonic tissue at constant location was fixed in 4% (w/v) paraformaldehyde, processed and embedded into paraffin. The remaining gut segments were cut opened, rinsed in PBS, frozen in liquid nitrogen, and stored at -80 ℃ till analysis.

In vivo intestinal permeability

Seven-week-old mice were fasted for 5 h with water provided, and then gavaged with FITC-dextran (Sigma, St Louis, MO) at 120 μg/kg body weight. Blood was collected 4 hours after gavage and centrifuged for 5 min at 4000 × g. The resulting serum was 1:5 diluted in PBS (pH 7.4), and the fluorescence intensity was measured at excitation 485 nm and emission 520 nm by a SpectraMax M5 Spectrophotometer (Molecular Device, Sunnyvale, CA)[16].

Glucose tolerance test

Mice at eight-week-old were subjected to intraperitoneal (*i.p.*) glucose tolerance test after overnight fasting with free access to water. D-glucose (2 mg/g body weight) was i.p. injected into mice. The blood glucose level was monitored at 0, 15, 30, 60 and 120 min after injection by tail tip bleeding using a Contour glucometer (Bayer Healthcare, Mishawaka, IN).

Measurement of GSH content

Glutathione *vs* glutathione disulfide ratio (GSH/GSSG) recycle assay was performed as previously described[17]. Briefly, 10 mg colon tissues were homogenized in 200 μL of 1.3% picric acid solution (Sigma) and followed by sonication and centrifugation. The supernatant was assayed for total GSH and GSSG by incubation with 2-vinylpyridine (Sigma), which conjugates any GSH present in the sample so that only GSSG is recycled to GSH without interference by GSH. The GSSG (as GSH × 2) was then subtracted from the total GSH to calculate the level of GSH.

Table 1 Primer sets used for quantitative reverse transcriptase-polymerase chain reaction analysis of mouse colonic tissue

Gene name	Accession No.	Product size	Direction	Sequence (5′ to 3′)	Ref.
Claudin2	NM_016675.4	120 bp	Forward	GGCGTCCAACTGGTGGGCTAC	[42]
			Reverse	AACCGCCGTCACAATGCTGGC	
Claudin3	NM_009902.4	132 bp	Forward	CAGGGGCAGTCTCTGTGCGAG	[42]
			Reverse	GCCGCTGGACCTGGGAATCAAC	
GAPDH	NM_008084.2	132 bp	Forward	AACTTTGGCATTGTGGAAGG	[42]
			Reverse	GGATGCAGGGATGATGTTCT	
IL-1β	NM_008361	73 bp	Forward	TCGCTCAGGGTCACAAGAAA	[43]
			Reverse	CATCAGAGGCAAGGAGGAAAAC	
INFγ	NM_008337.3	93 bp	Forward	AGGTCCAGCGCCAAGCATTCAA	[42]
			Reverse	AGCAGCGACTCCTTTTCCGCTT	
iNOS	U43428.1	76 bp	Forward	CAAAGTCTCAGACATGGCTTGC	This study
			Reverse	TTCCTCTGTCAGGTCACTTTGG	
NOX1	NM_172203.1	113 bp	Forward	CAGGCATCCTCATTTTGCGG	This study
			Reverse	CCTTCTGCTGGGAGCGATAA	
T-bet	NM_019507.2	138 bp	Forward	CCACTGGATGCGCCAGGAAGTT	[42]
			Reverse	TTCACCTCCACGATGTGCAGCC	
TNF-α	NM_013693.2	67 bp	Forward	TGGGACAGTGACCTGGACTGT	[43]
			Reverse	TTCGGAAAGCCCATTTGAGT	

Serum tumor necrosis factor α and interferon-γ level

Serum levels of tumor necrosis factor (TNF)-α and interferon (IFN)-γ were analyzed by ELISA (eBiosciences, San Diego, CA) according to the manufacturer's manual. The overall intra-assay and inter-assay coefficient of variation was < 5.0% and < 10%, respectively.

Histology

For pathobiological examination, embedded colonic tissue were cut into 5 μm thickness and subjected to hematoxylin-eosin (HE) staining. HE stained slides were scored using a scale as previously described[18]. Briefly, slides were scored for the presence of epithelial hyperplasia, the intensity and severity of inflammation. The maximum score of each colon section is 15. A higher number indicates more extensive/severe disease symptoms. To quantify the goblet cell density, the colonic tissue section was stained with alcian blue per published method[19]. The quantification of goblet cells (goblet cell area *vs* the tissue section area) of alcian blue stained section was performed using the Image J software (split color channels).

Quantitative reverse transcriptase PCR

Total RNA was extracted from colonic tissue using Trizol reagent (Sigma) and treated with DNase I (Qiagen, Valencia, CA) followed by purification with RNeasy® Mini Kit (Qiagen). The cDNA was synthesized with the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Reverse transcriptase PCR (qRT-PCR) was conducted on a Bio-Rad CFX96 thermocycler using glyceraldehyde 3-phosphate dehydrogenase as the housekeeping gene. The primers are listed in Table 1. SYBR Green Master Mix (Bio-Rad) was used in all PCR reactions. The amplification efficiency was 0.90 to 0.99. The qRT-PCR conditions were 95 ℃, 3 min; 35 cycles of 95 ℃ for 10 s, 56 ℃ for 10 s and 72 ℃ for 20 s. At the end of each run, dissociation melt curves were obtained to confirm the purity of PCR products, and the PCR products were electrophoresed to confirm the targeted sizes. Relative expression of mRNA was determined after normalization to GAPDH reference using ΔΔ-Ct method.

Gut microflora analysis

Bacterial genomic DNA was extracted from fecal samples using QIAamp® DNA Stool Mini Kit (Qiagen) per the manufacturer's instruction. The abundance of specific intestinal bacterial groups was quantified by qPCR using Bio-Rad CFX96 thermocycler as stated above. Genus or species specific 16S rRNA gene primers were listed in Table 2. The 16S rRNA of Eubacteria was used as the internal control.

Immunoblotting

Immunoblot analysis was conducted according to the procedures previously described[17]. Briefly, protein extracts from colonic tissues were separated by 5%-15% gradient SDS-PAGE gels and transferred to nitrocellulose membranes for immunoblotting analyses. Antibodies against myeloperoxidase, phospho-p65 and p65 were purchased from Cell Signaling Technology (Beverly, MA). Claudin2 and Claudin3 were purchased from Invitrogen (Camarillo, CA). GAPDH was purchased from GeneTex (Irvine, CA). Band density was quantified by Image J software and normalized according to the GAPDH content.

Serum total free fatty acid colorimetric assay

Plasma total free fatty acid (FFA) content was analyzed colorimetrically following the previous published methods[20,21]. Total FFA concentration was calculated based on the standard curve. Each sample was analyzed in duplicate and mean values were reported.

Statistical analysis

Statistical analyses were conducted as previously described[17,21]. Data were analyzed as a complete randomized design using GLM (General Linear Model of

Table 2 Primers for quantitative polymerase chain reaction analysis of selected fecal microbiota

Target organism	Primer set	Sequence (5′ to 3′)	Product size	Ref.
Bacteroides	BactF285	GGTTCTGAGAGGAGGTCCC	53	[44]
	UniR338	GCTGCCTCCCGTAGGAGT		
Ec-ssul	Ec-ssu1F	GGATAACACTTGGAAACAGG	115	[45]
	Ec-ssu1R	TCCTTGTTCTTCTCTAACAA		
Eubacteria	UniF340	ACTCCTACGGGAGGCAGCAGT	210	[46]
	UniR514	ATTACCGCGGCTGCTGGC		
Lactobacillus	LabF362	AGCAGTAGGGAATCTTCCA	315	[44]
	LabR677	CACCGCTACACATGGAG		
Ruminococcus albus (Ralb)	Ralb561F	CAGGTGTGAAATTTAGGGGC	246	[47]
	Ralb807R	GTCAGTCCCCCCACACCTAG		

Statistical Analysis System, SAS, 2000). Mean ± SE are reported. Statistical significance is considered as $P < 0.05$.

RESULTS

DKO mice showed aggravated colitis compared to their IL-10-deficient littermates

The severity of colitis was evaluated by examining pathological changes, goblet cell density, pro-inflammatory cytokine expression and neutrophil content. As shown in Figure 1A, colon section of DKO mice had a much higher pathological score than that of IL-10-deficient littermates. Meanwhile, the colonic tissue of DKO mice exhibited an increased expression of Th1 type inflammatory cytokines such as IL-1β and IFN-γ (Figure 1B, C), enhanced NF-κB inflammatory signaling (Figure 1D), and elevated neutrophil infiltration, as indicated by increased myeloperoxidase (MPO) content (Figure 1E). Being the major source of secreted mucin in the gastrointestinal tract, goblet cells play a vital role in regulating intestinal homeostasis. The depletion of goblet cells in the large intestine is another characterized feature of IBD. Alcian blue staining revealed that mast cell deletion resulted in decreased goblet cell staining in the colon of IL-10-deficient mice (Figure 1F).

DKO mice experienced increased oxidative stress in the colon compared to their IL-10-deficient littermates

Oxidative stress has arisen to be another crucial etiological event in colitis progression[22]. Consistent with aggravated colitis, GSH/GSSG recycle assay demonstrated that mast cell deficiency resulted in a marked decrease of GSH content (Figure 2A). Meanwhile, mRNA expression of NADPH oxidase 1 (NOX1) was increased in the colon of DKO mice compared to that of their IL-10-deficient littermates, but iNOS expression was unchanged (Figure 2B). These data indicated mast cell deletion resulted in a more severe oxidative stress in the colon of IL-10-deficient mice, which was consistent with the enhanced inflammatory responses observed in the colon of DKO mice.

DKO mice exhibited more mucosal damage than their IL-10-deficient littermates

Increased intestinal permeability is an important etiological event in the development of colitis in IL-10-deficient mice[23]. Consistent with aggravated colitis, the *in vivo* intestinal permeability of DKO mice was higher ($P < 0.01$) than that of their IL-10-deficient littermates (Figure 3A), indicating escalated mucosal barrier damage. In agreement with impaired intestinal permeability, mast cell deficiency decreased claudin-3 mRNA expression (Figure 3B) while increased "channel forming" claudin-2 protein content (Figure 3C). In addition, the myosin light chain 2 (MLC-2) phosphorylation and CK2α protein content were enhanced in the colon of DKO mice (Figure 3C).

Alteration of gut microflora composition

We further evaluated whether gut microflora could be a factor contributing to the enhanced inflammation in the DKO gut. Using genus or species specific 16s rRNA primers, quantitative PCR indicated that DKO mice had decreased *Ruminococcus albus* ($P < 0.05$) but no change in *Bacteroides, Lactic acid bacteria, Clostridium perfringens, Enterococcus* and *Faecalibacterium prausnitzii* compared to their IL-10-deficient littermates (Figure 4).

Mast cell deficiency in IL-10-deficient mice led to systemic inflammation

Besides exasperated colitis, DKO mice exhibited lower wean (4-wk-old) body weight (Figure 5A) compared to that of their IL-10-deficient littermates. Upon necropsy (10-wk-old), DKO mice showed higher spleen and liver weight (Table 3), associated with enhanced serum TNF-α and IFN-γ levels (Figure 5B). In addition, mast cell deficiency dramatically impeded systemic glucose tolerance in IL-10-deficient mice at 15 min, 30 min and 60 min post injection of glucose (Figure 5C).

Interestingly, the subcutaneous fat weight of DKO mice at necropsy was 32.3% ± 8.1% less than that of their IL-10-deficient littermates, while there was no difference in gonadal fat weight (Table 3). Subcutaneous fat is proposed to be the "sink" for free fatty acids (FFA)[24]. Therefore, we further analyzed the serum FFA level, which, however, did not differ between DKO mice and their IL-10-deficient littermates (Figure 5D).

DISCUSSION

Mast cells play a crucial role in innate immune responses

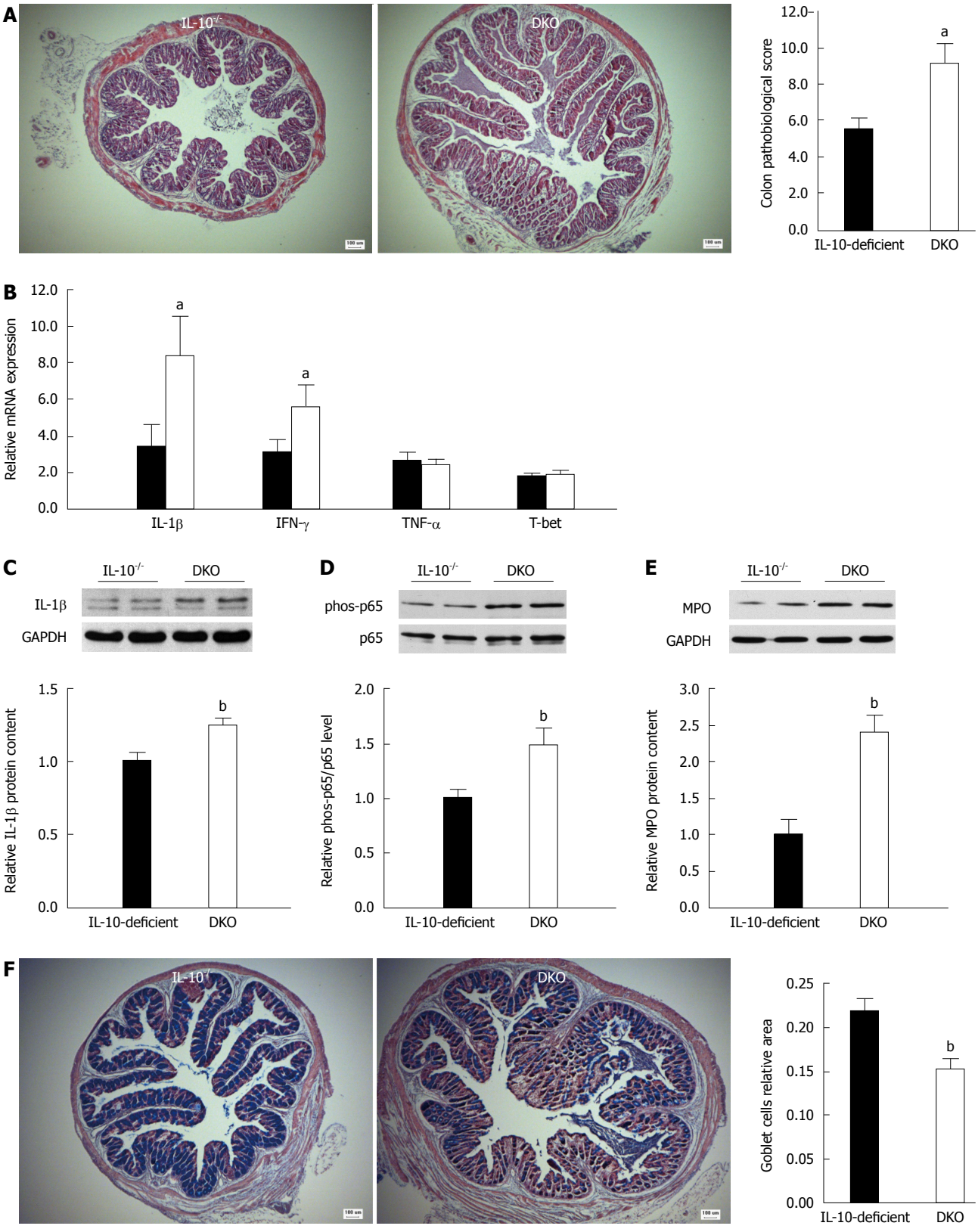


Figure 1 Mast cell deficiency aggravated colitis in the colon of interleukin-10-deficient mice. A: Pathological score; B: mRNA expression of inflammatory cytokines; C: Relative IL-1β protein content; D: NF-κB p65 inflammatory signaling; E: Relative myeloperoxidase content in colonic tissue; F: Goblet cell density. Mean ± SE, $^{a}P < 0.05$, DKO *vs* IL-10-deficient, $^{b}P < 0.01$, DKO *vs* IL-10-deficient, $n = 8$ for mRNA analysis, $n = 10$ for others. IL: Interleukin; DKO: Double knockout; MPO: Myeloperoxidase; NF-κB: Nuclear factor κB.

and IBD pathogenesis. Deletion of mast cells markedly attenuated multiple organ injury and damped systematic inflammation in response to trauma[25]. In chemical induced colitis, mast cells act as an initiator of innate immune response and likely aggravate disease indices[10]. However, our data showed that lack of mast cells exac-

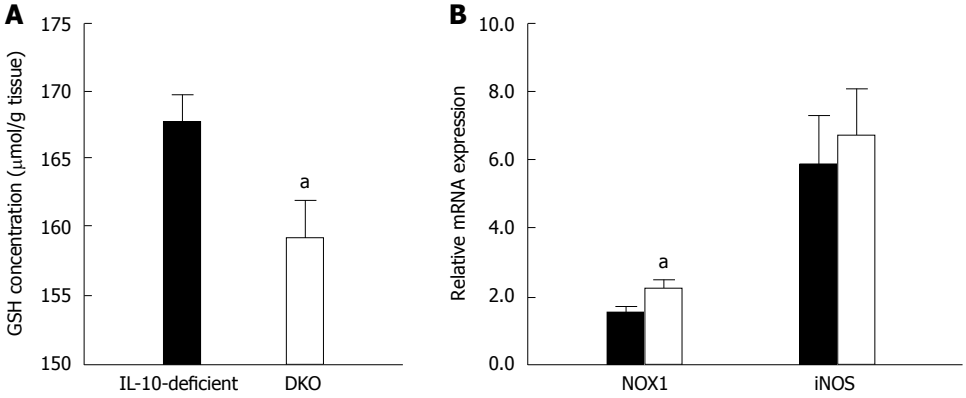


Figure 2 Oxidative stress in the colon tissue of interleukin-10-deficient and double knockout mice. A: Colonic glutathione concentration was measured by GSH/GSSG recycling assay; B: mRNA expression of NADPH oxidase 1 (NOX1) and inducible NO synthase (iNOS). Mean ± SE, $^{a}P < 0.05$, DKO *vs* IL-10-deficient, $n = 8$. IL: Interleukin; DKO: Double knockout.

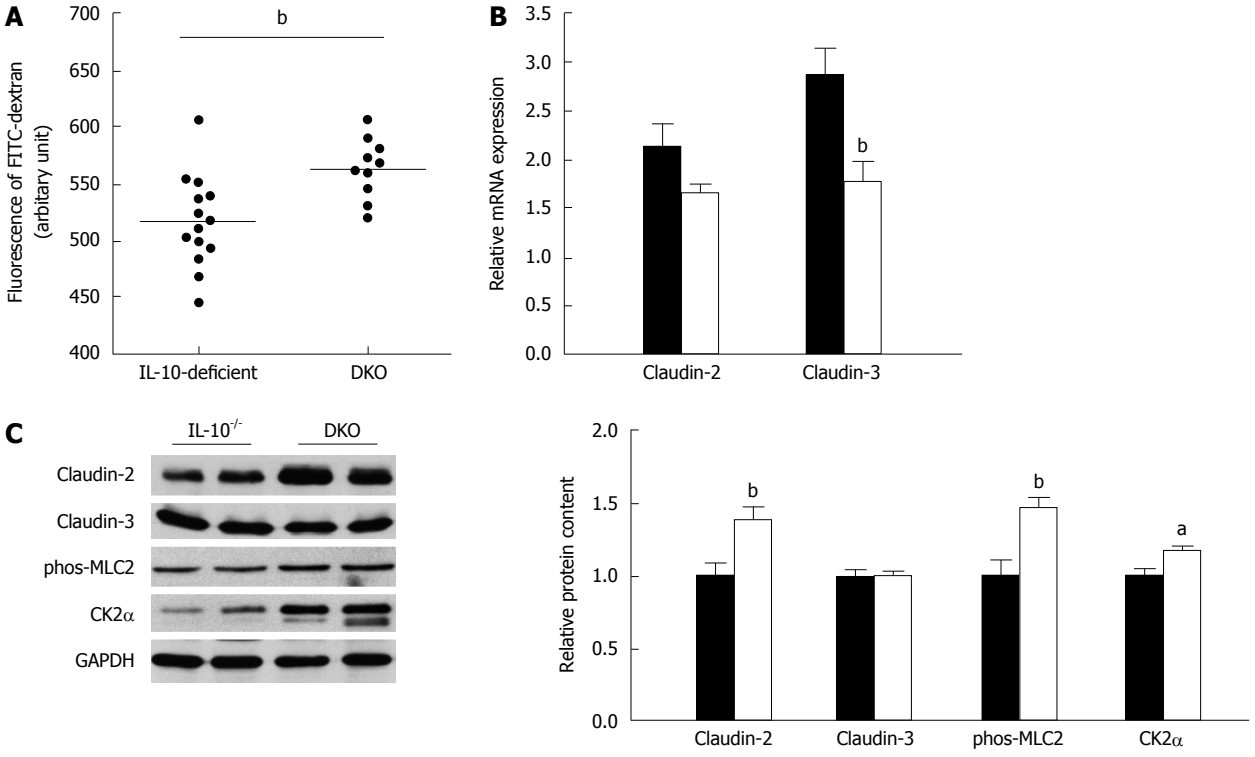


Figure 3 Mast cell deficiency exaggerated mucosal damage in interleukin-10-deficient. A: *In vivo* intestinal paracellular permeability; B: mRNA expression of Claudin-2 and Claudin-3; C: Relative protein content of Claudin-2, Claudin-3, phosphorylation of MLC-2 and CK2α in colonic tissues. Mean ± SE, $^{a}P < 0.05$, $^{b}P < 0.01$, DKO *vs* IL-10-deficient, $n = 8$ for mRNA analysis, $n = 10$ for western blotting analysis. IL: Interleukin; DKO: Double knockout.

erbated colitis in IL-10-deficient mice, associated with impaired mucosal barrier function, which was consistent with a previous study, where deletion of mast cell resulted in earlier onset of spontaneous colitis and associated with increased intestinal permeability in IL-10-deficient mice[15]. We speculate that mast cells might have bilateral roles under different circumstances. Mast cells may act as inflammatory mediators in intact immune system, but serve as sentinels under immune compromised conditions, which is currently underappreciated. As an anti-inflammatory cytokine, IL-10 plays a substantial role in intestinal immune regulation and homeostasis. The level of IL-10 was negatively correlated with the mucosal infiltration of inflammatory cells and the severity of IBD in the colon[26]. Loss of IL-10 signaling by itself is sufficient to drive changes in pro-inflammatory gene expression, but other existing endogenous compensatory mechanisms may be able to prevent robust inflammation. Indeed, inflammatory TLR4 signaling functions to maintain Treg cell populations and intestinal epithelial homeostasis in IL-10-deficient mice[27]. In this regard, mice lacking mast cells in addition to IL-10 deficiency would lose their immune regulation ability thus resulted in exasperated immune deregulation and aggravated colitis. In agreement,

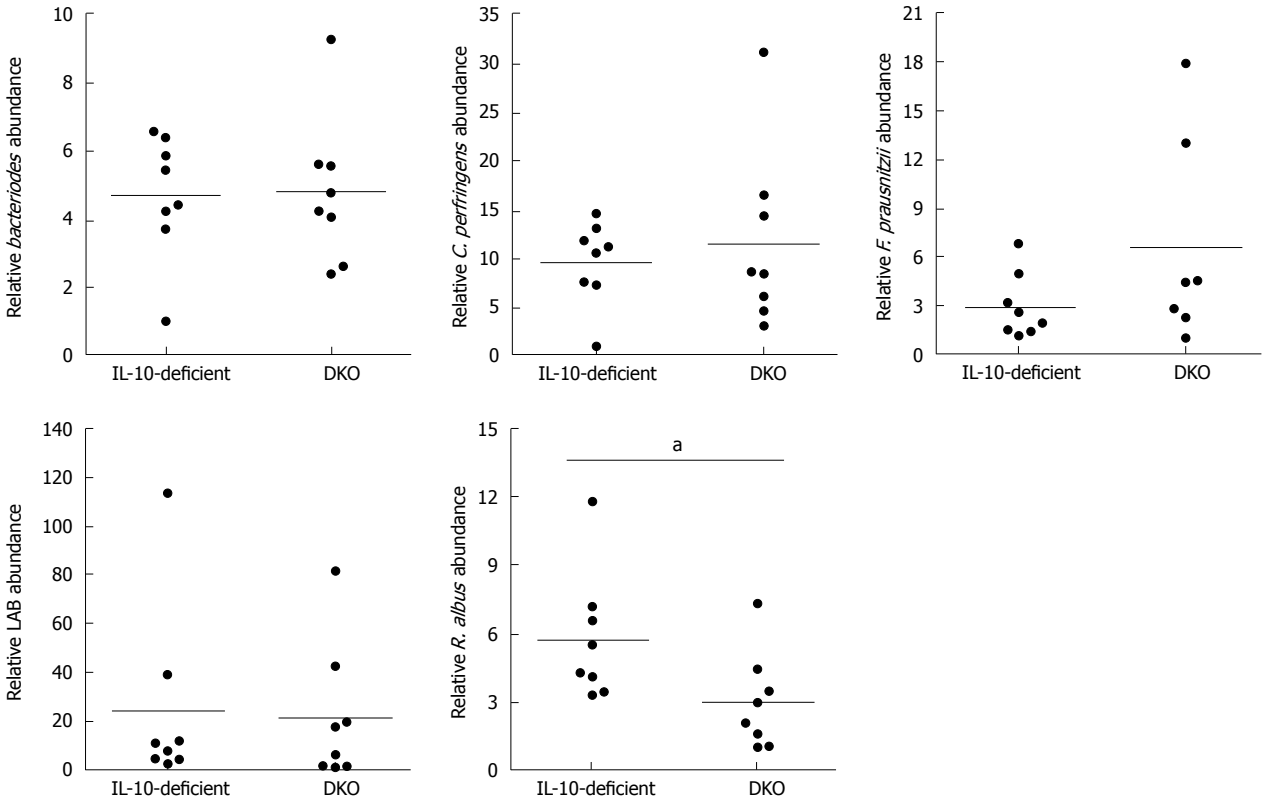


Figure 4 Abundance of selected fecal microflora in interleukin-10-deficient and double knockout mice. Mean ± SE, $^{a}P < 0.05$, DKO *vs* IL-10-deficient, $n = 8$. IL: Interleukin; DKO: Double knockout.

Table 3 Organ weights of 10-week-old interleukin-10-deficient and double knockout mice at necropsy

Organ weight (g)	IL-10-deficient	DKO	*P* value
Liver	1.12 ± 0.041	1.26 ± 0.03	0.0098
Heart	0.13 ± 0.01	0.13 ± 0.01	NS
Spleen	0.09 ± 0.01	0.16 ± 0.02	0.0006
Vastus muscle	0.24 ± 0.01	0.23 ± 0.02	NS
Gastrocnemius muscle	0.27 ± 0.01	0.25 ± 0.01	NS
Tibials muscle	0.081 ± 0.003	0.076 ± 0.003	NS
Subcutaneous fat	0.31 ± 0.03	0.21 ± 0.02	0.0052
Gonadal fat	0.33 ± 0.04	0.27 ± 0.03	NS

[1]Mean ± SE, $n = 10$. IL: Interleukin; DKO: Double knockout.

we found that mast cell deficiency induced exaggerated inflammatory responses in the gut as indicated by increased expression of inflammatory cytokines, enhanced NF-κB inflammatory signaling and elevated neutrophil infiltration. Such inflammation might directly contribute to the far severe colitis pathological changes observed in the colon of DKO mice. Aligned with enhanced gut inflammation, we also detected more severe oxidative stress in colonic tissues, which is another possible etiological factor in the initiation or progression of IBD[28].

Impairment of the epithelial barrier function allows the transmission of antigens, viruses and bacteria, which aggravates inflammation and forms a vicious circle to induce colitis. In IL-10-deficient mice, high intestinal permeability preceded the development of colitis, whereas improved epithelial barrier function alleviated colitis[23], clearly indicating that intestinal permeability is an important etiological factor in the development of colitis in IL-10-deficient mice. In alignment with enhanced gut inflammation, mast cell deficiency markedly enhanced the gut permeability in IL-10-deficient mice, which might propel the progress of colitis. Consistent with the enhanced gut permeability, we observed that IL-10-deficiency increased the "pore forming" Claudin-2 protein content, while decreased mRNA expression of barrier sealing protein, Claudin-3 in colonic tissue of DKO. Myosin light-chain kinase (MLCK) phosphorylates the regulatory light chain of myosin 2 (MLC2) and regulates actin-myosin contraction and further impairs tight junction formation to enhance paracellular permeability[29]. The dramatic increased phosphorylation of MLC2 in our study might serve as one of mechanisms for the impaired epithelial barrier function in DKO mice. Casein kinase 2 (CK2) is a key regulator of intestinal epithelial homeostasis in chronic intestinal inflammation, and enhanced intestinal epithelial cell CK2 protein content was observed in chronic experimental induced colitis[30]. Consistent with the previous report[30] and enhanced colitis, DKO mice showed increased CK2α protein content in the colon tissue. The impairment of gut epithelial barrier function and exaggerated gut inflammatory responses might reinforce each other to deteriorate colitis symptoms.

Besides local gut inflammation, we also observed systemic inflammation in DKO mice, as evidenced by

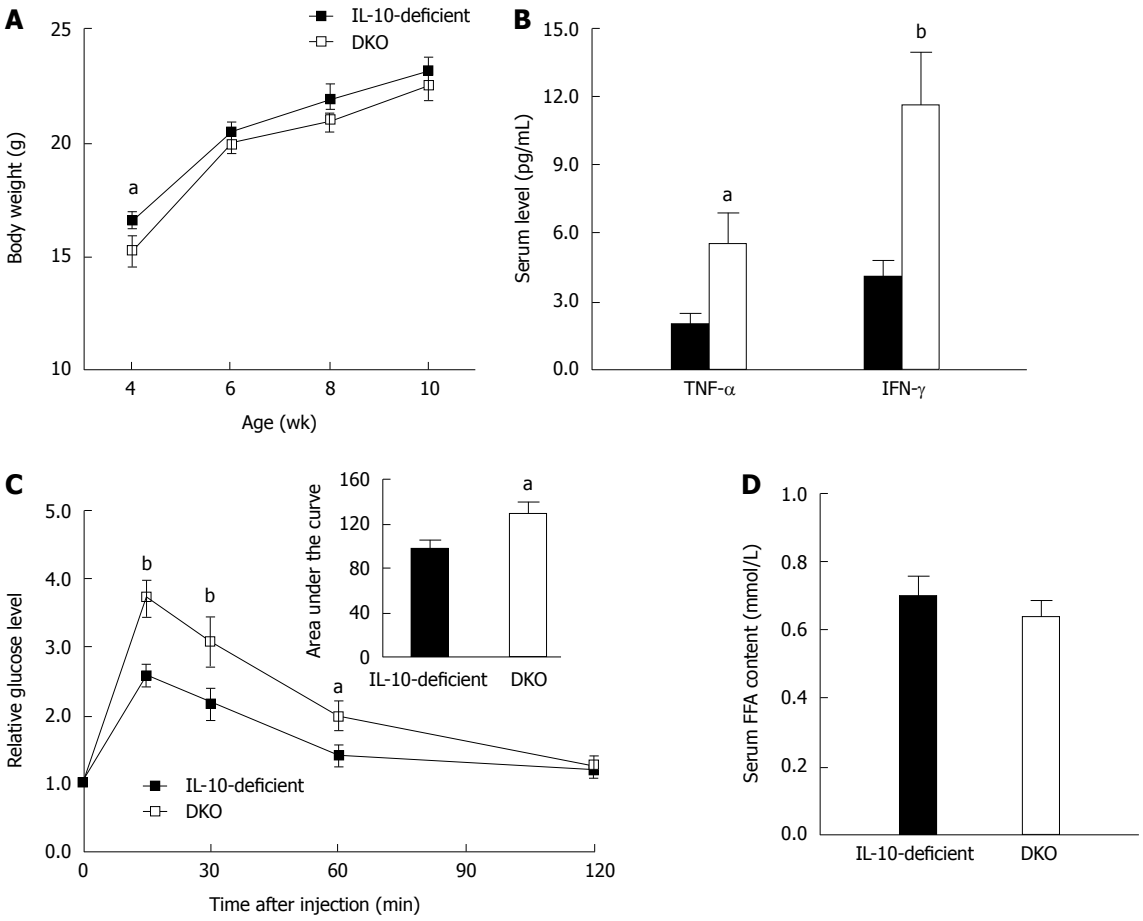


Figure 5 Systemic inflammation in interleukin-10-deficient and double knockout mice. A: Body weight; B: Serum tumor necrosis factor (TNF)-α and interferon (IFN)-γ level; C: Glucose tolerance test; D: Circulatory free fatty acids. Mean ± SE, $^{a}P < 0.05$, $^{b}P < 0.01$, $n = 8$. IL: Interleukin; DKO: Double knockout; FFA: Free fatty acid.

splenomegaly and hepatomegaly, as well as elevated serum TNF-α and IFN-γ levels. We speculated that splenomegaly and hepatomegaly were mediated by increased serum pro-inflammatory cytokines, which accompanied colitis as previously reported[31]. Increased serum pro-inflammatory cytokines could enhance glucose metabolism disorder[32]. Consistently, DKO mice showed glucose intolerance compared with their IL-10-deficient littermates, which could be attributed to the increased serum TNF-α and IFN-γ levels in DKO[32-34], but the exact mechanism remains to be determined.

It has been reported that mast cells are necessary for adipogenesis, while deficiency in mast cells reduces fat mass[35,36]. In the lean mice, mast cells are more prevalent in subcutaneous fat than visceral fat[37]. Indeed, we observed decreased subcutaneous fat in DKO mice while no change in gonadal fat. Because subcutaneous fat functions as FFA buffer[24], insufficient subcutaneous fat might limit the ability of absorbing circulating FFA, leading to elevated serum FFA level and systemic inflammation[24,38]. However, we did not observe significant difference in the serum FFA level between mice with/without mast cells, ruling out FFA as a source of observed exacerbated inflammatory response in DKO mice.

Gut microbiota is increasingly recognized as an important player in gut inflammation and IBD[39,40]. The current results showed increased *Ruminococcus albus* in DKO compared to their IL-10-deficient littermates. In support to our result, a previous report indicated that IBD patients had decreased *Ruminococcus albus* content in the gut[41]. The significance of *Ruminococcus albus* in colitis development needs to be further defined.

In conclusion, mast cell deficiency resulted in exaggerated colitis in IL-10-deficient mice, which was associated with enhanced gut and systematic inflammation, oxidative stress, altered gut microbiota and impaired gut barrier function. Enhanced inflammation and gut permeability likely form a vicious cycle to propel the aggravation of colitis in DKO mice. Our data suggest a protective role of mast cells in the development of colitis in IL-10-deficient mice through a balance of multiple factors.

COMMENTS

Background

Colitis is characterized by chronic inflammation and mast cells accumulate at the pathological sites, implicating their mediating roles, but the exact roles of mast cells in colitis remain poorly defined and controversial. The interleukin-10-deficient mice (IL-10$^{-/-}$) are one of the most frequently used models for studying inflammatory bowel diseases, which will be used to assess the role of mast cells in gut inflammation and colitis.

Research frontiers

In this study, the authors cross-bred mast cell-deficient mice with IL-10-deficient

mice to investigate the role of mast cells in gut inflammation and the onset of colitis. Data show that mast cells have protective roles in the development of colitis by suppressing Th1 type immune response and inflammation, altering gut microbiota composition, improving gut epithelial barrier function, and reducing epithelial damage in IL-10-deficient mice.

Innovations and breakthroughs

Up to now, the roles of mast cells in the development of colitis remain poorly defined and controversial. This study shows that mast cells protect gut epithelium from the development of colitis. Mast cell deficiency in IL-10-deficient mice resulted in systematic and gut inflammation, impaired gut barrier function, and severer Th1-mediated colitis when compared to mice with only IL-10 deficiency. Inflammation and impaired gut epithelial barrier function likely form a vicious cycle to worsen colitis in the double knockout mice. Therefore, both excess and deficiency of mast cells appear to be detrimental for the incidence of colitis.

Applications

The data suggest a protective role of mast cells in the development of colitis in IL-10-deficient mice through a balance of multiple factors. Thus mast cells likely provide a clinical target to mitigate the symptoms of inflammatory bowel disease.

Peer review

The authors are describing interesting results about the effects of mast cell deficiency on colitis in a double knockout mouse model obtained by cross-breeding mast cell-deficient mice with IL-10-deficient mice. The paper is well-written, the methods used are sound, and results established background for future research in this area.

REFERENCES

1 **Yen D**, Cheung J, Scheerens H, Poulet F, McClanahan T, McKenzie B, Kleinschek MA, Owyang A, Mattson J, Blumenschein W, Murphy E, Sathe M, Cua DJ, Kastelein RA, Rennick D. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest* 2006; **116**: 1310-1316 [PMID: 16670770 DOI: 10.1172/JCI21404]

2 **Okayama Y**, Kawakami T. Development, migration, and survival of mast cells. *Immunol Res* 2006; **34**: 97-115 [PMID: 16760571]

3 **Bischoff SC**, Wedemeyer J, Herrmann A, Meier PN, Trautwein C, Cetin Y, Maschek H, Stolte M, Gebel M, Manns MP. Quantitative assessment of intestinal eosinophils and mast cells in inflammatory bowel disease. *Histopathology* 1996; **28**: 1-13 [PMID: 8838115]

4 **Jacob C**, Yang PC, Darmoul D, Amadesi S, Saito T, Cottrell GS, Coelho AM, Singh P, Grady EF, Perdue M, Bunnett NW. Mast cell tryptase controls paracellular permeability of the intestine. Role of protease-activated receptor 2 and beta-arrestins. *J Biol Chem* 2005; **280**: 31936-31948 [PMID: 16027150 DOI: 10.1074/jbc.M506338200]

5 **McDermott JR**, Bartram RE, Knight PA, Miller HR, Garrod DR, Grencis RK. Mast cells disrupt epithelial barrier function during enteric nematode infection. *Proc Natl Acad Sci USA* 2003; **100**: 7761-7766 [PMID: 12796512 DOI: 10.1073/pnas.1231488100]

6 **Konturek PC**, Brzozowski T, Konturek SJ. Stress and the gut: pathophysiology, clinical consequences, diagnostic approach and treatment options. *J Physiol Pharmacol* 2011; **62**: 591-599 [PMID: 22314561]

7 **Bischoff SC**. Physiological and pathophysiological functions of intestinal mast cells. *Semin Immunopathol* 2009; **31**: 185-205 [PMID: 19533134 DOI: 10.1007/s00281-009-0165-4]

8 **De Winter BY**, van den Wijngaard RM, de Jonge WJ. Intestinal mast cells in gut inflammation and motility disturbances. *Biochim Biophys Acta* 2012; **1822**: 66-73 [PMID: 21497195 DOI: 10.1016/j.bbadis.2011.03.016]

9 **Stoyanova II**, Gulubova MV. Mast cells and inflammatory mediators in chronic ulcerative colitis. *Acta Histochem* 2002; **104**: 185-192 [PMID: 12086339]

10 **Hamilton MJ**, Sinnamon MJ, Lyng GD, Glickman JN, Wang X, Xing W, Krilis SA, Blumberg RS, Adachi R, Lee DM, Stevens RL. Essential role for mast cell tryptase in acute experimental colitis. *Proc Natl Acad Sci USA* 2011; **108**: 290-295 [PMID: 21173247 DOI: 10.1073/pnas.1005758108]

11 **Rijnierse A**, Koster AS, Nijkamp FP, Kraneveld AD. Critical role for mast cells in the pathogenesis of 2,4-dinitrobenzene-induced murine colonic hypersensitivity reaction. *J Immunol* 2006; **176**: 4375-4384 [PMID: 16547276]

12 **Vicario M**, Guilarte M, Alonso C, Yang P, Martínez C, Ramos L, Lobo B, González A, Guilà M, Pigrau M, Saperas E, Azpiroz F, Santos J. Chronological assessment of mast cell-mediated gut dysfunction and mucosal inflammation in a rat model of chronic psychosocial stress. *Brain Behav Immun* 2010; **24**: 1166-1175 [PMID: 20600818 DOI: 10.1016/j.bbi.2010.06.002]

13 **Söderholm JD**, Yang PC, Ceponis P, Vohra A, Riddell R, Sherman PM, Perdue MH. Chronic stress induces mast cell-dependent bacterial adherence and initiates mucosal inflammation in rat intestine. *Gastroenterology* 2002; **123**: 1099-1108 [PMID: 12360472]

14 **Groschwitz KR**, Ahrens R, Osterfeld H, Gurish MF, Han X, Abrink M, Finkelman FD, Pejler G, Hogan SP. Mast cells regulate homeostatic intestinal epithelial migration and barrier function by a chymase/Mcpt4-dependent mechanism. *Proc Natl Acad Sci USA* 2009; **106**: 22381-22386 [PMID: 20018751 DOI: 10.1073/pnas.0906372106]

15 **Chichlowski M**, Westwood GS, Abraham SN, Hale LP. Role of mast cells in inflammatory bowel disease and inflammation-associated colorectal neoplasia in IL-10-deficient mice. *PLoS One* 2010; **5**: e12220 [PMID: 20808919 DOI: 10.1371/journal.pone.0012220]

16 **Cani PD**, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, Geurts L, Naslain D, Neyrinck A, Lambert DM, Muccioli GG, Delzenne NM. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. *Gut* 2009; **58**: 1091-1103 [PMID: 19240062 DOI: 10.1136/gut.2008.165886]

17 **Zhu MJ**, Han B, Tong J, Ma C, Kimzey JM, Underwood KR, Xiao Y, Hess BW, Ford SP, Nathanielsz PW, Du M. AMP-activated protein kinase signalling pathways are down regulated and skeletal muscle development impaired in fetuses of obese, over-nourished sheep. *J Physiol* 2008; **586**: 2651-2664 [PMID: 18372306 DOI: 10.1113/jphysiol.2007.149633]

18 **Burich A**, Hershberg R, Waggie K, Zeng W, Brabb T, Westrich G, Viney JL, Maggio-Price L. Helicobacter-induced inflammatory bowel disease in IL-10- and T cell-deficient mice. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G764-G778 [PMID: 11518689]

19 **Pellegrinet L**, Rodilla V, Liu Z, Chen S, Koch U, Espinosa L, Kaestner KH, Kopan R, Lewis J, Radtke F. Dll1- and dll4-mediated notch signaling are required for homeostasis of intestinal stem cells. *Gastroenterology* 2011; **140**: 1230-1240.e1-7 [PMID: 21238454 DOI: 10.1053/j.gastro.2011.01.005]

20 **Tinnikov AA**, Boonstra R. Colorimetric micro-determination of free fatty acids in plasma using microplate readers. *Clin Chim Acta* 1999; **281**: 159-162 [PMID: 10217636]

21 **Zhu MJ**, Du M, Nathanielsz PW, Ford SP. Maternal obesity up-regulates inflammatory signaling pathways and enhances cytokine expression in the mid-gestation sheep placenta. *Placenta* 2010; **31**: 387-391 [PMID: 20185176 DOI: 10.1016/j.placenta.2010.02.002]

22 **Kamizato M**, Nishida K, Masuda K, Takeo K, Yamamoto Y, Kawai T, Teshima-Kondo S, Tanahashi T, Rokutan K. Interleukin 10 inhibits interferon gamma- and tumor necrosis factor alpha-stimulated activation of NADPH oxidase 1 in human colonic epithelial cells and the mouse colon. *J Gastroenterol* 2009; **44**: 1172-1184 [PMID: 19714290 DOI: 10.1007/s00535-009-0119-6]

23 **Arrieta MC**, Madsen K, Doyle J, Meddings J. Reducing small intestinal permeability attenuates colitis in the IL10 gene-deficient mouse. *Gut* 2009; **58**: 41-48 [PMID: 18829978 DOI:

10.1136/gut.2008.150888]
24 **Huffman DM**, Barzilai N. Contribution of adipose tissue to health span and longevity. *Interdiscip Top Gerontol* 2010; **37**: 1-19 [PMID: 20703052 DOI: 10.1159/000319991]
25 **Cai C**, Cao Z, Loughran PA, Kim S, Darwiche S, Korff S, Billiar TR. Mast cells play a critical role in the systemic inflammatory response and end-organ injury resulting from trauma. *J Am Coll Surg* 2011; **213**: 604-615 [PMID: 21920785 DOI: 10.1016/j.jamcollsurg.2011.08.009]
26 **Liu Z**, Feng BS, Yang SB, Chen X, Su J, Yang PC. Interleukin (IL)-23 suppresses IL-10 in inflammatory bowel disease. *J Biol Chem* 2012; **287**: 3591-3597 [PMID: 22158873 DOI: 10.1074/jbc.M111.304949]
27 **Matharu KS**, Mizoguchi E, Cotoner CA, Nguyen DD, Mingle B, Iweala OI, McBee ME, Stefka AT, Prioult G, Haigis KM, Bhan AK, Snapper SB, Murakami H, Schauer DB, Reinecker HC, Mizoguchi A, Nagler CR. Toll-like receptor 4-mediated regulation of spontaneous Helicobacter-dependent colitis in IL-10-deficient mice. *Gastroenterology* 2009; **137**: 1380-1390. e1-3 [PMID: 19596011 DOI: 10.1053/j.gastro.2009.07.004]
28 **Zhu H**, Li YR. Oxidative stress and redox signaling mechanisms of inflammatory bowel disease: updated experimental and clinical evidence. *Exp Biol Med* (Maywood) 2012; **237**: 474-480 [PMID: 22442342 DOI: 10.1258/ebm.2011.011358]
29 **Turner JR**, Rill BK, Carlson SL, Carnes D, Kerner R, Mrsny RJ, Madara JL. Physiological regulation of epithelial tight junctions is associated with myosin light-chain phosphorylation. *Am J Physiol* 1997; **273**: C1378-C1385 [PMID: 9357784]
30 **Koch S**, Capaldo CT, Hilgarth RS, Fournier B, Parkos CA, Nusrat A. Protein kinase CK2 is a critical regulator of epithelial homeostasis in chronic intestinal inflammation. *Mucosal Immunol* 2013; **6**: 136-145 [PMID: 22763408 DOI: 10.1038/mi.2012.57]
31 **Alhouayek M**, Lambert DM, Delzenne NM, Cani PD, Muccioli GG. Increasing endogenous 2-arachidonoylglycerol levels counteracts colitis and related systemic inflammation. *FASEB J* 2011; **25**: 2711-2721 [PMID: 21551239 DOI: 10.1096/fj.10-176602]
32 **Barbosa-da-Silva S**, Fraulob-Aquino JC, Lopes JR, Mandarim-de-Lacerda CA, Aguila MB. Weight cycling enhances adipose tissue inflammatory responses in male mice. *PLoS One* 2012; **7**: e39837 [PMID: 22848362 DOI: 10.1371/journal.pone.0039837]
33 **Gutierrez DA**, Puglisi MJ, Hasty AH. Impact of increased adipose tissue mass on inflammation, insulin resistance, and dyslipidemia. *Curr Diab Rep* 2009; **9**: 26-32 [PMID: 19192421]
34 **Lionetti L**, Mollica MP, Lombardi A, Cavaliere G, Gifuni G, Barletta A. From chronic overnutrition to insulin resistance: the role of fat-storing capacity and inflammation. *Nutr Metab Cardiovasc Dis* 2009; **19**: 146-152 [PMID: 19171470 DOI: 10.1016/j.numecd.2008.10.010]
35 **Liu J**, Divoux A, Sun J, Zhang J, Clément K, Glickman JN, Sukhova GK, Wolters PJ, Du J, Gorgun CZ, Doria A, Libby P, Blumberg RS, Kahn BB, Hotamisligil GS, Shi GP. Genetic deficiency and pharmacological stabilization of mast cells reduce diet-induced obesity and diabetes in mice. *Nat Med* 2009; **15**: 940-945 [PMID: 19633655 DOI: 10.1038/nm.1994]
36 **Tanaka A**, Nomura Y, Matsuda A, Ohmori K, Matsuda H. Mast cells function as an alternative modulator of adipogenesis through 15-deoxy-delta-12, 14-prostaglandin J2. *Am J Physiol Cell Physiol* 2011; **301**: C1360-C1367 [PMID: 21865589 DOI: 10.1152/ajpcell.00514.2010]
37 **Altintas MM**, Azad A, Nayer B, Contreras G, Zaias J, Faul C, Reiser J, Nayer A. Mast cells, macrophages, and crown-like structures distinguish subcutaneous from visceral fat in mice. *J Lipid Res* 2011; **52**: 480-488 [PMID: 21148461 DOI: 10.1194/jlr.M011338]
38 **Arsenault BJ**, Beaumont EP, Després JP, Larose E. Mapping body fat distribution: a key step towards the identification of the vulnerable patient? *Ann Med* 2012; **44**: 758-772 [PMID: 22149719 DOI: 10.3109/07853890.2011.605387]
39 **Sartor RB**. Microbial influences in inflammatory bowel diseases. *Gastroenterology* 2008; **134**: 577-594 [PMID: 18242222 DOI: 10.1053/j.gastro.2007.11.059]
40 **Albenberg LG**, Lewis JD, Wu GD. Food and the gut microbiota in inflammatory bowel diseases: a critical connection. *Curr Opin Gastroenterol* 2012; **28**: 314-320 [PMID: 22573192 DOI: 10.1097/MOG.0b013e328354586f]
41 **Kang S**, Denman SE, Morrison M, Yu Z, Dore J, Leclerc M, McSweeney CS. Dysbiosis of fecal microbiota in Crohn's disease patients as revealed by a custom phylogenetic microarray. *Inflamm Bowel Dis* 2010; **16**: 2034-2042 [PMID: 20848492 DOI: 10.1002/ibd.21319]
42 **Wang H**, Xue Y, Zhang H, Huang Y, Yang G, Du M, Zhu MJ. Dietary grape seed extract ameliorates symptoms of inflammatory bowel disease in IL10-deficient mice. *Mol Nutr Food Res* 2013; **57**: 2253-2257 [PMID: 23963706 DOI: 10.1002/mnfr.201300146]
43 **Cani PD**, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, Burcelin R. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* 2008; **57**: 1470-1481 [PMID: 18305141 DOI: 10.2337/db07-1403]
44 **Barman M**, Unold D, Shifley K, Amir E, Hung K, Bos N, Salzman N. Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. *Infect Immun* 2008; **76**: 907-915 [PMID: 18160481 DOI: 10.1128/IAI.01432-07]
45 **Matsuda K**, Tsuji H, Asahara T, Kado Y, Nomoto K. Sensitive quantitative detection of commensal bacteria by rRNA-targeted reverse transcription-PCR. *Appl Environ Microbiol* 2007; **73**: 32-39 [PMID: 17071791 DOI: 10.1128/AEM.01224-06]
46 **Amann RI**, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* 1990; **56**: 1919-1925 [PMID: 2200342]
47 **Bartosch S**, Fite A, Macfarlane GT, McMurdo ME. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. *Appl Environ Microbiol* 2004; **70**: 3575-3581 [PMID: 15184159 DOI: 10.1128/AEM.70.6.3575-3581.2004]

P- Reviewers: Hewicker-Trautwein M, Ribatti D, Singer SM
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9116

World J Gastroenterol 2014 July 21; 20(27): 9116-9120
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE CONTROL STUDY

Incidence of colorectal neoplasms among male pilots

Menachem Moshkowitz, Ohad Toledano, Lior Galazan, Aharon Hallak, Nadir Arber, Erwin Santo

Menachem Moshkowitz, Ohad Toledafno, Lior Galazan, Aharon Hallak, Nadir Arber, Erwin Santo, Integrated Cancer Prevention Center and Department of Gastroenterology, Tel- Aviv Sourasky Medical Center, and Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv 64239, Israel

Author contributions: Moshkowitz M, Arber N and Santo E designed research; Toledano O, Galazan L, Hallak A and Arber N performed research and analyzed data; Moshkowitz M and Arber N wrote the paper.

Correspondence to: Nadir Arber, MD, MSc, MHA, Head, Integrated Cancer Prevention Center and Department of Gastroenterology, Tel- Aviv Sourasky Medical Center, and Sackler Faculty of Medicine, Tel-Aviv University, 6 Weizmann St. Tel Aviv 64239, Israel. nadira@tlvmc.gov.il

Telephone: +972-3-6974968 Fax: +972-3-6974867
Received: October 21, 2013 Revised: December 11, 2013
Accepted: March 8, 2014
Published online: July 21, 2014

Abstract

AIM: To assess the prevalence of colorectal neoplasms (adenomas, advanced adenomas and colorectal cancers) among Israeli military and commercial airline pilots.

METHODS: Initial screening colonoscopy was performed on average-risk (no symptoms and no family history) airline pilots at the Integrated Cancer Prevention Center (ICPC) in the Tel-Aviv Medical Center. Visualized polyps were excised and sent for pathological examination. Advanced adenoma was defined as a lesion >10 mm in diameter, with high-grade dysplasia or villous histology. The results were compared with those of an age- and gender-matched random sample of healthy adults undergoing routine screening at the ICPC.

RESULTS: There were 270 pilots (mean age 55.2 ± 7.4 years) and 1150 controls (mean age 55.7 ± 7.8 years). The prevalence of colorectal neoplasms was 15.9% among the pilots and 20.6% among the controls (P = 0.097, χ^2 test). There were significantly more hyperplastic polyps among pilots (15.5% *vs* 9.4%, P = 0.004) and a trend towards fewer adenomas (14.8% *vs* 20.3% P = 0.06). The prevalence of advanced lesions among pilots and control groups was 5.9% and 4.7%, respectively (P = 0.49), and the prevalence of cancer was 0.7% and 0.69%, respectively (P = 0.93).

CONCLUSION: There tends to be a lower colorectal adenoma, advanced adenoma and cancer prevalence but a higher hyperplastic polyp prevalence among pilots than the general population.

Key words: Colorectal cancer; Adenomatous polyps; Colon neoplasms; Hyperplastic polyps

Core tip: Military and commercial airline pilots are exposed to cosmic radiation and other specific occupational factors. Several epidemiological studies on a possible elevated cancer risk, including colorectal cancer, among flight personnel have yielded contradictory results. We aimed to evaluate the incidence of colorectal neoplasms among Israeli military and commercial pilots and to compare it with the incidence of colorectal neoplasms among the general population. We found that the prevalence of colorectal adenomas, advanced adenoma and cancer is not higher, and tends to be even lower, among aircrew than that of the general population. It seems that ionizing radiation does not constitute a risk factor for colorectal cancer among air crew personal.

Moshkowitz M, Toledano O, Galazan L, Hallak A, Arber N, Santo E. Incidence of colorectal neoplasms among male pilots. *World J Gastroenterol* 2014; 20(27): 9116-9120 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9116.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9116

INTRODUCTION

Colorectal cancer (CRC) is the third most common can-

cer and the second leading cause of cancer death among both women and men in the Western countries[1]. It is estimated that up to 90% of CRC cases could be avoided with appropriate screening which allows the detection of asymptomatic cancers that are more amenable to curative therapy as well as the removal of adenomas that could subsequently develop into invasive cancer[2,3]. Several risk factors have been reported to be associated with colorectal adenomas, including obesity, impaired glucose tolerance, hypertension, low high-density lipoprotein cholesterol, and hypertriglyceridemia, as well as lifestyle factors, such as alcohol consumption, cigarette smoking, and lack of physical exercise[4,5]. Airline pilots are recognized as an occupational group at risk for various types of cancers[6]. Although they must be physically fit, mentally stabile and lead an appropriate lifestyle in addition to being under closer medical supervision than most other occupational groups, they are subject to several occupational exposures that may pose physiological challenges to their long-term health. The latter include circadian dysrhythmia, mild hypoxia, and exposure to reduced atmospheric pressure, low humidity, noise, vibration, cosmic radiation and magnetic fields[7,8].

Ionizing radiation in higher doses is known, or suspected, to be a cause of several types of cancer[9], and international recommendations regulate such exposure during flights[10,11]. Chromosomal analyses have showed significantly higher numbers of chromosomal translocations among pilots than among controls, and chromosomal translocation frequency was found to be significantly associated with flight years[12,13]. Health studies that have been conducted on this occupational group over the past three decades usually focused on radiation-associated cancer, and those investigations produced inconsistent results[14-20]. An excess mortality from a variety of lesions that were reported in some of these studies include tumors of brain[14], testis, urinary bladder[15], prostate and acute myeloid leukemia[14]. In addition, an excess risk of breast cancer has been observed among female cabin attendants[18], and several studies reported an increased risk for colorectal tumors with standardized incidence ratios ranging from 1.32 to 1.34[19-21].

The recognition that normal colorectal epithelium progresses to adenomas of increasing degrees of dysplasia and then to invasive cancer has been termed the "adenoma-carcinoma sequence", and it has proved to be an excellent model of human cancer[22]. According to this model, it can be assumed that an increased incidence of colorectal tumors would be associated with an increased incidence of adenomas.

This study aims to evaluate the incidence of colorectal neoplasms among Israeli military and commercial pilots and to compare it with the incidence of colorectal neoplasms among the general population.

MATERIALS AND METHODS

Study design

We performed a retrospective case-control study at the Tel-Aviv Sourasky Medical Center from 2003 to 2011. During this period, a total of 270 colonoscopies were performed on military and commercial pilots. All of these individuals were at average-risk for colorectal cancer and underwent colonoscopy for the first time and had all been referred for screening colonoscopy as a primary prevention procedure. Individuals were excluded if they reported a personal history of colorectal adenoma or carcinoma at any time; if they had a family history of colorectal adenoma or carcinoma (one first-degree relative aged < 70, or two or more family relatives at any age); if they had a personal history of inflammatory bowel diseases; if they reported symptoms suggestive of neoplasia (rectal bleeding, change in bowel habit, abdominal pain, or unexplained weight loss during the previous 6 mo); or if they had a positive fecal occult blood test, or laboratory abnormalities such as iron-deficiency anemia. A comparison group was randomly selected from an average-risk, age- and sex-matched control group (n = 1150) from the same institution who underwent colonoscopy during the same time period for similar indications.

Endoscopic procedures

All participants were interviewed and examined by a gastroenterologist. Colonoscopy was performed according to the usual protocol in our institute by experienced gastroenterologists using high-resolution Pentax endoscopes. It was complete to the cecum in 98.7% of patients, and the present analyses were restricted to subjects who underwent a complete colonoscopic examination. All the lesions that had been found during colonoscopy were sampled, removed and sent for pathological examination. The size of the polyp was estimated with the use of open-biopsy forceps. If more than one lesion was detected, the colonoscopic findings were classified according to the most advanced lesion. Advanced adenomas were defined by the presence of any of the following adenoma characteristics: size ⩾ 10 mm, any villous histology, high-grade dysplasia or colorectal cancer that was either invasive (through the muscularis mucosa) or in-situ. For analyses of advanced adenoma, the subjects were classified according to their most advanced pathological finding.

Statistical analysis

The proportion of individuals with polyps in the pilot and control groups was compared using the chi-square and Fisher's exact tests. A P value less than 0.05 was considered statistically significant, and the SPSS (Chicago, Illinois, United States) for Windows software, version 13.0 was used for the analysis.

RESULTS

The pilot group was comprised of 270 males whose average age was 55.2 years at the time of the index screening colonoscopy. They were compared to an age-matched control group of 1150 males (average age 55.7 years at time of the index screening colonoscopy) who were also

Table 1 Endoscopic findings in the study *vs* control groups *n* (%)

	Pilots Total *n* = 270	Controls Total *n* = 1150	*P* value
Age, yr (range)	55.2 (39-74)	55.7 (40-75)	
Endoscopic finding			
Normal	185 (68.5)	805 (70)	0.65
Hyperplastic polyps	42 (15.5)	108 (9.4)	0.004
Adenomatous polyps	41 (14.8)	234 (20.34)	0.06
Advanced adenoma	16 (5.9)	54 (4.7)	0.49
> 3 polyps	5 (1.85)	32 (2.8)	0.51
High-grade dysplasia	2 (0.7)	9 (0.78)	0.94
Cancer	2 (0.7)	8 (0.69)	0.93

undergoing routine colonoscopy.

Polyp, adenoma and advanced lesion detection

Eighty-three (30.7%) pilots were found to have polyps of any type (hyperplastic and/or adenomatous) compared to 342 (29.7%) controls (P = NS). There was a significant difference in the number of pilots and controls who had hyperplastic polyps [n = 42/270 (15.5%) *vs* n = 108/1150 (9.4%), respectively; P = 0.004]. Adenomas were detected in 41 (14.8%) pilots compared to 234 (20.3%) controls (P = 0.06). Similarly, the number of detected advanced adenoma was not significantly different between pilots and controls [n = 16/270 (5.9%) *vs* n = 54/1150 (4.7%); P = 0.49] (Table 1).

High-grade dysplasia and carcinoma detection

A total of 16 advanced lesions was detected in 270 pilots, 2 (0.7%) of which were carcinomas and 2 were high-grade dysplasia (0.7%). The control group had similar rates of advanced lesions: 8 of the 1150 subjects had carcinomas (0.69%) and 9 (0.78%) had high-grade dysplasia (P = 0.93) (Table 1).

DISCUSSION

Over the last 30 years, studies conducted on airline pilots usually focused on radiation-associated cancer, and their results were inconsistent. Nicholas *et al*[23] reported that United States pilots and navigators experienced significantly increased mortality due to cancer of the kidney and renal pelvis, and had a tendency towards increased mortality due to cancer of the prostate, brain, colon, lip, buccal cavity, and pharynx. In their first study which was based on small numbers of subjects, Band *et al*[20] had found excess deaths for brain cancer and for rectal cancer (n = 3; SMR = 4.35; P = 0.033; 95%CI: 1.20-11.20) and an excess cancer incidence for non-melanoma skin cancer, brain cancer and Hodgkin's disease. In a later study, however, the same investigators found a significantly decreased mortality among pilots for all types of cancers, except for prostate cancer and acute myeloid leukemia, which were significantly increased[21].

Unlike previous studies that examined the presence of CRC or death from CRC, the present study systematically compared the presence of premalignant lesions of CRC among pilots with that of age- and gender-matched subjects randomly chosen from the general population. Our study demonstrated no difference and an even lower rate of polyps and adenomas, regardless of size or polyp pathology, between the two groups. The findings of our study are compatible with those of a recent systematic review of the epidemiological literature on health of aircrew members since 1990, which included 65 relevant publications[6]. It reported that the overall cancer incidence and mortality was generally lower among aircrew members than in the comparison population, however, consistently elevated risks were reported for breast cancer incidence among female aircrew members and for melanoma among both male and female aircrew members. The conclusion of that review was that ionizing radiation was considered to contribute little if anything to elevated risks for cancers among an aircrew, whereas excess ultraviolet radiation was a probable cause of an increased melanoma risk among them.

One explanation for the low incidence of colorectal tumors among the crew members was the relative absence of risk factors among that professional group. Risk factors for colorectal cancer and adenomas have been extensively studied and include nutritional as well as lifestyle habits. Compelling evidence indicates that avoidance of smoking and heavy alcohol use, prevention of weight gain, and maintenance of a reasonable level of physical activity are associated with markedly lower risks of colorectal cancer[24]. The systematic monitoring of the health status of aircrew members presumably contributes to reducing the risk factors and pre-malignant as well as cancer rates.

One interesting finding of the current study is the significantly increased rate of hyperplastic polyps among air crew members compared to controls. Hyperplastic colonic polyps have traditionally been considered to have no malignant potential, and are generally regarded as being of little or no clinical consequence. However, recent evidence has suggested that some hyperplastic polyps may develop into cancer via the serrated or microsatellite instable pathways[24]. Chan *et al*[25] suggested that right-sided or large hyperplastic polyps appear to be the ones of concern. We have no explanation for the greater number of hyperplastic polyps among our cohort of pilots, however, they all were relatively small lesions, and none of them were on the right side of the colon.

In conclusion, we found that the prevalence of colorectal adenomas, advanced adenoma and cancer is not higher and tends to be even lower among aircrew than that of the general population. There was an increased number of small, left-sided hyperplastic polyps among the pilots compared to the general population, but the significance of this observation is not clear and further studies are needed to confirm it.

ACKNOWLEDGMENTS

The authors are grateful to Esther Eshkol for meticulous

editing of our paper.

COMMENTS

Background

Several occupational exposures may present physiological challenges to the long-term health of airline pilots. These include circadian dysrhythmia, mild hypoxia, exposure to reduced atmospheric pressure, low humidity, noise, vibration, and exposure to cosmic radiation and magnetic fields. Previous studies have shown an excess mortality from a variety of cancers such as brain, testis, urinary bladder, prostate, leukemia, breast and colorectal tumors.

Research frontiers

According the "adenoma-carcinoma sequence" model, normal colorectal epithelium progresses to adenomas of increasing degrees of dysplasia and then to invasive cancer. It can be assumed, therefore, that an increased incidence of colorectal tumors would be associated with an increased incidence of adenomatous polyps. In this study, the authors demonstrate that the prevalence of colorectal adenomatous polyps is not increased among male pilots.

Innovations and breakthroughs

In contrast to previous studies that examined the presence of colorectal cancer (CRC) or death from CRC among air crew, the present study is the first one that evaluated systematically the presence of premalignant lesions of CRC among pilots. This study demonstrated no difference and an even lower rate of polyps and adenomas, regardless of size or polyp pathology, between pilots and the general population.

Applications

Colorectal cancer screening program among pilots should be equal to that of general population

Terminology

Colorectal cancer is the third leading cause of cancer deaths among men and women in the Western world. Colorectal cancer is preventable, and curable, if detected early. Colorectal adenomas are considered as established precursors of colorectal cancers.

Peer review

The present study is the first to compare the rates of adenomas among pilots with that of age- and gender-matched subjects in the general population. It is a retrospective study with low number of pilots (n = 270), however a large control group (n = 1150) helps in the statistical analysis.

REFERENCES

1 **Jemal A**, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin* 2010; **60**: 277-300 [PMID: 20610543 DOI: 10.3322/caac.20073]

2 **Mandel JS**, Bond JH, Church TR, Snover DC, Bradley GM, Schuman LM, Ederer F. Reducing mortality from colorectal cancer by screening for fecal occult blood. Minnesota Colon Cancer Control Study. *N Engl J Med* 1993; **328**: 1365-1371 [PMID: 8474513]

3 **Rex DK**, Johnson DA, Anderson JC, Schoenfeld PS, Burke CA, Inadomi JM. American College of Gastroenterology guidelines for colorectal cancer screening 2009 [corrected]. *Am J Gastroenterol* 2009; **104**: 739-750 [PMID: 19240699 DOI: 10.1038/ajg.2009.104]

4 **Harriss DJ**, Atkinson G, George K, Cable NT, Reilly T, Haboubi N, Zwahlen M, Egger M, Renehan AG. Lifestyle factors and colorectal cancer risk (1): systematic review and meta-analysis of associations with body mass index. *Colorectal Dis* 2009; **11**: 547-563 [PMID: 19207714 DOI: 10.1111/j.1463-1318.2009.01766.x]

5 **Harriss DJ**, Atkinson G, Batterham A, George K, Cable NT, Reilly T, Haboubi N, Renehan AG. Lifestyle factors and colorectal cancer risk (2): a systematic review and meta-analysis of associations with leisure-time physical activity. *Colorectal Dis* 2009; **11**: 689-701 [PMID: 19207713 DOI: 10.1111/j.1463-1318.2009.01767.x]

6 **Hammer GP**, Blettner M, Zeeb H. Epidemiological studies of cancer in aircrew. *Radiat Prot Dosimetry* 2009; **136**: 232-239 [PMID: 19608578 DOI: 10.1093/rpd/ncp125]

7 **Bartlett DT**. Radiation protection aspects of the cosmic radiation exposure of aircraft crew. *Radiat Prot Dosimetry* 2004; **109**: 349-355 [PMID: 15273353 DOI: 10.1093/rpd/nch311]

8 **Vuković B**, Radolić V, Miklavcić I, Poje M, Varga M, Planinić J. Cosmic radiation dose in aircraft--a neutron track etch detector. *J Environ Radioact* 2007; **98**: 264-273 [PMID: 17600597]

9 **Sigurdson AJ**, Ron E. Cosmic radiation exposure and cancer risk among flight crew. *Cancer Invest* 2004; **22**: 743-761 [PMID: 15581056 DOI: 10.1081/CNV-200032767]

10 **Hammer GP**, Zeeb H, Tveten U, Blettner M. Comparing different methods of estimating cosmic radiation exposure of airline personnel. *Radiat Environ Biophys* 2000; **39**: 227-231 [PMID: 11200966 DOI: 10.1007/s004110000071]

11 **Mares V**, Maczka T, Leuthold G, Rühm W. Air crew dosimetry with a new version of EPCARD. *Radiat Prot Dosimetry* 2009; **136**: 262-266 [PMID: 19608574 DOI: 10.1093/rpd/ncp129]

12 **Yong LC**, Sigurdson AJ, Ward EM, Waters MA, Whelan EA, Petersen MR, Bhatti P, Ramsey MJ, Ron E, Tucker JD. Increased frequency of chromosome translocations in airline pilots with long-term flying experience. *Occup Environ Med* 2009; **66**: 56-62 [PMID: 19074211 DOI: 10.1136/oem.2008.038901]

13 **Nicholas JS**, Butler GC, Davis S, Bryant E, Hoel DG, Mohr LC. Stable chromosome aberrations and ionizing radiation in airline pilots. *Aviat Space Environ Med* 2003; **74**: 953-956 [PMID: 14503673]

14 **Blettner M**, Zeeb H, Auvinen A, Ballard TJ, Caldora M, Eliasch H, Gundestrup M, Haldorsen T, Hammar N, Hammer GP, Irvine D, Langner I, Paridou A, Pukkala E, Rafnsson V, Storm H, Tulinius H, Tveten U, Tzonou A. Mortality from cancer and other causes among male airline cockpit crew in Europe. *Int J Cancer* 2003; **106**: 946-952 [PMID: 12918075 DOI: 10.1002/ijc.11328]

15 **Langner I**, Blettner M, Gundestrup M, Storm H, Aspholm R, Auvinen A, Pukkala E, Hammer GP, Zeeb H, Hrafnkelsson J, Rafnsson V, Tulinius H, De Angelis G, Verdecchia A, Haldorsen T, Tveten U, Eliasch H, Hammar N, Linnersjö A. Cosmic radiation and cancer mortality among airline pilots: results from a European cohort study (ESCAPE). *Radiat Environ Biophys* 2004; **42**: 247-256 [PMID: 14648170 DOI: 10.1007/s00411-003-0214-7]

16 **Zeeb H**, Blettner M, Langner I, Hammer GP, Ballard TJ, Santaquilani M, Gundestrup M, Storm H, Haldorsen T, Tveten U, Hammar N, Linnersjö A, Velonakis E, Tzonou A, Auvinen A, Pukkala E, Rafnsson V, Hrafnkelsson J. Mortality from cancer and other causes among airline cabin attendants in Europe: a collaborative cohort study in eight countries. *Am J Epidemiol* 2003; **158**: 35-46 [PMID: 12835285 DOI: 10.1093/aje/kwg107]

17 **Pukkala E**, Aspholm R, Auvinen A, Eliasch H, Gundestrup M, Haldorsen T, Hammar N, Hrafnkelsson J, Kyyrönen P, Linnersjö A, Rafnsson V, Storm H, Tveten U. Cancer incidence among 10,211 airline pilots: a Nordic study. *Aviat Space Environ Med* 2003; **74**: 699-706 [PMID: 12862322]

18 **Pukkala E**, Auvinen A, Wahlberg G. Incidence of cancer among Finnish airline cabin attendants, 1967-92. *BMJ* 1995; **311**: 649-652 [PMID: 7549630 DOI: 10.1136/bmj.311.7006.649]

19 **Band PR**, Spinelli JJ, Ng VT, Moody J, Gallagher RP. Mortality and cancer incidence in a cohort of commercial airline pilots. *Aviat Space Environ Med* 1990; **61**: 299-302 [PMID: 2339962]

20 **Band PR**, Le ND, Fang R, Deschamps M, Coldman AJ, Gallagher RP, Moody J. Cohort study of Air Canada pilots: mortality, cancer incidence, and leukemia risk. *Am J Epidemiol* 1996; **143**: 137-143 [PMID: 8546114]

21 **Grayson JK**, Lyons TJ. Cancer incidence in United States Air Force aircrew, 1975-89. *Aviat Space Environ Med* 1996; **67**: 101-104 [PMID: 8834932]

22 **Cummings OW**. Pathology of the adenoma-carcinoma sequence: from aberrant crypt focus to invasive carcinoma. *Semin Gastrointest Dis* 2000; **11**: 229-237 [PMID: 11057950]
23 **Nicholas JS**, Lackland DT, Dosemeci M, Mohr LC, Dunbar JB, Grosche B, Hoel DG. Mortality among US commercial pilots and navigators. *J Occup Environ Med* 1998; **40**: 980-985 [PMID: 9830605 DOI: 10.1097/00043764-199811000-00008]
24 **Hawkins NJ**, Ward RL. Sporadic colorectal cancers with microsatellite instability and their possible origin in hyperplastic polyps and serrated adenomas. *J Natl Cancer Inst* 2001; **93**: 1307-1313 [PMID: 11535705 DOI: 10.1093/jnci/93.17.1307]
25 **Chan AT**, Giovannucci EL. Primary prevention of colorectal cancer. *Gastroenterology* 2010; **138**: 2029-2043.e10 [PMID: 20420944 DOI: 10.1053/j.gastro.2010.01.057]

P- Reviewers: De Nardi P, Meshikhes AWN, Supiot S
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9121

World J Gastroenterol 2014 July 21; 20(27): 9121-9127
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE CONTROL STUDY

Risk factors and surgical outcomes for spontaneous rupture of BCLC stages A and B hepatocellular carcinoma: A case-control study

Jing Li, Liang Huang, Cai-Feng Liu, Jie Cao, Jian-Jun Yan, Feng Xu, Meng-Chao Wu, Yi-Qun Yan

Jing Li, Liang Huang, Cai-Feng Liu, Jie Cao, Jian-Jun Yan, Feng Xu, Meng-Chao Wu, Yi-Qun Yan, Department of Hepatic Surgery I, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China

Author contributions: Li J and Huang L performed the study; Liu CF, Cao J, Yan JJ and Xu F collected the clinicopathological data and were involved in editing the manuscript; Li J and Huang L wrote the manuscript; Wu MC and Yan YQ designed the study; Li J and Huang L contributed equally to this work.

Supported by Grants of National Science and Technology Major Project Foundation, No. 2008ZX10002-025; and Scientific Research Fund Projects of Shanghai Health Bureau, No. 2009Y066

Correspondence to: Yi-Qun Yan, Professor, Department of Hepatic Surgery I, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, 800 Yinxiang Road, Shanghai 200438, China. ehbhyyq@163.com
Telephone: +86-21-81875501 Fax: +86-21-65562400

Received: January 5, 2014 Revised: March 12, 2014
Accepted: April 15, 2014
Published online: July 21, 2014

Abstract

AIM: To investigate the risk factors and surgical outcomes for spontaneous rupture of Barcelona Clinic Liver Cancer (BCLC) stages A and B hepatocellular carcinoma (HCC).

METHODS: From April 2002 to November 2006, 92 consecutive patients with spontaneous rupture of BCLC stage A or B HCC undergoing hepatic resection were included in a case group. A control arm of 184 cases (1:2 ratio) was chosen by matching the age, sex, BCLC stage and time of admission among the 2904 consecutive patients with non-ruptured HCC undergoing hepatic resection. Histological confirmation of HCC was available for all patients and ruptured HCC was confirmed by focal discontinuity of the tumor with surrounding perihepatic hematoma observed intraoperatively. Patients with microvascular thrombus in the hepatic vein branches were excluded from the study. Clinical data and survival time were collected and analysed.

RESULTS: Sixteen patients were excluded from the study based on exclusion criteria, of whom 3 were in the case group and 13 in the control group. Compared with the control group, more patients in the case group had underlying diseases of hypertension (10.1% *vs* 3.5%, $P = 0.030$) and liver cirrhosis (82.0% *vs* 57.9%, $P < 0.001$). Tumors in 67 (75.3%) patients in the case group were located in segments Ⅱ, Ⅲ and Ⅵ, and the figure in the control group was also 67 (39.7%) ($P < 0.001$). On multivariate analysis, hypertension (HR = 7.38, 95%CI: 1.91-28.58, $P = 0.004$), liver cirrhosis (HR = 6.04, 95%CI: 2.83-12.88, $P < 0.001$) and tumor location in segments Ⅱ, Ⅲ and Ⅵ (HR = 5.03, 95%CI: 2.70-6.37, $P < 0.001$) were predictive for spontaneous rupture of HCC. In the case group, the median survival time and median disease-free survival time were 12 mo (range: 1-78 mo) and 4 mo (range: 0-78 mo), respectively. The 1-, 3- and 5-year overall survival rates and disease-free survival rates were 66.3%, 23.4% and 10.1%, and 57.0%, 16.8% and 4.5%, respectively. Only radical resection remained predictive for overall survival (HR = 0.32, 95%CI: 0.08-0.61, $P = 0.015$) and disease-free survival (HR = 0.12, 95%CI: 0.01-0.73, $P = 0.002$).

CONCLUSION: Tumor location, hypertension and liver cirrhosis are associated with spontaneous rupture of HCC. One-stage hepatectomy should be recommended to patients with BCLC stages A and B disease.

Key words: Hepatocellular carcinoma; Rupture; Risk factor; Tumor location; Surgical outcome

Core tip: There are few reports concerning the risk factors associated with spontaneous rupture of hepatocel-

lular carcinoma (HCC) and the best approach in cases of ruptured HCC. This retrospective case-control study showed that three predictive factors including hypertension, liver cirrhosis and tumor location in segments Ⅱ, Ⅲ and Ⅵ were associated with spontaneous rupture of HCC. Especially, the relationship between tumor location and spontaneous rupture of HCC was identified for the first time.

Li J, Huang L, Liu CF, Cao J, Yan JJ, Xu F, Wu MC, Yan YQ. Risk factors and surgical outcomes for spontaneous rupture of BCLC stages A and B hepatocellular carcinoma: A case-control study. *World J Gastroenterol* 2014; 20(27): 9121-9127 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9121.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9121

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common neoplasms encountered clinically, and its incidence is increasing worldwide because of the increasing prevalence of hepatitis B and C virus infections[1,2]. One of the life-threatening complications of HCC is the spontaneous rupture of the tumor, with intra-peritoneal hemorrhage. Spontaneous rupture of HCC occurs in 3%-26% of all patients with HCC, and high mortality rates in the range of 32%-6.7% have been reported[3-7].

There were very few reports of risk factors associated with spontaneous rupture of HCC. Kim *et al*[5] reported that CT findings were associated with an increased risk of rupture, including a large tumor, a contour protrusion and portal vein thrombosis, which indicated advanced HCC. In addition, clinicians often feel helpless when facing these complicated situations, and previous studies have shown a very poor prognosis for HCC rupture, with a 30-d mortality rate in the range of 30%-70%[8-14].

There is still a debate concerning the best approach in cases of HCC rupture[9]. Many published studies have reported their various emergency treatments for freshly ruptured tumors and the outcomes afterwards[3,7-14]. The immediate management for ruptured HCC patients usually includes emergency hepatectomy, hepatic artery ligation, suture plication, packing hemostasis and transarterial embolization. However, at our hospital, which is a hepatobiliary specialty hospital without an emergency department, most HCC patients with ruptured tumors were admitted during the routine work in outpatient department. The ruptured tumors were usually indicated by a history of sudden abdominal pain, and confirmed by imaging diagnosis or even in operation. A large series of HCC patients with ruptured tumors were classified with stages A and B disease according to the Barcelona Clinic Liver Cancer (BCLC) classification[15], and most of them were suitable for hepatic resection. We believed that the ruptured BCLC stages A and B HCCs might differ from those at advanced stages in risk factors and clinical outcomes.

Recently, there was a patient with small HCC (3 cm in diameter, located in the left lateral lobe) admitted in our department. Surgery was prepared for him. To our surprise, his blood pressure dropped to 50/20 mmHg and his abdomen enlarged suddenly when he lay on the operating table. Tumor rupture was suspected and confirmed when his abdomen was opened. This case shocked us, and we hypothesized that the tumor location may be associated with spontaneous rupture of HCC. We therefore conducted this retrospective case-control study trying to investigate the risk factors associated with spontaneous rupture of BCLC stages A and B HCC, and to reveal the outcomes after primary hepatectomy during a 10-year period at a single center in China.

MATERIALS AND METHODS

BCLC classification

HCC was stratified according to the BCLC staging classification. The following categories were used. Stage A includes single tumors smaller than 5 cm in diameter or up to 3 tumors all smaller than 3 cm in diameter. Stage B includes up to 3 tumors (≥ 1 of which is > 3 cm in diameter) or more than 3 tumors of any size. Single tumors exceeding 5 cm in diameter are included in stage B as well based on the article by Bruix and Llovet[16].

Patients

From April 2002 to November 2006, a total of 200 consecutive patients with spontaneously ruptured HCC visited the Eastern Hepatobiliary Surgery Hospital. Among them, 92 were classified with stages A and B disease according to the BCLC classification and underwent hepatic resection. A control arm of 184 cases (1:2 ratio) was chosen by matching the age, sex, BCLC stage and time of admission among the 2904 consecutive patients seen in our hospital with non-ruptured HCC undergoing hepatic resection. Histological confirmation of HCC was available for all patients and ruptured HCC was confirmed by focal discontinuity of the tumor with surrounding perihepatic hematoma observed intraoperatively. The tumor tissue samples of all the 276 patients were reviewed by a pathologist, and those with microvascular thrombus in the hepatic vein branches were excluded from the study.

Clinical data of all patients were retrospectively collected, including underlying diseases, laboratory blood test results, intraoperative parameters and postoperative pathological findings. Overall survival time and disease-free survival time were obtained by follow-up. The liver function status was evaluated using the Child-Pugh score system. Tumor location was determined according to the Couinaud's classification to segment the liver.

Statistical analysis

Continuous data are expressed as mean ± SD or median (range) where appropriate and compared using the independent sample *t*-test. Categorical variables were compared using the χ^2 test with Yates correction or the Fisher

Table 1 Comparison of clinical data between patients with and without spontaneous rupture of hepatocellular carcinoma *n* (%)

Variable	Case group (*n* = 89)	Control group (*n* = 171)	*P* value
Age (yr)	48.5 ± 11.2	50.3 ± 11.5	0.215
Sex (M/F)	83/6	151/20	0.206
Diabetes			0.626
Yes	3	167	
No	86	4	
Hypertension			0.030
Yes	9	6	
No	80	165	
HBsAg status			0.195
Positive	82	151	
Negative	7	20	
Liver cirrhosis			< 0.001
Yes	73	99	
No	16	72	
Child-Pugh classification			0.148
A	84	159	
B	5	12	
Tumor BCLC classification			0.653
A	31	68	
B	58	103	
Tumor size (cm)	8.1 ± 3.1	6.5 ± 4.2	0.001
Tumor location[1]			< 0.001
Segment Ⅰ	2	1	
Segments Ⅱ and Ⅲ	20	16	
Segment Ⅵ	47	52	
Segments Ⅳ, Ⅴ, Ⅶ and Ⅷ	20	102	
Tumor protrudes from liver surface			0.138
Yes	45	70	
No	44	101	
Satellite nodule(s)			0.288
Yes	29	45	
No	60	126	
AFP (ng/mL)	138.5 (1.8, 20181.0)	73.8 (2.4, 224492.0)	0.102
WBC (× 10^9/L)	5.7 ± 2.5	5.9 ± 1.8	0.381
PLT (× 10^9/L)	154.7 ± 71.8	147.0 ± 61.3	0.112
Total bilirubin (umol/L)	21.67 ± 3.25	13.57 ± 4.33	0.104
Albumin (g/L)	39.18 ± 3.16	40.52 ± 3.45	0.624
ALT (IU/L)	55.5 ± 49.0	60.5 ± 51.0	0.447
PT (s)	13.0 ± 1.5	13.2 ± 1.5	0.286
BUN (mmol/L)	6.01 ± 0.39	5.93 ± 1.01	0.342
Cr (umol/L)	71.65 ± 12.51	74.72 ± 19.46	0.179
Types of liver resection			0.199
Minor ⩽ 3 liver segments	70	120	
Major ⩾ 4 liver segments	19	51	
Radical resection			0.011
Yes	80	163	
No	9	8	
Inflow blood occlusion time (min)	15.4 ± 6.9	15.4 ± 7.0	0.993
Blood loss (mL)	550 (300, 3200)	200 (50, 2000)	0.011
Blood transfusion (yes/no)	38/51	29/142	< 0.001
Tumor grade (Ⅰ and Ⅱ/Ⅲ and Ⅳ)[2]	3/86	20/151	0.025
30-d mortality	1 (1.1)	1 (0.6)	0.079

[1]Couinaud's classification to segment the liver; [2]Edmondson-Steiner classification. HCC: Hepatocellular carcinoma; BCLC: Barcelona Clinic Liver Cancer; AFP: Alpha-fetoprotein; WBC: White blood cell; PLT: Platelet; ALT: Alanine aminotransferase; PT: Prothrombin time; BUN: Blood urea nitrogen; Cr: Creatinine.

exact test where appropriate. $P < 0.05$ was considered significant. Hazard ratios (HRs) and their corresponding 95%CIs were calculated using simple logistic-regression analysis. Baseline factors associated with a P value < 0.1 in the univariate analysis were sequentially entered into the multivariate logistic regression analysis to indicate the relatively independent risk factors.

Survival rates were obtained by the Kaplan-Meier method and were compared using the log-rank test. A Cox regression model was used to analyse the prognostic predictors for survival. Survival time and disease-free survival time started from the date of hepatic resection until death and the diagnosis of recurrence or the closing date. The closing date of this study was March 31, 2011.

RESULTS

Clinical data

Sixteen patients were excluded from the study based on the exclusion criteria, of whom 3 were in the case group and 13 in the control group. Among the 89 patients with spontaneous rupture of HCC (case group), mean age was 48.5 ± 11.2 years and 83 (93.3%) were males. The corresponding figures for the 171 patients without rupture (control group) were 50.5 ± 11.4 years and a male prevalence of 88.3%. The most common initial symptoms of spontaneous rupture of HCC were sudden onset of abdominal pain (61 patients, 68.5%). There were 12 (13.5%) patients who developed hypovolemic shock at admission, while 16 (18.0%) patients had no symptoms. Emergency hepatic resection was performed in 12 (13.5%) patients, while the remaining 77 patients underwent limited one-stage operation.

The clinical data for the case group and control group are presented in Table 1. Compared with the control group, more patients in the case group had underlying diseases of hypertension (10.1% *vs* 3.5%, $P = 0.030$) and liver cirrhosis (82.0% *vs* 57.9%, $P < 0.001$). Tumors in 67 (75.3%) patients in the case group were located in segments Ⅱ, Ⅲ and Ⅵ, and the figure in the control group was also 67 (39.7%) ($P < 0.001$). No significant differences were observed between the case group and control group with respect to baseline levels of laboratory tests. Radical resection, defined as a negative margin and no residual tumor in the liver, was achieved in 80 (89.9%) patients in the case group, compared with 163 (95.3%) in the control group ($P = 0.011$). The 30-d mortality was 1.1% (1/89) in the case group and 0.5% (1/171) in the control group, and both patients died of liver failure.

Risk factors associated with spontaneous rupture of HCC

Four variables including hypertension, liver cirrhosis, tumor size ⩾ 10 cm, and tumor location in segments Ⅱ, Ⅲ and Ⅵ were selected on multivariate analysis to determine the independent risk factors associated with HCC rupture. The results showed that hypertension (HR = 7.38, 95%CI: 1.91-28.58, $P = 0.004$), liver cirrhosis (HR = 6.04, 95%CI: 2.83-12.88, $P < 0.001$) and tumor location in seg-

Table 2 Multivariate analysis of variables associated with spontaneous rupture of hepatocellular carcinoma

Variable	HR	95%CI for HR	*P* value
Hypertension			0.004
Yes	7.38	1.91-28.58	
No	1		
Liver cirrhosis			< 0.001
Yes	6.04	2.83-12.88	
No	1		
Tumor size			0.184
⩾ 10 cm	1.62	0.80-3.31	
< 10 cm	1		
Tumor locaction[1]			< 0.001
Segments Ⅱ, Ⅲ and Ⅵ	5.03	2.70-6.37	
Other segments	1		

[1]Couinaud's classification to segment the liver. HCC: Hepatocellular carcinoma; HR: Hazard ratio; CI: Confidence interval.

ments Ⅱ, Ⅲ and Ⅵ (HR = 5.03, 95%CI: 2.70-6.37, $P < 0.001$) were predictive for spontaneous rupture of HCC (Table 2).

Surgical outcomes

At the closing date of the study, 80 (89.9%) patients in the case group and 119 (69.6%) in the control group died. The median survival time (MST) and median disease-free survival time (MDST) of patients in the case group were 12 mo (range: 1-78 mo) and 4 mo (range: 0-78 mo), respectively, while the figures in the control group were 51 mo (range: 1-104 mo) and 24 mo (range: 1-87 mo), respectively. The 1-, 3- and 5-year overall survival rates were 66.3%, 23.4% and 10.1% in the case group, compared with 85.4%, 63.2% and 46.3% in the control group ($P < 0.001$). In addition, the 1-, 3-, and 5-year disease-free survival rates in the case group were 57.0%, 16.8% and 4.5%, respectively, while they were 65.7%, 39.2% and 27.5% in the control group ($P = 0.049$) (Figure 1).

Nine variables were selected on multivariate analysis to determine the prognostic predictors of survival in patients with spontaneously ruptured HCC (Table 3). Only radical resection remained statistically predictive for overall survival (HR = 0.32, 95%CI: 0.08-0.61, $P = 0.015$), as well as for disease-free survival (HR = 0.12, 95%CI: 0.01-0.73, $P = 0.002$).

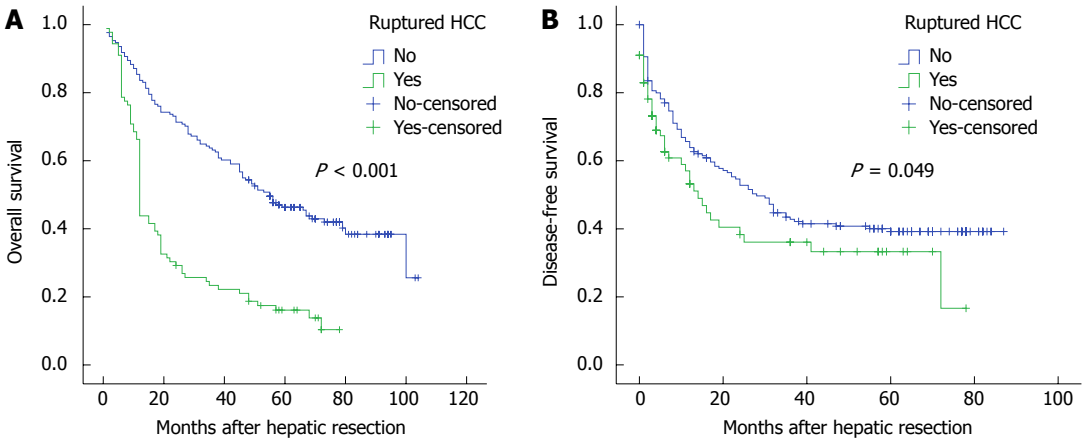
DISCUSSION

Risk factors for spontaneous rupture of HCC

Spontaneous rupture of HCC is one of the most common and life-threatening emergencies in liver surgery. However, there were very few studies investigating the risk factors associated with this dreaded complication of HCC. A commonly accepted mechanism of rupture described by Zhu *et al*[17] is that it is initiated by invasion and occlusion of the hepatic veins by tumor cells, which results in increased pressure within the tumor mass. The venous congestion in combination with various factors, such as central tumor necrosis, trauma and coagulopathy, leads to hemorrhage within the tumor. This further increases the pressure in the tumor and results in splitting of the overlying liver parenchyma and rupture at the surface. We think that this hypothesised mechanism may account for spontaneous rupture of advanced HCC when the hepatic vein is invaded by the tumor. Moreover, rupture of advanced HCC may cause heavy bleeding, and thus the patients are more likely to seek emergency care. In the present study, we included patients with BCLC stages A and B HCC which had no vascular invasion, and the results showed some other risk factors associated with spontaneous rupture of HCC.

In our series, we noticed that most ruptured tumors were located in the left lateral lobe (segments Ⅱ and Ⅲ, $n = 20$) and the right posterior inferior lobe (segment Ⅵ, $n = 47$), resulting in a proportion of 75.3%, and tumor location in segments Ⅱ, Ⅲ and Ⅵ were predictive for spontaneous rupture of HCC. According to gross anatomy, the shape of the liver is similar to a wedge. Compared with the other parts of the liver, either the left lateral lobe (segments Ⅱ and Ⅲ) or the right posterior inferior lobe (segment Ⅵ) is a "small room" restricted by the capsule of the liver. Therefore, when the tumor there becomes larger than the "room", the inner pressure of the tumor can break the local capsule. Sometimes a strike from outside may lead to the rise of the pressure on the basis of "small room and big guest", and cause rupture of the tumor as well. We also found that there was almost no overlying liver parenchyma on the ruptured tumor, neither was it showed by imaging examination nor found in operation. Especially, we did not find any "tunnel"-like tumor tissues at the most possible spot of rupture. Chen *et al*[8] also reported that left-lobe tumors presented a higher risk of rupture. So we doubt that hepatic vein occlusion is a main factor to initiate the rupture of HCC. Instead, we think that the differences in mechanics due to tumor location may play a major role in the pathogenesis of tumor rupture.

Previous studies have reported that the maximum tumor size > 5 cm was one of the risk factors predicting rupture of HCC[5,8]. Interestingly, in our study, tumor size ⩾ 10 cm was not a predictive factor for HCC rupture, while the underlying diseases of hypertension and liver cirrhosis were predictive for spontaneous rupture of HCC. Moreover, some tumors as small as 2 cm have been found to rupture, which was consistent with a report by Tanaka *et al*[18]. It is difficult to explain how a small HCC located in the periphery would rupture *via* the above mechanism. More recent studies suggested that underlying vascular dysfunction may play a role[10]. The vessels in the ruptured HCC tend to be more friable due to increased collagenase expression and increased collagen Ⅳ degradation[19]. Our results are consistent with this proposed mechanism, and we think the reasons may be as follows. A long history of hypertension always causes injury of the blood vessels, making them more friable. In addition, patients with liver cirrhosis always have underly-



	MST (mo)	MDST (mo)	1-, 3-, 5-yr OS	1-, 3-, 5-yr DFS
Case group	12 (1-78)	4 (0-78)	66.3%, 23.4%, 10.1%	57.0%, 16.8%, 4.5%
Control group	51 (1-104)	24 (1-87)	85.4%, 63.2%, 46.3%	65.7%, 39.2%, 27.5%

Figure 1 Overall survival (A) and disease-free survival (B) in patients with barcelona clinic liver cancer stages A and B hepatocellular carcinoma with or without rupture. HCC: Hepatocellular carcinoma; MST: Median survival time; MDST: Median disease-free survival time; OS: Overall survival; DFS: Disease-free survival.

Table 3 Predictors of survival on multivariate analysis using a Cox regression model in patients with spontaneous rupture of hepatocellular carcinoma

Variable	Overall survival			Disease-free survival		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age	1.01	0.99-1.03	0.348	1.02	0.99-1.05	0.097
Liver cirrhosis (yes *vs* no)	1.05	0.55-2.03	0.878	1.29	0.54-3.09	0.566
BCLC stage (stage A *vs* stage B)	1.35	0.60-3.06	0.470	1.13	0.41-3.07	0.817
Tumor size	0.96	0.88-1.06	0.456	0.92	0.81-1.05	0.235
Tumor location	1.02	0.59-1.76	0.936	1.24	0.64-2.42	0.524
Satellite nodule(s) (yes *vs* no)	0.77	0.46-1.27	0.299	0.76	0.39-1.47	0.413
Blood loss	1.00	1.00-1.00	0.085	1.00	1.00-1.00	0.940
Blood transfusion (yes *vs* no)	1.00	0.51-1.98	0.994	1.12	0.49-2.59	0.788
Radical resection (yes *vs* no)	0.32	0.08-0.61	0.015	0.12	0.01-0.73	0.002

HCC: Hepatocellular carcinoma; HR: Hazard ratio; CI: Confidence interval; BCLC: Barcelona clinic liver cancer.

ing coagulopathy. Both of them would lead to hemorrhage within the tumor and then initiate tumor rupture.

Survival of patients with spontaneous rupture of HCC

Spontaneous rupture of HCC can be a fatal complication of HCC. Previous studies have shown a very poor prognosis, with 30-d mortality rates in the range of 30%-70%[8-14,20,21]. The present study included patients with BCLC stages A and B HCC, and an overall 30-d mortality rate of 1.1% (one patient) was observed, which was much lower than those in previous reports.

Several studies have demonstrated that emergency hepatic resection for ruptured HCC may achieve long-term survival[20,21]. In another series, the 1- and 3-year survival rates for patients who underwent emergency resection were only 60% and 42%, respectively[22]. The MST of patients with ruptured HCC who had been treated by hepatectomy in the range of 1.2-25.7 mo has been reported[4,7]. In two case series of delayed resection for ruptured HCC from Japan, no in-hospital mortality was observed, and 1- and 3-year survival rates of 71%-77% and 48%-54%, respectively, were achieved[23,24]. It seems that hepatic resection was the most valuable treatment for patients with ruptured HCC, irrespective of emergency or delayed operation. However, there is still a disparity concerning the prognosis for these patients undergoing hepatic resection, because the surgical outcomes may be complicated by the stage of HCC and the inclusion of transarterial embolization in the treatment algorithm.

In our series, all the patients with ruptured HCC underwent one-stage hepatic resection without initial treatments. The MST and MDST were 12 mo and 4 mo, respectively, and the 1-, 3- and 5-year overall survival rates were 66.3%, 23.4% and 10.1%, respectively. The results were much worse than those patients with non-ruptured HCC undergoing hepatic resection, although recent studies have stated that the long-term survival for ruptured HCC may be equivalent to non-ruptured HCC[20]. We think the reason may be as follows. The ruptured HCC always stays in the "small room", the increased inner pressure of the tumor not only causes tumor rupture but also causes intrahepatic metastasis. Considering that

patients with ruptured HCC harbor advanced disease at presentation, the incidence of coexisting cirrhosis is high, and peritoneal seeding may occur at the time of rupture. The current tumor-node-metastasis staging system classifies ruptured HCC as T4 and as stage Ⅳ [25], which indicated advanced stage. Therefore, we think the results attained from our series are acceptable and one-stage hepatic resection should be recommended to patients with resectable ruptured HCC at BCLC stages A and B.

Furthermore, this study identified that radical resection remained predictive for surgical outcomes in patients with ruptured HCC. Inversely, some other acknowledged prognostic factors like BCLC stage, tumor size and satellite nodule(s) in non-ruptured HCC were not demonstrated to be statistically significant in ruptured HCC, suggesting that ruptured HCC may differ from non-rupture HCC in biological behaviour and pathological characteristics.

In summary, this retrospective case-control study showed that three predictive factors including hypertension, liver cirrhosis and tumor location in segments Ⅱ, Ⅲ and Ⅵ were associated with spontaneous rupture of HCC. Especially, the relationship between tumor location and spontaneous rupture of HCC was identified for the first time, which challenges the well-known concept that the congestion of hepatic venous vein is a main factor to initiate the rupture of HCC. Once the risk factors were determined, the next step we want to do is to determine whether we can adopt some measures to prevent tumor rupture in these patients. Although the surgical outcomes in patients with ruptured HCC was much worse than those without, we still think one-stage hepatic resection should be recommended to patients with ruptured HCC at BCLC stages A and B.

COMMENTS

Background

Spontaneous rupture of hepatocellular carcinoma (HCC) occurs in 3%-26% of all patients with HCC, and high mortality rates in the range of 32%-6.7% have been reported.

Research frontiers

There is still a debate concerning the best approach in cases of HCC rupture. Many published studies have reported their various emergency treatments for freshly ruptured tumors and the outcomes afterwards.

Innovations and breakthroughs

This retrospective case-control study tried to investigate the risk factors associated with spontaneous rupture of barcelona clinic liver cancer stages A and B HCC, and to reveal the outcomes after primary hepatectomy during a 10-year period at a single center in China.

Applications

This study identified that radical resection remained predictive for surgical outcomes in patients with ruptured HCC.

Peer review

A good series of a single institutional experience of patients with localised spontaneously ruptured HCC treated by surgical resection. It explores biologically plausible risk factors for rupture with appropriate statistical analysis. Important message is that outcomes are not necessarily grim as some 5-year survivors are possible following resection with a low surgical mortality.

REFERENCES

1 **McGlynn KA**, London WT. The global epidemiology of hepatocellular carcinoma: present and future. *Clin Liver Dis* 2011; **15**: 223-243, vii-x [PMID: 21689610 DOI: 10.1016/j.cld.2011.03.006]

2 **Kobayashi M**, Ikeda K, Hosaka T, Sezaki H, Someya T, Akuta N, Suzuki F, Suzuki Y, Saitoh S, Arase Y, Miyakawa Y, Kumada H. Natural history of compensated cirrhosis in the Child-Pugh class A compared between 490 patients with hepatitis C and 167 with B virus infections. *J Med Virol* 2006; **78**: 459-465 [PMID: 16482557 DOI: 10.1002/jmv.20562]

3 **Miyoshi A**, Kitahara K, Kohya N, Noshiro H, Miyazahi K. Outcomes of patients with spontaneous rupture of hepatocellular carcinoma. *Hepatogastroenterology* 2011; **58**: 99-102 [PMID: 21510294]

4 **Liu CL**, Fan ST, Lo CM, Tso WK, Poon RT, Lam CM, Wong J. Management of spontaneous rupture of hepatocellular carcinoma: single-center experience. *J Clin Oncol* 2001; **19**: 3725-3732 [PMID: 11533094]

5 **Kim HC**, Yang DM, Jin W, Park SJ. The various manifestations of ruptured hepatocellular carcinoma: CT imaging findings. *Abdom Imaging* 2008; **33**: 633-642 [PMID: 18172704 DOI: 10.1007/s00261-007-9353-7]

6 **Tan FL**, Tan YM, Chung AY, Cheow PC, Chow PK, Ooi LL. Factors affecting early mortality in spontaneous rupture of hepatocellular carcinoma. *ANZ J Surg* 2006; **76**: 448-452 [PMID: 16768766 DOI: 10.1111/j.1445-2197.2006.03750.x]

7 **Al-Mashat FM**, Sibiany AM, Kashgari RH, Maimani AA, Al-Radi AO, Balawy IA, Ahmad JE. Spontaneous rupture of hepatocellular carcinoma. *Saudi Med J* 2002; **23**: 866-870 [PMID: 12174244]

8 **Chen CY**, Lin XZ, Shin JS, Lin CY, Leow TC, Chen CY, Chang TT. Spontaneous rupture of hepatocellular carcinoma. A review of 141 Taiwanese cases and comparison with nonrupture cases. *J Clin Gastroenterol* 1995; **21**: 238-242 [PMID: 8648061 DOI: 10.1097/00004836-199510000-00015]

9 **Rossetto A**, Adani GL, Risaliti A, Baccarani U, Bresadola V, Lorenzin D, Terrosu G. Combined approach for spontaneous rupture of hepatocellular carcinoma. *World J Hepatol* 2010; **2**: 49-51 [PMID: 21160956 DOI: 10.4254/wjh.v2.i1.49]

10 **Zhu LX**, Geng XP, Fan ST. Spontaneous rupture of hepatocellular carcinoma and vascular injury. *Arch Surg* 2001; **136**: 682-687 [PMID: 11387009 DOI: 10.1001/archsurg.136.6.682]

11 **Tarantino L**, Sordelli I, Calise F, Ripa C, Perrotta M, Sperlongano P. Prognosis of patients with spontaneous rupture of hepatocellular carcinoma in cirrhosis. *Updates Surg* 2011; **63**: 25-30 [PMID: 21258886 DOI: 10.1007/s13304-010-0041-8]

12 **Yoshida H**, Mamada Y, Taniai N, Mizuguchi Y, Kakinuma D, Ishikawa Y, Kanda T, Matsumoto S, Bando K, Akimaru K, Tajiri T. Long-term results of elective hepatectomy for the treatment of ruptured hepatocellular carcinoma. *J Hepatobiliary Pancreat Surg* 2008; **15**: 178-182 [PMID: 18392711 DOI: 10.1007/s00534-007-1239-0]

13 **Buczkowski AK**, Kim PT, Ho SG, Schaeffer DF, Lee SI, Owen DA, Weiss AH, Chung SW, Scudamore CH. Multidisciplinary management of ruptured hepatocellular carcinoma. *J Gastrointest Surg* 2006; **10**: 379-386 [PMID: 16504883 DOI: 10.1016/j.gassur.2005.10.012]

14 **Hai L**, Yong-Hong P, Yong F, Ren-Feng L. One-stage liver resection for spontaneous rupture of hepatocellular carcinoma. *World J Surg* 2005; **29**: 1316-1318 [PMID: 16132405 DOI: 10.1007/s00268-005-7626-2]

15 **Marrero JA**. Staging systems for hepatocellular carcinoma: should we all use the BCLC system? *J Hepatol* 2006; **44**: 630-632 [PMID: 16503077 DOI: 10.1016/j.jhep.2006.02.003]

16 **Bruix J**, Llovet JM. Prognostic prediction and treatment strategy in hepatocellular carcinoma. *Hepatology* 2002; **35**: 519-524 [PMID: 11870363 DOI: 10.1053/jhep.2002.32089]

17 **Zhu LX**, Wang GS, Fan ST. Spontaneous rupture of hepatocellular carcinoma. *Br J Surg* 1996; **83**: 602-607 [PMID:

8689200 DOI: 10.1002/bjs.1800830507]
18 **Tanaka A**, Takeda R, Mukaihara S, Hayakawa K, Shibata T, Itoh K, Nishida N, Nakao K, Fukuda Y, Chiba T, Yamaoka Y. Treatment of ruptured hepatocellular carcinoma. *Int J Clin Oncol* 2001; **6**: 291-295 [PMID: 11828948 DOI: 10.1007/s10147-001-8030-z]
19 **Zhu LX**, Liu Y, Fan ST. Ultrastructural study of the vascular endothelium of patients with spontaneous rupture of hepatocellular carcinoma. *Asian J Surg* 2002; **25**: 157-162 [PMID: 12376238 DOI: 10.1016/s1015-9584(09)60166-4]
20 **Yeh CN**, Lee WC, Jeng LB, Chen MF, Yu MC. Spontaneous tumour rupture and prognosis in patients with hepatocellular carcinoma. *Br J Surg* 2002; **89**: 1125-1129 [PMID: 12190677]
21 **Chiappa A**, Zbar A, Audisio RA, Paties C, Bertani E, Staudacher C. Emergency liver resection for ruptured hepatocellular carcinoma complicating cirrhosis. *Hepatogastroenterology* 1999; **46**: 1145-1150 [PMID: 10370682]
22 **Chen MF**, Hwang TL, Jeng LB, Jan YY, Wang CS. Clinical experience with hepatic resection for ruptured hepatocellular carcinoma. *Hepatogastroenterology* 1995; **42**: 166-168 [PMID: 7672765]
23 **Shimada R**, Imamura H, Makuuchi M, Soeda J, Kobayashi A, Noike T, Miyagawa S, Kawasaki S. Staged hepatectomy after emergency transcatheter arterial embolization for ruptured hepatocellular carcinoma. *Surgery* 1998; **124**: 526-535 [PMID: 9736905 DOI: 10.1016/S0039-6060(98)70099-6]
24 **Shuto T**, Hirohashi K, Kubo S, Tanaka H, Hamba H, Kubota D, Kinoshita H. Delayed hepatic resection for ruptured hepatocellular carcinoma. *Surgery* 1998; **124**: 33-37 [PMID: 9663249 DOI: 10.1016/S0039-6060(98)70072-8]
25 **Collaborative Staging Task Force of the American Committee on Cancer**. Collaborative Staging Manual and Coding Instructions, version 1.0. Published jointly by the American Joint Committee on Cancer (Chicago, IL) and the U.S. Department of Health and Human Services (Bethesda, Maryland). Bethesda: NIH Publication, 2004: No. 04-5496

P- Reviewers: Cheng A, Olah A, Yip D **S- Editor**: Qi Y
L- Editor: Wang TQ **E- Editor**: Zhang DN

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9128

World J Gastroenterol 2014 July 21; 20(27): 9128-9137
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RETROSPECTIVE STUDY

Relevance of α-defensins (HNP1-3) and defensin β-1 in diabetes

Balázs Csaba Németh, Tamás Várkonyi, Ferenc Somogyvári, Csaba Lengyel, Katalin Fehértemplomi, Szabolcs Nyiraty, Péter Kempler, Yvette Mándi

Balázs Csaba Németh, Ferenc Somogyvári, Yvette Mándi, Department of Medical Microbiology and Immunology, University of Szeged, H-6720 Szeged, Hungary
Balázs Csaba Németh, Department of Molecular and Cell Biology, Boston University Henry M. Goldman School of Dental Medicine, Boston, MA 02118, United States
Tamás Várkonyi, Csaba Lengyel, Katalin Fehértemplomi, Szabolcs Nyiraty, Péter Kempler, First Department of Internal Medicine, University of Szeged, H6720 Szeged, Hungary
Author contributions: Németh BC performed all laboratory experiments; Várkonyi T designed clinical research and analyzed the clinical data; Somogyvári F designed the genetical experiments; Fehértemplomi K, Lengyel C and Nyiraty S collected all the human material and analyzed the clinical data; Kempler P provided the scientific clinical background; Mándi Y designed and coordinated the study, provided the immunological background, ensured financial support for the research and wrote the manuscript.
Supported by TÁMOP-4.2.2.A-11-1-KONV-2012-0035 Research Grant
Correspondence to: Yvette Mándi, MD, PhD, DSc, Department of Medical Microbiology and Immunobiology, University of Szeged, Dóm tér 10, H-6720 Szeged, Hungary. mandi.yvette@med.u-szeged.hu
Telephone: +36-62-545115 Fax: +36-62-545113
Received: December 10, 2013 Revised: February 7, 2014
Accepted: April 8, 2014
Published online: July 21, 2014

Abstract

AIM: To investigate the genetic background of human defensin expression in type 1 and 2 diabetes.

METHODS: Associations between *DEFA1/DEFA3* gene copy number polymorphism and diabetes as well as between the promoter polymorphisms of *DEFB1* and diabetes were studied. The copy number variation of the *DEFA1/DEFA3* genes was determined in 257 diabetic patients (117 patients with type 1 and 140 with type 2 diabetes). The control group consisted of 221 age- and gender-matched healthy blood donors. The cumulative copy numbers of the *DEFA1/DEFA3* genes were detected by using quantitative PCR analysis. To evaluate the HNP 1-3 (human neutrophil peptide 1-3 or α-defensin) levels in the circulation, plasma HNP 1-3 concentrations were measured by ELISA. The expression of *DEFA1/A3* in peripheral leukocytes of the diabetic patients was measured by quantitative RT PCR analysis. Three SNPs of the human *DEFB1* (human defensin β-1) gene: *DEFB1* G-20A (rs11362), *DEFB1* C-44G (rs1800972) and *DEFB1* G-52A (rs1799946) were genotyped by Custom TaqMan® Real Time PCR assay.

RESULTS: Significant differences were observed in HNP1-3 levels between the healthy subjects and both groups of diabetic patients. The mean ± SE was 28.78 ± 4.2 ng/mL in type 1 diabetes, and 29.82 ± 5.36 ng/mL in type 2 diabetes, *vs* 11.94 ± 2.96 ng/mL in controls; $P < 0.01$ respectively. There was no significant difference between patients with type 1 and type 2 diabetes in the high plasma concentrations of HNP1-3. The highest concentrations of α-defensin were found in diabetic patients with nephropathy (49.4 ± 4.8 ng/mL), neuropathy (38.7 ± 4.8 ng/mL) or cardiovascular complications (45.6 ± 1.45 ng/L). There was no significant difference in the cumulative copy numbers of *DEFA1/DEFA3* genes between controls and patients, or between patients with the two types of diabetes. Comparisons of HNP 1-3 plasma level and *DEFA1/A3* copy number of the same patient did not reveal significant relationship between defensin-α levels and the gene copy numbers (r^2 = 0.01). Similarly, no positive correlation was observed between the copy numbers and the mRNA expression levels of *DEFA1/A3*. Regarding the C-44G polymorphism of *DEFB1*, the GG "protective" genotype was much less frequent (1%-2%) among both groups of patients than among controls (9%).

CONCLUSION: Elevated HNP1-3 levels in diabetes are

independent of *DEFA1/DEFA3* copy numbers, but GG genotype of C-44G SNP in *DEFB1* gene may result in decreased defensin β-1 production.

Key words: α-defensins; HNP1-3; β-defensin 1; Diabetes; Copy number polymorphism; Single nucleotide polymorphism

Core tip: There is growing evidence of the role of innate immunity in diabetes To our knowledge our data provide the first report on a complex investigation of defensin-α and defensin β-1 in type 1 and type 2 diabetes. The main conclusion of our manuscript is, that the elevated HNP1-3 levels in diabetes are independent of the *DEFA1/DEFA3* copy numbers, but the GG genotype of C-44G SNP in the *DEFB1* gene may result in a decreased level of defensin β-1 production. Our data support the view that both alpha and beta-defensins may have an important role in the pathogenesis of diabetes and diabetic complications.

Németh BC, Várkonyi T, Somogyvári F, Lengyel C, Fehértemplomi K, Nyiraty S, Kempler P, Mándi Y. Relevance of α-defensins (HNP1-3) and defensin β-1 in diabetes. *World J Gastroenterol* 2014; 20(27): 9128-9137 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9128.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9128

INTRODUCTION

Defensins are members of small antimicrobial peptides of the innate immune system[1,2]. However, today these peptides are also known as danger signals or "alarmins" playing important roles in inflammation and immunity[3]. Mammalian defensins are divided into two major families, the α- and β-defensins. Human α-defensins include human neutrophil peptide 1-4 (HNP1-4) and intestinal human defensins (HD-5 and HD-6) produced by Paneth cells. Besides the antimicrobial effects, alpha defensins display chemotactic activity and induce proinflammatory cytokines[4-6]. HNPs increase the binding of low density lipoprotein (LDL) cholesterol to the endothelial surface suggesting that alpha defensins may modulate the development of atherosclerosis[7]. Neutrophil granulocytes are considered to be the primary cellular origin of α-defensins; HNP 1-3 comprise 30%-50% of the granule proteins. HNPs can be released into the extracellular milieu following granulocyte activation as a consequence of degranulation, leakage, cell death, and lysis during inflammation[8]. α-defensins are also involved in the formation of neutrophil extracellular traps[9].

Human β-defensins make up another family of antimicrobial peptides[1,10]. In addition to their antibacterial and antiviral effects, the chemoattractive function of these defensins has been shown to play a role in immunological reactions that protect the host from various pathogens[11]. While the expression of human defensin beta-1 (HBD1) is generally constitutive, the levels of human defensin beta-2 (HBD2) are inducible by proinflammatory cytokines and bacteria[10,12,13]. Human beta defensins are expressed by epithelial cells of the skin, gut, respiratory and urogenital tissues, the pancreas and the kidneys. HBD1 is also constitutively expressed by leukocytes[14-16].

The level of defensin expression varies among individuals, and it has been suggested that this variation is due to genetic differences in the genes encoding defensins. Defensin genes have been mapped to 8p22-p23[17]. Two types of genetic polymorphisms have been identified in genes encoding defensins: copy number polymorphisms and single nucleotide poymorphisms (SNPs). Human defensin beta-1 (HBD-1) is encoded by the gene *DEFB1* (OMIM: 602056), in which several SNPs (single nucleotide polymorphisms) have been characterized. Three frequent SNPs at positions G-20A (rs11362), C-44G (rs 1800972) and G-52A (rs1799946) in the 5' -untranslated region (UTR) of *DEFB1* were described[18]. The untranslated variants influence HBD-1 expression or function[19].

The cluster of human alpha-defensin genes on chromosome 8 includes the genes *DEFA1* (*OMIM:* 125220) and *DEFA3* (*OMIM:* 604522), which are copy-variables. The genes *DEFA1* and *DEFA3* differ only in a single base substitution in the coding sequence, corresponding to a single amino acid difference between the peptides encoded[20]. HNP 1-3 differs only in a single N-terminal acid, and the HNP-2 peptide lacks this residue and might be a proteolytic product of the other two peptides because no separate gene has been identified to encode HNP-2[21]. Several copy number polymorphisms form the major source of genetic polymorphism of α-defensin genes *DEFA1* and *DEFA3*, encoding human neutrophil peptides HNP-1, -2 and 3[20,22]. These genes are present in a cluster that is close to but independent from the β-defensin cluster on 8p23. The total *DEFA1/DEFA3* copy number has been found to range between 4 and 11 copies per diploid genome with 5 to 9 copies being the most common[22].

To date, little is known about the genetic basis and the functions of α- and β-defensins in diabetes. Infections are frequent in diabetic patients because the antimicrobial function of their immune response is impaired. It has been reported that mRNA levels of rat β-defensin-1 are significantly low in the kidneys, which may explain the high incidence of urinary tract infections in diabetes mellitus[23]. The effects of glucose and insulin on the β-defensin expression have recently been demonstrated[24], but no connection has been found between genetic polymorphisms of the *HBD1* gene and diabetes in a Brazilian study on diabetic children[25].

Increased levels of alpha-defensin -1, -2 and -3 have recently been reported in patients with type 1 diabetes with nephropathy and in cardiovascular complica-

tions[26,27]. It is tempting to speculate whether copy number polymorphisms and the *DEFA1/DEFA3* mRNA in the granulocytes may influence the levels of HNP1-3 in patients with types 1 and 2 diabetes.

The aim of our study was to investigate the genetic background of human defensin-α and human defensin β-1 production in adult patients with type 1 and type 2 diabetes, especially with complications. Therefore, we carried out an association study between *DEFA1/DEFA3* copy number polymorphism and diabetes, and between the promoter polymorphisms of DEFB1 and diabetes. We also measured the plasma levels of HNP 1-3 in both types of diabetes, and the mRNA expression of *DEFA1/DEFA3* in leukocytes.

MATERIALS AND METHODS

Patients

257 diabetic patients (122 men and 135 women) were enrolled in our study, which included 117 patients with type 1 and 140 patients with type 2 diabetes. All patients participating in the study were diagnosed according to the ADA criteria: Diagnosis and classification of diabetes mellitus. Diabetes Care 36 (Suppl 1) 2013. S64-S74.

The mean age of type 1 diabetic patients was 40.6 years ± 1.51 years, the mean duration of diabetes was 17.7 ± 1.12 years, and their mean HbA1c was 8.86% ± 0.17%. In type 2 diabetic subjects, the mean age was 58.4 ± 1.27 years, the mean duration of diabetes was 14.5 ± 0.8 years, and the mean HbA1c was 8.03% ± 0.13%.

Seventy-one subjects in the cohort had diabetic nephropathy (32 with type 1 and 39 with type 2 diabetes) defined as an albumin-to-creatinine ratio in a random spot collection being higher than 3.4 mg/mmol, or the protein content being over 300 mg/d in collected urine. Abnormal kidney function was described when the glomerular filtration rate (GFR) was lower than 60 mL/min per 1.73 m^2.

One hunred twenty-one patients suffered from retinopathy (47 with type 1 and 68 with type 2 diabetes). This complication was evidenced as the presence of background or proliferative retinopathy, macular edema or diabetes-related blindness, or the administration of retinal photocoagulation therapy. The retinopathy status was checked by color stereo-ophthalmography and fluorescence angiography. Neuropathy was diagnosed in 95 patients (35 with type 1 and 60 with type 2 diabetes). Neuropathy was proven when abnormal peripheral sensory functions or altered lower limb tendon reflexes as well as impaired cardiovascular reflex tests were detected. 54 diabetic patients (14 with type 1 diabetes and 40 with type 2 diabetes) had previously been diagnosed with macrovascular disease including major coronary events, stroke or a transient ischemic attack, peripheral artery disease or amputation. A high number of the patients (182) had controlled hypertension (50 with type 1 and 132 with type 2 diabetes).

The control group consisted of 221 age- and gender-matched healthy blood donors. These control subjects were selected from blood donors at the regional Center of Hungarian National Blood Transfusion Service, Szeged, Hungary. The exclusion criteria for blood donors were diabetes, nephropathy, hypertension, or ischemic heart disease. All cases and controls were of Hungarian ethnic origin and resident in Hungary. Informed consent was obtained from all patients and controls, and the local Ethics Committee gave prior approval to the study. All patients consented to the study and were treated according to the Patient Right Protection Act of our institutions and according to international guidelines.

Assay of HNP 1-3 concentration

Blood samples containing EDTA were obtained from patients and controls. Plasma was isolated after the blood was centrifuged at 3000 g for 3 min and stored at -80 ℃ for further analysis. The HNP1-3 concentrations in plasma were determined by ELISA (Hycult-Biotech HK324, Uden, The Netherlands) according to the instructions of the manufacturer.

DNA isolation

Genomic DNA purified from peripheral blood was used. Leukocyte DNA was isolated using the High Pure PCR Template Preparation Kit according to the instructions of the manufacturer (Roche Diagnostic GmbH, Mannheim, Germany). DNA concentrations were measured with a Qubit™ fluorometer (Invitrogen, Carlsbad, CA, United States) according to the instructions of the manufacturer. Genomic DNA was stored at -20 ℃ until further use.

Determination of DEFA1/DEFA3 gene copy numbers by quantitative real-time PCR

Genomic DNA purified from peripheral blood was used. Gene copy number determination was carried out as previously described by Linzmeier[20] with slight modifications. BIO-RAD CFX 96 instrument (Bio-Rad, Hercules, CA, United States) was used for quantitation. The reaction volume was 15 μL, containing 3 μL of DNA, 1 μmol L^{-1} each of the primers, 7.5 μL of reaction buffer (Fermentas Probe/ROX qPCR MasterMix, Fermentas, Lithuania) and 0.6 μL EVAGreen (20 × EVAGreen™ Biotium Inc., Hayward, CA, United States). Forward primer: DEFA1 1 F (5' TAC CCA CTG CTA ACT CCA TAC 3'), reverse primer: DEFA1 1 R (5' GAA TGC CCA GAG TCT TCC C 3'); MPO (myeloperoxidase) reference gene primer set MPO 1 F (5' CCA GCC CAG AAT ATC CTT GG 3'), MPO 1 R (5' GGT GAT GCC TGT GTT GTC G 3'). PCR conditions were as follows: initial denaturation at 95 ℃ for 10 min followed by 40 cycles of denaturation (95 ℃ for 15 s) and extension (54 ℃ for 1 min).

Quantification was performed by monitoring the emitted fluorescence after each cycle of PCR reaction of genomic DNA samples in order to identify the exact time point at which the log-linear phase could be dis-

tinguished from the background (crossing point). The precise amount of DNA added to each reaction mix was based on optical density. Each DNA sample was analyzed in triplicate, in 2 independent experiments.

DEFA1/DEFA3 mRNA quantification by RT PCR

We collected further 2 mL of venous blood in EDTA tubes from patients with diabetes and controls. Leukocytes from blood were separated by centrifugation at 1200 rpm/min for 15 min. By using the reverse transcription polymerase chain reaction (RT-PCR), we examined the expression of *DEFA1/DEFA3* mRNA in 24 patients with diabetes (12 cases of type 1 and 12 cases of type 2 diabetes). Total RNA was extracted with High Pure RNA isolation kit (Roche) according the manufacturer's instruction. RNA concentration was determined by the A_{260} value of the sample. Complementary DNA (cDNA) was generated from 1 μg total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a final volume of 20 μL. After reverse transcription, amplification was carried out by using Light Cycler Fast Start DNA Master^{PLUS} SYBR Green I mix (Roche). Samples were loaded into capillary tubes and placed in the fluorescence thermocycler (LightCycler). Initial denaturation at 95 ℃ for 10 min was followed by 45 cycles of 95 ℃ for 10 s, annealing at 58 ℃ for 8 s, and elongation at 72 ℃ for 12 s. DEFA1/DEFA3 sense, 5'-TCC CAG AAG TGG TTG TTT CC-3'; and antisense, 5'-GCA GAA TGC CCA GAG TCT TC-3', and for the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) sense, 5' -AAG GTC GGA GTC AAC GGA TTT-3'; antisense, 5'-TGG AAG ATG GTG ATG GGA TTT-3' primers were used to amplify specific products from cDNA samples. At the end of each run, melting-curve profiles were achieved by cooling the sample to 40 ℃ for 15 s, and then heating the sample slowly at 0.20 ℃/s up to 95 ℃ with continuous measurement of the fluorescence to confirm the amplification of specific transcripts. Cycle-to-cycle fluorescence emission readings were monitored and analyzed by using LightCycler software (Roche Diagnostics GmbH). All quantifications were normalized to the housekeeping *GAPDH* gene. Relative gene expression was determined by using the ΔΔCt method.

Genotyping of SNPs of human defensin beta-1 (DEFB1)

Genotyping was performed by means of Custom TaqMan® SNP Genotyping Assays (Applied Biosystems, CA). Fluorogenic minor groove binder probes were used for each case using the dyes 6-carboxyfluorescein (FAM; excitation, 494 nm) and VIC (excitation, 538 nm): beta-defensin-1 polymorphisms *DEFB1* G-20A (rs11362) Applied Biosystems code c_11636793_20, *DEFB1* C-44G (rs1800972) c_11636794_10 and *DEFB1* G-52A (rs1799946) c_11636795_20. Thermal cycling was performed on ABI Prism 7000 sequence-detection PCR systems. The amplification mix contained the following ingredients: 7.5 μL of TaqMan® universal PCR master mix (Applied Biosystems, CA), 0.375 μL of primer-probe mix, 6.375 μL of RNase- and DNase-free water (Sigma), and 0.8 μL of sample DNA, in a total volume of 15 μL per single tube reaction. Assay conditions were 2 min at 50 ℃, 10 min at 95 ℃, and 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. Each 96-well plate contained 90 samples of an unknown genotype and six reactions with reagents but no DNA. DNase-free water was used as nontemplate control. Initial and postassay analysis was performed by using the Sequence Detection System (SDS) version 2.1 software (Applied Biosystems, CA) as outlined in the TaqMan Allelic Discrimination Guide. Genotypes were determined visually based on the dye-component fluorescent emission data depicted in the X-Y scatter plot of the SDS software. Genotypes were also determined automatically by the signal processing algorithms in the software. Results of each scoring method were saved in two separate output files for later comparison.

Statistical analysis

Comparisons of plasma concentrations were carried out by the Mann-Whitney test and with two-tailed paired Student test. The level of significance of the genotype frequency of different *DEFB1* SNPs was analyzed by using the χ^2 test and the Fischer test. Levels $P < 0.05$ indicated statistical significance. All statistical calculations were performed with the Graph Pad Prism 5.0 statistical program (GraphPad Software, San Diego CA. United States). The genotype frequencies for each polymorphism of DEFB1 were tested for deviation from the Hardy-Weinberg equilibrium by the χ^2 test with 1 degree of freedom.

RESULTS

Plasma levels of HNP1-3 in patients with type 1 and type 2 diabetes

In a pilot study, plasma levels of α-defensin, HNP1-3, in 50 patients with type 1 diabetes and in 60 patients with type 2 diabetes were determined and compared with those of 50 healthy blood donors. There was a high individual variation in the plasma levels of α-defensin, but significant differences were observed between the healthy subjects and both groups of diabetic patients. The mean value ± SE was 28.78 ± 4.2 ng/mL in patients with type 1 diabetes and 29.82 ± 5.36 ng/mL in patients with type 2 diabetes *vs* 11.94 ± 2.96 ng/mL in controls ($P < 0.01$ respectively). The difference between the high plasma concentrations of HNP1-3 in patients with type 1 or type 2 diabetes was not significant (Figure 1).

Plasma levels of HNP1-3 in diabetic patients with complications

After that, we checked whether the generally high level of HNP1-3 in the peripheral blood of both groups (type 1 and type 2) of diabetic patients was connected to diabetic complications. Nephropathy was diagnosed in 71

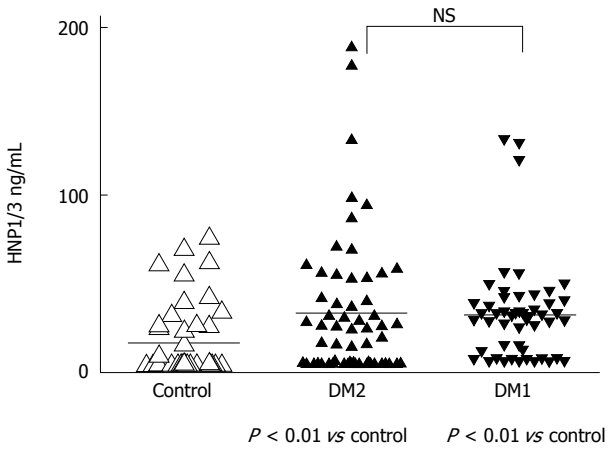


Figure 1 Plasma levels of HNP1-3 in patients with type 1 and type 2 diabetes (DM1 and DM2) and healthy controls. The line represents the mean plasma levels of HNP1-3. Significant differences as determined by Mann-Whitney test are indicated. NS: Not significant.

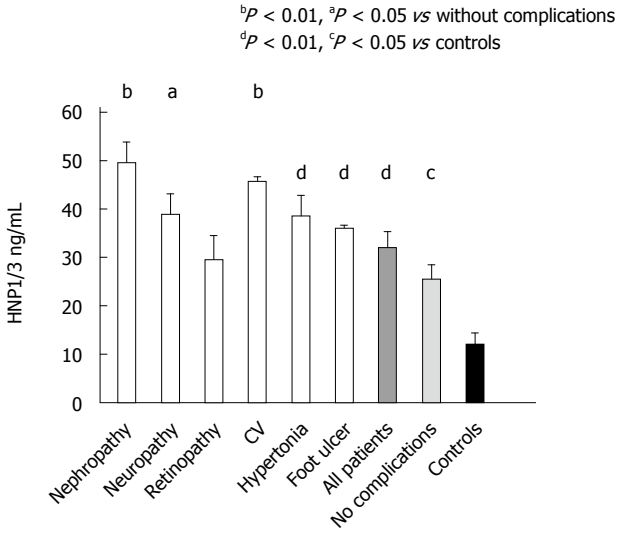


Figure 2 Plasma levels of human neutrophil peptides in diabetic patients with different complications relative to those without complications, and to healthy controls. Mean ± SE are indicated; significant differences between groups were determined by unpaired *t*-test.

patients (32 with type 1 and 39 with type 2 diabetes) and neuropathy in 95 ones (35 with type 1 and 60 with type 2 diabetes). 115 patients suffered from retinopathy (47 with type 1 and 68 with type 2 diabetes), 54 patients had cardiovascular diseases (14 with type 1 diabetes and 40 with type 2 diabetes), and 182 had hypertension (50 with type 1 and 132 with type 2 diabetes). Their data concerning the HNP1-3 levels were compared with those of the 67 patients who did not suffer from these complications, and with the data of the 100 healthy subjects (Figure 2). The highest HNP 1-3 concentrations were found in the diabetic patients with nephropathy (49.4 ± 4.8 ng/mL) and with neuropathy (38.7 ± 4.8 ng/mL) or with cardiovascular complications (45.6 ± 1.4 ng/mL). These concentrations were significantly higher than those in the diabetic patients without complications (25.4 ± 3.5

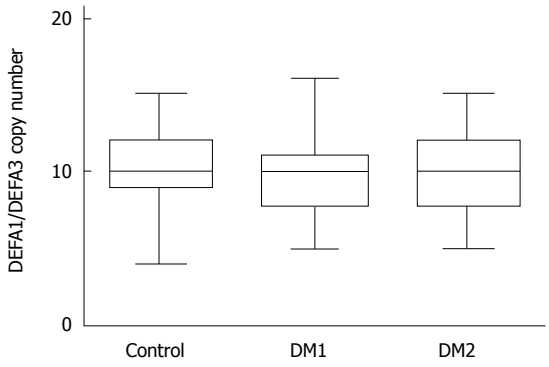


Figure 3 Genomic copy number of *DEFA1/DEFA3* in patients with diabetes and in healthy blood donors. Quantitative box-plot analysis (median, minimum, maximum value, 25% and 75% percentiles) of *DEFA1/A3* copy numbers determined in DNA samples from 133 controls, 100 patients with type 1 diabetes (DM1), and 100 patients with type 2 diabetes (DM2).

ng/mL). These data are in accordance with previous observations[26,27] of high levels of HNP 1-3 in type 1 diabetic patients with cardiovascular diseases. In a relatively smaller group of patients (n = 28) with diabetic foot ulcer (20 with type 1 diabetes and 8 with type 2 diabetes), the HNP1-3 plasma levels were 35.9 ± 1.1 ng/mL. These high HNP1-3 levels might be the consequence of the degranulation of recruited neutrophils from the skin frequently following infections. Our results suggest that in diabetic complications such as nephropathy, neuropathy and cardiovascular diseases, the HNP1-3 level in the circulation is elevated independently of the type of diabetes. All diabetic patients, with or without complications, exhibited significantly higher plasma levels of HNP1-3 than the control subjects (Figure 2).

Copy number polymorphism of DEFA1/DEFA3

The cumulative copy numbers of *DEFA1/DEFA3* were determined by using quantitative PCR analysis. In the control group, 133 DNA samples were used for copy number determination and 100 DNA samples of diabetic patients with type 1 or type 2 diabetes. There was no significant difference in copy number between the controls and the patients or between the patients with the two types of diabetes (Figure 3). In the control group, *DEFA1/DEFA3* copy numbers ranged from 4 to 15 per genome with a median number of 10 copies. The median copy number of *DEFA1/DEFA3* in the patients with type 1 diabetes was 10 copies per genome (range 5 to 16 copies), and that in the patients with type 2 diabetes was also 10 (range 5 to 15). Comparing the HNP 1-3 plasma level and the *DEFA1/A3* copy number of the same patient, no significant correlation was observed between defensin levels and genomic copy numbers (r^2 = 0.01; Figure 4.).

DEFA1/DEFA3 gene expression

The expression of *DEFA1/A3* was measured in the peripheral leukocytes of diabetic patients. *DEFA1/DEFA3* mRNA was determined in blood samples from 12 pa-

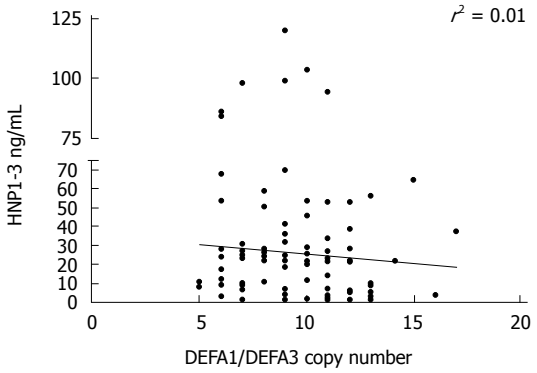


Figure 4 Plasma levels of HNP1-3 in diabetic patients *vs* copy numbers of *DEFA1/DEFA3*. The *DEFA1/A3* copy numbers were determined by quantitative PCR analysis and compared to the HNP1-3 plasma level of the same patient (50 with type 1 and 50 with type 2 diabetes).

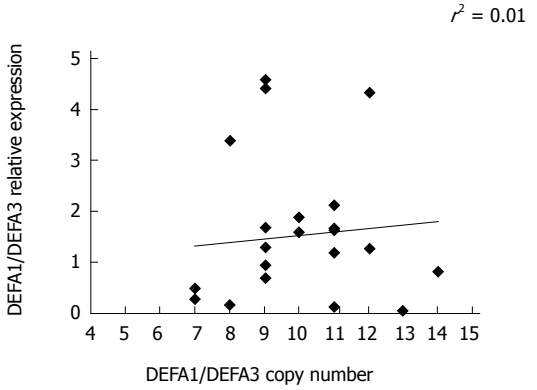


Figure 5 Relative mRNA expression levels of *DEFA1/DEFA3* in diabetic patients *vs* copy numbers of *DEFA1/DEFA3*. The *DEFA1/A3* copy numbers were determined by quantitative PCR analysis and compared to the *DEFA1/DEFA3* mRNA measured by RT-PCR of the same patient (12 with type 1 and 12 with type 2 diabetes).

tients with type 1 and another 12 with type 2 diabetes, and the relative expressions of *DEFA1/DEFA3* were compared with the *DEFA1/DEFA3* copy numbers of the same patients (Figure 5). The data indicated that peripheral leukocytes had the ability to transcribe *DEFA1/DEFA3* genes (mean ± SE of relative expression 1.5 ± 0.28) and to biosynthetize HNP1-3 peptides. However, no positive correlation was observed between the copy numbers and the expression levels of the human neutrophil peptide 1-3 (Figure 5). The variation in expression levels between individuals did not exhibit a positive correlation with the copy number. Similarly, the expression of specific mRNA in the leukocytes for HNP 1-3 did not parallel the HNP 1-3 plasma levels (data not shown).

DEFB1 G-20A, DEFB1 G-52A and DEFB1 C-44G polymorphisms

The genotypic distributions of *DEFB1* G-20A, and *DEFB1* G-52A and *DEFB1* C-44G polymorphisms are presented in Table 1.

The distribution of the *DEFB1* G-20A genotypes was in accordance with the Hardy-Weinberg equilibrium both in the control population and in the patients (P = 0.912 and P = 0.795, respectively). There was no significant difference in genotype distribution between the patients overall and the healthy controls. Similarly, no significant differences in genotypes were observed when the patients were grouped according to type 1 and type 2 diabetes.

Table 1 Genotypes of DEFB1 G-20A, DEFB1 G-52A, and DEFB1 C-44G polymorphisms in patients with diabetes *n* (%)

DEFB1 G-20A	GG	GA	AA	χ^2 test[1]
Patients with diabetes *n* = 257	82 (32)	131 (51)	44 (17)	0.568
Type 1 diabetes *n* = 117	36 (31)	60 (51)	21 (18)	0.775
Type 2 diabetes *n* = 140	46 (33)	71 (51)	23 (16)	0.573
Controls *n* = 200	62 (31)	96 (48)	42 (21)	
DEFB1 G-52A	GG	GA	AA	χ^2 test[1]
Patients with diabetes *n* = 257	114 (44)	104 (40)	39 (15)	0.572
Type 1 diabetes *n* = 117	52 (44)	47 (40)	18 (17)	0.702
Type 2 diabetes *n* = 140	62 (44)	57 (41)	21 (15)	0.658
Controls *n* = 200	80 (40)	84 (42)	36 (18)	
DEFB1 C-44G	CC	CG	GG	χ^2 test[1]
Patients with diabetes *n* = 257	156 (61) +	95 (37)	6 (2)[2]	0.002
Type 1 diabetes *n* = 117	70 (60)	44 (37)	3 (2.5)	0.01
Type 2 diabetes *n* = 140	86 (61)	51 (36)	3 (2)	0.003
Controls *n* = 200	90 (45)	92 (46)	18 (9)	

[1]χ^2 test *vs* controls; [2]Fisher test *vs* controls, P = 0.001, OR = 9.136, 95%CI: 3.512-23.82; +: Fisher test *vs* controls, P = 0.0009, OR = 2.005, 95%CI: 1.218-2.746.

As concerns the *DEFB1* G-52A SNP, distribution of the genotypes was in accordance with the Hardy-Weinberg equilibrium both in the control population and in the patients (P = 0.252 and P = 0.181, respectively). We did not detect any significant difference in genotypes between the patients and the controls, either in type 1 or type 2 diabetes.

The genotypic distribution of DEFB1 C- 44G polymorphism is shown in Table 1.

The distribution of genotypes was in accordance with the Hardy-Weinberg equilibrium among the patients with diabetes (P = 0.151) and also in the control population (P = 0.722). But there was a significant difference in genotype distribution between the patients overall and the healthy controls (χ^2 test, P = 0.002). The frequency of the GG genotype was significantly lower in both types of diabetes (2.5% and 2%, respectively) than in the healthy controls (9%) (Fisher test *vs* control, P = 0.001, OR = 9.136, 95%CI: 3.512-23.82). Conversely, the prevalence of the *DEFB1* CC genotype was 61 % in the group of diabetic patients *vs* 45% in the controls (Fisher

test: $P = 0.0009$, OR = 2.055, 95%CI: 1.248-2.746). When the patients were grouped according to the diabetic complications, there was a lower frequency of the GG genotype among the patients with nephropathy and among those with neuropathy (1.4% and 1%, respectively).

DISCUSSION

HNP1-3 levels in the circulation were measured, and the copy number variation of *DEFA1/A3* genes was determined in diabetic patients. The diabetic patients exhibited overall higher plasma levels of HNP 1-3 (α-defensin) with either type 1 or type 2 form of the disease than the healthy controls. The highest concentrations of HNPs were detected in patients with nephropathic or neuropathic and cardiovascular complications. An essential question arises as to why an increased concentration of plasma α-defensin (HNP1-3) level is associated with type 1 and type 2 diabetes, especially in the event of diabetic complications such as nephropathy, neuropathy or cardiovascular problems. The explanation might be that the elevation in the plasma HNP1-3 level is the consequence of the decreased renal degradation of the peptides in patients with advanced nephropathy[26]. HNP 1-3 promote the accumulation of low density lipoprotein in the vasculature, inhibit fibrinolytic activity on the surface of vascular cells, and accumulate in the intima of atherosclerotic plaques. Therefore, HNP 1-3 may have clinical implications in diabetic patients with hypercholesteremia or vascular dysfunction[7,27].

The association of high levels of HNP1-3 in patients with neuropathy in type 1 and type 2 diabetes is yet to be clarified. The next question is whether there is an increased gene expression responsible for the elevated plasma levels of α-defensins. Our study revealed that there was no correlation between HNP1-3 plasma levels and the copy numbers of *DEFA1/A3* genes. The effect of copy number variations in *DEFA1/DEFA3* on the disease or even on the plasma concentrations of the peptides remain unclear. Controversal data have been published about the correlation between *DEFA1/A3* copy number and the α-defensin peptide concentration. Linzmeier and Ganz[20] have shown that the intracellular HNP1-3 levels in human neutrophils are proportional to the copy numbers of the *DEFA1* and *DEFA3* genes Copy numbers of *DEFA1/A3* may be proportional to the intracellular levels of HNP1-3 in neutrophil granulocytes but possibly not to the circulating HNP1-3 levels. Additionally, a discrepancy between gene copy number and the HNP1-3 protein levels has recently been reported in septic patients[28]. There are several potential explanations of the discrepancy between the gene copy number and the plasma levels. HNP1-3 is stored primarily in the granules of neutrophils and is released into the circulation during the activation of the neutrophils. Moreover, it might also be due to different transcriptional mechanisms modulating these genes or to an increased distance between the regulators of the genes[28,29].

The fact that in our study no significant correlation was observed between the genomic copy number variation of *DEFA1/DEFA3* and the mRNA expression levels (Figure 5) suggests that the degranulation rather than the increased gene expression may be responsible for the increased plasma HNP 1-3 levels in diabetes.

Similar observations have been published about the copy number polymorphism and expression level variation of *DEFA1* and *DEFA3* genes[22]. In that study, the combined expression levels of *DEFA1/A3* and the genomic copy number have not been correlated, suggesting the superimposed influence of trans-acting factors.

It is noteworthy that there are several examples of the absence of a correlation between copy number polymorphisms and the relative transcription level[29].

There are no direct data showing that exaggerated degranulation is linked to diabetic complications, or it is higher in DM patients than in controls. However, it has recently been published that pro-inflammatory conditions during hyperglycemia favor NET - neutrophil extracellular traps - formation[30], and HNP 1-3 are also involved in the formation of neutrophil extracellular traps[9]. It is noteworthy that diabetes is associated with low grade, sub-clinical and chronic inflammation characterized by abnormal cytokine production. Therefore, the diabetic microenvironment can induce NET formation, which may result in a basic high HNP-1 concentration in the circulations.

In order to detect whether increased mRNA expression is responsible for elevated defensin levels in diabetic patients, quantitative RT-PCR reactions were performed. Expression of specific mRNA in the leukocytes was observed for HNP 1-3 but not parallel with HNP1-3 plasma levels. The mRNA values between patients and controls were rather equal (mean ± SE of relative expression 1.5 ± 0.28 *vs* 1.49 ± 0.35, respectively) suggesting that not an increased gene expression may be responsible for increased plasma levels of HNP 1-3. Our findings were in good correlation with the observations of Fang *et al*[31], that is, α-defensin genes were constitutively transcribed at low level in mature neutrophils, but they were not inducible.

Eosinophils with transcriptionally active α-defensin production have recently been detected in the capillary blood of diabetic patients[32]. Eosinophils but not neutrophils displayed the augmentation of transcriptional activation of α-defensin expression. In our study, the majority of the cells in peripheral venous blood were neutrophils; therefore, our purified DNA and RNA samples were derived mostly from neutrophils.

The present study demonstrated that the distributions of the C-44G genotypes were different between patients with diabetes and healthy controls, whereas the frequency of the GG genotype was significantly higher in the control population. It indicates that the presence of G allele probably leads to strengthened HBD1 antimicrobial activity, which is less frequent in patients with

diabetes. The G allele of C-44G SNP generates a putative binding site for nuclear factor κB (NF-κB), and it is very likely to induce an overexpression. The proposed effect of this SNP could partially explain why the GG genotype was considered to be a protective genotype in atopic dermatitis[33] and also in the susceptibility to Candida colonization in diabetic patients[34]. Conversely, in these studies, subjects carrying the CC genotype at the -44 locus site of the gene were at a greater risk of acquiring infection. It has been recently suggested that the C allele of *DEFB1* C-44G SNP probably abrogates NF-κB-dependent *DEFB1* upregulation[35].

These data are consistent with our present observation that the GG phenotype could also be protective in diabetes, and *vice versa*, the higher frequency of CC genotype might be connected with lower expression of human defensin β-1. Among the 257 patients with diabetes, only 6 (2%) were GG homozygotes, and 61% of the patients were CC homozygotes, as compared with 45% of CC homozygotes in the control group. Furthermore, the number of GG homozygotes was even lower (1%) among patients with nephropathy and neuropathy. These observations draw the attention to the importance of *DEFB1* polymorphisms in diabetes, especially in the cases with nephropathy and neuropathy. Foot ulcerations in diabetic patients are often combined with infections. None of the 28 patients with foot ulcer displayed GG genotype of C- 44G SNP of DEFB1 gene. A high blood glucose level itself can result in low levels of β-defensin[23], and it might be further downregulated in humans as a consequence of C-44G polymorphism. It is noteworthy that insulin is an important factor mediating hBD-1 expression[24].

Taken together, our study demonstrated elevated levels of α-defensin (HNP1-3) in type 1 and type 2 diabetes, which were more pronounced when there were diabetic complications. However, there was no correlation between the circulating HNP1-3 levels and the *DEFA1/DEFA3* copy number. Similarly, no correlation was found between the mRNA expression and the copy number variation. Further studies are needed to explore whether the elevated α-defensin levels of the plasma in diabetes are causally linked to this disease and its complications, or they are simply the consequences of the degranulation of neutrophils under pathologic conditions. Whatever the mechanism, the elevated HNP1-3 level might not be genetically determined or at least independent of the copy number variation of the *DEFA1/DEFA3* genes. In contrast, the CC genotype of the C-44G SNP of *DEFB1* was more frequent in diabetic patients than in healthy controls, which draws the attention to the genetic background of a potentially impaired function of hBD1 (human defensin β-1) in diabetes. These data support the view that both alpha and beta-defensins may have important roles in the pathogenesis of diabetes and diabetic complications. Our results should be regarded as preliminary results, which should be confirmed on a larger series of patients in a future multicenter study.

ACKNOWLEDGMENTS

We thank Mrs.Györgyi Müller for expert technical assistance.

COMMENTS

Background

There is a growing evidence of the role of innate immunity in diabetes. Defensins are members of small antimicrobial peptides of the innate immune system. In addition to their antibacterial and antiviral effects, immunologic functions of defensins has been shown to play a role in the homeostasis. To date, little is known about the genetic basis and the functions of α- and β-defensins in diabetes. The aim of this study was to investigate the genetic background of human defensin-α and human defensin β-1 production in adult patients with type1 and type 2 diabetes, especially with complications.

Research Frontiers

Neutrophil granulocytes are considered to be the primary cellular origin of α-defensins; HNP 1-3. HNPs can be released into the extracellular milieu following granulocyte activation as a consequence of degranulation, leakage, cell death, and lysis during inflammation. Human beta defensins are expressed mainly by epithelial cells of the skin, gut, respiratory and urogenital tissues, the pancreas and the kidneys. The level of defensin expression varies among individuals, and it has been suggested that this variation is due to genetic differences in the genes encoding defensins.

Innovations and breakthroughs

These data provide the first report about a complex investigation of defensin-alpha and defensin β-1 in type 1 and type 2 diabetes. Increased levels of alpha-defensin -1, -2 and -3 have recently been reported in patients with type 1 diabetes with nephropathy and in cardiovascular complication. In this study not only HNP1-3 levels in the circulation were measured, but also the copy number variation of *DEFA1/A3* genes was determined in diabetic patients, together with the expression of *DEFA1/A3* in peripheral leukocytes. Several SNPs (single nucleotide polymorphisms) have been characterized of human *DEFB1* (human defensin β-1) gene in previous studies. In this complex study the authors demonstrated that elevated HNP1-3 levels in diabetes are independent of *DEFA1/DEFA3* copy numbers, but the GG genotype of C-44G SNP in *DEFB1* gene may result in decreased β1-defensin production.

Applications

The data support the view, that both alpha and beta-defensins may have an important role in the pathogenesis of diabetes and diabetic complications. The results may contribute to a better understanding of the roles of defensins in the pathomechanism of diabetes and may represent a future possibility toward broadening of the prognostic laboratory markers.

Terminology

HNP 1-3 are Human Neutrophil Peptides, members of the human α-defensin family. Human defensin β-1 (HBD1) is the member of another family of antimicrobial peptides. Two types of genetic polymorphisms have been identified in genes encoding defensins: copy number polymorphisms and single nucleotide poymorphisms (SNPs). Several copy number polymorphisms form the major source of genetic polymorphism of α-defensin genes DEFA1 and *DEFA3*, encoding human neutrophil peptides Human defensin beta-1 (HBD-1) is encoded by the gene *DEFB1*. Three frequent SNPs at positions G-20A (rs11362), C-44G (rs 1800972) and G-52A (rs1799946) in the 5'-untranslated region (UTR) of *DEFB1* were described. The untranslated variants influence HBD-1 expression or function.

Peer review

The current manuscript addresses the role of defensins in patients with type 1 and type 2 diabete. The authors, in addition to the protein levels, correlate absolute mRNA expression to the diabetic patients. The authors explain the biological role/relevance of the defensine increase.

REFERENCES

1 **Ganz T**. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* 2003; **3**: 710-720 [PMID: 12949495

DOI: 10.1038/nri1180]
2 **Chen H**, Xu Z, Peng L, Fang X, Yin X, Xu N, Cen P. Recent advances in the research and development of human defensins. *Peptides* 2006; **27**: 931-940 [PMID: 16226346 DOI: 10.1016/j.peptides.2005.08.018]
3 **Oppenheim JJ**, Yang D. Alarmins: chemotactic activators of immune responses. *Curr Opin Immunol* 2005; **17**: 359-365 [PMID: 15955682 DOI: 10.1016/j.coi.2005.06.002]
4 **Bowdish DM**, Davidson DJ, Hancock RE. A re-evaluation of the role of host defence peptides in mammalian immunity. *Curr Protein Pept Sci* 2005; **6**: 35-51 [PMID: 15638767 DOI: 10.2174/1389203053027494]
5 **Chertov O**, Yang D, Howard OM, Oppenheim JJ. Leukocyte granule proteins mobilize innate host defenses and adaptive immune responses. *Immunol Rev* 2000; **177**: 68-78 [PMID: 11138786 DOI: 10.1034/j.1600-065X.2000.17702.x]
6 **Yang D**, Biragyn A, Kwak LW, Oppenheim JJ. Mammalian defensins in immunity: more than just microbicidal. *Trends Immunol* 2002; **23**: 291-296 [PMID: 12072367 DOI: 10.1016/s1471-4906(02)02246-9]
7 **Quinn K**, Henriques M, Parker T, Slutsky AS, Zhang H. Human neutrophil peptides: a novel potential mediator of inflammatory cardiovascular diseases. *Am J Physiol Heart Circ Physiol* 2008; **295**: H1817-H1824 [PMID: 18805897 DOI: 10.1152/ajpheart.00472.2008]
8 **Ganz T**. Extracellular release of antimicrobial defensins by human polymorphonuclear leukocytes. *Infect Immun* 1987; **55**: 568-571 [PMID: 3643886]
9 **Papayannopoulos V**, Zychlinsky A. NETs: a new strategy for using old weapons. *Trends Immunol* 2009; **30**: 513-521 [PMID: 19699684 DOI: 10.1016/j.it.2009.07.011]
10 **Pazgier M**, Hoover DM, Yang D, Lu W, Lubkowski J. Human beta-defensins. *Cell Mol Life Sci* 2006; **63**: 1294-1313 [PMID: 16710608 DOI: 10.1007/s00018-005-5540-2]
11 **Dürr M**, Peschel A. Chemokines meet defensins: the merging concepts of chemoattractants and antimicrobial peptides in host defense. *Infect Immun* 2002; **70**: 6515-6517 [PMID: 12438319 DOI: 10.1128/iai.70.12.6515-6517.2002]
12 **Boman HG**. Antibacterial peptides: basic facts and emerging concepts. *J Intern Med* 2003; **254**: 197-215 [PMID: 12930229 DOI: 10.1046/j.1365-2796.2003.01228.x]
13 **De Smet K**, Contreras R. Human antimicrobial peptides: defensins, cathelicidins and histatins. *Biotechnol Lett* 2005; **27**: 1337-1347 [PMID: 16215847 DOI: 10.1007/s10529-005-0936-5]
14 **Zhao C**, Wang I, Lehrer RI. Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells. *FEBS Lett* 1996; **396**: 319-322 [PMID: 8915011 DOI: 10.1016/0014-5793(96)01123-4]
15 **Schnapp D**, Reid CJ, Harris A. Localization of expression of human beta defensin-1 in the pancreas and kidney. *J Pathol* 1998; **186**: 99-103 [PMID: 9875146]
16 **Duits LA**, Ravensbergen B, Rademaker M, Hiemstra PS, Nibbering PH. Expression of beta-defensin 1 and 2 mRNA by human monocytes, macrophages and dendritic cells. *Immunology* 2002; **106**: 517-525 [PMID: 12153515 DOI: 10.1046/j.1365-2567.2002.01430.x]
17 **Hollox EJ**, Barber JC, Brookes AJ, Armour JA. Defensins and the dynamic genome: what we can learn from structural variation at human chromosome band 8p23.1. *Genome Res* 2008; **18**: 1686-1697 [PMID: 18974263 DOI: 10.1101/gr.080945.108]
18 **Dörk T**, Stuhrmann M. Polymorphisms of the human beta-defensin-1 gene. *Mol Cell Probes* 1998; **12**: 171-173 [PMID: 9664579 DOI: 10.1006/mcpr.1998.0165]
19 **Sun CQ**, Arnold R, Fernandez-Golarz C, Parrish AB, Almekinder T, He J, Ho SM, Svoboda P, Pohl J, Marshall FF, Petros JA. Human beta-defensin-1, a potential chromosome 8p tumor suppressor: control of transcription and induction of apoptosis in renal cell carcinoma. *Cancer Res* 2006; **66**: 8542-8549 [PMID: 16951166 DOI: 10.1158/0008-5472.can-06-0294]
20 **Linzmeier RM**, Ganz T. Human defensin gene copy number polymorphisms: comprehensive analysis of independent variation in alpha- and beta-defensin regions at 8p22-p23. *Genomics* 2005; **86**: 423-430 [PMID: 16039093 DOI: 10.1016/j.ygeno.2005.06.003]
21 **Selsted ME**, Ouellette AJ. Mammalian defensins in the antimicrobial immune response. *Nat Immunol* 2005; **6**: 551-557 [PMID: 15908936 DOI: 10.1038/ni1206]
22 **Aldred PM**, Hollox EJ, Armour JA. Copy number polymorphism and expression level variation of the human alpha-defensin genes DEFA1 and DEFA3. *Hum Mol Genet* 2005; **14**: 2045-2052 [PMID: 15944200 DOI: 10.1093/hmg/ddi209]
23 **Froy O**, Hananel A, Chapnik N, Madar Z. Differential effect of insulin treatment on decreased levels of beta-defensins and Toll-like receptors in diabetic rats. *Mol Immunol* 2007; **44**: 796-802 [PMID: 16740310 DOI: 10.1016/j.molimm.2006.04.009]
24 **Barnea M**, Madar Z, Froy O. Glucose and insulin are needed for optimal defensin expression in human cell lines. *Biochem Biophys Res Commun* 2008; **367**: 452-456 [PMID: 18178160 DOI: 10.1016/j.bbrc.2007.12.158]
25 **Guimarães RL**, Segat L, Rocha CR, Brandão LA, Zanin V, Araujo J, Naslavsky MS, de Lima Filho JL, Crovella S. Functional polymorphisms of DEFB1 gene in type 1 diabetes Brazilian children. *Autoimmunity* 2009; **42**: 406-413 [PMID: 19811257]
26 **Saraheimo M**, Forsblom C, Pettersson-Fernholm K, Flyvbjerg A, Groop PH, Frystyk J. Increased levels of alpha-defensin (-1, -2 and -3) in type 1 diabetic patients with nephropathy. *Nephrol Dial Transplant* 2008; **23**: 914-918 [PMID: 18003664 DOI: 10.1093/ndt/gfm711]
27 **Joseph G**, Tarnow L, Astrup AS, Hansen TK, Parving HH, Flyvbjerg A, Frystyk J. Plasma alpha-defensin is associated with cardiovascular morbidity and mortality in type 1 diabetic patients. *J Clin Endocrinol Metab* 2008; **93**: 1470-1475 [PMID: 18211979 DOI: 10.1210/jc.2007-1910]
28 **Chen Q**, Hakimi M, Wu S, Jin Y, Cheng B, Wang H, Xie G, Ganz T, Linzmeier RM, Fang X. Increased genomic copy number of DEFA1/DEFA3 is associated with susceptibility to severe sepsis in Chinese Han population. *Anesthesiology* 2010; **112**: 1428-1434 [PMID: 20502117 DOI: 10.1097/ALN.0b013e3181d968eb]
29 **Henrichsen CN**, Chaignat E, Reymond A. Copy number variants, diseases and gene expression. *Hum Mol Genet* 2009; **18**: R1-R8 [PMID: 19297395 DOI: 10.1093/hmg/ddp011]
30 **Joshi MB**, Lad A, Bharath Prasad AS, Balakrishnan A, Ramachandra L, Satyamoorthy K. High glucose modulates IL-6 mediated immune homeostasis through impeding neutrophil extracellular trap formation. *FEBS Lett* 2013; **587**: 2241-2246 [PMID: 23735697 DOI: 10.1016/j.febslet.2013.05.053]
31 **Fang XM**, Shu Q, Chen QX, Book M, Sahl HG, Hoeft A, Stuber F. Differential expression of alpha- and beta-defensins in human peripheral blood. *Eur J Clin Invest* 2003; **33**: 82-87 [PMID: 12492457 DOI: 10.1046/j.1365-2362.2003.01076]
32 **Neuwirth A**, Dobeš J, Oujezdská J, Ballek O, Benešová M, Sumník Z, Včeláková J, Koloušková S, Obermannová B, Kolář M, Stechová K, Filipp D. Eosinophils from patients with type 1 diabetes mellitus express high level of myeloid alpha-defensins and myeloperoxidase. *Cell Immunol* 2012; **273**: 158-163 [PMID: 22248881 DOI: 10.1016/j.cellimm.2011.12.001]
33 **Prado-Montes de Oca E**, García-Vargas A, Lozano-Inocencio R, Gallegos-Arreola MP, Sandoval-Ramírez L, Dávalos-Rodríguez NO, Figuera LE. Association of beta-defensin 1 single nucleotide polymorphisms with atopic dermatitis. *Int Arch Allergy Immunol* 2007; **142**: 211-218 [PMID: 17108702 DOI: 10.1159/000097023]
34 **Jurevic RJ**, Bai M, Chadwick RB, White TC, Dale BA. Single-nucleotide polymorphisms (SNPs) in human beta-defensin 1: high-throughput SNP assays and association with Candida carriage in type I diabetics and nondiabetic controls. *J Clin*

Microbiol 2003; **41**: 90-96 [PMID: 12517831 DOI: 10.1128/jcm.41.1.90-96.2003]

35 **Prado-Montes de Oca E**, Velarde-Félix JS, Ríos-Tostado JJ, Picos-Cárdenas VJ, Figuera LE. SNP 668C (-44) alters a NF-kappaB1 putative binding site in non-coding strand of human beta-defensin 1 (DEFB1) and is associated with lepromatous leprosy. *Infect Genet Evol* 2009; **9**: 617-625 [PMID: 19460328 DOI: 10.1016/j.meegid.2009.03.006]

P- Reviewers: Kanda N, Kietzmann T
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9138

World J Gastroenterol 2014 July 21; 20(27): 9138-9145
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RETROSPECTIVE STUDY

Abdominoperineal excision following preoperative radiotherapy for rectal cancer: Unfavorable prognosis even with negative circumferential resection margin

Lin Wang, Guo-Li Gu, Zhong-Wu Li, Yi-Fan Peng, Jin Gu

Lin Wang, Yi-Fan Peng, Jin Gu, Department of Colorectal Surgery, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Peking University Cancer Hospital and Institute, Beijing 100142, China
Zhong-Wu Li, Department of Pathology, Department of Gastroenterology, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Peking University Cancer Hospital and Institute, Beijing 100142, China
Guo-Li Gu, Department of General Surgery, Air Force General Hospital, Beijing 100142, China
Author contributions: Wang L, Gu GL, Li ZW and Peng YF contributed equally to this study; Wang L contributed to collecting data, designing research and writing article; Gu GL contributed to drafting and revising the article; Li ZW contributed to pathological quality control and assessment; Peng YF contributed to references management; Gu J contributed to the final approval of this article.
Supported by Beijing Municipal Administration of Hospitals Clinical Medicine Development of Special Funding Support (code ZY201410)
Correspondence to: Jin Gu, MD, FACS, Department of Colorectal Surgery, Key laboratory of Carcinogenesis and Translational Research (Ministry of Education), Peking University Cancer Hospital and Institute, 52 Fuchenglu Road, Beijing 100142, China. zlgujin@126.com
Telephone: +86-10-88196085 Fax: +86-10-88196085
Received: January 26, 2014 Revised: March 10, 2014
Accepted: April 15, 2014
Published online: July 21, 2014

Abstract

AIM: To evaluate whether an abdominoperineal excision (APE) is associated with increased local recurrence (LR) and shortened disease-free survival (DFS) in mid-low rectal cancer with a negative circumferential resection margin (CRM).

METHODS: 283 consecutive cases of mid-low rectal cancer underwent preoperative 30 Gy/10 F radiotherapy and surgery in Peking University Cancer Hospital between August 2003 and August 2009. Patients with positive CRM and intraoperative distant metastasis were precluded according to exclusion criteria. Survival analyses were performed in patients with APE or non-APE procedures.

RESULTS: 256 of the 283 (90.5%) cases were enrolled in the analysis, including 78 (30.5%) and 178 (69.5%) cases who received APE and non-APE procedures. Fewer female patients (P = 0.016), lower level of tumor (P = 0.000) and higher body mass index (P = 0.006) were found in the APE group. On univariate analysis, the APE group had a higher LR rate (5.1% *vs* 1.1%, P = 0.036) and decreased DFS (73.1% *vs* 83.4%, P = 0.021). On multivariate analysis, APE procedure was also an independent risk factor for LR (HR = 5.960, 1.085-32.728, P = 0.040) and decreased DFS (HR = 2.304, 1.298-4.092, P = 0.004). In stratified analysis for lower rectal cancer, APE procedure was still an independent risk factor for higher LR rate (5.6% *vs* 0%, P = 0.024) and shortened DFS (91.5% *vs* 73.6%, P = 0.002).

CONCLUSION: Following preoperative 30 Gy/10 F radiotherapy, APE procedure was still a predictor for LR and decreased DFS even with negative CRM. More intensive preoperative treatment should be planned for the candidates who are scheduled to receive APE with optimal imaging assessment.

Key words: Abdominoperineal excision; Preoperative radiotherapy; Circumferential resection margin; Survival

Core tip: The present study focused on survival differences between rectal cancer treated with abdominoperineal excision or non-abdominoperineal excision (APE) following preoperative radiotherapy, with the adjustments of the circumferential resection margin (CRM) to preclude the influence of surgical radicality. The results

revealed the more aggressive oncological behavior of low-lying or fixed tumors, which were unavailable for the sphincter preservation procedure even with negative CRM. We also emphasized the importance of preoperative staging and decision-making before APE procedure, and reviewed the related hypotheses for the unfavorable local control of APE in the discussion.

Wang L, Gu GL, Li ZW, Peng YF, Gu J. Abdominoperineal excision following preoperative radiotherapy for rectal cancer: Unfavorable prognosis even with negative circumferential resection margin. *World J Gastroenterol* 2014; 20(27): 9138-9145 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9138.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9138

INTRODUCTION

Treatment for mid-low rectal cancer has substantially improved in the last few decades with the introduction of total mesorectal excision (TME) combined with neoadjuvant radiotherapy (nRT). Locally advanced rectal cancer could have better local control with a combination treatment of nRT and TME, which had been proven to be effective in many large-scale trials[1-5]. With the down-staging and downsize effect of nRT and a better understanding of tumor spread, sphincter-preserving surgery for low-lying tumor now can be safely performed with 1-cm distal margin[6]. Nevertheless, abdominoperineal excision (APE) still has indications for low-lying tumors which invade levator ani or are resistant to nRT[7].

The current literature reports a poorer outcome following APE than non-APE surgery[7-12]. High frequency of the circumferential resection margin (CRM) in patients who underwent APE was identified in series of studies, and it had been suggested as the main reason for worse outcome after APE[11,13-18]. In the results of a 12 year period follow-up of the Dutch TME trial, short-course nRT was not effective in patients with a positive CRM[1]. However, the unfavorable oncological results of APE might be multifactorial, excluding the increased CRM involvement rate. For instance, bulkier tumors with locally aggressive characteristics received more APE. In a previous report[9], a worse prognosis after APE was observed in patients even with clear CRM in the subgroups analyses. It is unclear whether surgical quality, APE procedure itself, or tumor biological behavior is responsible for the higher rate of treatment failure. There is currently little research comparing outcomes after APE or non-APE procedures with the adjustment of surgical radicality following nRT.

In our unit, nRT was a modified short-course regimen as 30 Gy in 10 fractions (biological equivalent dose: 36 Gy) with a prolonged interval of 2-4 wk to surgery, which has been promoted by the Committee of the Chinese Anti-Cancer Association (CACA) from 2001[19-22].

The present retrospective study was designed to address the following question: in patients with pathologically identified negative CRM, is the APE procedure is still relevant with an unfavorable prognosis when compared with non-APE surgery following 30 Gy/10 F nRT?

MATERIALS AND METHODS

Patient selection

We retrospectively reviewed the data of 283 rectal cancer patients who received 30 Gy/10 F neoadjuvant radiotherapy (nRT) and total mesorectal excision at Peking University Cancer Hospital between August 2003 and August 2009.

Neoadjuvant radiotherapy

All involved patients received preoperative neoadjuvant radiotherapy followed by TME. The radiotherapy regimen consisted of a 30 Gy dose delivered in 10 fractions for 2 wk. The biological equivalent dose of this regimen is 36 Gy, according to linear-quadratic formula. Three-dimensional conformal radiotherapy (3D-CRT) was employed routinely.

Surgery and adjuvant treatment

Surgery was performed 2-4 wk after following the principle of total mesorectal excision[23]. The decision to perform APE was made before surgery for low-lying or levator threatening rectal cancer, but made intraoperatively for mid rectal cancer depending on whether sufficient length of muscle tube could be mobilized for a tumor-free anastomosis. The abdominoperineal excision was performed in the lithotomy position without an extended resection of levator ani or coccyx. After surgery, 5-fluorouracil-based chemotherapy was administered for 6 mo if patients could tolerate.

Pathological evaluation

The 7th edition of the American Joint Committee on the Cancer TNM system was used for tumor staging. Following nRT and surgery, the results of histopathologic examination of the specimens were reviewed by the same group of experienced pathologists; CRM involvement was assessed following the protocol laid out by Quirke *et al*[24-26]. Tumor regression grade (TRG) was evaluated by a 3-points system assessing residual tumor cell and fibrosis, with less than 5% residual tumor being identified as major regression[27,28].

Inclusive and exclusive criteria

Each subject conformed to the following entry criteria: (1) patient was diagnosed as having rectal adenocarcinoma by biopsy; (2) cancerous lesion was located within 10 cm from the anal verge; (3) the cancer was staged as T3-4 or any T, N+ by endorectal ultrasound, pelvic magnetic resonance imaging (MRI), or computed tomography (CT); and (4) presence of distant metastasis excluded by imaging exams.

Patients with the following characteristics were ex-

Table 1 Clinical and pathological features of abdominoperineal excision and non- abdominoperineal excision groups *n* (%)

Variates		Non-APE (*n* = 178)	APE (*n* = 78)	*P* value
Gender				
	Male	92 (51.7)	53 (67.9)	0.016
	Female	86 (48.3)	25 (32.1)	
Age (yr)				
	< 65	119 (66.9)	56 (71.8)	0.434
	⩾ 65	59 (33.1)	22 (28.2)	
Level of tumor				
	⩽ 5 cm	82 (46.1)	72 (92.3)	0.000
	> 5 cm	96 (53.9)	6 (7.7)	
BMI (kg/m^2)				
	⩽ 23.5	97 (54.5)	28 (35.9)	0.006
	> 23.5	81 (45.5)	50 (64.1)	
Pre-operative CEA[1]				
	⩽ 5 ng/mL	97 (63.0)	41 (66.1)	0.664
	> 5 ng/mL	57 (37.0)	21 (33.9)	
Differentiation				
	ypCR	12 (6.7)	6 (7.7)	0.651
	G1-2	120 (67.4)	56 (71.8)	
	G3-4	46 (25.8)	16 (20.5)	
TRG				
	Complete regression	12 (6.7)	6 (7.7)	0.402
	Major regression	27 (15.2)	7 (9.0)	
	Minor regression	139 (78.1)	65 (83.3)	
ypT				
	T0	12 (6.7)	6 (7.7)	0.511
	T1	11 (6.2)	5 (6.4)	
	T2	56 (31.5)	29 (37.2)	
	T3	98 (551)	36 (46.2)	
	T4	1 (0.6)	2 (2.6)	
ypN				
	N0	114 (64.0)	45 (57.7)	0.209
	N1/N1c	37 (20.8)	24 (30.8)	
	N2a/b	27 (15.2)	9 (11.5)	
ypTNM stage				
	ypCR	12 (6.7)	4 (5.1)	0.642
	Ⅰ	57 (32.0)	26 (33.3)	
	Ⅱ	45 (25.3)	15 (19.2)	
	Ⅲ	64 (36.0)	33 (42.3)	

[1]Pre-treatment CEA assessment was unavailable in 40 cases. APE: Abdominoperineal excision. CEA: Carcino-embryonic antigen.

cluded: (1) previous chemotherapy, or pelvic radiation; (2) previous history (within 5 years) of malignant tumor; (3) intraoperative confirmed metastasis; and (4) pathologically-confirmed circumferential resection margin (CRM) by Quirke's protocol[24,29].

Follow-up

Patients were followed at three-month intervals for the first two years and then at six-month intervals for the next three years. Evaluations consisted of physical examination, serum CEA, a complete blood count, and blood chemical analysis. Proctoscopy, abdominal ultrasonography, CT of the abdomen and pelvis, and chest radiography was also routinely performed every 6-12 mo.

Endpoints

Endpoints of the research were 3-year disease-free survival (3 years DFS)[30] and local recurrence (LR) rate.

Statistical analyses

The categorical variables were analyzed with the Pearson chi-squared or Fisher's exact test, and the level of significance was set at 0.05. DFS curves were compared among groups. The Kaplan-Meier survival curve (method: log-rank test) was used for time-to-event parameters. Multivariate Cox proportional hazards regression was used to analyze the major factors affecting DFS and LR, with the level of significance set at 0.1. The software IBM SPSS Statistics for Mac, Version 22.0 (Armonk, NY: IBM Corp.) was used for the analyses.

RESULTS

Patient demographics

The records of 283 patients were reviewed. Positive CRM was identified in 26 of 283 patients (9.2%). The incidence of CRM involvement had no statistical difference between the APE and non-APE groups [10.3% (9/87) *vs* 8.7% (17/196), $P = 0.653$]. Intraoperative liver metastasis occurred in one patient (0.3%).

There were 256 cases with negative CRM and without synchronous distant metastasis that were entered into the analysis following the inclusion and exclusion criteria. The median age was 58 years (range: 22-85 years), with 56.7% (145/256) male patients. The median body mass index (BMI) was 23.5 (range: 15.6-36.3). The median distance of tumors from the anal verge was 5.0 cm (range: 1-10 cm).

Initial clinical pre-staging was cⅡ in 18.8% (48/256) and cⅢ in 81.2% (208/256) of cases. The median operation time was 120 min (range: 60-240 min) and the median blood loss was 200 mL (range: 50-4000 mL). 30.5% (78/256), 67.6% (173/256), and 1.9% (5/256) of patients received APE, LAR, and the Hartmann procedure.

The distribution of ypTNM stages was: complete response (no microscopic residual tumor cell), 6.3% (16/256); stage Ⅰ, 32.4% (83/256); stage Ⅱ, 23.4% (60/256); and stage Ⅲ, 37.8% (97/256).

Clinical and pathological features

Patients were divided into APE and non-APE groups according to types of surgery. The characteristics of the two groups, including preoperative variables and postoperative histologic/pathologic stages, are listed in Table 1.

Statistical analysis showed that fewer female patients ($P = 0.016$), lower level of tumors ($P = 0.000$), and higher median BMI ($P = 0.006$) were found in the APE group compared to the non-APE group. Other patient characteristics showed no statistical significance between these two groups.

Local recurrence

The LR rate was 5.1% (4/78) and 1.1% (2/178) in the APE and non-APE groups, respectively, with a significant difference in univariate analysis ($P = 0.036$) (Figure

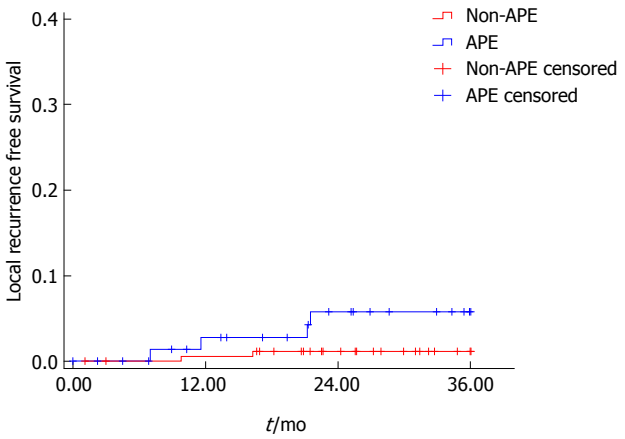


Figure 1 Kaplan-Meier curves of local recurrence free survival in abdominoperineal excision and non-abdominoperineal excision groups (*P* = 0.036). APE: Abdominoperineal excision.

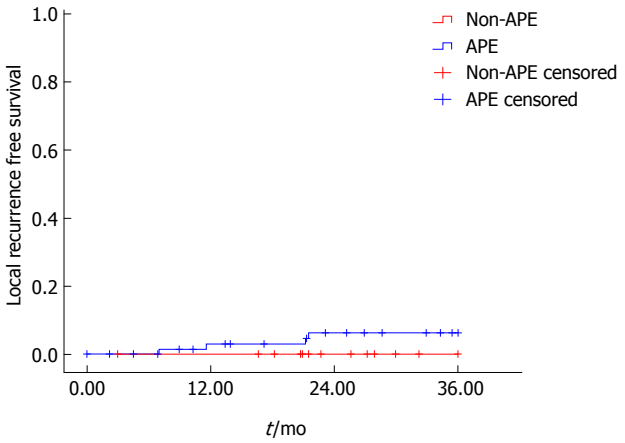


Figure 2 Kaplan-Meier curves of local recurrence free survival in abdominoperineal excision and non-abdominoperineal excision groups for low rectal cancer (≤ 5 cm) (*P* = 0.024). APE: Abdominoperineal excision.

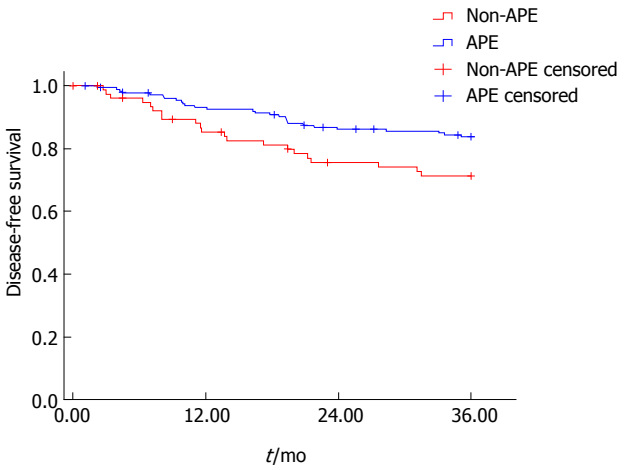


Figure 3 Kaplan-Meier curves of disease-free survival in abdominoperineal excision and non- abdominoperineal excision groups (*P* = 0.021). APE: Abdominoperineal excision.

1). On stratified analysis in the low rectal cancer group (level of tumor ≤ 5 cm), the LR rate was also significantly higher in the APE group than the non-APE group [5.6% (4/72) *vs* 0% (0/72), *P* = 0.024]. Multivariate analysis was not performed for limited events of local failure (Figure 2).

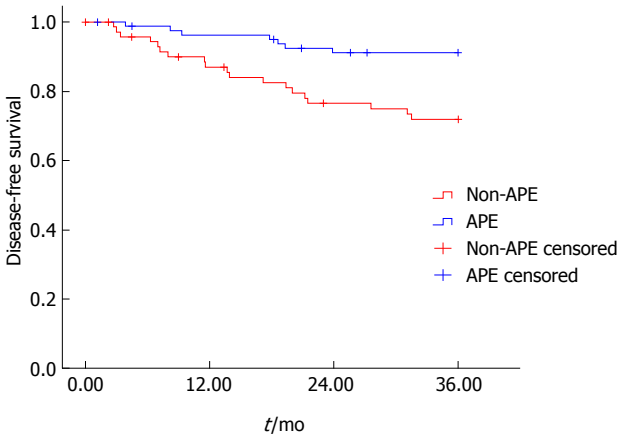


Figure 4 Kaplan-Meier curves of disease-free survival in abdominoperineal excision and non-abdominoperineal excision groups for low rectal cancer (≤ 5 cm) (*P* = 0.002). APE: Abdominoperineal excision.

Disease-free survival

On univariate analysis, there was a significantly shortened 3 years DFS in the APE group compared to the non-APE group (74.1% *vs* 84.3%, *P* = 0.021) (Figure 3). On the multivariate COX regression model, APE procedure (HR = 2.304, 1.298-4.092, *P* = 0.004), ypN stage (HR = 2.288, 1.593-3.284, *P* = 0.000), and tumor differentiation (HR = 2.044, 1.178-3.545, *P* = 0.011) were independently associated DFS.

On stratified analysis in the group of low rectal cancer (level of tumor ≤ 5 cm), the 3 years DFS was also significantly lower in the APE group than the non-APE group (91.5% *vs* 73.6%, *P* = 0.002) (Figure 4). On the multivariate COX regression model, APE procedure (HR = 3.810, 1.544-9.403, *P* = 0.004), gender (HR = 2.318, 0.983-5.464, *P* = 0.055), ypT stage (HR = 2.671, 1.303-5.474, *P* = 0.007), and ypN stage (HR = 3.839, 2.181-6.757, *P* = 0.000) were independently associated DFS in lower rectal cancer.

DISCUSSION

APE has been the standard surgery for the lower rectum. In last few decades, more sphincter preserving surgeries could be performed with a negative distal margin following the introduction of total mesorectal excision and novel stapling devices. Furthermore, the rate of APE has decreased with the application of nRT, which caused tumor regression and the downstaging effect. Good response to nRT enhances anal sphincter preservation in rectal cancer patients[4,31-34]. Nevertheless, for distal bulky tumors which invade the levator ani or sphincter, APE remains the first choice for treatment.

Previous studies concluded that APE was associated with a higher local recurrence rate[8,10,12,13,15,35,36] and de-

creased disease-free or cancer-specific survival than the non-APE procedure[10,12,15,35,37]. In many studies, the unfavorable prognosis of APE compared with non-APE could be explained by the high incidence of CRM involvement. Wibe *et al*[13] reported that the APE procedure was associated with a significantly higher incidence of positive CRM involvement (12% *vs* 5%, $P = 0.01$) when compared with anterior resection (AR). Consequently, patients who received APE had worse local control (10% *vs* 15%, $P = 0.008$) and poorer overall survival (55% *vs* 68%, $P < 0.001$). Nagtegaal *et al*[14] also investigated the results from the Dutch TME trial and found more positive CRMs presented in the patients operated with APE compared to AR (30.4% *vs* 10.7%, $P = 0.002$). Overall survival differed greatly between APE and AR (38.5% *vs* 57.6%, $P = 0.008$). Similar results were reported in many other studies[11,15-17].

Despite the higher involved CRM rate being accepted as the main cause for the poorer prognosis of APE, the strategy for performing APE is still multifactorial. Apparently lower and advanced tumors are treated by APE rather than non-APE. Some authors indicated that patients who received APE had more advanced T stage than those received non-APE[7,37], while others found more advanced TNM stage in the APE group[13,15,18,38-40]. Consequently, neoadjuvant radiotherapy was more frequently adopted in these high-risk patients for better local control, even without the expectation of sphincter preservation[10,13,18,40]. For the heterogeneous factors above that might induce selection bias, it was difficult to analyze whether the technique of the APE procedure or pre-existing oncological risks would be responsible for the poorer outcome of APE.

The present study was designed to analyze the prognostic value of type of surgery in patients following nRT when excluding CRM positive cases. In our study, a significant higher incidence of lower tumor (92.3% *vs* 46.1%, $P = 0.000$), higher median BMI (64.1% *vs* 45.5%, $P = 0.006$), and male patients (51.7% *vs* 67.9%, $P = 0.016$) was observed in the APE group compared to the non-APE group. It is not surprising that the location of tumor predominates over other factors when making a decision regarding type of surgery. Male gender and a higher BMI were mainly associated with increased difficulty to perform sphincter preservation procedure with a safe margin. Tumor-related factors such as tumor differentiation, TRG, LVI, T stage, N stage, and TNM stage were not significantly different between the APE and non-APE groups following nRT.

Our results revealed that APE itself was an independent risk factor in patients with a negative margin. Although with pathological confirmed surgical radicality, the local recurrence rate in the APE group was statistically higher than the non-APE group (5.1% *vs* 1.1%, $P = 0.040$). On survival analysis, a difference of 3 years DFS was also significant between the APE and non-APE groups. In stratified analysis of lower rectal cancer, APE procedure is also a risk factor for local failure and disease relapse.

Reshef *et al*[9] reported the similar phenomenon that a higher local recurrence rate (7% *vs* 3%, $P = 0.02$) and shortened disease-free survival (54% *vs* 70%, $P < 0.001$) after APE, rather than non-APE, persisted in patients with clear CRM. Meanwhile, the proportion of patients who underwent nRT was significant higher in the APE group. Patients in the APE group also more frequently had tumors with an advanced TNM stage.

In our study, we eliminated the bias of tumor biological factors and CRM involvement for pertinent analyses. Following short-course nRT and surgery with negative CRM, the prognosis of APE was still less favorable than the non-APE group. In the Dutch TME and CR07 trial[1-3,5], the local recurrence rate of patients with negative CRM was 3.2% (22/691) and 3.3% (22/674), respectively, following short-course nRT, which was closely consistent with our results (3.3%, 6/256).

Some authors advocate that candidates for APE procedure should receive long-course chemoradiation to enhance downstaging and the downsize effect. Nevertheless, Bujko *et al*[6] found there was no significant difference in local recurrence (15.6% *vs* 10.6%, $P = 0.210$), despite patients having a higher incidence of complete response (16.1% *vs* 0.7%, *P* value not given) and lesser CRM involvement (4.4% *vs* 12.9%, $P = 0.017$) following long-course chemoradiotherapy than short-course nRT. New nRT regimens which have been reported with the addition of oxaliplatin or induction chemotherapy were more intensive, but failed to reveal oncological superiority[41-44].

Radiotherapy regimen in the present study was 30 Gy in 10 fractions, which was designed to have a similar biological equivalent dose as 5 × 5 Gy regimen and a prolonged interval of 2-4 wk prior to surgery to increase response rate and clinical efficiency[19-22]. Our results revealed objective pathologic downstaging and response rate, which was higher than that of published data of the traditional short-course regimen. This combination of short-course regimen and delayed surgery was also adopted in newly published studies with 8%-10% ypCR rate[45,46].

Apart from nRT, surgical technique was another factor that might influence patient prognosis. Heald *et al*[8] postulated that a lack of precisely definable planes for perineal dissection increased the incidence of implantation of shed cancer cells on large areas of raw surfaces and soft tissue residues in APE procedure.

In this study, APE was conventionally performed without extended resection of levator ani and coccyx. Recently, Holm *et al*[47,48] introduced extended APE in a prone jack-knife position and performed more cylindrical resection of low rectal cancer. With this technique, the amount of tissue beyond the internal sphincter or muscularis propria significantly increased.

Some authors recommended preoperative decision-making of type of surgery by MRI to avoid intraoperative "coning-in" of the mesorectum for those due to receive APE[48,49]. Bebenek *et al*[50] also evaluated low rectal specimens and preliminarily revealed extramesorectal

lymphatic drainage besides the mesorectal route. These findings may elucidate the cause of local recurrence in patients with negative CRM following APE procedure. However, extended APE is still controversial in the literature[51,52].

MRI has shown a diagnostic advantage over other radiological methods[53]. In the Mercury trial, patients were classified to have a good or bad prognosis by mesorectal invasion on MRI[54]. By this means, patients with low rectal cancer should be recognized as a high-risk group because the mesorectum gradually tapers at the anorectal junction. When staging rectal cancer, MRI can visualize the construction of the anal-canal region and provide a more accurate description of the pelvic structures than was previously available[55]. Patients should also be restaged by MRI following neoadjuvant therapy for reassessment before making surgical plan[53].

In summary, our study proved the unfavorable prognosis of rectal cancer following 30 Gy/10 F nRT and APE, even with negative CRM. More intensive neoadjuvant regimen, improved surgical technique, and accurate preoperative assessment should be recommended for low rectal cancer in the future.

The main limitation of this study was that the pathology department in our hospital did not routinely perform macroscopic assessment of resected mesorectum or record tumor perforation. Therefore, it is limited to surgical quality that was comprehensively revealed, except for CRM assessment. Despite this limitation, our data was obtained from a high volume hospital with large number of cases. We believe these findings would benefit clinical practice.

COMMENTS

Background

Rectal cancer following abdominoperineal excision (APE) was thought to be associated with a poorer outcome for the high incidence of positive circumferential resection margin (CRM). However, the poorer outcome of low rectal cancer following abdominoperineal excision is multifactorial. The inherent biological behavior of low rectal cancer should be evaluated with the adjustment of surgical radicality.

Research frontiers

The research hotspot in this field is whether the inferior prognosis of low rectal cancer treated with APE is caused by the surgical procedure itself, is attributable to other factors as inherent biological behavior, or both.

Innovations and breakthroughs

The innovation and breakthrough of the present study is that we precluded CRM positive cases and compared local recurrent rate and disease-free survival in patients received APE or non-APE procedures, mitigated the influence by surgical technique related bias in analyses, and revealed the inherent biological behavior of low-lying or levator threatening rectal cancer.

Applications

The results of the present study proved the unfavorable survival of low-lying or levator threatening rectal cancer following APE and preoperative radiotherapy. More intensive neoadjuvant treatment and optimized pathways for performing APE might be needed in order to improve treatment outcomes for these patients.

Peer review

This is a retrospective study focusing on the outcomes in pre-radiated rectal cancer followed by APE or non-APE. The authors precluded margin positive cases and revealed the biological behavior of low rectal cancer without the influence of technique-related factors. Their results prove that local control and disease-free survival in APE cases is worse than in non-APE cases, even with neoadjuvant radiotherapy and R0 resection.

REFERENCES

1 **van Gijn W**, Marijnen CA, Nagtegaal ID, Kranenbarg EM, Putter H, Wiggers T, Rutten HJ, Påhlman L, Glimelius B, van de Velde CJ. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer: 12-year follow-up of the multicentre, randomised controlled TME trial. *Lancet Oncol* 2011; **12**: 575-582 [PMID: 21596621 DOI: 10.1016/S1470-2045(11)70097-3]

2 **Sebag-Montefiore D**, Stephens RJ, Steele R, Monson J, Grieve R, Khanna S, Quirke P, Couture J, de Metz C, Myint AS, Bessell E, Griffiths G, Thompson LC, Parmar M. Preoperative radiotherapy versus selective postoperative chemoradiotherapy in patients with rectal cancer (MRC CR07 and NCIC-CTG C016): a multicentre, randomised trial. *Lancet* 2009; **373**: 811-820 [PMID: 19269519]

3 **Peeters KC**, Marijnen CA, Nagtegaal ID, Kranenbarg EK, Putter H, Wiggers T, Rutten H, Pahlman L, Glimelius B, Leer JW, van de Velde CJ. The TME trial after a median follow-up of 6 years: increased local control but no survival benefit in irradiated patients with resectable rectal carcinoma. *Ann Surg* 2007; **246**: 693-701 [PMID: 17968156 DOI: 10.1097/01.sla.0000257358.56863.ce]

4 **Sauer R**, Becker H, Hohenberger W, Rödel C, Wittekind C, Fietkau R, Martus P, Tschmelitsch J, Hager E, Hess CF, Karstens JH, Liersch T, Schmidberger H, Raab R. Preoperative versus postoperative chemoradiotherapy for rectal cancer. *N Engl J Med* 2004; **351**: 1731-1740 [PMID: 15496622]

5 **Kapiteijn E**, Marijnen CA, Nagtegaal ID, Putter H, Steup WH, Wiggers T, Rutten HJ, Pahlman L, Glimelius B, van Krieken JH, Leer JW, van de Velde CJ. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. *N Engl J Med* 2001; **345**: 638-646 [PMID: 11547717 DOI: 10.1056/NEJMoa010580]

6 **Bujko K**, Rutkowski A, Chang GJ, Michalski W, Chmielik E, Kusnierz J. Is the 1-cm rule of distal bowel resection margin in rectal cancer based on clinical evidence? A systematic review. *Ann Surg Oncol* 2012; **19**: 801-808 [PMID: 21879269 DOI: 10.1245/s10434-011-2035-2]

7 **Chuwa EW**, Seow-Choen F. Outcomes for abdominoperineal resections are not worse than those of anterior resections. *Dis Colon Rectum* 2006; **49**: 41-49 [PMID: 16283562 DOI: 10.1007/s10350-005-0227-1]

8 **Heald RJ**, Smedh RK, Kald A, Sexton R, Moran BJ. Abdominoperineal excision of the rectum--an endangered operation. Norman Nigro Lectureship. *Dis Colon Rectum* 1997; **40**: 747-751 [PMID: 9221846]

9 **Reshef A**, Lavery I, Kiran RP. Factors associated with oncologic outcomes after abdominoperineal resection compared with restorative resection for low rectal cancer: patient- and tumor-related or technical factors only? *Dis Colon Rectum* 2012; **55**: 51-58 [PMID: 22156867 DOI: 10.1097/DCR.0b013e3182351c1f]

10 **Law WL**, Chu KW. Abdominoperineal resection is associated with poor oncological outcome. *Br J Surg* 2004; **91**: 1493-1499 [PMID: 15455362 DOI: 10.1002/bjs.4723]

11 **Marr R**, Birbeck K, Garvican J, Macklin CP, Tiffin NJ, Parsons WJ, Dixon MF, Mapstone NP, Sebag-Montefiore D, Scott N, Johnston D, Sagar P, Finan P, Quirke P. The modern abdominoperineal excision: the next challenge after total mesorectal excision. *Ann Surg* 2005; **242**: 74-82 [PMID: 15973104]

12 **den Dulk M**, Putter H, Collette L, Marijnen CA, Folkesson J, Bosset JF, Rödel C, Bujko K, Påhlman L, van de Velde CJ. The abdominoperineal resection itself is associated with an adverse outcome: the European experience based on a pooled analysis of five European randomised clinical trials on rectal cancer. *Eur J Cancer* 2009; **45**: 1175-1183 [PMID:

19128956 DOI: 10.1016/j.ejca.2008.11.039]
13 **Wibe A**, Syse A, Andersen E, Tretli S, Myrvold HE, Søreide O. Oncological outcomes after total mesorectal excision for cure for cancer of the lower rectum: anterior vs. abdominoperineal resection. *Dis Colon Rectum* 2004; **47**: 48-58 [PMID: 14719151 DOI: 10.1007/s10350-003-0012-y]
14 **Nagtegaal ID**, van de Velde CJ, Marijnen CA, van Krieken JH, Quirke P. Low rectal cancer: a call for a change of approach in abdominoperineal resection. *J Clin Oncol* 2005; **23**: 9257-9264 [PMID: 16361623]
15 **Ptok H**, Marusch F, Kuhn R, Gastinger I, Lippert H. Influence of hospital volume on the frequency of abdominoperineal resection and long-term oncological outcomes in low rectal cancer. *Eur J Surg Oncol* 2007; **33**: 854-861 [PMID: 17933024]
16 **Tilney HS**, Tekkis PP, Sains PS, Constantinides VA, Heriot AG. Factors affecting circumferential resection margin involvement after rectal cancer excision. *Dis Colon Rectum* 2007; **50**: 29-36 [PMID: 17115338 DOI: 10.1007/s10350-006-0744-6]
17 **Youssef H**, Collantes EC, Rashid SH, Wong LS, Baragwanath P. Rectal cancer: involved circumferential resection margin - a root cause analysis. *Colorectal Dis* 2009; **11**: 470-474 [PMID: 18637930 DOI: 10.1111/j.1463-1318.2008.01640.x]
18 **Shihab OC**, Brown G, Daniels IR, Heald RJ, Quirke P, Moran BJ. Patients with low rectal cancer treated by abdominoperineal excision have worse tumors and higher involved margin rates compared with patients treated by anterior resection. *Dis Colon Rectum* 2010; **53**: 53-56 [PMID: 20010351 DOI: 10.1007/DCR.0b013e3181c70465]
19 **Du CZ**, Xue WC, Cai Y, Li M, Gu J. Lymphovascular invasion in rectal cancer following neoadjuvant radiotherapy: a retrospective cohort study. *World J Gastroenterol* 2009; **15**: 3793-3798 [PMID: 19673022]
20 **Wang L**, Gu J. Risk factors for symptomatic anastomotic leakage after low anterior resection for rectal cancer with 30 Gy/10 f/2 w preoperative radiotherapy. *World J Surg* 2010; **34**: 1080-1085 [PMID: 20145926 DOI: 10.1007/s00268-010-0449-9]
21 **Du C**, Xue W, Li J, Cai Y, Gu J. Morphology and prognostic value of tumor budding in rectal cancer after neoadjuvant radiotherapy. *Hum Pathol* 2012; **43**: 1061-1067 [PMID: 22204710 DOI: 10.1016/j.humpath.2011.07.026]
22 **Peng Y**, Wang L, Du C, Gu J. Expression of vascular endothelial growth factor can predict distant metastasis and disease-free survival for clinical stage III rectal cancer following 30-Gy/10-f preoperative radiotherapy. *Int J Colorectal Dis* 2012; **27**: 1555-1560 [PMID: 22645074 DOI: 10.1007/s00384-012-1485-8]
23 **Heald RJ**. Total mesorectal excision is optimal surgery for rectal cancer: a Scandinavian consensus. *Br J Surg* 1995; **82**: 1297-1299 [PMID: 7489148]
24 **Nagtegaal ID**, van de Velde CJ, van der Worp E, Kapiteijn E, Quirke P, van Krieken JH. Macroscopic evaluation of rectal cancer resection specimen: clinical significance of the pathologist in quality control. *J Clin Oncol* 2002; **20**: 1729-1734 [PMID: 11919228]
25 **Quirke P**. Training and quality assurance for rectal cancer: 20 years of data is enough. *Lancet Oncol* 2003; **4**: 695-702 [PMID: 14602250]
26 **Quirke P**, Steele R, Monson J, Grieve R, Khanna S, Couture J, O'Callaghan C, Myint AS, Bessell E, Thompson LC, Parmar M, Stephens RJ, Sebag-Montefiore D. Effect of the plane of surgery achieved on local recurrence in patients with operable rectal cancer: a prospective study using data from the MRC CR07 and NCIC-CTG CO16 randomised clinical trial. *Lancet* 2009; **373**: 821-828 [PMID: 19269520]
27 **Wheeler JM**, Warren BF, Mortensen NJ, Ekanyaka N, Kulacoglu H, Jones AC, George BD, Kettlewell MG. Quantification of histologic regression of rectal cancer after irradiation: a proposal for a modified staging system. *Dis Colon Rectum* 2002; **45**: 1051-1056 [PMID: 12195189]
28 **Wheeler JM**, Dodds E, Warren BF, Cunningham C, George BD, Jones AC, Mortensen NJ. Preoperative chemoradiotherapy and total mesorectal excision surgery for locally advanced rectal cancer: correlation with rectal cancer regression grade. *Dis Colon Rectum* 2004; **47**: 2025-2031 [PMID: 15657650 DOI: 10.1007/s10350-004-0713-x]
29 **Nagtegaal ID**, Marijnen CA, Kranenbarg EK, van de Velde CJ, van Krieken JH. Circumferential margin involvement is still an important predictor of local recurrence in rectal carcinoma: not one millimeter but two millimeters is the limit. *Am J Surg Pathol* 2002; **26**: 350-357 [PMID: 11859207]
30 **de Gramont A**, Hubbard J, Shi Q, O'Connell MJ, Buyse M, Benedetti J, Bot B, O'Callaghan C, Yothers G, Goldberg RM, Blanke CD, Benson A, Deng Q, Alberts SR, Andre T, Wolmark N, Grothey A, Sargent D. Association between disease-free survival and overall survival when survival is prolonged after recurrence in patients receiving cytotoxic adjuvant therapy for colon cancer: simulations based on the 20,800 patient ACCENT data set. *J Clin Oncol* 2010; **28**: 460-465 [PMID: 20008641 DOI: 10.1200/JCO.2009.23.1407]
31 **Janjan NA**, Khoo VS, Abbruzzese J, Pazdur R, Dubrow R, Cleary KR, Allen PK, Lynch PM, Glober G, Wolff R, Rich TA, Skibber J. Tumor downstaging and sphincter preservation with preoperative chemoradiation in locally advanced rectal cancer: the M. D. Anderson Cancer Center experience. *Int J Radiat Oncol Biol Phys* 1999; **44**: 1027-1038 [PMID: 10421535]
32 **Luna-Pérez P**, Rodríguez-Ramírez S, Rodriguez-Coria DF, Fernández A, Labastida S, Silva A, López MJ. Preoperative chemoradiation therapy and anal sphincter preservation with locally advanced rectal adenocarcinoma. *World J Surg* 2001; **25**: 1006-1011 [PMID: 11571965]
33 **Gavioli M**, Losi L, Luppi G, Iacchetta F, Zironi S, Bertolini F, Falchi AM, Bertoni F, Natalini G. Preoperative therapy for lower rectal cancer and modifications in distance from anal sphincter. *Int J Radiat Oncol Biol Phys* 2007; **69**: 370-375 [PMID: 17524570 DOI: 10.1016/j.ijrobp.2007.03.049]
34 **Kim NK**, Baik SH, Min BS, Pyo HR, Choi YJ, Kim H, Seong J, Keum KC, Rha SY, Chung HC. A comparative study of volumetric analysis, histopathologic downstaging, and tumor regression grade in evaluating tumor response in locally advanced rectal cancer following preoperative chemoradiation. *Int J Radiat Oncol Biol Phys* 2007; **67**: 204-210 [PMID: 17084555 DOI: 10.1016/j.ijrobp.2006.08.020]
35 **Bonadeo FA**, Vaccaro CA, Benati ML, Quintana GM, Garione XE, Telenta MT. Rectal cancer: local recurrence after surgery without radiotherapy. *Dis Colon Rectum* 2001; **44**: 374-379 [PMID: 11289283]
36 **García-Granero E**, Martí-Obiol R, Gómez-Barbadillo J, García-Armengol J, Esclapez P, Espí A, Jiménez E, Millán M, Lledó S. Impact of surgeon organization and specialization in rectal cancer outcome. *Colorectal Dis* 2001; **3**: 179-184 [PMID: 12790986]
37 **Weiser MR**, Quah HM, Shia J, Guillem JG, Paty PB, Temple LK, Goodman KA, Minsky BD, Wong WD. Sphincter preservation in low rectal cancer is facilitated by preoperative chemoradiation and intersphincteric dissection. *Ann Surg* 2009; **249**: 236-242 [PMID: 19212176 DOI: 10.1097/SLA.0b013e318195e17c]
38 **Nakagoe T**, Ishikawa H, Sawai T, Tsuji T, Tanaka K, Hidaka S, Nanashima A, Yamaguchi H, Yasutake T. Survival and recurrence after a sphincter-saving resection and abdominoperineal resection for adenocarcinoma of the rectum at or below the peritoneal reflection: a multivariate analysis. *Surg Today* 2004; **34**: 32-39 [PMID: 14714226 DOI: 10.1007/s00595-003-2637-9]
39 **Chiappa A**, Biffi R, Bertani E, Zbar AP, Pace U, Crotti C, Biella F, Viale G, Orecchia R, Pruneri G, Poldi D, Andreoni B. Surgical outcomes after total mesorectal excision for rectal cancer. *J Surg Oncol* 2006; **94**: 182-193; discussion 181 [PMID:

16900534 DOI: 10.1002/jso.20518]
40 **Chambers W**, Hancock L, McKenzie R, Buchel O, Lindsey I, Cunningham C, George B, Mortensen N. Changes in the management and outcome of rectal cancer over a 10-year period in Oxford. *Colorectal Dis* 2011; **13**: 1004-1008 [PMID: 20608947 DOI: 10.1111/j.1463-1318.2010.02360.x]
41 **Aschele C**, Cionini L, Lonardi S, Pinto C, Cordio S, Rosati G, Artale S, Tagliagambe A, Ambrosini G, Rosetti P, Bonetti A, Negru ME, Tronconi MC, Luppi G, Silvano G, Corsi DC, Bochicchio AM, Chiaulon G, Gallo M, Boni L. Primary tumor response to preoperative chemoradiation with or without oxaliplatin in locally advanced rectal cancer: pathologic results of the STAR-01 randomized phase III trial. *J Clin Oncol* 2011; **29**: 2773-2780 [PMID: 21606427]
42 **Fernández-Martos C**, Pericay C, Aparicio J, Salud A, Safont M, Massuti B, Vera R, Escudero P, Maurel J, Marcuello E, Mengual JL, Saigi E, Estevan R, Mira M, Polo S, Hernandez A, Gallen M, Arias F, Serra J, Alonso V. Phase II, randomized study of concomitant chemoradiotherapy followed by surgery and adjuvant capecitabine plus oxaliplatin (CAPOX) compared with induction CAPOX followed by concomitant chemoradiotherapy and surgery in magnetic resonance imaging-defined, locally advanced rectal cancer: Grupo cancer de recto 3 study. *J Clin Oncol* 2010; **28**: 859-865 [PMID: 20065174]
43 **Gérard JP**, Azria D, Gourgou-Bourgade S, Martel-Laffay I, Hennequin C, Etienne PL, Vendrely V, François E, de La Roche G, Bouché O, Mirabel X, Denis B, Mineur L, Berdah JF, Mahé MA, Bécouarn Y, Dupuis O, Lledo G, Montoto-Grillot C, Conroy T. Comparison of two neoadjuvant chemoradiotherapy regimens for locally advanced rectal cancer: results of the phase III trial ACCORD 12/0405-Prodige 2. *J Clin Oncol* 2010; **28**: 1638-1644 [PMID: 20194850 DOI: 10.1200/JCO.2009.25.8376]
44 **Gérard JP**, Azria D, Gourgou-Bourgade S, Martel-Lafay I, Hennequin C, Etienne PL, Vendrely V, François E, de La Roche G, Bouché O, Mirabel X, Denis B, Mineur L, Berdah JF, Mahé MA, Bécouarn Y, Dupuis O, Lledo G, Seitz JF, Bedenne L, Juzyna B, Conroy T. Clinical outcome of the ACCORD 12/0405 PRODIGE 2 randomized trial in rectal cancer. *J Clin Oncol* 2012; **30**: 4558-4565 [PMID: 23109696 DOI: 10.1200/JCO.2012.42.8771]
45 **Faria S**, Kopek N, Hijal T, Liberman S, Charlebois P, Stein B, Meterissian S, Meguerditchian A, Fawaz Z, Artho G. Phase II trial of short-course radiotherapy followed by delayed surgery for locoregionally advanced rectal cancer. *Colorectal Dis* 2014; **16**: O66-O70 [PMID: 24148225 DOI: 10.1111/codi.12466]
46 **Pettersson D**, Holm T, Iversen H, Blomqvist L, Glimelius B, Martling A. Preoperative short-course radiotherapy with delayed surgery in primary rectal cancer. *Br J Surg* 2012; **99**: 577-583 [PMID: 22241246 DOI: 10.1002/bjs.7796]
47 **West NP**, Finan PJ, Anderin C, Lindholm J, Holm T, Quirke P. Evidence of the oncologic superiority of cylindrical abdominoperineal excision for low rectal cancer. *J Clin Oncol* 2008; **26**: 3517-3522 [PMID: 18541901 DOI: 10.1200/JCO.2007.14.5961]
48 **Holm T**, Ljung A, Häggmark T, Jurell G, Lagergren J. Extended abdominoperineal resection with gluteus maximus flap reconstruction of the pelvic floor for rectal cancer. *Br J Surg* 2007; **94**: 232-238 [PMID: 17143848 DOI: 10.1002/bjs.5489]
49 **Shihab OC**, Heald RJ, Holm T, How PD, Brown G, Quirke P, Moran BJ. A pictorial description of extralevator abdominoperineal excision for low rectal cancer. *Colorectal Dis* 2012; **14**: e655-e660 [PMID: 22788385 DOI: 10.1111/j.1463-1318.2012.03181.x]
50 **Bebenek M**, Wojnar A. Infralevator lymphatic drainage of low-rectal cancers: preliminary results. *Ann Surg Oncol* 2009; **16**: 887-892 [PMID: 19165544 DOI: 10.1245/s10434-009-0324-9]
51 **Moore TJ**, Moran BJ. Precision surgery, precision terminology: the origins and meaning of ELAPE. *Colorectal Dis* 2012; **14**: 1173-1174 [PMID: 22966814 DOI: 10.1111/j.1463-1318.2012.03082.x]
52 **Asplund D**, Haglind E, Angenete E. Outcome of extralevator abdominoperineal excision compared with standard surgery: results from a single centre. *Colorectal Dis* 2012; **14**: 1191-1196 [PMID: 22221401 DOI: 10.1111/j.1463-1318.2012.02930.x]
53 **MERCURY Study Group**. Extramural depth of tumor invasion at thin-section MR in patients with rectal cancer: results of the MERCURY study. *Radiology* 2007; **243**: 132-139 [PMID: 17329685]
54 **Taylor FG**, Quirke P, Heald RJ, Moran B, Blomqvist L, Swift I, Sebag-Montefiore DJ, Tekkis P, Brown G. Preoperative high-resolution magnetic resonance imaging can identify good prognosis stage I, II, and III rectal cancer best managed by surgery alone: a prospective, multicenter, European study. *Ann Surg* 2011; **253**: 711-719 [PMID: 21475011 DOI: 10.1097/SLA.0b013e31820b8d52]
55 **Guo M**, Gao C, Li D, Guo W, Shafik AA, Zbar AP, Pescatori M. MRI anatomy of the anal region. *Dis Colon Rectum* 2010; **53**: 1542-1548 [PMID: 20940604 DOI: 10.1007/DCR.0b013e3181f05256]

P- Reviewers: Kang SB, Lam AK, Riss S, Zhou PH
S- Editor: Qi Y **L- Editor**: Rutherford A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9146

World J Gastroenterol 2014 July 21; 20(27): 9146-9153
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RETROSPECTIVE STUDY

Perioperative advantages of modified laparoscopic *vs* open splenectomy and azygoportal disconnection

Guo-Qing Jiang, Ping Chen, Jian-Jun Qian, Jie Yao, Xiao-Dong Wang, Sheng-Jie Jin, Dou-Sheng Bai

Guo-Qing Jiang, Ping Chen, Jian-Jun Qian, Jie Yao, Xiao-Dong Wang, Sheng-Jie Jin, Dou-Sheng Bai, Department of Hepatobiliary Surgery, Clinical Medical College of Yangzhou University, Yangzhou 225001, Jiangsu Province, China

Author contributions: Jiang GQ and Chen P contributed equally to this work; Jiang GQ, Chen P, and Bai DS designed the research; Bai DS, Chen P, Qian JJ, Yao J, and Jin SJ performed the research; Jiang GQ and Wang XD analyzed the data; and Jiang GQ and Chen P wrote the manuscript.

Correspondence to: Dou-Sheng Bai, MD, Department of Hepatobiliary Surgery, Clinical Medical College of Yangzhou University, 98 West Nantong Rd, Yangzhou 225001, Jiangsu Province, China. bdsno1@hotmail.com

Telephone: +86-514-87373272 Fax: +86-514-87990188
Received: January 21, 2014 Revised: March 16, 2014
Accepted: April 15, 2014
Published online: July 21, 2014

Abstract

AIM: To investigate perioperative outcomes in patients undergoing modified laparoscopic splenectomy or open splenectomy and azygoportal disconnection for portal hypertension.

METHODS: This study included 44 patients who underwent modified laparoscopic splenectomy and azygoportal disconnection (MLSD) and 71 who underwent open procedures for portal hypertension. Blood samples were collected before surgery and on days 1, 3, and 7 after surgery. Markers of liver and renal function, C-reactive protein (CRP), interleukin-6 (IL-6), and procalcitonin (PCT) were measured, and perioperative variables were compared between the two groups.

RESULTS: The modified laparoscopic group showed significantly better and faster recovery, better liver and renal function, and fewer complications than the open group. CRP, IL-6, and PCT concentrations on postoperative days 1, 3, and 7 were significantly lower in the modified laparoscopic group than in the open group.

CONCLUSION: MLSD was associated with lower inflammatory immune responses, less impairment of liver and renal function, and faster and better recovery.

Key words: Portal hypertension; Laparoscopy; Splenectomy; Azygoportal disconnection; Inflammatory response

Core tip: Minimal surgical trauma is an important goal to both surgeons and patients. A novel technique, in which massively enlarged spleens are removed from the abdominal cavity with an electromechanical morcellator through an existing 12-mm port, was first developed by our surgical team for laparoscopic splenectomy and azygoportal disconnection, and greatly reduces surgical trauma for cirrhotic patients with bleeding portal hypertension and secondary hypersplenism. This technique resulted in minimal postoperative pain and scarring, faster and better postoperative recovery, and lower inflammatory immune responses.

Jiang GQ, Chen P, Qian JJ, Yao J, Wang XD, Jin SJ, Bai DS. Perioperative advantages of modified laparoscopic *vs* open splenectomy and azygoportal disconnection. *World J Gastroenterol* 2014; 20(27): 9146-9153 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9146.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9146

INTRODUCTION

Surgical trauma results in the activation of systemic immunologic and inflammatory responses; a process called surgical stress. Acute inflammatory responses are initiated by direct tissue trauma caused by incisions, dissections, organ manipulation, and vascular compromise[1-4]. The minimally invasive nature of laparoscopic surgery is thought to generate weaker systemic immune and inflam-

matory responses than traditional open surgery. These weaker responses are likely caused by the minimal manipulation of organs, as well as smaller surgical incisions.

Modified laparoscopic splenectomy and azygoportal disconnection (MLSD), which was first developed by our surgical team, is a novel technique in which massively enlarged spleens are removed from the abdominal cavity with an electromechanical morcellator through an existing 12-mm port[5]. Although resulting in less surgical trauma than open procedures, little is known about the exact pathophysiologic mechanisms that occur during MLSD. Other types of laparoscopic surgical techniques have shown immunologic advantages over traditional open surgery[1,3,6], with lower concentrations of interleukin-6 (IL-6) and C-reactive protein (CRP) induced by laparoscopic than by open surgical procedures[7-10]. Laparoscopic splenectomy and azygoportal disconnection (LSD) has become increasingly popular for the treatment of cirrhotic patients with bleeding portal hypertension and secondary hypersplenism. However, the immunologic effects of LSD, and especially MLSD, have not yet been well clarified. To our knowledge, this report is the first to compare the immunologic effects of MLSD *vs* open splenectomy and azygoportal disconnection (OSD). We therefore compared systemic inflammatory indices and perioperative outcomes in patients undergoing MLSD or OSD for portal hypertension, focusing specifically on the immunologic markers IL-6, the main indicator of surgical trauma[11]; CRP, as an important acute phase reactant produced by the liver; and procalcitonin (PCT), a pro-peptide of calcitonin produced by the thyroid gland and an early and specific biologic marker of infection.

MATERIALS AND METHODS

Patients

All cirrhotic patients who underwent LSD or MLSD for bleeding portal hypertension and secondary hypersplenism at the Clinical Medical College of Yangzhou University in China from January 2010 and May 2013 were eligible for inclusion. Data on these patients were retrospectively entered into a database. Of the 115 cirrhotic patients with bleeding portal hypertension and secondary hypersplenism, 44 elected to undergo MLSD and 71 chose OSD.

This study was not a randomized trial. During the pre-operation discussion, all patients were informed that MLSD is a minimally invasive procedure, but still in the experimental stage compared with the more typical OSD. Procedures were selected by individual patients, who provided written informed consent. This study was approved by the Ethics Committee of the Clinical Medical College of Yangzhou University.

Data collected included patient gender, age, etiology of cirrhosis, Acute Physiology and Chronic Health Evaluation (APACHE) Ⅱ score, Child-Pugh class, longitudinal diameter of the spleen, operation time, estimated intraoperative blood loss, and volume of intraoperative blood transfusion. Other factors analyzed included Visual Analogue Scale (VAS) pain score on the first day after surgery, time to first oral intake, first passage of flatus, and off-bed activity, postoperative hospital stay, perioperative complications, number of days of postoperative body temperature > 38.0 ℃, and incidence of non-fever and normal white blood cell (WBC) counts on postoperative days 1, 3, and 7. Blood analysis included WBC count, hemoglobin (Hb) concentration, platelet (PLT) count; and concentrations of aspartate transaminase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine (CER), CRP, IL-6, and PCT determined preoperatively and 1, 3, and 7 d after surgery.

VAS pain score was evaluated by direct interview using a questionnaire that rated pain intensity on a scale of 0-10[12-14], with 0 representing no pain and 10 representing very severe pain.

Surgical procedures

MLSD: After induction and intubation, patients received general anesthesia and were placed in the supine and parted-legs position. A pneumoperitoneum of 13 mm Hg was obtained with a Veress needle. A five-port method (Figure 1A) was used, including one 5-mm, three 10-mm, and one 12-mm port. The splenic artery was dissociated and clipped with a hem-o-lok, and the ligaments surrounding the spleen were divided with a LigaSure vessel-sealing device (Covidien, Boulder, CO, United States). The splenic artery and vein were transected en bloc through the 12-mm port using a linear laparoscopic vascular stapler (EndoGIA). During laparoscopic azygoportal disconnection, all paraesophageal venous collaterals were divided by the LigaSure vessel-sealing device, from back to front, from below to above, and from left to right.

The spleen was removed from the abdominal cavity through the 12-mm port using an electromechanical morcellator (TSCS, Hangzhou, China), consisting of a motor-driven cutting tube and large claw forceps. The spleen was grasped by these forceps through the cutting tube and extracted by rolling the tube (Figure 1B), allowing a cylindrical spleen sample (Figure 1C) to be cut and removed from the tube using a mild pulling force. These steps were repeated until the entire spleen was removed. The entire upper quadrant was irrigated and carefully inspected for residual tissue and bleeding.

OSD: OSD was performed through either a midline laparotomy or a left subcostal incision using traditional methods. Splenectomy was performed before azygoportal disconnection.

Statistical analysis

Data are presented as the mean ± SD, median (range), or number (%). Group means were compared using Student's *t*-test or the Mann-Whitney *U* test, as appropriate, and χ^2 tests were used to compare percentages. A *P* val-

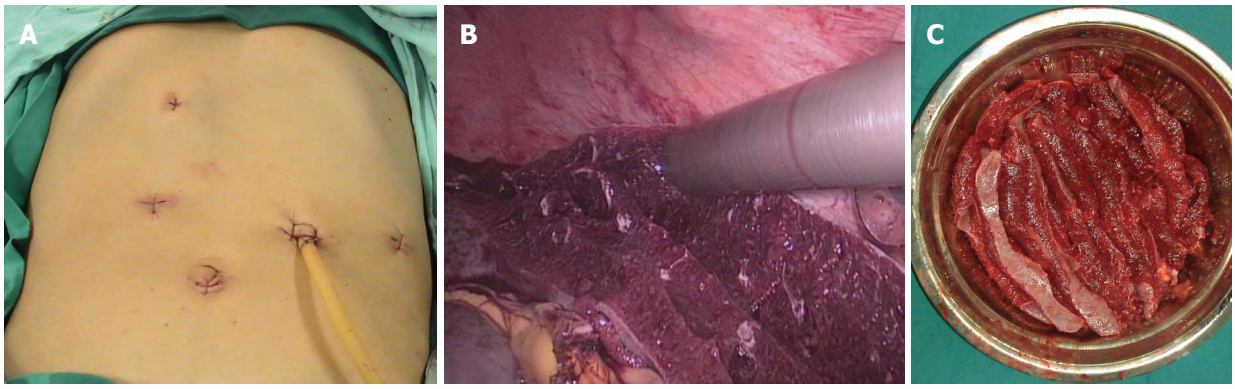


Figure 1 Five-port position for modified laparoscopic splenectomy and azygoportal disconnection using an electromechanical morcellator. A: Five-port position; B: Electromechanical morcellator portraying the spleen tissue; C: Cylindrical splenic tissue.

Table 1 Baseline demographic and clinical characteristics of the modified laparoscopic splenectomy and azygoportal disconnection and open splenectomy and azygoportal disconnection groups

Variable	MLSD (n = 44)	OSD (n = 71)	P value
Gender, M/F, n	27/17	41/30	0.701
Age, mean ± SD, yr	54.98 ± 10.41	52.46 ± 10.33	0.209
Etiology, n			
HBV cirrhosis	23	46	0.183
HCV cirrhosis	3	5	0.963
Schistosome cirrhosis	6	9	0.882
Alcoholic cirrhosis	3	4	0.796
Autoimmunity liver cirrhosis	9	7	0.111
APACHE Ⅱ score, mean ± SD	3.32 ± 2.27	3.49 ± 2.57	0.712
Child-Pugh classification, A/B, n	29/15	39/32	0.244
Longitudinal diameter of spleen, mean ± SD, mm	179.45 ± 27.88	185.44 ± 33.24	0.321
WBC, mean ± SD, 10^9/L	2.89 ± 1.65	3.07 ± 1.85	0.596
Hb , mean ± SD, g/dL	104.16 ± 26.95	95.34 ± 28.14	1.000
PLT, mean ± SD, 10^9/L	40.32 ± 7.74	38.24 ± 9.18	0.214
TBIL, mean ± SD, μmol/L	19.21 ± 9.76	20.88 ± 12.66	0.454
AST, mean ± SD, U/L	34.93 ± 21.26	35.89 ± 15.08	0.779
ALT, mean ± SD, U/L	31.14 ± 23.91	29.41 ± 15.28	0.637
BUN, mean ± SD, mmol/L	5.93 ± 2.25	5.38 ± 1.95	0.168
CER, mean ± SD, μmol/L	72.32 ± 17.63	71.15 ± 19.81	0.748

ALT: Alanine aminotransferase; AST: Aspartate transaminase; BUN: Urea nitrogen; CER: Creatinine; Hb: Hemoglobin; HBV: Hepatitis B virus; HCV: Hepatitis C virus; MLSD: Modified laparoscopic splenectomy and azygoportal disconnection; OSD: Open splenectomy and azygoportal disconnection; PLT: Platelet; WBC: White blood cell.

ue < 0.05 was considered significant. SPSS 13.0 software (SPSS, Chicago, IL, United States) was used for statistical analysis.

RESULTS

Of 115 cirrhotic patients with bleeding portal hypertension and secondary hypersplenism, 44 underwent MLSD and 71 underwent OSD. There were no significant differences between groups in terms of patient gender, age, etiology of cirrhosis, APACHE Ⅱ score, Child-Pugh class, longitudinal spleen diameter, preoperative WBC counts, or preoperative Hb, PLT, TBIL, AST, ALT, BUN, and CER concentrations (Table 1).

Operation

The median operation time was significantly longer for the MLSD than for the OSD group (P < 0.0001; Table 2). Median intraoperative estimated blood loss was significantly lower for MLSD than for OSD (P < 0.0001), as was median intraoperative volume of blood transfused (P < 0.05) (Table 2).

Recovery after surgery

The mean ± SD VAS pain score on the first day after surgery was significantly lower in the MLSD than the OSD group (P < 0.0001; Table 2). Mean times to first oral intake, first flatus, and off-bed activity were significantly shorter in the MLSD than the OSD group (all P < 0.001), as was median hospital stay (P < 0.0001) (Table 2).

Complications

Seven of the 44 patients (15.91%) in the MLSD group and 26 of 71 (36.62%) in the OSD group experienced

Table 2 Intraoperative and postoperative characteristics of the modified laparoscopic splenectomy and azygoportal disconnection and open splenectomy and azygoportal disconnection groups

Variable	MLSD (n = 44)	OSD (n = 71)	P value
Operation time, median (range), min	210 (140-390)	180 (110-300)	< 0.0001
Estimated blood loss, median (range), mL	150 (50-800)	300 (50-1200)	< 0.0001
Blood transfused, median (range), mL	0 (0-400)	0 (0-700)	0.024
VAS pain score on the first day, mean ± SD	2.50 ± 0.85	5.06 ± 1.08	< 0.0001
Time to first oral intake, mean ± SD, d	1.52 ± 0.63	2.76 ± 0.62	< 0.0001
Time to first flatus, mean ± SD, d	2.36 ± 0.97	3.18 ± 1.00	0.0004
Time to off-bed activity, mean ± SD, d	2.59 ± 0.69	5.96 ± 0.93	< 0.0001
Postoperative hospital stay, median (range), d	10 (7-18)	15 (7-28)	< 0.0001
Perioperative complications, n	7	26	0.017
Incision complications	0	9	0.035
Incision hernia	0	1	
Superficial SSI	0	5	
Deep SSI	0	3	
Pneumonia	0	3	0.436
Organ space SSI	0	2	0.697
Emergency operation for bleeding	0	3	0.436
Pancreatic fistula	2	1	0.672
Asymptomatic portal vein thrombosis	5	8	1.000

MLSD: Modified laparoscopic splenectomy and azygoportal disconnection; OSD: Open splenectomy and azygoportal disconnection; VAS: Visual analog scale; SSI: Surgical site infection.

Table 3 Postoperative fever and white blood cell counts of the modified laparoscopic splenectomy and azygoportal disconnection and open splenectomy and azygoportal disconnection groups

Variable	MLSD (n = 44)	OSD (n = 71)	P value
Postoperative fever, mean ± SD, d	4.09 ± 3.16	6.89 ± 3.55	< 0.0001
No fever, n	7	2	0.029
WBC 0 d, mean ± SD, 10^9/L	2.89 ± 1.65	3.07 ± 1.85	0.596
WBC 1 d, median (range), 10^9/L	11.45 (5.2-17.4)	16.9 (6.1-33.90)	< 0.0001
WBC 3 d, mean ± SD, 10^9/L	11.63 ± 3.29	13.72 ± 4.67	0.011
WBC 7 d, median (range), 10^9/L	8.4 (5-23.1)	9.5 (4.9-38)	0.035
Normal WBC, 1 d, n	15	3	< 0.0001
Normal WBC, 3 d, n	14	10	0.023
Normal WBC, 7 d, n	36	39	0.003

MLSD: Modified laparoscopic splenectomy and azygoportal disconnection; OSD: Open splenectomy and azygoportal disconnection; Postoperative fever, the number of days of postoperative body temperature > 38.0 ℃; WBC: White blood cell count; day 0: Day of admission; day 1: Postoperative day 1; day 3: Postoperative day 3; day 7: Postoperative day 7.

postoperative complications ($P < 0.05$). The seven complications in the MSLD group included two patients with pancreatic fistulae and five with asymptomatic portal vein thrombosis. In the OSD group, one patient had a pancreatic fistula, eight had asymptomatic portal vein thrombosis, nine had incision complications [including five with superficial surgical site infections (SSI), three with deep SSI, and one with an incision hernia], three had pneumonia, two had organ space SSI, and three required emergency laparotomy for bleeding. All complications were successfully managed. The incision complication rate was significantly lower in the MLSD than in the OSD group (0% *vs* 12.68%, $P < 0.05$). Emergency laparotomy operation for bleeding was not necessary following MLSD (Table 2).

Body temperature and white blood cell counts

Before surgery, none of the patients in either group had fever. The mean ± SD number of days of postoperative body temperature > 38.0 ℃ was significantly lower after MLSD than after OSD ($P < 0.0001$; Table 3). Seven patients in the MLSD and two in the OSD group did not have fever postoperatively, making the rate significantly higher in the MLSD than in the OSD group (15.91% *vs* 2.82%, $P < 0.05$; Table 3). Although WBC count at admission was similar in both the MLSD and OSD groups ($P > 0.05$ each), median WBC counts on postoperative days 1 ($P < 0.0001$), 3 ($P < 0.05$), and 7 ($P < 0.05$) were significantly lower after MLSD than after OSD (Table 3). The percentage of patients with normal WBC counts on postoperative days 1 (34.09% *vs* 4.22%, $P < 0.0001$), 3 (31.82% *vs* 14.08%, $P < 0.05$), and 7 (81.82% *vs* 54.93%, $P < 0.01$) were all significantly higher after MLSD than after OSD (Table 3).

Postoperative liver and renal function

Preoperative AST and ALT concentrations were similar in the two groups (Table 1). Although AST concentrations were similar on postoperative day 1 ($P > 0.05$), mean AST on postoperative days 3 ($P < 0.05$) and 7 ($P < 0.001$) were significantly lower after MLSD than after OSD (Table 4). Moreover, although ALT concentrations on postoperative days 1 and 3 were similar in the two groups, ALT was significantly lower after MLSD than after OSD on day 7 ($P < 0.01$) (Table 4).

Preoperative BUN and CER concentrations were similar in the two groups (Table 1). Although mean BUN was significantly lower after MLSD than after OSD on postoperative day 1 ($P < 0.05$), BUN concentrations were similar in the two groups on postoperative days 3 and 7 (Table 4). Similarly, mean CER was significantly lower after MLSD than after OSD on day 1 ($P < 0.05$), but was similar in the two groups on postoperative days 3 and 7 (Table 4).

CRP, IL-6, and PCT

Median CRP concentrations were similar preoperatively in the MLSD and OSD groups, but were significantly lower in the MLSD group on postoperative days 1 ($P <$

Table 4 Postoperative liver and renal functions of the modified laparoscopic splenectomy and azygoportal disconnection and open splenectomy and azygoportal disconnection groups

Variable	MLSD (n = 44)	OSD (n = 71)	P value
AST 1 d, mean ± SD, U/L	57.32 ± 27.89	87.14 ± 135.13	0.152
AST 3 d, mean ± SD, U/L	33.84 ± 22.16	50.86 ± 43.83	0.018
AST 7 d, mean ± SD, U/L	25.66 ± 11.28	33.63 ± 16.32	0.005
ALT 1 d, mean ± SD, U/L	40.14 ± 18.81	61.66 ± 105.43	0.183
ALT 3 d, mean ± SD, U/L	31.93 ± 25.21	46.93 ± 56.60	0.100
ALT 7 d, mean ± SD, U/L	21.14 ± 12.63	29.68 ± 17.36	0.003
BUN 1 d, mean ± SD, mmol/L	5.52 ± 1.76	6.54 ± 2.33	0.014
BUN 3 d, mean ± SD, mmol/L	6.63 ± 2.20	7.33 ± 2.85	0.171
BUN 7 d, mean ± SD, mmol/L	4.76 ± 2.02	5.52 ± 2.21	0.069
CER 1 d, mean ± SD, μmol/L	75.27 ± 16.22	84.99 ± 20.92	0.010
CER 3 d, mean ± SD, μmol/L	59.75 ± 16.64	66.04 ± 19.08	0.074
CER 7 d, mean ± SD, μmol/L	60.88 ± 13.92	60.83 ± 16.17	0.986

ALT: Alanine aminotransferase; AST: Aspartate transaminase; BUN: Blood urea nitrogen; CER: Creatinine; MLSD: Modified laparoscopic splenectomy and azygoportal disconnection; OSD: Open splenectomy and azygoportal disconnection; day 1: Postoperative day 1; day 3: Postoperative day 3; day 7: Postoperative day 7.

Table 5 Perioperative C-reactive protein, interleukin-6, and procalcitonin concentrations of the modified laparoscopic splenectomy and azygoportal disconnection and open splenectomy and azygoportal disconnection groups

Variable	MLSD (n = 44)	OSD (n = 71)	P value
CRP day 0, median (range), mg/L	1.03 (0.02-49.21)	0.96 (0.03-13.32)	0.715
CRP day 1, mean ± SD, mg/L	28.39 ± 16.30	36.86 ± 12.86	0.002
CRP day 3, mean ± SD, mg/L	92.64 ± 53.16	114.06 ± 44.37	0.022
CRP day 7, mean ± SD, mg/L	41.14 ± 27.61	52.49 ± 29.70	0.043
IL-6 day 0, mean ± SD, pg/mL	6.14 ± 5.61	6.60 ± 7.26	0.715
IL-6 day 1, mean ± SD, pg/mL	8.09 ± 6.97	11.78 ± 9.18	0.024
IL-6 day 3, mean ± SD, pg/mL	7.65 ± 6.95	10.84 ± 8.42	0.037
IL-6 day 7, mean ± SD, pg/mL	6.58 ± 5.66	9.83 ± 8.26	0.024
PCT day 0, mean ± SD, ng/mL	0.45 ± 0.25	0.47 ± 0.27	0.722
PCT 1 d, median (range), ng/mL	0.97 (0.36-3.2)	1.96 (0.54-10.08)	< 0.0001
PCT 3 d, median (range), ng/mL	0.795 (0.3-2.67)	1.45 (0.6-4.88)	< 0.0001
PCT 7 d, median (range), ng/mL	0.5 (0.28-4.01)	0.77 (0.38-5.87)	< 0.0001

CRP: C-reactive protein; IL-6: Interleukin-6; MLSD: Modified laparoscopic splenectomy and azygoportal disconnection; OSD: Open splenectomy and azygoportal disconnection; PCT: Procalcitonin; day 0: Day of admission; day 1: Postoperative day 1; day 3: Postoperative day 3; day 7: Postoperative day 7.

0.001), 3 ($P < 0.05$), and 7 ($P < 0.05$) (Table 5). Similarly, IL-6 concentrations did not differ preoperatively, but were significantly lower in the MLSD group on postoperative days 1, 3, and 7 ($P < 0.05$ each) (Table 5). Additionally, PCT concentrations were similar in the two groups preoperatively, but were significantly lower in the MLSD than in the OSD group on postoperative days 1, 3, and 7 ($P < 0.0001$ each) (Table 5).

DISCUSSION

LSD has become more frequently used at some institutions to treat portal hypertension and has been shown to be superior to OSD in reducing postoperative pain severity, time to first flatus, and the duration of hospital stay and convalescence[15,16]. We developed an even less invasive technique, MLSD, for portal hypertension, extending the advantages of LSD to patients likely to most benefit from it (namely cirrhotic patients with bleeding portal hypertension and hypersplenism). This study compared both subjective and objective parameters, including measures of surgical trauma, convalescence, and burden on the immune system, in patients undergoing MLSD and OSD. To our knowledge, this is the first retrospective clinical study comparing these techniques in patients with portal hypertension.

None of the patients included in this study had a liver transplant or TIPS. In China, there is a shortage of donor livers, whereas TIPS is usually used to treat portal hypertension, especially as a bridge to transplantation. Although TIPS can reduce portal pressure to prevent recurrent gastroesophageal variceal bleeding, it cannot be used to treat secondary hypersplenism. Furthermore, TIPS has been associated with portosystemic encephalopathy.

Traditional LSD utilizes several methods to remove massively enlarged spleens, including the creation of an enlarged incision to morcellate and remove the spleen enveloped in a cumbersome intracorporeal bag or hand-assisted laparoscopy to remove the spleen through a hand-assisted incision[15-21]. During MLSD, a massively enlarged spleen is removed through the existing 12 mm port using an electromechanical morcellator. Therefore, MLSD appears to involve less surgical trauma than LSD. Moreover, to avoid damage to organs or tissues when using the electromechanical morcellator to cut the spleen, the patient is placed in the reverse Trendelenburg position, the dissected spleen is placed in the left subphrenic space, and a splenic retractor or forceps is used to lift the upper pole of the spleen to maintain it in an ideal location.

Although the operation time of LSD was longer than that of OSD, LSD was associated with lower estimated blood loss and a lower volume of blood transfused[15]. We observed similar outcomes when comparing MLSD with OSD. Only one patient in the MLSD group required a blood transfusion. These advantages of MLSD and LSD are due to their good operational view and exposure, as well as the use of a LigaSure vessel-sealing device, which is efficient in dividing the splenogastric ligament and esophagogastric varices.

We also found that convalescence was more rapid after MLSD than after OSD. VAS pain score on the first postoperative day was significantly lower, and times to first oral intake, passage of flatus, and off-bed activity,

as well as postoperative hospital stay, were significantly shorter in the MLSD than the OSD group. Similarly, LSD resulted in faster recovery than OSD, as shown by a reduced time to first flatus and postoperative hospital stay[15,16].

MLSD also showed other benefits, including fewer numbers of days of postoperative body temperature > 38.0 ℃; a lower rate of no fever postoperatively; lower WBC counts on postoperative days 1, 3, and 7; and higher rates of normal WBC counts on postoperative days 1, 3, and 7. In contrast, a previous study found no significant differences in WBC count on postoperative day 7 between patients undergoing LSD and OSD[16]. These findings suggest that MLSD results in weaker inflammatory responses and better recovery than OSD.

The absence of incision complications in patients undergoing LSD may be due to the small sizes of the incisions, and earlier ambulation after surgery may result from decreased postoperative pain, but the postoperative rates of total and incision-associated complications did not differ significantly in patients undergoing LSD and OSD[16]. The incisions resulting from MLSD were less invasive than those resulting from LSD, with no incision complications after MLSD. However, we found that the postoperative rates of total and incision-associated complications differed in our MLSD and OSD groups. The difference between studies was likely due to the smaller sample size in the previous study, which included 24 patients in the LSD and 30 in the OSD group[16].

The earlier study found that ALT and AST concentrations on postoperative day 7 were significantly lower in the LSD than in the OSD group[16]. Our findings were similar, except that AST concentrations on postoperative day 3 were significantly lower in the MLSD than in the OSD group. We also found that BUN and CER concentrations on postoperative day 1 were lower in the MLSD group, providing further evidence for the benefits of MLSD. These findings also suggest a difference in the rate of recovery from surgical trauma to the liver and kidneys.

The immune response to surgical trauma involves a complex cascade of many types of mediators and immune cells. IL-6 is thought to play a pivotal role in the pathogenesis of surgical trauma, with increased IL-6 concentrations found to correlate with the magnitude of surgical trauma[22,23], as well as contributing to fever[24]. CRP is an acute phase molecule produced in the liver during tissue injury, whereas PCT is a pro-peptide of calcitonin produced by the thyroid gland and a highly specific marker of clinically relevant bacterial infections and sepsis[25,26].

Systemic stress responses are lower after laparoscopic than after conventional open surgery[4,27], with differences in cytokine concentrations and cell-mediated immune responses observed both in animal experiments and clinical trials[28-30]. Laparoscopic techniques have been shown to have an immunologic advantage over traditional open surgery[1,3,6], with lower concentrations of IL-6 and CRP induced during laparoscopic than during open general and urinary surgery[7-10,24]. These differences may be due to CO_2 pneumoperitoneum; CO_2 insufflation has a protective function during laparoscopic procedures, with CO_2 acidification of the peritoneal cavity attenuating overall inflammatory response and suppressing peritoneal macrophages, which are cells that initiate inflammatory responses during surgery[31,32].

We observed similar findings, in that the concentrations of IL-6, CRP, and PCT on postoperative days 1, 3, and 7 were all lower after MLSD than after OSD. These objective measurements showed that MLSD resulted in improved immunologic function compared with OSD. Because liver function in these patients is usually very poor, postoperative immune dysfunction is an important aspect of treatment for patients with liver cirrhosis, bleeding portal hypertension, and hypersplenism. IL-6, CRP, and PCT may be better markers of immune recovery than WBC count in patients with liver cirrhosis, since patients with leukocytopenia due to hypersplenism frequently have WBC counts below the lower limit of the normal range.

Despite longer operation times, MLSD resulted in better and faster recovery, less liver and renal dysfunction, less fever, and less stimulation of host immunity than OSD. These findings may be due to: (1) the smaller size of the incision used to remove the enlarged spleen; (2) the reduced estimated blood loss during surgery; (3) CO2 pneumoperitoneum; and/or (4) fewer postoperative complications.

In conclusion, this study indicated that MLSD had numerous advantages over OSD and is technically feasible and safe. MLSD consisted of a combination of LSD and a novel technique with an electromechanical morcellator. MLSD was associated with minimal postoperative pain and scarring, faster and better postoperative recovery, and lower inflammatory immune responses than OSD. MLSD can therefore extend the advantages of LSD to patients with portal hypertension. It represents a promising and minimally-invasive treatment that may become the gold standard of surgical procedures for liver cirrhosis patients with bleeding portal hypertension and hypersplenism.

ACKNOWLEDGMENTS

The authors thank Yao Tang, biostatistician at the Clinical Medical College of Yangzhou University, for his assistance with statistical analyses.

COMMENTS

Background

Although many types of laparoscopic surgery have shown immunologic benefits, the effects of modified laparoscopic splenectomy and azygoportal disconnection (MLSD) for portal hypertension have not been investigated thoroughly.

Research frontiers

Systemic stress responses are lower after laparoscopic than after conventional open surgery, with differences in cytokine concentrations and cell-mediated immune responses observed both in animal experiments and clinical trials. Laparoscopic techniques have been shown to have an immunologic advantage

over traditional open surgery, with lower concentrations of interleukin-6 (IL-6) and C-reactive protein induced during laparoscopic than during open general and urinary surgery.

Innovations and breakthroughs

The authors developed an even less invasive technique, MLSD. During the procedure, an electromechanical morcellator allows for easy extraction of the entire massive splenic tissue through the existing 12 mm port without the cumbersome intracorporeal bag, enlarged incision, or hand-assisted incision used in traditional laparoscopic splenectomy and azygoportal disconnection (LSD). Therefore, MLSD appears to involve less surgical trauma than LSD.

Applications

MLSD is technically feasible, safe, and resulted in better and faster recovery, less liver and renal dysfunction, less fever, and less stimulation of host immunity than open splenectomy and azygoportal disconnection. MLSD represents a promising and minimally-invasive treatment for liver cirrhosis patients with bleeding portal hypertension and hypersplenism.

Terminology

Surgical trauma, a process also called surgical stress, activates systemic immunologic and inflammatory responses, which are characterized by (1) release of the pro-inflammatory cytokines tumor necrosis factor α, interleukin (IL)-1, and IL-6; (2) neutrophil activation and microvascular adherence; and (3) uncontrolled polymorphonuclear and macrophage oxidative burst.

Peer review

The authors investigated the perioperative advantages of modified laparoscopic *vs* open splenectomy and azygoportal disconnection. The paper and the results are interesting. The manuscript is original and may be useful to clinicians.

REFERENCES

1 **Novitsky YW**, Litwin DE, Callery MP. The net immunologic advantage of laparoscopic surgery. *Surg Endosc* 2004; **18**: 1411-1419 [PMID: 15791361 DOI: 10.1007/s00464-003-8275-x]

2 **Biffl WL**, Moore EE, Moore FA, Peterson VM. Interleukin-6 in the injured patient. Marker of injury or mediator of inflammation? *Ann Surg* 1996; **224**: 647-664 [PMID: 8916880]

3 **Sylla P**, Kirman I, Whelan RL. Immunological advantages of advanced laparoscopy. *Surg Clin North Am* 2005; **85**: 1-18, vii [PMID: 15619524 DOI: 10.1016/j.suc.2004.09.005]

4 **Buunen M**, Gholghesaei M, Veldkamp R, Meijer DW, Bonjer HJ, Bouvy ND. Stress response to laparoscopic surgery: a review. *Surg Endosc* 2004; **18**: 1022-1028 [PMID: 15136930 DOI: 10.1007/s00464-003-9169-7]

5 **Jiang G**, Qian J, Yao J, Wang X, Jin S, Bai D. A New Technique for Laparoscopic Splenectomy and Azygoportal Disconnection. *Surg Innov* 2013; **21**: 256-262 [PMID: 23804998 DOI: 10.1177/1553350613492587]

6 **Vittimberga FJ**, Foley DP, Meyers WC, Callery MP. Laparoscopic surgery and the systemic immune response. *Ann Surg* 1998; **227**: 326-334 [PMID: 9527054 DOI: 10.1097/00000658-199803000-00003]

7 **Schwenk W**, Jacobi C, Mansmann U, Böhm B, Müller JM. Inflammatory response after laparoscopic and conventional colorectal resections - results of a prospective randomized trial. *Langenbecks Arch Surg* 2000; **385**: 2-9 [PMID: 10664112 DOI: 10.1007/s004230050002]

8 **Sietses C**, Wiezer MJ, Eijsbouts QA, Beelen RH, van Leeuwen PA, von Blomberg BM, Meijer S, Cuesta MA. A prospective randomized study of the systemic immune response after laparoscopic and conventional Nissen fundoplication. *Surgery* 1999; **126**: 5-9 [PMID: 10418585 DOI: 10.1067/msy.1999.98702]

9 **Scheepers JJ**, Sietses C, Bos DG, Boelens PG, Teunissen CM, Ligthart-Melis GC, Cuesta MA, van Leeuwen PA. Immunological consequences of laparoscopic versus open transhiatal resection for malignancies of the distal esophagus and gastroesophageal junction. *Dig Surg* 2008; **25**: 140-147 [PMID: 18446036 DOI: 10.1159/000128171]

10 **Grande M**, Tucci GF, Adorisio O, Barini A, Rulli F, Neri A, Franchi F, Farinon AM. Systemic acute-phase response after laparoscopic and open cholecystectomy. *Surg Endosc* 2002; **16**: 313-316 [PMID: 11967686 DOI: 10.1007/s00464-001-9042-5]

11 **Cruickshank AM**, Fraser WD, Burns HJ, Van Damme J, Shenkin A. Response of serum interleukin-6 in patients undergoing elective surgery of varying severity. *Clin Sci* (Lond) 1990; **79**: 161-165 [PMID: 2167805]

12 **Chapman CR**, Casey KL, Dubner R, Foley KM, Gracely RH, Reading AE. Pain measurement: an overview. *Pain* 1985; **22**: 1-31 [PMID: 4011282 DOI: 10.1016/0304-3959(85)90145-9]

13 **Hoffmann NE**, Bischof JC. The cryobiology of cryosurgical injury. *Urology* 2002; **60**: 40-49 [PMID: 12206847 DOI: 10.1016/S0090-4295(02)01683-7]

14 **Gage AA**, Baust J. Mechanisms of tissue injury in cryosurgery. *Cryobiology* 1998; **37**: 171-186 [PMID: 9787063 DOI: 10.1006/cryo.1998.2115]

15 **Jiang XZ**, Zhao SY, Luo H, Huang B, Wang CS, Chen L, Tao YJ. Laparoscopic and open splenectomy and azygoportal disconnection for portal hypertension. *World J Gastroenterol* 2009; **15**: 3421-3425 [PMID: 19610145 DOI: 10.3748/wjg.15.3421]

16 **Zheng X**, Liu Q, Yao Y. Laparoscopic splenectomy and esophagogastric devascularization is a safe, effective, minimally invasive alternative for the treatment of portal hypertension with refractory variceal bleeding. *Surg Innov* 2013; **20**: 32-39 [PMID: 22461108 DOI: 10.1177/1553350612441863]

17 **Helmy A**, Abdelkader Salama I, Schwaitzberg SD. Laparoscopic esophagogastric devascularization in bleeding varices. *Surg Endosc* 2003; **17**: 1614-1619 [PMID: 12874686 DOI: 10.1007/s00464-002-8928-1]

18 **Wang Y**, Ji Y, Zhu Y, Xie Z, Zhan X. Laparoscopic splenectomy and azygoportal disconnection with intraoperative splenic blood salvage. *Surg Endosc* 2012; **26**: 2195-2201 [PMID: 22278104 DOI: 10.1007/s00464-012-2159-x]

19 **Yamamoto J**, Nagai M, Smith B, Tamaki S, Kubota T, Sasaki K, Ohmori T, Maeda K. Hand-assisted laparoscopic splenectomy and devascularization of the upper stomach in the management of gastric varices. *World J Surg* 2006; **30**: 1520-1525 [PMID: 16855808 DOI: 10.1007/s00268-005-0243-2]

20 **Wang Y**, Zhan X, Zhu Y, Xie Z, Zhu J, Ye Z. Laparoscopic splenectomy in portal hypertension: a single-surgeon 13-year experience. *Surg Endosc* 2010; **24**: 1164-1169 [PMID: 20033218 DOI: 10.1007/s00464-009-0744-4]

21 **Xin Z**, Qingguang L, Yingmin Y. Total laparoscopic versus open splenectomy and esophagogastric devascularization in the management of portal hypertension: a comparative study. *Dig Surg* 2009; **26**: 499-505 [PMID: 20090339 DOI: 10.1159/000236033]

22 **Allendorf JD**, Bessler M, Kayton ML, Oesterling SD, Treat MR, Nowygrod R, Whelan RL. Increased tumor establishment and growth after laparotomy vs laparoscopy in a murine model. *Arch Surg* 1995; **130**: 649-653 [PMID: 7763175 DOI: 10.1001/archsurg.1995.01430060087016]

23 **Hildebrandt U**, Kessler K, Plusczyk T, Pistorius G, Vollmar B, Menger MD. Comparison of surgical stress between laparoscopic and open colonic resections. *Surg Endosc* 2003; **17**: 242-246 [PMID: 12399854 DOI: 10.1007/s00464-001-9148-9]

24 **Aminsharifi A**, Salehipoor M, Arasteh H. Systemic immunologic and inflammatory response after laparoscopic versus open nephrectomy: a prospective cohort trial. *J Endourol* 2012; **26**: 1231-1236 [PMID: 22471699 DOI: 10.1089/end.2012.0110]

25 **Takakura Y**, Hinoi T, Egi H, Shimomura M, Adachi T, Saito Y, Tanimine N, Miguchi M, Ohdan H. Procalcitonin as a predictive marker for surgical site infection in elective colorectal cancer surgery. *Langenbecks Arch Surg* 2013; **398**: 833-839 [PMID: 23784676 DOI: 10.1007/s00423-013-1095-0]

26 **Ha YE**, Kang CI, Wi YM, Chung DR, Kang ES, Lee NY, Song JH, Peck KR. Diagnostic usefulness of procalcitonin as a marker of bacteremia in patients with acute pyelonephritis. *Scand J Clin Lab Invest* 2013; **73**: 444-448 [PMID: 23772894 DOI: 10.3109/00365513.2013.803231]

27 **Gupta A**, Watson DI. Effect of laparoscopy on immune

function. *Br J Surg* 2001; **88**: 1296-1306 [PMID: 11578282 DOI: 10.1046/j.0007-1323.2001.01860.x]
28 **Hajri A**, Mutter D, Wack S, Bastien C, Gury JF, Marescaux J, Aprahamian M. Dual effect of laparoscopy on cell-mediated immunity. *Eur Surg Res* 2000; **32**: 261-266 [PMID: 11111169 DOI: 10.1159/000008773]
29 **Holub Z**. Impact of laparoscopic surgery on immune function. *Clin Exp Obstet Gynecol* 2002; **29**: 77-81 [PMID: 12171321]
30 **Tang CL**, Eu KW, Tai BC, Soh JG, MacHin D, Seow-Choen F. Randomized clinical trial of the effect of open versus laparoscopically assisted colectomy on systemic immunity in patients with colorectal cancer. *Br J Surg* 2001; **88**: 801-807 [PMID: 11412248 DOI: 10.1046/j.1365-2168.2001.01781.x]
31 **Hanly EJ**, Aurora AA, Shih SP, Fuentes JM, Marohn MR, De Maio A, Talamini MA. Peritoneal acidosis mediates immunoprotection in laparoscopic surgery. *Surgery* 2007; **142**: 357-364 [PMID: 17723887 DOI: 10.1016/j.surg.2007.02.017]
32 **Shimotakahara A**, Kuebler JF, Vieten G, Kos M, Metzelder ML, Ure BM. Carbon dioxide directly suppresses spontaneous migration, chemotaxis, and free radical production of human neutrophils. *Surg Endosc* 2008; **22**: 1813-1817 [PMID: 18074179 DOI: 10.1007/s00464-007-9703-0]

P- Reviewer: Papparella A
S- Editor: Qi Y **L- Editor**: Rutherford A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9154

World J Gastroenterol 2014 July 21; 20(27): 9154-9161
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CLINICAL TRIALS STUDY

Pain sensation in pancreatic diseases is not uniform: The different facets of pancreatic pain

Jan G D'Haese, Mark Hartel, Ihsan Ekin Demir, Ulf Hinz, Frank Bergmann, Markus W Büchler, Helmut Friess, Güralp O Ceyhan

Jan G D'Haese, Mark Hartel, Ihsan Ekin Demir, Helmut Friess, Güralp O Ceyhan, Department of Surgery, Klinikum Rechts der Isar, Technische Universität München, D-81675 Munich, Germany
Ulf Hinz, Unit for Documentation and Statistics, Department of General Surgery, University of Heidelberg, 69117 Heidelberg, Germany
Frank Bergmann, Institute of Pathology, University of Heidelberg, 69117 Heidelberg, Germany
Markus W Büchler, Department of General Surgery, University of Heidelberg, 69117 Heidelberg, Germany
Author contributions: D'Haese JG and Hartel M contributed equally to this manuscript; Ceyhan GO, and Friess H designed and supervised the study; D'Haese JG and Hartel M performed the data collection and analysis; Demir IE and Hinz U performed and verified the statistical analyses; Friess H and Büchler MW provided the clinical data for correlation analyses; Bergmann F made the histopathological verification of the diagnosis and cancer classification; D'Haese JG, Hartel M, Demir IE, and Ceyhan GO wrote the manuscript; all authors read and approved the final version of the manuscript.
Correspondence to: Güralp O Ceyhan, MD, Department of Surgery, Klinikum Rechts der Isar, Technische Universität München, Ismaninger Str 22, D-81675 Munich, Germany. gueralp.ceyhan@tum.de
Telephone: +49-89-41405091 Fax: +49-89-41404870
Received: November 12, 2013 Revised: January 13, 2014
Accepted: April 2, 2014
Published online: July 21, 2014

Abstract

AIM: To systematically characterize specific pain patterns in the most frequent pancreatic diseases.

METHODS: Pain in patients with chronic pancreatitis (n = 314), pancreatic cancer (n = 469), and other pancreatic tumors (n = 249) including mucinous (n = 20) and serous cystadenoma (n = 31), invasive (n = 37) and non-invasive intraductal papillary mucinous neoplasia (IPMN; n = 48), low stage (n = 18) and high stage neuroendocrine neoplasia (n = 44), and ampullary cancer (n = 51) was registered and correlated with clinicopathological data. Survival times were estimated by the Kaplan-Meier method. Patients alive at the follow-up time were censored. Survival curves were compared statistically using the log-rank test.

RESULTS: Forty-nine point one percent of pancreatic cancer patients revealed no pain, whereas in chronic pancreatitis only 18.3% were pain free. In contrary, moderate/severe pain was registered in 15.1% in pancreatic cancer patients that was increased in chronic pancreatitis with up to 34.2%. Serous cystadenoma was asymptomatic in most cases (58.1%), whereas 78.9% of all mucinous cystadenoma patients suffered pain. In neuroendocrine neoplasia pain was not a key clinical symptom since 64% of low stage neuroendocrine neoplasia and 59% of high stage neuroendocrine neoplasia patients were pain free. Cancer localization in the pancreatic body and patients with malignant pancreatic neoplasms were associated with more severe pain. Tumor grading and stage did not show any impact on pain. Only in pancreatic cancer, pain was directly associated with impaired survival.

CONCLUSION: Pancreatic pain depicts different patterns of abdominal pain sensation according to the respective pancreatic disorder and does not allow a unification of the term pancreatic pain.

Key words: Abdominal pain; Pancreatic neoplasm; Chronic pancreatitis; Intraductal papillary mucinous neoplasia; Pancreatic cancer

Core tip: Abdominal pain is a major clinical feature in chronic pancreatitis and pancreatic cancer. Little is

known about pain sensations in other, less frequent pancreatic tumors. This study is to our knowledge the first to systematically record pain patterns in all pancreatic tumors. Additionally, these were correlated with clinicopathological data. Pain patterns in pancreatic diseases turned out to be very diverse and mainly dependent on tumor type, anatomic localization and dignity. In pancreatic cancer pain was significantly associated with impaired survival.

D'Haese JG, Hartel M, Demir IE, Hinz U, Bergmann F, Büchler MW, Friess H, Ceyhan GO. Pain sensation in pancreatic diseases is not uniform: The different facets of pancreatic pain. *World J Gastroenterol* 2014; 20(27): 9154-9161 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9154.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9154

INTRODUCTION

Chronic enduring visceral pain frequently develops in pancreatic diseases, especially in chronic pancreatitis and advanced pancreatic cancer. However, little is known about the impact of pain in less frequent pancreatic diseases like cystadenomas, intraductal papillary mucinous neoplasia (IPMN), neuroendocrine neoplasia and ampullary cancer.

Abdominal pain is the most (80%-94%) common and the most difficult to manage clinical feature among chronic pancreatitis patients[1,2]. It is often characterized as burning, intermittent, and shooting pain but can as well occur as constant or continuous pain with superimposed acute flares. The pathophysiology of chronic pancreatitis pain is poorly understood. A large recent prospective study including 540 chronic pancreatitis patients demonstrated that disease duration did not correlate with pain severity or frequency in their cohort[1]. Hence, this observation contradicts the opposite hypothesis: *i.e.*, "burn-out-hypothesis" of chronic pancreatitis progression during which pain improves with progressive pancreatic insufficiency[3].

Pancreatic cancer is a devastating disease with poor long-term survival, which is often accompanied by abdominal or back pain limiting the quality of life. Pancreatic pain in pancreatic cancer is associated with a poor prognosis and is typically described as deep-delving pain penetrating to the back[4]. Early stage pancreatic cancer is associated with abdominal pain in only 30% of patients, while 60% of patients with limited and 80% of patients with advanced pancreatic cancer complain of abdominal and/or back pain[5]. At the time of diagnosis, the disease is often advanced and up to 73% of patients suffer from abdominal pain[6]. The localization of the tumor seems to play a major role, since patients with tumors in the head of the pancreas had less pain than patients with cancer in the body or tail of the pancreas, independent of tumor stage or size[6].

Mechanisms of pain generation in pancreatic cancer and chronic pancreatitis have not been completely understood. At first glance, pain was reported to occur by completely different mechanisms in pancreatic cancer and chronic pancreatitis. In chronic pancreatitis, pain generation was attributed to ductal strictures, increased intraductal pressure, interstitial hypertension, and pancreatic pseudocysts[7,8]. The initial hypothesis on the generation of abdominal pain in pancreatic cancer was based on the mechanical pressure and/or invasion of neighboring organs, and especially by cancer cell invasion of the neural plexus. Nowadays a variety of ligands and their respective receptors have been identified to play roles in the initiation of pancreatic pain[9]. It is now widely accepted that pain sensation in both pancreatic cancer and chronic pancreatitis has been identified as neuropathic due to the prominent neuroplastic alterations which cannot be seen to that extent in any other gastrointestinal disorders[10,11]. This specific pancreatic neuropathy is characterized by enlarged intrapancreatic nerves which are increased in number and frequently infiltrated by inflammatory and/or cancer cells, leading to pancreatic neuritis and perineural cancer cell invasion[12-15]. Furthermore, pancreatic neuropathy is characterized by numerous molecular and morphological alterations at both the peripheral and the central nervous system level[10]. Increased peripheral nociceptive signals mediated by neurotransmitters and neurotrophic factors together with neural damage and neuroplastic alterations are paralleled by hypersensitive dorsal root ganglia and spinal cord neurons[10]. At lasts, the cerebral cortex adapts to these changes by increasing its basal activity[16]. These phenomena are closely associated with increased abdominal pain sensation in the respective patients[4]. Such neuropathic changes or neuropathic pain sensation were not registered in other pancreatic tumors like, cystadenomas, IPMN, neuroendocrine neoplasia and ampullary cancer.

Little is known about pain sensation and their mechanisms in pancreatic neoplasms other than pancreatic cancer and chronic pancreatitis and their pain patterns have not yet been fully characterized. Therefore, the current study aimed at systematically analyzing pain sensation in patients with different pancreatic neoplasms. Both chronic pancreatitis and all pancreatic tumors including pancreatic cancer, mucinous and serous cystadenoma, IPMN, neuroendocrine neoplasia, and ampullary cancer were evaluated regarding their pain intensity and frequency and associated with tumor localization, dignity, stage and grading.

MATERIALS AND METHODS

Patients and tissues

Pancreatic tissue samples were collected from patients following pancreatic resection due to the following pathologies: Pancreatic adenocarcinoma (n = 469), chronic pancreatitis (n = 314) and other pancreatic tumors/PTm

Table 1 Pancreatic disease entities and the severity of pain *n* (%)

Diagnosis	Pain			*P* value
	Pain 0	Pain Ⅰ	Pain Ⅱ	
Pancreatic cancer	211 (49.0)	154 (35.8)	65 (15.1)	< 0.0001
Chronic pancreatitis	54 (18.3)	140 (47.5)	101 (34.2)	
Serous cystadenoma	18 (58.0)	12 (38.7)	1 (3.2)	< 0.02
Mucinous cystadenoma	4 (21.0)	11 (57.9)	4 (21.0)	
Non-invasive IPMN	19 (46.3)	20 (48.8)	2 (4.9)	0.235
Invasive IPMN	11 (32.4)	18 (52.9)	5 (14.7)	
Low stage neuroendocrine neoplasia	9 (64.3)	5 (35.7)	0 (0.0)	0.565
High stage neuroendocrine neoplasia	23 (59.0)	13 (33.3)	3 (7.7)	
AmpC	27 (61.4)	13 (29.6)	4 (9.1)	
Total	322 (49.4)	246 (37.7)	84 (12.9)	

IPMN: Intraductal papillary mucinous neoplasia.

(n = 249) including mucinous (n = 20) and serous cystadenoma (n = 31), invasive intraductal papillary mucinous neoplasia (IPMN; n = 37) and non-invasive IPMN (n = 48), low stage (UICC Stage 1-2) neuroendocrine neoplasia (n = 18) and high stage (UICC Stage 2-4) neuroendocrine neoplasia (n = 44), and ampullary cancer (n = 51). The stage of pancreatic cancer, invasive IPMN, neuroendocrine neoplasia and ampullary cancer was graded according to the international classification of the UICC. The study protocol was approved by the ethics committee of the University of Heidelberg (Germany), Tissue preservation was performed as reported previously[13].

Clinicopathological analysis

Consecutive sections obtained from paraformaldehyde-fixed and paraffin-embedded pancreatic tissue from each patient were stained with hematoxylin and eosin for concomitant histomorphological examination. Histopathological verification of the diagnosis and cancer classification was performed by an expert in pancreatic pathology.

Pain analysis

In all patients, the individual pain score was prospectively recorded prior to the operation in hospital one day before surgery, including pain intensity and frequency. The intensity of pain was graded by using the following scale: 0 = none, 1 = mild, 2 = moderate (abdominal discomfort or pain which is non-disabling but requires analgesics) and 3 = severe pain (pain which is disabling and controlled only by narcotic analgesics) In addition, the frequency of pain was graded as 3 = daily, 2 = weekly, and 1 = monthly. To calculate the severity of pain, pain intensity and pain frequency of each individual were multiplied. According to the final pain score, the patients were categorized into 3 groups: Pain 0 (0) representing the group of patients who did not have any pain; Pain I (1-3) representing the group of patients with mild pain; and Pain Ⅱ (4-9), the group that suffered from moderate to severe pain, as demonstrated previously[13,17,18].

Survival

Overall survival was defined as the time from the date of tumor resection to either death from any cause or last follow-up. At the time of analysis, 384 patients with pancreatic cancer (81.9%) were dead, and 85 (18.1%) were alive.

Statistical analysis

Statistical analysis was performed by an expert statistician (UH) using the SAS (Release 9.1; SAS Institute, Inc, Cary, NC, United States) and GraphPad Prism 4 Software. Survival times were estimated by the Kaplan-Meier method. Patients alive at the follow-up time were censored. Survival curves were compared statistically using the log-rank test. The χ^2 test, if appropriate or the Fisher's exact test was used to compare subgroups with respect to pain severity. Two-sided P values were always computed, and a P value was considered statistically significant at the 5% level.

RESULTS

Distinct differences in pancreatic pain depending on pancreatic disease entity

Almost 50% of patients with pancreatic cancer did not have any pain (Pain 0), while only 15.1% of patients suffered from moderate to severe pain (Pain Ⅱ; Table 1). Distinct differences in pancreatic pain are evident when especially comparing the pain pattern of patients with chronic pancreatitis to those with pancreatic cancer. Nearly half of all pancreatic cancer patients (49.1%) revealed no pain at all, whereas this figure was noticeably less in chronic pancreatitis patients with only 18.3% of the total chronic pancreatitis population (Table 1). In other words, while almost 50% of pancreatic cancer patients were pain free, more than 80% of chronic pancreatitis patients had at least mild pain sensations. In detail, patients with pancreatic cancer showed generally less pain, with 154 cases of mild pain (Pain Ⅰ: 35.8%) and 65 cases of moderate to severe pain (Pain Ⅱ: 15.1%) in direct comparison to significantly greater mild pain (Pain Ⅰ: 47.5%) and moderate to severe pain sensation (Pain Ⅱ: 34.2%, P < 0.0001, Table 1) in chronic pancreatitis.

Particularly striking were the differences in pain sensation comparing the two different cystadenomas of the pancreas. Whereas, patients with serous cystadenoma seem to be almost free of pain in the majority of cases (Pain 0: 58.1%), nearly 80% of all patients with mucinous cystadenoma suffered at least from mild to severe

Table 2 Intrapancreatic localization of all included pancreatic tumors *n* (%)

Diagnosis	Tumor localization					
	Pancreatic head	Pancreatic body	Pancreatic tail	> 1 pancreatic area	Pancreas unspecified	Papilla vateri
Pancreatic cancer	362	50	44	11	1	1
	(77.2)	(10.7)	(9.4)	(2.4)	(0.2)	(0.2)
Serous cystadenoma	8	9	9	4	1	0
	(25.8)	(29.0)	(29.0)	(12.9)	(3.2)	(0.0)
Mucinous cystadenoma	2	6	9	1	1	1
	(10.0)	(30.0)	(45.0)	(5.0)	(5.0)	(5.0)
Non-invasive IPMN	33	2	3	6	2	2
	(68.8)	(4.2)	(6.3)	(12.5)	(4.2)	(4.2)
Invasive IPMN	26	3	4	4	0	0
	(70.3)	(8.1)	(10.8)	(10.8)	(0.0)	(0.0)
Low stage neuroendocrine neoplasia	5	4	4	2	2	1
	(27.8)	(22.2)	(22.2)	(11.1)	(11.1)	(5.6)
High stage neuroendocrine neoplasia	16	7	14	1	2	4
	(36.4)	(15.9)	(31.8)	(2.3)	(4.5)	(9.1)
Ampullary cancer	0	0	0	0	0	51
	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(100)
Total	452	81	87	29	9	60
	(63.0)	(11.3)	(12.1)	(4.0)	(1.3)	(8.4)

IPMN: Intraductal papillary mucinous neoplasia.

pain ($P < 0.02$, Table 1). There was no significant difference in the presence of pain between the non-invasive vs. invasive IPMN but with a trend towards higher pain sensations in patients with invasive IPMN (14.7% *vs* 4.9%, Table 1). Interestingly, abdominal pain in patients with neuroendocrine neoplasia seems not to be a key clinical symptom since 64% of patients with low stage neuroendocrine neoplasia and 59% of patients with high stage neuroendocrine neoplasia did not report of any pain at all (Table 1). Although not significant, it was again noticeable that patients with the more invasive disorder/high stage neuroendocrine neoplasia showed greater moderate to severe pain sensations than low stage neuroendocrine neoplasia (7.7% *vs* 0.0%, Table 1). Similar to neuroendocrine neoplasia of the pancreas most patients with ampullary cancer did not report any symptoms of pain (61.4%, Table 1).

Tumors in the pancreatic body and tail are associated with more severe pain

The pancreatic head was by far the most common tumor localization (63%) among all pancreatic tumors and represented the most frequent tumor site for pancreatic cancer and IPMN (Table 2). On the other hand, serous and mucinous cystadenomas were the only pancreatic tumors that were most commonly observed in the pancreatic body and tail (Table 2). To clarify whether anatomical tumor localization is affecting the patient's pain status, pancreatic tumor localization was related to individual pain sensation. Here, patients with ampullary cancer were excluded due to their solely localization at the ampullary region and patients with chronic pancreatitis since these special subtype of patients display an inflammatory disease which is frequently affecting several parts of the pancreas. Pancreatic tumor localization tends to influence pain sensation independent of tumor histology. The percentage of patients with no pain (Pain 0) was considerably lower in patients with tumors in the pancreatic body (39.0%) or tail (42.0%) compared to those in the pancreatic head (51.0%). In the entire pancreatic tumor population the most severe pain was detected in patients with a tumor located in the pancreatic body (16.9% *vs* 13.5% in the pancreatic head and 14.8% in the pancreatic tail), but this difference showed no statistical significance. Thirty-five point nine percent of patients with a tumor in the pancreatic head showed mild pain (Pain Ⅰ) sensations (*vs* 44.2% of patients with a tumor in the pancreatic body and 43.2% of patients with a tumor in the pancreatic tail). But regarding the largest subgroup of patients with pancreatic cancer, it was evident ($P < 0.02$) that 29.8% of these pancreatic cancer patients with a tumor at the pancreatic body were pain free (Pain 0), 46.8% revealed mild (Pain Ⅰ) and 23.4% moderate/severe (Pain Ⅱ) pain. In contrast, the majority of patients with pancreatic cancer in the pancreatic head had no pain (53.0% *vs* 33.0% Pain Ⅰ and 14% Pain Ⅱ). Accordingly, the rate of patients with moderate to severe pain (Pain Ⅱ) was considerably higher for pancreatic cancer patients with a pancreatic body tumor when compared to pancreatic cancer patients with a pancreatic head or tail cancer (23.4% *vs* 14.0% and 17.5% respectively).

Cancer classification and pancreatic pain

Regarding the tumor dignity, it was evident that within the malignant and benign pancreatic tumors (excluding chronic pancreatitis patients) a comparable number of patients existed who had no abdominal pain at all (Pain 0: 49.2% *vs* 47.6%). However, patients with more malignant tumors showed still a significantly higher rate of moderate to severe pain (Pain Ⅱ) when compared to patients with benign pancreatic lesions (14.4% *vs* 6.7%, $P < 0.05$).

Tumor grading and staging did not show any signifi-

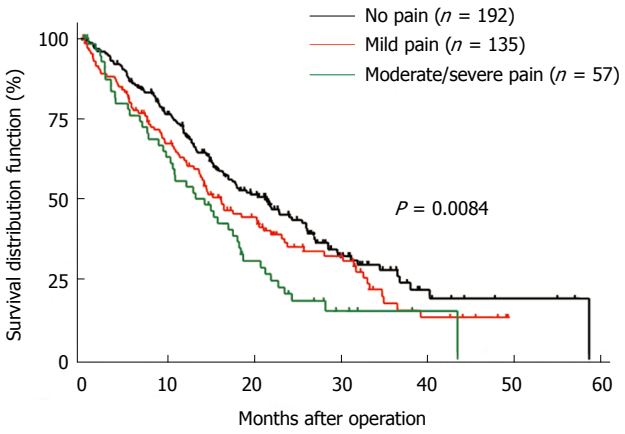


Figure 1 Kaplan-Meier analysis of postoperative survival according to pain in patients with pancreatic adenocarcinoma.

cant correlation to pain sensation in all of the analyzed pancreatic cancer cases. Patients with more advanced disease showed a tendency to suffer from more severe pain than those with earlier tumor stages (moderate/severe pain in 5.9% of pT2 tumors *vs* 16.0% of pT3 and 36.4% of pT4 tumors). On the other hand, most patients with pT2 tumors were pain free (61.8%) while only 47.9% of patients with pT3 and 36.4% of patients with pT4 tumors did not have any pain. We could not detect any influence of the number of lymph nodes and/or the number of metastases on the existence or severity of pancreatic pain in pancreatic cancer patients (data not shown).

Diabetes and pain

Out of 314 patients with chronic pancreatitis, 225 patients had normal endocrine pancreatic function with no diabetes mellitus. 14 patients presented with latent diabetes and 58 patients with manifest diabetes. In 17 patients, the endocrine function was unknown. The additional presence of diabetes did not have any effect on pancreatic pain sensation since pain pattern of chronic pancreatitis patients with or without diabetes mellitus did not differ (data not shown).

Survival and pain

Since recently, pain was found to be associated with impaired survival in pancreatic cancer[6], we have re-investigated this interesting phenomenon in a nearly 3 times larger study group of pancreatic cancer patients and also in the other pancreatic tumors. The survival of pancreatic cancer patients was significantly influenced by the severity of pain ($P = 0.0084$). Patients without pain had a 3-year survival rate of 27.9%, with mild pain 15.3%, and with moderate to severe pain a 3 year survival rate of 15.2% (Figure 1). The survival of patients with other pancreatic neoplasms was not influenced by the severity of pain (data not shown).

DISCUSSION

The present study is the currently largest study to extensively assess and compare pain sensation among the most frequent pancreatic diseases. With the attained data it becomes obvious that pancreatic pain depicts different facets and patterns of abdominal pain sensation according to the respective pancreatic disorder and does not allow a unification of the term pancreatic pain.

It has been shown that pain in pancreatic cancer is probably not as frequent as commonly stated[6]. In our study population, only 51% of patients with pancreatic cancer suffered from pain, which is even somewhat lower than the majority of previously published frequencies. But once pain is detected in pancreatic adenocarcinoma, it serves as a predictor of poor outcome, which was also the case in the presented study[4,19,20]. In all other pancreatic malignancies, where neural invasion of cancer cells is not a key pathomorphological phenomenon, no association of pain and survival was registered. We can only speculate why pancreatic cancer patients with pain do have a dismal prognosis. Besides increased neural cancer cell invasion, patients with severe pain may develop more comorbidities. They will most likely be on analgesics and narcotics and probably be not as mobile and physically fit as patients without any pain. They may therefore easier develop thrombosis and pneumonia which may further immobilize and worsen prognosis. However, since pain is a predictor of poor outcome, the sole presence of pain and especially severe pain may be used as an indication for more aggressive regional therapies such as radiotherapy or prolonged adjuvant therapies in pancreatic cancer but not in other pancreatic malignancies. When interpreting these results, however, one has to bear in mind that in the present study we only analyzed patients that were resected, meaning that there may be a bias towards less advanced, resectable tumors in our study cohort. On the other hand, 82% of patients with chronic pancreatitis suffered from pain in our study population which is similar to previously published data[1]. Also here, a possible bias towards more painful chronic pancreatitis leading to surgery has to be considered. Nevertheless, it becomes evident that pancreatic pain is very distinct and much more frequent and severe in chronic pancreatitis when compared to pancreatic cancer. It was interesting to detect, that the great majority of pancreatic disorders like serous cystadenoma, low stage neuroendocrine neoplasia, high stage neuroendocrine neoplasia, and ampullary cancer were not associated with abdominal pain at all. To our knowledge, there are no previous reports on pain patterns in these pancreatic diseases. One may speculate on the reasons why these entities are not associated with significant pain. It seems most likely, that these entities are detected rather early through symptoms other than pain (*e.g.*, jaundice in ampullary cancer) and are consequently treated and

may never get to a stage where they can cause significant pain. On the other hand it may also be the difference in tumor biology that leads to less neuropathic changes as we have previously shown[4].

The anatomic localization of the primary pancreatic tumor has a major impact on the respective pain pattern. We were able to confirm previously published data showing the highest prevalence of pain in patients with pancreatic cancer in the pancreatic body when compared to tumors in the pancreatic head or tail[21]. The underlying mechanisms for this evident phenomenon may be due to the close anatomical affiliation of the pancreatic body with the coeliac plexus and with the respective peripancreatic neural network. Abdominal pain in pancreatic cancer has a strong neuropathic component due to the characteristic intrapancreatic neuropathic alterations like neural hypertrophy, increased neural density and especially due to the prominent perineural cancer cell invasion[4,14,15,17,22,23]. The increase in neurite formation suggests that neurotropic factors, growth factors, and axonal guidance molecules can be key molecules in the development and maintenance of these phenomena. A few mediators of these neuroplastic changes have been identified, like artemin and neurturin as the members of the glial cell-derived neurotrophic factor family of ligands[17,23,24]. We have recently demonstrated that the severity of pain increases in parallel with the degree of neural invasion and that therefore the anatomic affiliation of the pancreatic body with the close nerve plexus may be one of the leading reasons for increased pain sensation in tumors at this special anatomic location. This hypothesis is underlined by the fact that in up to 70% of the cases intrapancreatic neural invasion in pancreatic cancer is directly associated with an evident extrapancreatic plexus invasion of cancer cells[25]. Furthermore, in a post mortem cadaver study pancreatic body tumors featured a special neural route to the coeliac plexus showing a direct anatomic connection[26]. Keeping these data in mind, it is not over speculated when regarding abdominal pain sensation as a reliable parameter that mirrors neuro-cancer interactions in pancreatic cancer patients. Interestingly, the other investigated pancreatic tumors showed a similar correlation between the localization in the pancreatic body and pain sensation even though these tumors are known to show significantly less neural invasion and neuroplastic changes when compared to pancreatic cancer[4]. Even though these neuropathic alterations were detected significantly less often, they were still all present in these pancreatic neoplasms[4]. Therefore, it is possible that the observed pain pattern in other pancreatic tumors may be explained by similar mechanism as in pancreatic cancer. This is moreover underlined by our finding that pain was more frequently present and more severe in patients with malignant pancreatic neoplasms as compared to their benign counterparts. Therefore, pain history should be carefully investigated since malignancy could be suspected in patients with especially severe pain states and apparently benign pancreatic neoplasms. Pain should therefore be integrated in the diagnostic workup and may help us in our indications for surgery.

Neither the TNM status nor tumor grading of the malignant tumor entities showed any significant correlation to the presence or severity of abdominal pain sensation. However, it has been previously shown that even T1 tumors can show the features of neural invasion and pain in pancreatic cancer[27]. Therefore, it seems that the size of the tumor is not terminating the extent of neuropathic alterations and that the sole presence of these neuropathic changes can lead to pancreatic pain irrespective of tumor size, grade or location.

Chronic alcohol abuse is the most common etiological factor of chronic pancreatitis. Alcoholic chronic pancreatitis was shown to be associated with more constant pain sensation as compared to non-alcoholic chronic pancreatitis which could be reproduced also in our series (data not shown)[1,28]. However, previous studies could not find a difference in the severity of pain between alcoholic and non-alcoholic chronic pancreatitis[1,28], as we found in our study. The underlying mechanisms for increased abdominal pain in alcoholic chronic pancreatitis remain to be unravelled. Although diabetes and consequent peripheral neural damage is known to largely influence general pain sensation, this does unexpectedly not seem to be the case for pancreatic pain in our study. There was no difference in pain sensation between diabetic and non-diabetic chronic pancreatitis patients in our study population. A recently published study investigated the association of diabetes in pancreatic pain in the case of pancreatic cancer. Here, the authors report that patients with diabetes had a significantly lower frequency of abdominal pain[29] with at the same time significantly higher prevalence of perineural invasion. Further studies will be needed to investigate whether diabetes as such can induce neural plasticity in pancreatic diseases.

In summary, the results of the presented study demonstrate that pain patterns in pancreatic diseases are very diverse and mainly dependent on tumor type, anatomic localization and dignity. Abdominal pain sensation in pancreatic diseases should therefore be observed and documented much more carefully since they may be used as an additional diagnostic tool to estimate pancreatic tumor dignity and patient's prognosis.

COMMENTS

Background

Pain is an important symptom of both pancreatitis and pancreatic cancer, which are the most common pancreatic diseases. There are numerous other, more seldom pancreatic diseases and little is known about pain patterns in these rare conditions.

Research frontiers

Pancreatic pain in pancreatic cancer and in chronic pancreatitis is increasingly acknowledged as of neuropathic origin. The neuroplastic changes seen in pancreatic cancer and chronic pancreatitis have not been observed to that extend in other pancreatic diseases.

Innovations and breakthroughs

This is the first study evaluating pain patterns in patients with less common pancreatic diseases. The results of the presented study demonstrate that pain patterns in pancreatic diseases are very diverse and mainly dependent on tumor type, anatomic localization and dignity.

Applications

Pain history in pancreatic diseases may be used as an additional diagnostic tool to estimate disease dignity.

Peer review

Abdominal pain is a major clinical feature in chronic pancreatitis and pancreatic cancer. It is the first study about the pain patterns in all pancreatic tumors and correlate pain with the respective clinicopathological data. This study is well designed and properly performed. The statistical analysis is reasonable. The result is credible. The conclusion is helpful in clinical practice.

REFERENCES

1 **Mullady DK**, Yadav D, Amann ST, O'Connell MR, Barmada MM, Elta GH, Scheiman JM, Wamsteker EJ, Chey WD, Korneffel ML, Weinman BM, Slivka A, Sherman S, Hawes RH, Brand RE, Burton FR, Lewis MD, Gardner TB, Gelrud A, DiSario J, Baillie J, Banks PA, Whitcomb DC, Anderson MA. Type of pain, pain-associated complications, quality of life, disability and resource utilisation in chronic pancreatitis: a prospective cohort study. *Gut* 2011; **60**: 77-84 [PMID: 21148579 DOI: 10.1136/gut.2010.213835]

2 **Andrén-Sandberg A**, Hoem D, Gislason H. Pain management in chronic pancreatitis. *Eur J Gastroenterol Hepatol* 2002; **14**: 957-970 [PMID: 12352215 DOI: 10.1097/00042737-200209000-00006]

3 **Ammann RW**, Akovbiantz A, Largiader F, Schueler G. Course and outcome of chronic pancreatitis. Longitudinal study of a mixed medical-surgical series of 245 patients. *Gastroenterology* 1984; **86**: 820-828 [PMID: 6706066]

4 **Ceyhan GO**, Bergmann F, Kadihasanoglu M, Altintas B, Demir IE, Hinz U, Müller MW, Giese T, Büchler MW, Giese NA, Friess H. Pancreatic neuropathy and neuropathic pain--a comprehensive pathomorphological study of 546 cases. *Gastroenterology* 2009; **136**: 177-186.e1 [PMID: 18992743]

5 **Krech RL**, Walsh D. Symptoms of pancreatic cancer. *J Pain Symptom Manage* 1991; **6**: 360-367 [PMID: 1880437 DOI: 10.1016/0885-3924(91)90027-2]

6 **Grahm AL**, Andrén-Sandberg A. Prospective evaluation of pain in exocrine pancreatic cancer. *Digestion* 1997; **58**: 542-549 [PMID: 9438600 DOI: 10.1159/000201499]

7 **Ebbehøj N**, Borly L, Bülow J, Rasmussen SG, Madsen P. Evaluation of pancreatic tissue fluid pressure and pain in chronic pancreatitis. A longitudinal study. *Scand J Gastroenterol* 1990; **25**: 462-466 [PMID: 2359973 DOI: 10.3109/00365529009095516]

8 **Di Sebastiano P**, di Mola FF, Buchler MW, Friess H. Pathogenesis of pain in chronic pancreatitis. *Dig Dis* 2004; **22**: 267-272 [PMID: 15753609 DOI: 10.1159/000082798]

9 **Barreto SG**, Saccone GT. Pancreatic nociception--revisiting the physiology and pathophysiology. *Pancreatology* 2012; **12**: 104-112 [PMID: 22487519 DOI: 10.1016/j.pan.2012.02.010]

10 **Demir IE**, Tieftrunk E, Maak M, Friess H, Ceyhan GO. Pain mechanisms in chronic pancreatitis: of a master and his fire. *Langenbecks Arch Surg* 2011; **396**: 151-160 [PMID: 21153480 DOI: 10.1007/s00423-010-0731-1]

11 **Drewes AM**, Krarup AL, Detlefsen S, Malmstrøm ML, Dimcevski G, Funch-Jensen P. Pain in chronic pancreatitis: the role of neuropathic pain mechanisms. *Gut* 2008; **57**: 1616-1627 [PMID: 18566105 DOI: 10.1136/gut.2007.146621]

12 **Bockman DE**, Buchler M, Malfertheiner P, Beger HG. Analysis of nerves in chronic pancreatitis. *Gastroenterology* 1988; **94**: 1459-1469 [PMID: 3360267]

13 **Ceyhan GO**, Bergmann F, Kadihasanoglu M, Erkan M, Park W, Hinz U, Giese T, Müller MW, Büchler MW, Giese NA, Friess H. The neurotrophic factor artemin influences the extent of neural damage and growth in chronic pancreatitis. *Gut* 2007; **56**: 534-544 [PMID: 17047099 DOI: 10.1136/gut.2006.105528]

14 **Ceyhan GO**, Demir IE, Rauch U, Bergmann F, Müller MW, Büchler MW, Friess H, Schäfer KH. Pancreatic neuropathy results in „neural remodeling" and altered pancreatic innervation in chronic pancreatitis and pancreatic cancer. *Am J Gastroenterol* 2009; **104**: 2555-2565 [PMID: 19568227 DOI: 10.1038/ajg.2009.380]

15 **Demir IE**, Ceyhan GO, Rauch U, Altintas B, Klotz M, Müller MW, Büchler MW, Friess H, Schäfer KH. The microenvironment in chronic pancreatitis and pancreatic cancer induces neuronal plasticity. *Neurogastroenterol Motil* 2010; **22**: 480-490, e112-113 [PMID: 19912545]

16 **Drewes AM**, Gratkowski M, Sami SA, Dimcevski G, Funch-Jensen P, Arendt-Nielsen L. Is the pain in chronic pancreatitis of neuropathic origin? Support from EEG studies during experimental pain. *World J Gastroenterol* 2008; **14**: 4020-4027 [PMID: 18609686 DOI: 10.3748/wjg.14.4020]

17 **Ceyhan GO**, Giese NA, Erkan M, Kerscher AG, Wente MN, Giese T, Büchler MW, Friess H. The neurotrophic factor artemin promotes pancreatic cancer invasion. *Ann Surg* 2006; **244**: 274-281 [PMID: 16858191 DOI: 10.1097/01.sla.0000217642.68697.55]

18 **D'Haese JG**, Demir IE, Kehl T, Winckler J, Giese NA, Bergmann F, Giese T, Büchler MW, Friess H, Hartel M, Ceyhan GO. The impact of MFG-E8 in chronic pancreatitis: potential for future immunotherapy? *BMC Gastroenterol* 2013; **13**: 14 [PMID: 23324439 DOI: 10.1186/1471-230X-13-14]

19 **Müller MW**, Friess H, Köninger J, Martin D, Wente MN, Hinz U, Ceyhan GO, Blaha P, Kleeff J, Büchler MW. Factors influencing survival after bypass procedures in patients with advanced pancreatic adenocarcinomas. *Am J Surg* 2008; **195**: 221-228 [PMID: 18154768 DOI: 10.1016/j.amjsurg.2007.02.026]

20 **Okusaka T**, Okada S, Ueno H, Ikeda M, Shimada K, Yamamoto J, Kosuge T, Yamasaki S, Fukushima N, Sakamoto M. Abdominal pain in patients with resectable pancreatic cancer with reference to clinicopathologic findings. *Pancreas* 2001; **22**: 279-284 [PMID: 11291930 DOI: 10.1097/00006676-200104000-00009]

21 **Eyigor C**, Karaca B, Kuzeyli-Yildirim Y, Uslu R, Uyar M, Coker A. Does the tumor localization in advanced pancreatic cancer have an influence on the management of symptoms and pain? *J BUON* 2010; **15**: 543-546 [PMID: 20941825]

22 **Ceyhan GO**, Demir IE, Altintas B, Rauch U, Thiel G, Müller MW, Giese NA, Friess H, Schäfer KH. Neural invasion in pancreatic cancer: a mutual tropism between neurons and cancer cells. *Biochem Biophys Res Commun* 2008; **374**: 442-447 [PMID: 18640096 DOI: 10.1016/j.bbrc.2008.07.035]

23 **Ceyhan GO**, Schäfer KH, Kerscher AG, Rauch U, Demir IE, Kadihasanoglu M, Böhm C, Müller MW, Büchler MW, Giese NA, Erkan M, Friess H. Nerve growth factor and artemin are paracrine mediators of pancreatic neuropathy in pancreatic adenocarcinoma. *Ann Surg* 2010; **251**: 923-931 [PMID: 20395845 DOI: 10.1097/SLA.0b013e3181d974d4]

24 **Wang K**, Demir IE, D'Haese JG, Tieftrunk E, Kujundzic K, Schorn S, Xing B, Kehl T, Friess H, Ceyhan GO. The neurotrophic factor neurturin contributes toward an aggressive cancer cell phenotype, neuropathic pain and neuronal plasticity in pancreatic cancer. *Carcinogenesis* 2014; **35**: 103-113 [PMID: 24067900]

25 **Nakao A**, Harada A, Nonami T, Kaneko T, Takagi H. Clinical significance of carcinoma invasion of the extrapancreatic nerve plexus in pancreatic cancer. *Pancreas* 1996; **12**: 357-361 [PMID: 8740402 DOI: 10.1097/00006676-199605000-00006]

26 **Yi SQ**, Miwa K, Ohta T, Kayahara M, Kitagawa H, Tanaka A, Shimokawa T, Akita K, Tanaka S. Innervation of the pancreas from the perspective of perineural invasion of pancreatic cancer. *Pancreas* 2003; **27**: 225-229 [PMID: 14508126 DOI:

10.1097/00006676-200310000-00005]
27 **Reiser-Erkan C**, Gaa J, Kleeff J. T1 pancreatic cancer with lymph node metastasis and perineural invasion of the celiac trunk. *Clin Gastroenterol Hepatol* 2008; **6**: e41-e42 [PMID: 18848906 DOI: 10.1016/j.cgh.2008.07.033]
28 **Ammann RW**, Muellhaupt B. The natural history of pain in alcoholic chronic pancreatitis. *Gastroenterology* 1999; **116**: 1132-1140 [PMID: 10220505 DOI: 10.1016/S0016-5085(99)70016-8]
29 **Sahin IH**, Shama MA, Tanaka M, Abbruzzese JL, Curley SA, Hassan M, Li D. Association of diabetes and perineural invasion in pancreatic cancer. *Cancer Med* 2012; **1**: 357-362 [PMID: 23342285 DOI: 10.1002/cam4.43]

P- Reviewers: Camara-Lemarroy CR, Mezhir JJ, Merrett ND, Sun LM
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9162

World J Gastroenterol 2014 July 21; 20(27): 9162-9169
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CLINICAL TRIALS STUDY

Biofeedback-guided pelvic floor exercise therapy for obstructive defecation: An effective alternative

Ma-Mu-Ti-Jiang A ba-bai-ke-re, Ni-Re Wen, Yun-Long Hu, Liang Zhao, Tuerhongjiang Tuxun, Aierhati Husaiyin, Yalikun Sailai, Alimujiang Abulimiti, Yun-Hai Wang, Peng Yang

Ma-Mu-Ti-Jiang A ba-bai-ke-re, Tuerhongjiang Tuxun, Aierhati Husaiyin, Yalikun Sailai, Alimujiang Abulimiti, Liang Zhao, General Surgical Department of First Affiliated Hospital of Xinjiang Medical University, Urumqi 830011, Xinjiang Uygur Autonomous Region, China
Ni-Re Wen, Otolaryngological Department of Urumqi Military Hospital of Lanzhou Military Center, Urumqi 830011, Xinjiang Uygur Autonomous Region, China
Yun-Long Hu, Peng Yang, Surgical Department, First Affiliated Hospital, Xinjiang Medical University, Urumqi 830011, Xinjiang Uygur Autonomous Region, China
Yun-Hai Wang, Digestive and Vascular Surgical Center of First Affiliated Hospital of Xinjiang Medical University, Urumqi 830011, Xinjiang Uygur Autonomous Region, China
Author contributions: A ba-bai-ke-re MMTJ contributed to the conception, design, and revision of this article for critically important intellectual content; Tuxun T, Husaiyin A and Abulimiti A performed control, recording, and data acquisition; Hu YL and Abulimiti A provided substantial contributions, including statistical calculations and study design; Wen NR and Zhao L performed intervention control, recording, data acquisition, and final approval of the version to be published; Sailai Y, Wang YH, and Yang P performed analysis and interpretation of the data.
Supported by Research Award Fund of First Affiliated Hospital of Xinjiang Medical University, Fund Serial No. 2012YFY30
Correspondence to: Yalikun Sailai, Surgical Assistant Professor, MD, General Surgical Department of First Affiliated Hospital of Xinjiang Medical University, Liyushan Road, No 1, Urumqi 830011, Xinjiang Uygur Autonomous Region, China. 834140633@qq.com
Telephone: +86-991-4366142 Fax: +86-991-4366142
Received: December 30, 2013 Revised: February 26, 2014
Accepted: April 15, 2014
Published online: July 21, 2014

Abstract

AIM: To compare biofeedback-guided pelvic floor exercise therapy (BFT) with the use of oral polyethylene glycol (PEG) for the treatment of obstructive defecation.

METHODS: A total of 88 subjects were assigned to treatment with either BFT (n = 44) or oral PEG (n = 44). Constipation symptoms (including difficult evacuation, hard stool, digitation necessity, incomplete emptying sensation, laxative dependence, perianal pain at defecation, and constipation satisfaction), Wexner Scores, and quality of life scores were assessed after 1, 3, and 6 mo.

RESULTS: At the 6 mo follow-up, the symptoms of the BFT group patients showed significantly greater improvements compared with the PEG group regarding difficult evacuation, hard stools, digitation necessity, laxative dependence, perianal pain at defecation, constipation satisfaction, Wexner Constipation Score, and quality of life score (P < 0.05). The quality of life score of the BFT group at the final follow-up time (6 mo) was 80 ± 2.2. After a complete course of training, improvements in the clinical symptoms of the BFT group were markedly improved (P < 0.05), and the Wexner Constipation Scores were greatly decreased compared with the oral PEG group (P < 0.05).

CONCLUSION: We concluded that manometric biofeedback-guided pelvic floor exercise training is superior to oral polyethylene glycol therapy for obstructive defecation.

Key words: Obstructive defecation; Biofeedback; Polyethylene glycol

Core tip: Until now, there have been no standard treatment methods for obstructed defecation. Although we believe biofeedback is more beneficial for obstructive defecation, recent controlled studies indicate that the efficacy of manometric biofeedback treatment for ob-

structive defecation remains controversial. The main purpose of this research was to assess and compare the quality of life scores of patients diagnosed with obstructive defecation following treatment with biofeedback therapy or oral polyethylene glycol management. Biofeedback had the clear effect of teaching patients how to squeeze and relax their anorectal and pelvic floor muscles during defecation. The data in this study show a clear superiority of biofeedback related to oral polyethylene glycol for the treatment of this subtype of constipation. If this research was extended to large multicenter randomized trials and its efficiency proven, biofeedback could become the standard treatment method for obstructive defecation.

A ba-bai-ke-re MMTJ, Wen NR, Hu YL, Zhao L, Tuxun T, Husaiyin A, Sailai Y, Abulimiti A, Wang YH, Yang P. Biofeedback-guided pelvic floor exercise therapy for obstructive defecation: An effective alternative. *World J Gastroenterol* 2014; 20(27): 9162-9169 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9162.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9162

INTRODUCTION

Constipation in adults is a common disorder of the gastrointestinal area. It affects nearly everyone in the general population at different points in their lifetime. Currently, constipation has a profound impact on adult patients' quality of life and has been considered a major social and psychological disability. Chronic functional constipation influences 2%-30% of individuals in Western countries[1]. Although some of these patients can be managed with conservative treatments such as a high fiber diet, laxatives, suppositories, or oral polyethylene glycol, others are not sensitive to these options. According to large scale epidemiological research by Talley *et al*[2], medical treatment is ineffective in 39% of adult patients with chronic functional constipation. Levitt[3] reported that a significant number of patients with a single treatment failure instead seek, and subsequently receive, a surgical alternative. Primary constipation can be further classified into slow-transit constipation (STC), normal-transit constipation (NTC), and obstructed defecation (OD). Among the subtypes of chronic functional constipation, OD seems particularly common, occurring in 7%-35% of the adult population[4]. Traditional treatment is well-established and safe for OD; however, it does not provide satisfying improvement for many patients, prompting interest in other therapeutic strategies[5,6]. Treatment with high dietary fiber and anti-constipation drugs for many OD patients is usually ineffective. OD patients who do not respond to regular medication seek further medical treatment; however, there have been no worldwide standard treatment methods until now. OD is commonly considered to be a form of maladaptive behavior, as there is no clear anatomical problem around the anorectum and it can be solved by behavioral action, anorectal squeezing, or relaxative training[7]. Although PEG has shown certain effects on adult OD, it can have diverse effects in patients from different countries or areas. Biofeedback-guided pelvic floor exercise training is an alternative treatment where physiologic process information can be converted into a visual signal, which ultimately should allow patients to learn to control their impaired defecation process. Compared with the results of previous clinical studies on biofeedback therapy in functional pelvic floor disorders, which have yielded conflicting results with efficacy rates ranging from 18% to 100%[8-15], biofeedback has been shown to be rather effective in treating OD in recent clinical studies[1,16-19]. Although these randomized controlled trials were performed to clarify the actual efficiency of BFT for OD, considering the methodological diversity of the research, the actual efficiency of BFT for OD still requires clarification. Although some studies show contrary results regarding the use of biofeedback in patients with normal transit during straining, the biofeedback technique has a clear therapeutic benefit for OD, including patients with STC[20].

Although BFT is more beneficial for OD, recent controlled studies have indicated that the efficacy of manometric biofeedback treatment for different types of OD remains controversial and has been associated with pathogenesis or learning obstacles. Our present study should hopefully solve the difficult dilemma of OD management and offer significant accordance to anorectal surgeons. In patients with OD, the role of manometric biofeedback-guided pelvic floor training is to teach patients to relax their pelvic and anorectal muscles while simultaneously applying downward intra-abdominal pressure to generate a propulsive force toward the anus. The primary purpose of this research was to compare the quality of life scores of patients diagnosed with OD after BFT or oral polyethylene glycol management. The other aims of the study were to determine whether BFT training affects the pelvic physiologic mechanism and to try to establish a new alternative management method for OD.

MATERIALS AND METHODS

Patients and study design

Eighty-eight consecutive patients, referred to the General Surgical Department of the First Affiliated Hospital in Xinjiang Medical University during the period from September 2011 to June 2013, were included in the trial. The diagnoses of all participants were confirmed by history, general medical examination, anorectal testing, and biochemical techniques. All research subjects received the same standard medication (*e.g.*, suppositories), treatment strategies, and necessary behavioral education before biofeedback exercise or polyethylene glycol (PEG) treatment. The patients were clinically assessed using Wexner Constipation and quality of life scores by a specialist

Table 1 Clinical, physiologic, and psychological characteristics of biofeedback pelvic floor training and polyethylene glycol groups

Baseline	BFT	PEG
n	44	44
Age (yr)	54	57
Symptoms	6.4	6.6
Physician visits (past six months)	3.1	3.3
Mean duration of disease (yr)	3.5	3.6
Previous treatment Times (yr)	2.3	2.6
First sensation (mL)	19.3	18.7
Rest pressure (mm Hg)	38	40
Squeeze pressure (mm Hg)	63	61
Wexner constipation score	29 ± 3.9	30 ± 4.3
Quality of life score	42 ± 2.9	43 ± 3.2

BFT: Biofeedback pelvic floor training; PEG: Polyethylene glycol.

who was blinded to treatment assignment; the information collected included constipation onset age, bowel frequency, precipitation factors, laxative use, family history, gynecologic history, and other relevant disease history. A protocol synopsis for this study was used as supporting information. A total of 88 patients who satisfied the inclusion criteria were distributed by computerized randomization into the BFT (biofeedback) group (n = 44) or the oral PEG group (n = 44). The sample calculation of this research was performed based on SPSS statistical software (version 15.0)[21]. The investigators and biofeedback operators were not able to anticipate the patients' informed consent assignments. The sample size calculation was completed at the Medical Statistical Center, First Affiliated Hospital of Xinjiang Medical University. Demographic data (*i.e.*, age and gender), constipation grade, constipation status, mean disease duration, and previous treatment times were recorded for each patient (Table 1). To assess the post-treatment efficiency of each group, the Wexner Scores (Table 2), followed by the Patient Assessment of Constipation Quality of Life questionnaire (PACQOL) of the patients were completed. PACQOL is a validated 28-item questionnaire[22,23] used to assess the severity of chronic functional constipation on the quality of the patient's life. The PACQOL is scored for four domains: Worries and Concerns, Satisfaction, Psychosocial Discomfort, and Physical Discomfort. One of the researchers who were unaware of the patients' treatment assignments collected the patient satisfaction, Wexner Constipation Score, and quality of life score results. There were no significant differences between the two groups in terms of demographics (Baseline data, Table 1). After the management of the two compared methods had been completed, assessment of the Wexner Scores and quality of life scores were performed. The anorectal resting pressure and squeeze pressure of the patients were measured after each treatment.

Medical research ethics and patient informed consent

This research was performed according to the principles of the Declaration of Helsinki[24], the Guidelines for Good Clinical Practice (GCP) for trials on pharmaceutical products[25], and the local regulations of Xinjiang Uygur Autonomous Region. The research protocol was approved by the Medical Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University. Because the research was conducted as an investigational study, a strict policy of informed consent was followed and obtained from each of the patients before the study began. The research consisted of three periods of observation (1, 3, and 6 mo after treatment). The last visit was to be completed 180 d after either of the two treatment arms. There were no missing data during the 6-mo follow-up period. The analysis was based on the intention-to-treat principle. All patients were informed that two alternative managements were being compared: manomet-

Table 2 Wexner Constipation Score system[23] (Minimum score 0; maximum score 30)[24]

Frequency of bowel movements	Score
1-2 times per 1-2 d	0
2 times per week	1
Once per week	2
Less than once per week	3
Less than once per month	4
Difficulty: painful evacuation effort	Score
Never	0
Rarely	1
Sometimes	2
Usually	3
Always	4
Completeness: (feeling incomplete evacuation)	Score
Never	0
Rarely	1
Sometimes	2
Usually	3
Always	4
Failure: (unsuccessful attempts at evacuation per 24 h)	Score
Never	0
1-3	1
3-6	2
6-9	3
More than 9	4
Pain: abdominal	Score
Never	0
Rarely	1
Sometimes	2
Usually	3
Always	4
Time: minutes in lavatory per attempt	Score
Less than 5	0
5-10	1
10-20	2
20-30	3
More than 30	4
History: duration of constipation (yr)	Score
0	0
1-5	1
5-10	2
10-20	3
More than 20	4
Assistance: type of assistance	Score
Without assistance	0
Stimulant laxatives	1
Digital assistance/enema	2

ric biofeedback-guided pelvic floor training *vs* oral polyethylene glycol. The patients assigned to the biofeedback group received a detailed interpretation of the principle for biofeedback exercises, while patients assigned to the oral PEG group additionally received related information about the advantages of PEG treatment.

Research support

This study was supported by the Research Award Fund of the First Affiliated Hospital of Xinjiang Medical University. Fund serial number: 2012YFY30.

Inclusion/exclusion criteria

The patients were included in the study if they fulfilled the Rome Ⅲ criteria, consensus statement[26], and underwent standard anorectal testing. Anorectal manometry (resting pressure and maximum squeeze pressure), balloon expulsion test, electromyography, anoscope, and anorectal digital examination were evaluated to verify the diagnoses, according to the recommended guidelines, from face-to-face interviews performed by the treating physician. All patients were required to have experienced constipation symptoms for more than 6 mo. In addition to satisfying the Rome Ⅲ criterion of functional constipation, patients also had to meet these inclusion criteria: (1) report of < 3 bowel movements per week by patient history; (2) failure of treatment with a high-fiber diet; (3) history of excessive straining on defecation with normal bowel frequency; (4) absence of secondary causes of constipation; and (5) absence of a surgically treatable cause. Patients were excluded from the trial if they had the following underlying conditions: (1) previous abdominal surgery or trauma of the pelvic area; (2) megacolon or megarectum; (3) symptom resolution upon conservative medical management; or (4) systemic disease.

Sample size and power

Before research was initiated, the sample size was calculated using SPSS software version 15.0. The total number of patients needed to show a 30% success rate difference from the previously established success rate of approximately 50% was determined in order to detect a clinically significant difference. A sample of at least 44 patients in each group was judged necessary to demonstrate such a difference ($P < 0.05$), with a power of 90%.

Biofeedback pelvic floor training

All 44 patients in the biofeedback-guided pelvic floor exercise therapy (BFT) group were explained that their constipation may be related to a certain degree of defecation disorders. Patients were told the possible respective advantages of biofeedback and oral PEG. All patients in the BFT group underwent 5-wk manometric (Medtronic Med Ltd)-guided biofeedback training sessions that lasted 30 min each in the outpatient manometric unit. The physiology of the anorectal and pelvic floor were interpreted to the attenders using their own testing results. The main purpose and possible results of biofeedback and oral PEG were explained to the attenders. All biofeedback training was performed by one of the researchers. Another researcher who was unaware of the management style collected the data. Biofeedback-guided anorectal training consisted of three different parts. In the first phase of training, the participants were guided to squeeze the pelvic muscles according to their breathing rate. In the second part, electromyographic detection was performed and biofeedback was undertaken according to researchers' guidance. In the final part, patients practiced defecating a water balloon catheter. One of the researchers recorded all patient data using a computer-video system. All patients were trained to finish biofeedback pelvic floor exercises by verbal instructions. As this skill developed, the participants were instructed to practice pelvic floor exercises during their usual daily activities and while standing. All participants were required to continue on a high-fiber diet as much as possible during the biofeedback training.

Oral PEG treatment

Oral polyethylene glycol treatment was administered according to a previously reported method[27]. The Wexner Constipation Score and quality of life score of patients were collected before and after treatment. Oral polyethylene glycol (PEG) is a large polymer that is poorly absorbed and not easily degraded by microorganisms. The PEG laxative was given to all PEG group patients, who were instructed to take 17 g of the laxative orally, with water, 3 times a day. The patients in the PEG group participated in a 14-d treatment period according to the method used in a previous report of a PEG trial[28]. All of the PEG group participants were required to continue on a high-fiber diet as much as possible during the treatment.

Follow-up

The constipation symptom relief, Wexner score, and quality of life score were investigated at 1, 3, and 6 mo after each type of treatment. The follow-up dates ranged from June 2013 to December 2013. All patients finished the related questionnaire at 1, 3, and 6 mo after each type of treatment. The patients accepted 6-mo follow-up evaluations regardless of their symptomatic improvement. During the follow-up evaluation, anorectal manometry, post-treatment symptom evaluation, Wexner Score, and quality of life score were completed. Each of the patients was contacted by telephone within 6 mo by an investigator and asked whether they had experienced adequate constipation relief. All patients were scheduled for 6-mo follow-up evaluations.

Statistical analysis

The statistical analysis was performed using SPSS software version 15.0. The intention-to-treat principle was

Table 3 Comparison of biofeedback pelvic floor training and polyethylene glycol groups at each follow-up time after treatment *n* (%)

Symptoms	PEG	BFT	Statistic value	*P* value
Difficult evacuation				
1-mo follow-up	39 (88.64)	31 (70.45)	4.4698	0.0345
3-mo follow-up	31 (70.45)	18 (40.90)	8.0173	0.0046
6-mo follow-up	24 (54.54)	11 (25.00)	8.0173	0.0046
Hard stools				
1-mo follow-up	34 (77.27)	28 (63.64)	1.9653	0.1610
3- mo follow-up	29 (65.91)	10 (22.73)	16.6238	0.0000
6- mo follow-up	21 (47.73)	7 (15.91)	10.2667	0.0014
Need for digitation				
1-mo follow-up	31 (70.45)	7 (15.55)	26.6779	0.0000
3-mo follow-up	25 (56.82)	5 (11.36)	20.2299	0.0000
6-mo h follow-up	20 (45.45)	3 (6.82)	17.0114	0.0000
Sensation of incomplete emptying				
1-mo follow-up	28 (63.64)	15 (34.09)	7.6858	0.0056
3-mo follow-up	21 (47.73)	18 (40.90)	6.4144	0.5197
6-mo follow-up	14 (31.82)	10 (22.73)	0.9167	0.3384
Laxative dependence				
1-mo follow-up	37 (84.09)	25 (56.82)	7.8610	0.0051
3-mo follow-up	31 (70.45)	19 (43.18)	6.6695	0.0098
6-mo follow-up	28 (63.64)	10 (22.73)	15.0063	0.0001
Perianal pain at defecation				
1-mo follow-up	28 (63.64)	12 (27.27)	11.7333	0.0006
3-mo follow-up	24 (54.54)	15 (34.09)	3.7300	0.0534
6-mo follow-up	18 (40.91)	9 (20.45)	4.3279	0.0375
Satisfaction				
1-mo follow-up	8 (18.18)	19 (42.22)	6.4651	0.0110
3-mo follow-up	11 (25.00)	25 (56.81)	9.2137	0.0024
6-mo follow-up	9 (20.45)	35 (79.54)	7.0625	0.0010

PEG: Polyethylene glycol; BFT: Biofeedback-guided pelvic floor exercise therapy.

Table 4 Comparison of biofeedback pelvic floor training and polyethylene glycol groups wexner scores and quality of life scores

	Symptoms (mo)	PEG	BFT	Statistic value	*P* value
Wexner scores	1	16 ± 4.1	22 ± 4.2	14.518	0
	3	18 ± 3.9	23 ± 3.2	29.3749	0
	6	19 ± 3.4	25 ± 3.1	68.2902	0
Quality of life scores	1	50 ± 2.5	62 ± 3.8	16.4269	0
	3	57 ± 2.1	71 ± 3.2	22.5295	0
	6	64 ± 1.9	80 ± 2.2	36.5105	0

BFT: Biofeedback pelvic floor training; PEG: Polyethylene glycol.

applied in this study. Non-normal data were expressed as medians and full ranges. Normal data were expressed as the mean ± SD. Student's *t*-test was used to compare the treatment results, and the χ^2 test was used for the comparison of proportions. $P < 0.05$ was considered statistically significant.

RESULTS

At the concluding times of the 1, 3, and 6 mo follow-up periods, the symptoms of the BFT group patients, compared with the oral PEG group, showed significantly greater improvements in the aspects of difficult evacuation, digitation necessity, laxative dependence, and constipation satisfaction. At the time of the 1-mo follow-up, the symptom of hard stool showed no significant improvement. At the time of the 3-mo follow-up, the sensation of incomplete emptying and perianal pain at defecation showed no significant improvements. At the 6-mo follow-up, the sensation of incomplete emptying also showed no significant improvement. (Table 3).

At the time of each follow-up, the Wexner Score data of the BFT group patients were significantly higher than the PEG group (Table 4). The quality of life score of the BFT group at the final follow-up time was 80 ± 2.2. The quality of life scores of the BFT group were also significantly improved (Table 4). At the final follow-up time, the BFT group's average quality of life score was 80 ± 2.2. There was a clear and significant difference between the two groups in anorectal resting and squeeze pressure after the BFT treatment.

DISCUSSION

Obstructed defecation is a common disorder that clearly influences the life quality of patients. Although Ellis CN[29] concluded that there were multiple treatment methods for OD, such as botulinum toxin, transvaginal repair, transrectal repair, transperineal repair, and rectal intussusception, there was no standard treatment method up to now. OD is frequently related to other disorders of anorectal and pelvic function. Any therapy that does not address all components will result in less than optimal outcomes and quality of life both before and after treatment[29]. The primary purpose of our study was to improve the quality of life score after BFT in patients diagnosed with OD. The final result of this research shows that, in contrast to recently reported views, biofeedback training is more effective for the treatment of OD compared with oral PEG administration. In the three month and final follow-up evaluations, difficult evacuation, hard stools, digitation necessity, laxative dependence, perianal pain at defecation, and bowel satisfaction results of the BFT group were significantly superior to those of the PEG group. In all follow-up evaluations, BFT demonstrated superior results to the oral PEG group. The average Wexner Constipation Score of the BFT group in the final visit was 25 ± 3.1, and the quality of life score was 80 ± 2.2. This result indicates that BFT can produce a clearer effect in the management of OD than PEG treatment.

In this study, 79.54% of patients in the BFT group resolved their constipation symptoms, compared with 20.45% in the PEG group. The results also show that patients in the BFT group have a clearer significance than patients in the PEG group in terms of difficult evacuation, hard stool, and sensation of incomplete emptying. The main purpose of biofeedback exercise is guiding patients to perform normal defecation activity

and improve their quality of life. The average Wexner Score of the BFT group was 22 ± 4.2, 23 ± 3.2, and 25 ± 3.1 in 1-mo, 3-mo, and 6-mo follow-up time, respectively. This result was closely associated with the biofeedback resolution of OD. The improvement was seen in the improved ability to defecate and in the increases in anorectal pressure that were measured in the rectum when patients performed defecation. Our results indicate that biofeedback-guided pelvic exercise training was helpful in improving the life quality of pelvic floor dysfunction patients. The results show that constipation improvement of the OD group may correlate with the frequency of biofeedback training.

We considered that the effectiveness of biofeedback-guided anorectal training depends on the skills of the biofeedback performer. Therefore, we strongly suggest that all performers should receive the standard biofeedback guidance course. Considering the diversity of biofeedback in different countries or areas, we consider that biofeedback-guided pelvic floor exercise training techniques should be standardized. Moreover, all participants in the BFT group were guided by one highly-experienced performer. Recent studies in some countries show that clinical benefits can still be obtained despite any diversity in performers' skill.

The comparison of hard stool and incomplete emptying sensation did not demonstrate statistical significance. A possible reason for this result may be associated with a difference in the patients' recognition of either a hard stool or an incomplete emptying sensation in the questionnaire. We propose that anorectal researchers should design a more practical questionnaire and formulate it based on patients' characteristics.

There are at least 8 randomized placebo-controlled trials of PEG compounds. There are two randomized control trials comparing PEG with lactulose. PEG was superior to placebo in increasing stool frequency and stool consistency[27]. A study reported relief of constipation in 52% of patients on PEG-3350 compared with 11% of patients on placebo[27]. In our study, the patients' quality of life score was 64 ± 1.9. We considered that PEG was beneficial to some portion of the OD patients. The actual efficiency of PEG for OD requires further investigation and larger controlled trials.

Battaglia *et al*[30] indicated the clear benefits of biofeedback for OD patients. They found no differences in the patients' satisfaction ratings with the treatment. Our data indicate that biofeedback does provide a specific benefit to this subtype of constipation. The final quality of life score of the BFT group was significantly improved compared with the PEG group. We consider that further large, multicenter, double blind, randomized controlled trials will demonstrate the actual efficiency of BFT for OD.

The data in our study indicate clear advantages of biofeedback related to PEG for the treatment of this subtype of constipation. Some aspects of our results were similar to previous studies[31,32]. Five sessions training led to a major improvement for 79.54% of patients. According to our findings, biofeedback training should be applied as effective treatment for patients with this form of constipation. By contrast, PEG treatment was relatively ineffective, poorly-tolerated, and required continuous treatment.

Chiarioni *et al*[33] performed 14.6 g of PEG with five weekly biofeedback sessions in patients who did not respond to conservative therapy. At the 6-mo follow-up, major improvements were reported by the patients enrolled in the biofeedback arm (80%) compared with those in the PEG arm. They reported that biofeedback also produced greater reductions in straining, incomplete evacuation sensation, enema use, and abdominal pain ($P < 0.01$). Rao *et al*[34] compared biofeedback with sham biofeedback or a standard therapy of diet, exercise, and laxatives in 77 randomly assigned patients. In their study, dyssynergia of 79% patients was solved using biofeedback training. The overall defecation satisfaction was also improved in the biofeedback group. These findings were contrary to the results of our study. Therefore, whether biofeedback can be established as the first line treatment for obstructed defecation still requires further multicenter, large, randomized controlled trials.

COMMENTS

Background

Chronic constipation has a clear impact on the quality of life of adult patients, and has been considered a major social and psychological disability. Although obstructive defecation patients who do not respond to regular medications can seek further medical treatment, there have been no worldwide standard treatment methods for this type of condition until now. Although the authors consider biofeedback to be a more beneficial treatment for obstructive defecation, recent controlled studies have indicated that the efficiency of manometric biofeedback treatment for different types of obstructive defecation remains controversial and may be associated with pathogenesis or learning obstacles.

Research frontiers

Biofeedback had a clear effect on obstructed defecation. The main purpose of this research was to assess the quality of life scores of patients diagnosed with obstructive defecation after treatment comparing biofeedback training with oral polyethylene glycol management. The purpose of this study was also to determine whether biofeedback training affects the pelvic physiologic mechanism and to establish a new alternative management method.

Innovation and breakthroughs

Biofeedback-guided pelvic floor exercise training is an alternative treatment where physiologic process information can be converted into a visual signal. In most studies, simple visual, auditory EMG, or pressure signals of sphincter activity provide feedback to the patients. Most techniques used were based on the method of simulated evacuation, such as the expulsion of a balloon to demonstrate to the patient normal coordination for successful expulsion. Previous studies on biofeedback therapy in obstructive defecation have yielded conflicting results, with efficacy rates that range from 18% to 100%. Based on these reports, biofeedback training requires further research to elucidate the actual efficiency for patients with this form of constipation.

Applications

The data in this study show a clear superiority for biofeedback relative to oral polyethylene glycol for the treatment of this subtype of constipation. If this research was extended to large multicenter randomized trials and its efficiency proven, biofeedback could become the standard treatment method for obstructive defecation.

Terminology

Obstructive defecation: Obstructive defecation is a state of impaired inhibition

of the pelvic floor while straining to defecate. Biofeedback: Biofeedback-guided pelvic floor exercise training is an alternative treatment where physiologic process information can be converted into a visual signal.

Peer review

This is an interesting study. If biofeedback therapy could be added as another treatment in further research, new findings for functional obstructive defecation may arise.

REFERENCES

1 **Lembo A**, Camilleri M. Chronic constipation. *N Engl J Med* 2003; **349**: 1360-1368 [PMID: 14523145 DOI: 10.1056/NEJMra020995]
2 **Talley NJ**, Weaver AL, Zinsmeister AR, Melton LJ. Onset and disappearance of gastrointestinal symptoms and functional gastrointestinal disorders. *Am J Epidemiol* 1992; **136**: 165-177 [PMID: 1415139]
3 **Levitt MA**, Peña A. Surgery and constipation: when, how, yes, or no? *J Pediatr Gastroenterol Nutr* 2005; **41** Suppl 1: S58-S60 [PMID: 16131973]
4 **D'Hoore A**, Penninckx F. Obstructed defecation. *Colorectal Dis* 2003; **5**: 280-287 [PMID: 12814403 DOI: 10.1046/j.1463-1318.2003.00497.x]
5 **Preston DM**, Lennard-Jones JE. Anismus in chronic constipation. *Dig Dis Sci* 1985; **30**: 413-418 [PMID: 3987474 DOI: 10.1007/BF01318172]
6 **Bongers ME**, Benninga MA, Maurice-Stam H, Grootenhuis MA. Health-related quality of life in young adults with symptoms of constipation continuing from childhood into adulthood. *Health Qual Life Outcomes* 2009; **7**: 20 [PMID: 19254365 DOI: 10.1186/1477-7525-7-20]
7 **Longstreth GF**, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology* 2006; **130**: 1480-1491 [PMID: 16678561 DOI: 10.1053/j.gastro.2005.11.061]
8 **Rao SS**. Biofeedback therapy for dyssynergic (obstructive) defecation. *J Clin Gastroenterol* 2000; **30**: 115-116 [PMID: 10730916 DOI: 10.1016/j.bpobgyn.2005.08.010]
9 **Pourmomeny AA**, Emami MH, Amooshahi M, Adibi P. Comparing the efficacy of biofeedback and balloon-assisted training in the treatment of dyssynergic defecation. *Can J Gastroenterol* 2011; **25**: 89-92 [PMID: 21321680]
10 **Varma MG**, Hart SL, Brown JS, Creasman JM, Van Den Eeden SK, Thom DH. Obstructive defecation in middle-aged women. *Dig Dis Sci* 2008; **53**: 2702-2709 [PMID: 18340532 DOI: 10.1007/s10620-008-0226-x]
11 **Chiotakakou-Faliakou E**, Kamm MA, Roy AJ, Storrie JB, Turner IC. Biofeedback provides long-term benefit for patients with intractable, slow and normal transit constipation. *Gut* 1998; **42**: 517-521 [PMID: 9616314]
12 **Emmanuel AV**, Kamm MA. Response to a behavioural treatment, biofeedback, in constipated patients is associated with improved gut transit and autonomic innervation. *Gut* 2001; **49**: 214-219 [PMID: 11454797]
13 **Wang J**, Luo MH, Qi QH, Dong ZL. Prospective study of biofeedback retraining in patients with chronic idiopathic functional constipation. *World J Gastroenterol* 2003; **9**: 2109-2113 [PMID: 12970917]
14 **Rao SS**, Valestin J, Brown CK, Zimmerman B, Schulze K. Long-term efficacy of biofeedback therapy for dyssynergic defecation: randomized controlled trial. *Am J Gastroenterol* 2010; **105**: 890-896 [PMID: 20179692 DOI: 10.1038/ajg.2010.53]
15 **Rao SS**, Welcher KD, Leistikow JS. Obstructive defecation: a failure of rectoanal coordination. *Am J Gastroenterol* 1998; **93**: 1042-1050 [PMID: 9672327]
16 **Koh CE**, Young CJ, Young JM, Solomon MJ. Systematic review of randomized controlled trials of the effectiveness of biofeedback for pelvic floor dysfunction. *Br J Surg* 2008; **95**: 1079-1087 [PMID: 18655219 DOI: 10.1002/bjs.6303]
17 **Rao SS**. Dyssynergic defecation and biofeedback therapy. *Gastroenterol Clin North Am* 2008; **37**: 569-586, viii [PMID: 18793997 DOI: 10.1016/j.gtc.2008.06.011]
18 **Koh D**, Lim JF, Quah HM, Tang CL. Biofeedback is an effective treatment for patients with dyssynergic defaecation. *Singapore Med J* 2012; **53**: 381-384 [PMID: 22711036]
19 **Chiarioni G**, Heymen S, Whitehead WE. Biofeedback therapy for dyssynergic defecation. *World J Gastroenterol* 2006; **12**: 7069-7074 [PMID: 17131466]
20 **Hart SL**, Lee JW, Berian J, Patterson TR, Del Rosario A, Varma MG. A randomized controlled trial of anorectal biofeedback for constipation. *Int J Colorectal Dis* 2012; **27**: 459-466 [PMID: 22159695]
21 SPSS advanced statistics [computer program]. Version 11.0. Chicago, IL: SPSS Inc, 2003
22 **Dubois D**, Gilet H, Viala-danten M, Tack J. Psychometric performance and clinical meaningfulness of the Patient Assessment of Constipation – Quality of Life questionnaire in prucalopride (RESOLOR®) trials for chronic constipation. *Neurogastroenterology & Motility* 2010; **22**: e54-e63 [DOI: 10.1111/j.1365-2982.2009.01408.x]
23 **Agachan F**, Chen T, Pfeifer J, Reissman P, Wexner SD. A constipation scoring system to simplify evaluation and management of constipated patients. *Dis Colon Rectum* 1996; **39**: 681-685 [PMID: 8646957]
24 World Medical Association Declaration of Helsinki. Recommendations guiding physicians in biomedical research involving human subjects. *Cardiovasc Res* 1997; **35**: 2-3 [PMID: 9302340]
25 **Idänpään-Heikkilä JE**. WHO guidelines for good clinical practice (GCP) for trials on pharmaceutical products: responsibilities of the investigator. *Ann Med* 1994; **26**: 89-94 [PMID: 8024733]
26 **Bove A**, Pucciani F, Bellini M, Battaglia E, Bocchini R, Altomare DF, Dodi G, Sciaudone G, Falletto E, Piloni V, Gambaccini D, Bove V. Consensus statement AIGO/SICCR: diagnosis and treatment of chronic constipation and obstructed defecation (part I: diagnosis). *World J Gastroenterol* 2012; **18**: 1555-1564 [PMID: 22529683 DOI: 10.3748/wjg.v18.i14.1555]
27 **DiPalma JA**, DeRidder PH, Orlando RC, Kolts BE, Cleveland MB. A randomized, placebo-controlled, multicenter study of the safety and efficacy of a new polyethylene glycol laxative. *Am J Gastroenterol* 2000; **95**: 446-450 [PMID: 10685748 DOI: 10.1111/j.1572-0241.2000.01765]
28 **Ramkumar D**, Rao SS. Efficacy and safety of traditional medical therapies for chronic constipation: systematic review. *Am J Gastroenterol* 2005; **100**: 936-971 [PMID: 15784043 DOI: 10.1111/j.1572-0241.2005.40925.x]
29 **Bove A**, Pucciani F, Bellini M, Battaglia E, Bocchini R, Altomare DF, Dodi G, Sciaudone G, Falletto E, Piloni V, Gambaccini D, Bove V. Consensus statement AIGO/SICCR: diagnosis and treatment of chronic constipation and obstructed defecation (part I: diagnosis). *World J Gastroenterol* 2012; **18**: 1555-1564 [PMID: 22529683 DOI: 10.3748/wjg.v18.i14.1555]
30 **Battaglia E**, Serra AM, Buonafede G, Dughera L, Chistolini F, Morelli A, Emanuelli G, Bassotti G. Long-term study on the effects of visual biofeedback and muscle training as a therapeutic modality in pelvic floor dyssynergia and slow-transit constipation. *Dis Colon Rectum* 2004; **47**: 90-95 [PMID: 14702645 DOI: 10.2494/photopolymer.20.767]
31 **Heymen S**, Scarlett Y, Jones K. Randomized Controlled Trial Shows Biofeedback to be Superior to Alternative Treatments for Patients with Pelvic Floor Dyssynergia type Constipation. *Dis Colon Rectum* 2009; **52**: 10 [DOI: 10.1007/DCR.0b013e3181b55455]
32 **Corazziari E**, Badiali D, Bazzocchi G. Long term efficacy, safety, and tolerability of low daily doses of isosmotic polyethylene glycol electrolyte balanced solution (PMF-100) in the treatment of functional chronic constipation. *Gut* 2000;

46: 522–526 [DOI: 10.1136/gut.46.4.522]
33 **Chiarioni G**, Salandini L, Whitehead WE. Biofeedback benefits only patients with outlet dysfunction, not patients with isolated slow transit constipation. *Gastroenterology* 2005; **129**: 86-97 [PMID: 16012938 DOI: 10.1053/j.gastro.2005.05.015]
34 **Rao SS**, Seaton K, Miller M, Brown K, Nygaard I, Stumbo P, Zimmerman B, Schulze K. Randomized controlled trial of biofeedback, sham feedback, and standard therapy for dyssynergic defecation. *Clin Gastroenterol Hepatol* 2007; **5**: 331-338 [PMID: 17368232]

P- Reviewers: Ker CG, Kasawara KT, Zubarik R
S- Editor: Qi Y **L- Editor**: Rutherford A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9170

World J Gastroenterol 2014 July 21; 20(27): 9170-9177
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

OBSERVATIONAL STUDY

Need for infliximab dose intensification in Crohn's disease and ulcerative colitis

Carlos Taxonera, David Olivares, Juan L Mendoza, Manuel Díaz-Rubio, Enrique Rey

Carlos Taxonera, David Olivares, Juan L Mendoza, Inflammatory Bowel Disease Unit, Department of Gastroenterology, Hospital Clínico San Carlos, 28040 Madrid, Spain
Carlos Taxonera, David Olivares, Juan L Mendoza, Manuel Díaz-Rubio, Enrique Rey, Instituto de Investigación del Hospital Clínico San Carlos (IdISSC), 28040 Madrid, Spain
Manuel Díaz-Rubio, Enrique Rey, Department of Gastroenterology, Hospital Clínico San Carlos, 28040 Madrid, Spain
Author contributions: Taxonera C performed the research, analyzed the data, designed the research study and wrote the paper; Olivares D collected and analyzed the data and designed the research; Mendoza JL performed the research and critical review of the manuscript; Díaz-Rubio M and Rey E designed the research study and critical review of the manuscript.
Correspondence to: Carlos Taxonera, MD, PhD, Inflammatory Bowel Disease Unit, Department of Gastroenterology, Hospital Clínico San Carlos, c/Profesor Martín Lagos s/n, 28040 Madrid, Spain. carlos.taxonera@salud.madrid.org
Telephone: +34-91-3303713 Fax: +34-91-3303785
Received: November 13, 2013 Revised: January 24, 2014
Accepted: April 2, 2014
Published online: July 21, 2014

Abstract

AIM: To compare the need for infliximab dose intensification in two cohorts of patients with Crohn's disease (CD) or ulcerative colitis (UC).

METHODS: Single centre, uncontrolled, observational study. Consecutive patients with CD and UC who responded to infliximab induction doses were included. Data collected in a prospectively maintained database were retrospectively analysed. Differences in the rates of dose intensification per patient-month and the intensification-free survival time were compared. We also evaluated the interval between the first infliximab induction dose and the first infliximab escalated dose. The weight-adjusted infliximab administration costs were also calculated.

RESULTS: Fifty nine patients with CD and 38 patients with UC were enrolled. The rate of intensification per patient-month was 3.9% for UC and 1.4% for CD (P = 0.005). The median time from baseline to intensification was significantly shorter in UC compared to CD [6.6 mo (IQR: 4.2-9.5 mo) *vs* 10.7 mo (IQR: 8.9-11.7 mo), P = 0.005]. In the survival analysis, the cumulative probability of avoiding infliximab dose intensification was significantly higher in CD (P = 0.002). In the multivariate analysis, disease (UC *vs* CD) was the only factor significantly associated with dose intensification. The infiximab administration costs during the first year were significantly higher for UC compared to CD (mean ± SD 234.9 ± 53.3 Euros/kg *vs* 212.3 ± 15.1 Euros/kg, P = 0.03).

CONCLUSION: The rate of infliximab dose intensification per patient-month is significantly higher in UC patients. The infliximab administration costs are also significantly higher in patients with UC.

Key words: Crohn's disease; Ulcerative colitis; Infliximab; Dose intensification; Costs

Core tip: Infliximab dose intensification to counteract loss of response is well established in the management of patients with Crohn's disease (CD). In ulcerative colitis, the need for infliximab dose intensification is less well established. The study compares for the first time the need for infliximab dose intensification for ulcerative colitis and CD in the same clinical setting. The need for infliximab dose intensification was significantly higher in patients with ulcerative colitis compared to patients with Crohn's disease. The drug administration costs were also higher in patients with ulcerative colitis. Our data provide a rational basis for economic planning in patients with ulcerative colitis selected for anti-tumor necrosis factor-α therapy.

Taxonera C, Olivares D, Mendoza JL, Díaz-Rubio M, Rey E. Need for infliximab dose intensification in Crohn's disease and ulcerative colitis. *World J Gastroenterol* 2014; 20(27): 9170-9177 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9170.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9170

INTRODUCTION

Infliximab is a monoclonal antibody that targets tumour necrosis factor (TNF)-α and it is indicated for Crohn's disease (CD) and moderate-to-severe ulcerative colitis (UC), among other conditions[1]. In the pivotal clinical trials of infliximab in CD, patients were randomized to placebo, 5 or 10 mg/kg maintenance dosing after an initial response[2,3]. In the ACCENT I trial for luminal CD, dose intensification was allowed in patients who had lost response during maintenance therapy. An analysis of the results of this trial showed that approximately 90% of those in the 5 mg/kg maintenance arm and 80% of those in the 10 mg/kg maintenance arm re-established response on switching to a higher dose (10 and 15 mg/kg), respectively[4]. In the case of UC, the pivotal studies used for approval did not allow dose intensification on loss of response during maintenance[5]. Although the pivotal clinical trials provide less support for dose intensification in UC, in clinical practice, the need for infliximab dose intensification as a result of loss of response has been reported by a number of authors[6-10].

In a systematic review in CD patients, the annual risk of loss of infliximab response and need for infliximab dose intensification was consistently established at around 13% per patient-year[11]. In UC patients the need for infliximab dose intensification is less well defined.

No studies have directly compared the need for dose intensification in CD and UC, even though loss of response is a problem in clinical practice common to both diseases. The primary objective of this study was to compare the need for and time to infliximab dose intensification in patients with CD and patients with UC in the same clinical setting. The drug administration costs were also compared between cohorts.

MATERIALS AND METHODS

Study design and selection of patients

In this single centre, uncontrolled observational study, data collected prospectively as part of a well-established treatment protocol (see below) were retrospectively analyzed by chart review. All consecutive patients with CD or UC who started infliximab in the Inflammatory Bowel Disease Unit of our hospital between July 2008 and January 2010 were included. Only patients who responded to the three infliximab induction doses according to standard criteria and received at least the first infliximab maintenance dose were eligible. Infliximab was administered for CD or UC according to the indications accepted in the summary of product characteristics. The main reasons for prescription of infliximab were steroid-refractory disease and steroid dependence for both cohorts and perianal disease in the case of CD patients. The study was approved by the local hospital ethics committee.

Criteria for dose intensification

Patients were assessed for the need of infliximab dose intensification by two specialists in inflammatory bowel disease with more than 15 years of experience in this field. Prescription of infliximab in our unit follows a standard protocol that requires systematic recording in the database of demographic and disease characteristics prior to starting treatment, as well as information about therapy (doses administered and any adverse reactions). The demographic characteristics and disease characteristics of the patients on initiating treatment were extracted from this database. For the purposes of the analysis, the initiation of treatment was taken as baseline.

The need for dose intensification, whether by increased dose or decreased dosing interval, was noted. For patients who required dose intensification, the time relative to baseline (that is, the first induction dose) was recorded. For patients who did not require dose intensification, time on infliximab was calculated as the interval between the first infliximab induction dose and either the last follow-up visit or the time of infliximab discontinuation. Other treatment parameters such as concomitant corticosteroid or immunomodulatory therapy were also extracted. Adverse events were recorded throughout the infliximab treatment (both before and, if applicable, after intensification) in the clinical database.

Loss of response to infliximab was evaluated at each visit during maintenance therapy. In patients with luminal CD or UC the need for infliximab intensification was supported by measuring the Harvey-Bradshaw index[12] or the 9-point partial Mayo score[13], respectively. Loss of response was defined in those patients who responded to the infliximab induction doses but were not in remission as follows: Harvey-Bradshaw score $\geqslant$ 5 for luminal CD or 9-point partial Mayo score $\geqslant$ 4 for UC. Loss of response in fistulising CD was evaluated by assessing the number of draining fistulae, the amount of discharge and the presence of pain and the restriction of daily activities. Although these measures, together with the C-reactive protein (CRP) values, were used to guide the decision to intensify the dose, the final decision was made by the investigators taking into account the overall clinical situation of the patient. Before intensification in patients in whom an infection was suspected, other causes of persistent symptoms including coexistent cytomegalovirus or *Clostridium difficile* were ruled out.

Infliximab administration costs

The infiximab administration costs were calculated as the purchase price paid by the hospital together with the total number of infusions and the number of vials used

Table 1 Baseline characteristics of the patients enrolled *n* (%)

Baseline characteristics	Ulcerative colitis (*n* = 38)	Crohn's disease (*n* = 59)	*P*
Sex			
Men	16 (42.1)	33 (55.9)	0.184
Women	22 (57.9)	26 (44.1)	
Age (mean ± SD, yr)	41.9 (14.2)	38.9 (38.9)	0.312
Duration of disease [median (interquartile range), yr]	4.5 (2-10.3)	6 (1-12)	0.738
Smoking status			
Non-smoker	15 (39.5)	21 (35.6)	0.003
Smoker	8 (21.1)	30 (50.8)	
Ex-smoker	15 (39.5)	8 (13.6)	
Steroid status			
Neither steroid dependent nor refractory	0	19 (32.2)	< 0.001
Steroid dependent	21 (55.3)	33 (55.9)	
Steroid refractory	17 (44.7)	7 (11.9)	
Steroid use at induction			
No	10 (26.3)	38 (64.4)	< 0.001
Yes	28 (73.7)	21 (35.6)	
Immunomodulator therapy			
No	7 (18.4)	14 (23.7)	0.536
Yes	31 (81.6)	45 (76.3)	

per administration. The cost of the drug treatment was derived from the Catalogue of Pharmaceutical Specialties of the Spanish General Council of Pharmacists for the year 2010. The costs of the remaining resources, mainly day-care hospitalizations for infliximab administration, were obtained from the Spanish health-care costs database SOIKOS. The total administration costs (infliximab, pre-medication and day-care hospitalization costs) were calculated for each patient who were in treatment for at least 1 year. Results were weight-adjusted and expressed as cost (Euros) per kilogram for the first year of treatment.

Outcomes

The co-primary endpoints were the differences in the rates of patients requiring infliximab dose intensification per month and the intensification-free survival time between the cohorts of patients with CD or UC. We also evaluated the interval between the first infliximab induction dose and the first infliximab escalated dose. Potential predictors of the need for infliximab dose intensification such as age, gender, type of disease (CD or UC), disease duration, reason for infliximab prescription (steroid dependence, steroid-refractory disease, or perianal disease in the case of CD) and steroid or immunosuppressant use at baseline were investigated. We also calculated the impact of the type of disease and the need for dose intensification on infliximab administration costs.

Statistical analysis

Study variables were summarized descriptively using number and percentage for discrete variables and mean ± SD or medians (IQR) as appropriate for continuous variables. Demographic, disease and treatment characteristics were explored using the χ^2 test for qualitative variables and the Student *t*-test and the median test if applicable for quantitative variables. The rates of intensification per patient-month of treatment were calculated. Intensification-free survival was estimated using the Kaplan-Meier method and differences between curves evaluated using the Breslow exact test. A Cox proportional hazards survival regression analysis was employed to estimate the adjusted hazard ratios and their 95%CI. Variables with $P < 0.10$ in the univariate analysis were included in the model. The null hypothesis was rejected in each statistical test when $P < 0.05$. Analysis was performed using the SPSS version 15.0 (Chicago, IL, United States) statistical package for Microsoft Windows.

RESULTS

Ninety-seven patients from our prospectively maintained database of about 1400 patients with inflammatory bowel disease were evaluated. Demographic characteristics and the use of steroids or immunosuppressants at baseline (time of first infliximab induction dose) are shown in Table 1. The two cohorts showed no differences regarding sex, age and disease duration. At the time of diagnosis, more patients with CD were smokers, whereas more patients with UC were ex-smokers. Infliximab was indicated due to steroid-refractory disease in a higher proportion of UC patients. In one-third of CD patients ($n = 19$), infliximab was prescribed to treat complex perianal disease. Twenty-six patients (70%) had extensive UC, and 12 (30%) left-sided colitis. A greater proportion of UC patients were receiving steroids at baseline ($P < 0.001$). Infliximab was never administered as salvage therapy to hospitalized patients with severe UC refractory to intravenous steroids. There were no significant differences in the proportions of patients who were receiving immunomodulator treatment at baseline (Table 1).

Thirty-two patients required infliximab dose intensification, 16 of 38 (42%) patients with UC and 16 of 59

Table 2 Summary of dose intensification

Dose intensification	Ulcerative colitis (n = 38)	Crohn's disease (n = 59)	P value
Time on infliximab[1] [median (interquartile range), mo]	9 (5.2-12.5)	13.1 (8.1-23.3)	0.006
Rate of intensification per patient-month	3.9%	1.4%	0.005
Time to infliximab intensification [median (interquartile range), mo]	6.6 (4.2-9.5)	10.7 (8.9-15.7)	0.005

[1]Time on infliximab: Interval of time between the first infliximab induction dose and the first infliximab intensified dose, or for patients who did not require dose intensification time between the initiation of infliximab and either the last follow-up visit or the time of infliximab discontinuation.

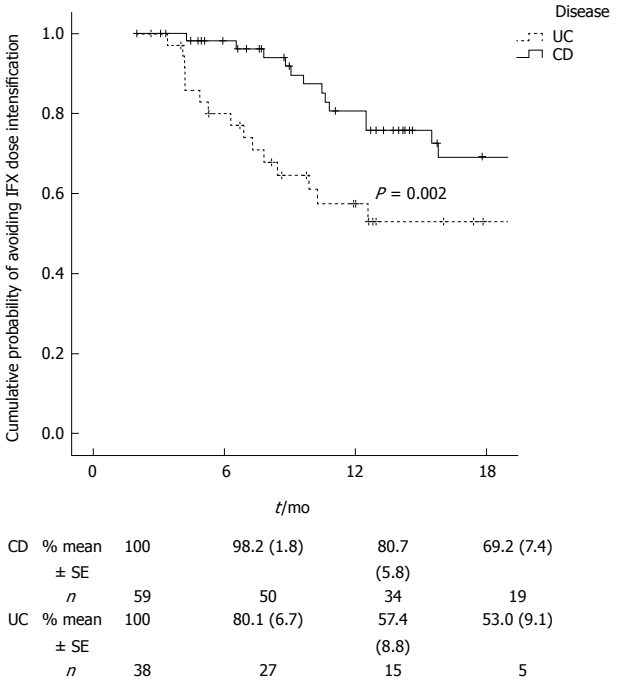


Figure 1 Cumulative probability of avoiding infliximab dose intensification. The data below indicate the number and percent of patients at risk. Comparison using the Breslow test. CD: Crohn's disease; UC: Ulcerative colitis.

(27%) patients with CD. At the time of intensification, patients with luminal CD had a Harvey-Bradshaw score of 8.2 ± 4.6 (range 5-13) and the patients with UC a 9-point partial Mayo score of 5.9 ± 1.5 (range 4-8). Of these 32 patients with dose intensification, the dose was increased to 10 mg/kg every 8 wk in 10 patients and the dosing interval was shortened in the remaining 22 (to 5 mg/kg every 6 wk in 20 patients and to 5 mg/kg every 4 wk in two patients). There were no significant differences according to type of intensification between the two cohorts. At time of intensification, 18 out of 32 (56.3%) patients were on immunomodulators; the proportion was similar regardless of type of disease. Before the first intensified dose of infliximab, the mean ± SD CRP (mg/dL) was 1.8 (2.9) for patients with CD and 1.4 (3.2) for patients with UC.

Need for infliximab dose intensification

The duration of exposure to infliximab was longer in patients with CD than in patients with UC [median 13.1 mo (IQR: 8.1-23.3 mo) *vs* 9 mo (IQR: 5.2-12.5 mo), P = 0.006] (Table 2). Total exposure to infliximab was 404 mo for the 38 patients with UC compared to 1133 mo for the 59 patients with CD. The rates of patients requiring dose intensification per month with infliximab were significantly higher for UC compared with CD (3.9% *vs* 1.4% per month, P = 0.005). The rate of infliximab dose intensification per patient-month was not significantly different between perianal and luminal CD (1.2% *vs* 1.6% per month, P = 0.4). Patients with UC showed a significantly higher rate of infliximab dose intensification per patient-month when compared with the cohort of patients with luminal CD (3.9% *vs* 1.6% per month, P = 0.03). No significant differences in the rate of infliximab dose intensification per patient-month were observed for luminal CD according to Montreal localization (L1 *vs* L2+L3, P = 0.6).

In patients who needed infliximab intensification, the median time from baseline to intensification was significantly shorter in the UC cohort compared to the CD cohort [6.6 mo (IQR: 4.2-9.5 mo) *vs* 10.7 mo (IQR: 8.9-11.7 mo), P = 0.005]. As shown in Figure 1, the Kaplan-Meier curves for cumulative probability of avoiding dose intensification rapidly separated for the two cohorts. The cumulative probability of avoiding infliximab dose intensification was higher in CD patients, with the Breslow exact test showing a highly significant difference (P = 0.002).

Predictors of the need for infliximab dose intensification

The only factor significantly associated with the rates of patients requiring dose intensification per month was disease, with intensification being more likely with UC (HR = 2.73, 95%CI: 1.31-5.69, P = 0.007) (Table 3). Patients who were receiving immunomodulator treatment at baseline showed a trend toward a lower adjusted rate of infliximab intensification (HR = 0.51, 95%CI: 0.24-1.07, P = 0.08). Neither the need for steroids at baseline nor having steroid-refractory disease at baseline were associated with the need for infliximab dose intensification.

Infliximab administration costs

The infiximab administration costs during the first year were significantly higher for UC patients compared to CD patients (mean ± SD 234.9 ± 53.3 Euros/kg *vs* 212.3 ± 15.1 Euros/kg, P = 0.03). The first-year infiximab administration costs to patients weighting 70 kg were 16443 Euros for UC and 14861 Euros for CD. In

Table 3 Summary of factors associated with dose intensification in the multivariate analysis

Factor	Adjusted Hazard ratio	95%CI	*P* value
Corticosteroid dependence (yes/no)	1.359	0.425-4.343	0.605
Age	1.015	0.991-1.040	0.216
Induction with corticosteroids (yes/no)	1.258	0.521-3.041	0.610
0Immunomodulator use (yes/no)	0.510	0.242-1.073	0.076
Disease (ulcerative colitis *vs* Crohn´s disease)	2.732	1.313-5.686	0.007

Table 4 Summary of studies of infliximab dose intensification in ulcerative colitis: Proportion of patients who needed infliximab intensification

Ref.	Patients[1]	Median duration of follow-up (mo)	Dose intensification
Rostholder *et al*[7]	50	14[2]	54%
Oussalah *et al*[8]	80	18	45%
Seow *et al*[9]	93	14	58%
Arias *et al*[10]	136	14	46%
Present study	38	9	42%

[1]Only patients who responded and started maintenance therapy; [2]Mean duration.

the multivariate analysis, only the type of disease (P = 0.02) and the need for infliximab dose intensification (P = 0.008) were associated with increased drug costs.

Safety

Twenty-five patients experienced a total of 42 adverse events during the whole follow-up period. Five adverse events were classified as severe: 2 herpes zoster, 1 severe delayed hypersensitivity reaction, 1 viral meningitis and 1 leishmaniosis. Six adverse events led to the discontinuation of infliximab: 1 herpes zoster, 1 severe delayed hypersensitivity reaction, 1 viral meningitis and 3 acute infusion reactions. Two of the severe adverse events that led to discontinuation occurred in patients with intensified doses.

DISCUSSION

This study compares for the first time the need for infliximab dose intensification between patients with UC and CD in the same clinical setting. Our study also provides an updated comparison of the drug administration costs between cohorts, which is necessary in understanding the economic burden of inflammatory bowel disease. Infliximab is a chimeric IgG1 monoclonal antibody with a high affinity for TNF-α, which is an important cytokine in the pathogenesis of inflammatory bowel disease[14]. The validity of using TNF-α as a therapeutic target has been demonstrated in randomized clinical trials with infliximab in both the induction and maintenance setting of luminal[2] and fistulising CD[3] and in UC[5].

Despite the proven efficacy of infliximab in the maintenance setting, loss of response is a real problem. Gisbert *et al*[11] reviewed 16 studies that assessed loss of response in CD. For follow-up durations of between 5 and 72 mo, the loss of response ranged from 11% to 54%. The authors calculated that the annual risk of loss of infliximab response was 13.1%, which is similar to the rate found in our study (1.4% per patient-month or 16.8% annually of patients who lost response and required dose intensification). Gisbert *et al*[11] also studied the outcomes of infliximab dose intensification and found evidence of the effectiveness of the approach. For example, in the ACCENT 1 study, dose intensification to 10 mg/kg was effective in 90% of the patients in the 5 mg/kg dose arm who had loss response[4]. In a multicentre study, dose intensification, whether by doubling the infliximab dose or by shortening the dosing interval to 5 mg/kg every 4 wk, enabled response to be regained in 73% of patients[15].

In the case of UC, the need for infliximab dose intensification is less well defined. The extension study of the pivotal ACT trial reported that only 7% of patients required dose intensification[6]. The situation in clinical practice, however, would seem to be very different. In observational studies, the proportion of patients who needed infliximab dose intensification ranged between 42% and 58% for follow-ups between 14 and 18 mo (Table 4)[7-10]. The need for intensification in our study (42% after a median of 9 mo of follow-up) is within the range reported in the other studies performed in a clinical practice setting[7-10].

To the best of our knowledge there are no studies published comparing the need for infliximab dose intensification for UC and CD in the same hospital or by the same specialists. Given that the rationale for dose intensification in UC is not supported by randomized studies, such a comparison provides further support for this therapeutic approach. There is also a need for long term observational data on the costs incurred by patients selected for anti-TNF-α therapy, without forgetting that the intensification of the drug is one of the main drivers of the increased direct costs[16]. Our study confirmed that the drug intensification rates had a significant im-

pact on infiximab administration costs. The infiximab administration costs were higher in patients with UC, and this was related to the increased need for drug intensification in this cohort of patients.

We observe a higher rate of dose intensification per patient-month in patients with UC, and this dose intensification is also required earlier than in patients with CD. Although it could be argued that the size of the cohort and follow-up are limited, we have compared more than 400 and 1100 mo of follow-up for patients with UC and CD, respectively, resulting in highly significant differences in the primary endpoints. In addition the differences were established very early in time, and do not seem reasonable that it would change with longer follow-ups. Loss of response to infliximab and other anti-TNF-α agents in CD is generally thought to arise because of the immunogenic nature of these drugs. For example, in a paediatric study, 22% of responders at the end of follow-up had developed anti-infliximab antibodies compared to 75% of children who had lost response[17]. In patients with UC, repeated administration may lead to the development of anti-infliximab antibodies over time, inducing a drop in infliximab trough serum levels and hence the need for dose intensification[9]. Arias *et al*[10] showed that patients with UC who displayed low infliximab trough levels demonstrated shorter time to dose intensification. However, in our study, the Kaplan-Meier curve of time to intensification (Figure 1) clearly shows that a proportion of patients with UC require dose intensification earlier in follow-up than is the case for Crohn's patients. This is a reflection that more patients with UC need infliximab dose intensification at the start of the maintenance period. It may be that UC, which is a different disease entity, may require higher doses of infliximab for an initial control of the disease. Seow *et al*[9] described a high proportion of patients with UC with absent trough levels of infliximab that contrast with other studies in CD. They suggested that the explanation could be a more rapid clearance of infliximab in UC patients.

Several reasons might explain the reported high rates of infliximab dose intensification in patients with UC. First, such an approach in CD patients has been shown to be effective, while administration every 8 wk of the 10 mg/kg has been shown to have an equivalent safety profile compared to the lower dose, both in CD[2-4] and UC[6]. Second, in a subset of patients with UC, infliximab could be used as a last resort to avoid the need for colectomy. In a subanalysis of pooled data from the ACT1 and ACT2 trials, there was a significant difference between the 10 mg/kg dose group and placebo in terms of reduced need for colectomy but not for the 5 mg/kg dose group[18]. Third, when the study was performed, infliximab was the only anti-TNF agent approved for use in UC. In clinical practice, adalimumab has only been used as an alternative treatment in UC after discontinuation of infliximab due to an adverse effect or after loss of response despite intensification[19]. Cyclosporine is also useful when used as rescue therapy in acute severe steroid-refractory UC, but patients need to be hospitalized[20,21]. Interestingly, a recent survey of clinical practice showed that not only adult gastroenterologist but also paediatric gastroenterologist who prescribed infliximab considered that intensified doses of infliximab have a recognized role and perceived benefit in the treatment of some paediatric UC patients[22].

The study is subject to a number of limitations. First, this was a retrospective study. However, the data were collected prospectively by the same two inflammatory bowel disease specialists according to the infliximab treatment protocol. Another limitation of the study was the discretional criteria used to decide infliximab dose intensification. In the ACCENT 1 study the loss of response was defined by an increase in CDAI for patients with CD[4]. In the ULTRA 1 study the need for adalimumab dose intensification in patients with UC was defined according to the Partial Mayo Score values[23]. Therefore in clinical trials the definition of loss of response or inadequate response during the maintenance phase has been based on clinical activity indexes, and not on c-reactive protein values nor on endoscopic assessment. In our study, the physician global assessment, supported by the Harvey-Bradshaw score or the Partial Mayo Score, was used to guide the decision to intensify the infliximab dose. This assessment is subject to potential bias. CRP values were also taken into account in the final decision. In conclusion, in contrast to the case in clinical trials, the decision to escalate the dose of infliximab is based on "real life" clinical practice.

Another weakness of the study is that, in our clinical practice, infliximab trough serum levels and anti-infliximab antibodies titres are not usually determined. Such information could be useful for explaining the differences in the need for infliximab dose intensification between the two diseases, and also to understand the possible role of combination therapy in the reduction of anti-infliximab immunogenicity[24]. Genetic test were not available in our study. Genetic polymorphisms may contribute to predict efficacy of infliximab[25,26].

As regards the adverse effects profile, our results are similar to those of large controlled series in patients with CD or UC treated with infliximab[2,3,6].

In conclusion, in clinical practice, the rate of patient-months of treatment with need for infliximab dose intensification was higher in patients with UC compared to patients with CD. Patients with UC required intensification of infliximab dosing earlier and infliximab intensification-free survival was also lower in these patients. The infiximab administration costs were higher in patients with UC. Our data provide a rational basis for economic planning in patients with inflammatory bowel disease selected for anti-TNF-α therapy.

ACKNOWLEDGMENTS

The authors would like to thank Cristina Fernandez for her assistance in the statistical analysis and Dr. Gregory

Morley for writing support and for reviewing the English manuscript.

COMMENTS

Background

Despite the proven efficacy of infliximab in the maintenance setting, loss of response is a real problem. Infliximab dose intensification to counteract loss of response is well established in the management of patients with CD. In UC, the need for infliximab dose intensification is less well established.

Research frontiers

The results provide a rational basis for economic planning in patients with inflammatory bowel disease selected for anti-TNF-α therapy.

Innovations and breakthroughs

The study compares for the first time the need for infliximab dose intensification for UC and CD in the same clinical setting.

Applications

The study also provides an updated comparison of the drug administration costs between cohorts, which is necessary in understanding the economic burden of inflammatory bowel disease. The infiximab administration costs were higher in patients with UC.

Terminology

Infliximab dose intensification: need for infliximab dose intensification, whether by increased dose or decreased dosing interval.

Peer review

The investigation has profound therapeutically implication highlighting the problem of intensification of infliximab in the phenotypes of inflammatory bowel disease, CD and UC, incorporating the administration costs as well.

REFERENCES

1 **Rutgeerts P**, Van Assche G, Vermeire S. Review article: Infliximab therapy for inflammatory bowel disease--seven years on. *Aliment Pharmacol Ther* 2006; **23**: 451-463 [PMID: 16441465 DOI: 10.1111/j.1365-2036.2006.02786.x]

2 **Hanauer SB**, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Rutgeerts P. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 2002; **359**: 1541-1549 [PMID: 12047962 DOI: 10.1016/S0140-6736(02)]

3 **Sands BE**, Anderson FH, Bernstein CN, Chey WY, Feagan BG, Fedorak RN, Kamm MA, Korzenik JR, Lashner BA, Onken JE, Rachmilewitz D, Rutgeerts P, Wild G, Wolf DC, Marsters PA, Travers SB, Blank MA, van Deventer SJ. Infliximab maintenance therapy for fistulizing Crohn's disease. *N Engl J Med* 2004; **350**: 876-885 [PMID: 14985485 DOI: 10.1056/NEJMoa030815]

4 **Rutgeerts P**, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Hanauer SB. Comparison of scheduled and episodic treatment strategies of infliximab in Crohn's disease. *Gastroenterology* 2004; **126**: 402-413 [PMID: 14762776 DOI: 10.1053/j.gastro.2003.11.014]

5 **Rutgeerts P**, Sandborn WJ, Feagan BG, Reinisch W, Olson A, Johanns J, Travers S, Rachmilewitz D, Hanauer SB, Lichtenstein GR, de Villiers WJ, Present D, Sands BE, Colombel JF. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med* 2005; **353**: 2462-2476 [PMID: 16339095 DOI: 10.1056/NEJMoa050516]

6 **Reinisch W**, Sandborn WJ, Rutgeerts P, Feagan BG, Rachmilewitz D, Hanauer SB, Lichtenstein GR, de Villiers WJ, Blank M, Lang Y, Johanns J, Colombel JF, Present D, Sands BE. Long-term infliximab maintenance therapy for ulcerative colitis: the ACT-1 and -2 extension studies. *Inflamm Bowel Dis* 2012; **18**: 201-211 [PMID: 21484965 DOI: 10.1002/ibd.21697]

7 **Rostholder E**, Ahmed A, Cheifetz AS, Moss AC. Outcomes after escalation of infliximab therapy in ambulatory patients with moderately active ulcerative colitis. *Aliment Pharmacol Ther* 2012; **35**: 562-567 [PMID: 22239070 DOI: 10.1111/j.1365-2036.2011.04986.x]

8 **Oussalah A**, Evesque L, Laharie D, Roblin X, Boschetti G, Nancey S, Filippi J, Flourié B, Hebuterne X, Bigard MA, Peyrin-Biroulet L. A multicenter experience with infliximab for ulcerative colitis: outcomes and predictors of response, optimization, colectomy, and hospitalization. *Am J Gastroenterol* 2010; **105**: 2617-2625 [PMID: 20736936 DOI: 10.1038/ajg.2010.345]

9 **Seow CH**, Newman A, Irwin SP, Steinhart AH, Silverberg MS, Greenberg GR. Trough serum infliximab: a predictive factor of clinical outcome for infliximab treatment in acute ulcerative colitis. *Gut* 2010; **59**: 49-54 [PMID: 19651627 DOI: 10.1136/gut.2009.183095]

10 **Arias MT**, Van de Casteele N, Drobne D, Ferrante M, Cleynen I, Ballet V, Rutgeerts P, Gils A, Vermeire S. Importance of trough serum levels and antibodies on long-term efficacy of infliximab therapy in ulcerative colitis. *Gut* 2012; **6** (Suppl 1): S5 [DOI: 10.1016/S1873-9946(12)60011-7]

11 **Gisbert JP**, Panés J. Loss of response and requirement of infliximab dose intensification in Crohn's disease: a review. *Am J Gastroenterol* 2009; **104**: 760-767 [PMID: 19174781 DOI: 10.1038/ajg.2008.88]

12 **Vermeire S**, Schreiber S, Sandborn WJ, Dubois C, Rutgeerts P. Correlation between the Crohn's disease activity and Harvey-Bradshaw indices in assessing Crohn's disease severity. *Clin Gastroenterol Hepatol* 2010; **8**: 357-363 [PMID: 20096379 DOI: 10.1016/j.cgh.2010.01.001]

13 **Lewis JD**, Chuai S, Nessel L, Lichtenstein GR, Aberra FN, Ellenberg JH. Use of the noninvasive components of the Mayo score to assess clinical response in ulcerative colitis. *Inflamm Bowel Dis* 2008; **14**: 1660-1666 [PMID: 18623174 DOI: 10.1002/ibd.20520]

14 **van Dullemen HM**, van Deventer SJ, Hommes DW, Bijl HA, Jansen J, Tytgat GN, Woody J. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). *Gastroenterology* 1995; **109**: 129-135 [PMID: 7797011 DOI: 10.1016/0016-5085(95)90277-5]

15 **Katz L**, Gisbert JP, Manoogian B, Lin K, Steenholdt C, Mantzaris GJ, Atreja A, Ron Y, Swaminath A, Shah S, Hart A, Lakatos PL, Ellul P, Israeli E, Svendsen MN, van der Woude CJ, Katsanos KH, Yun L, Tsianos EV, Nathan T, Abreu M, Dotan I, Lashner B, Brynskov J, Terdiman JP, Higgins PD, Chaparro M, Ben-Horin S. Doubling the infliximab dose versus halving the infusion intervals in Crohn's disease patients with loss of response. *Inflamm Bowel Dis* 2012; **18**: 2026-2033 [PMID: 22294554 DOI: 10.1002/ibd.22902]

16 **Bodger K**. Cost effectiveness of treatments for inflammatory bowel disease. *Pharmacoeconomics* 2011; **29**: 387-401 [PMID: 21271748]

17 **Candon S**, Mosca A, Ruemmele F, Goulet O, Chatenoud L, Cézard JP. Clinical and biological consequences of immunization to infliximab in pediatric Crohn's disease. *Clin Immunol* 2006; **118**: 11-19 [PMID: 16125467 DOI: 10.1016/j.clim.2005.07.010]

18 **Sandborn WJ**, Rutgeerts P, Feagan BG, Reinisch W, Olson A, Johanns J, Lu J, Horgan K, Rachmilewitz D, Hanauer SB, Lichtenstein GR, de Villiers WJ, Present D, Sands BE, Colombel JF. Colectomy rate comparison after treatment of ulcerative colitis with placebo or infliximab. *Gastroenterology* 2009; **137**: 1250-1260; quiz 1520 [PMID: 19596014 DOI: 10.1053/j.gastro.2009.06.061]

19 **Taxonera C**, Estellés J, Fernández-Blanco I, Merino O, Marín-Jiménez I, Barreiro-de Acosta M, Saro C, García-Sánchez V, Gento E, Bastida G, Gisbert JP, Vera I, Martinez-Montiel P, Garcia-Morán S, Sánchez MC, Mendoza JL. Adalimumab induction and maintenance therapy for patients with ulcerative colitis previously treated with infliximab. *Aliment Phar-*

macol Ther 2011; **33**: 340-348 [PMID: 21133961 DOI: 10.1111/j.1365-2036.2010.04531.x]
20 **Laharie D**, Bourreille A, Branche J, Allez M, Bouhnik Y, Filippi J, Zerbib F, Savoye G, Nachury M, Moreau J, Delchier JC, Cosnes J, Ricart E, Dewit O, Lopez-Sanroman A, Dupas JL, Carbonnel F, Bommelaer G, Coffin B, Roblin X, Van Assche G, Esteve M, Färkkilä M, Gisbert JP, Marteau P, Nahon S, de Vos M, Franchimont D, Mary JY, Colombel JF, Lémann M. Ciclosporin versus infliximab in patients with severe ulcerative colitis refractory to intravenous steroids: a parallel, open-label randomised controlled trial. *Lancet* 2012; **380**: 1909-1915 [PMID: 23063316 DOI: 10.1016/S0140-6736(12)61084-8]
21 **Chang KH**, Burke JP, Coffey JC. Infliximab versus cyclosporine as rescue therapy in acute severe steroid-refractory ulcerative colitis: a systematic review and meta-analysis. *Int J Colorectal Dis* 2013; **28**: 287-293 [PMID: 23114475 DOI: 10.1007/s00384-012-1602-8]
22 **Nattiv R**, Wojcicki JM, Garnett EA, Gupta N, Heyman MB. High-dose infliximab for treatment of pediatric ulcerative colitis: a survey of clinical practice. *World J Gastroenterol* 2012; **18**: 1229-1234 [PMID: 22468086 DOI: 10.3748/wjg.v18.i11.1229]
23 **Sandborn WJ**, van Assche G, Reinisch W, Colombel JF, D' Haens G, Wolf DC, Kron M, Tighe MB, Lazar A, Thakkar RB. Adalimumab induces and maintains clinical remission in patients with moderate-to-severe ulcerative colitis. *Gastroenterology* 2012; **142**: 257-265.e1-3 [PMID: 22062358 DOI: 10.1053/j.gastro.2011.10.032]
24 **Bortlik M**, Duricova D, Malickova K, Machkova N, Bouzkova E, Hrdlicka L, Komarek A, Lukas M. Infliximab trough levels may predict sustained response to infliximab in patients with Crohn's disease. *J Crohns Colitis* 2013; **7**: 736-743 [PMID: 23200919 DOI: 10.1016/j.crohns.2012.10.019]
25 **Steenholdt C**, Enevold C, Ainsworth MA, Brynskov J, Thomsen OØ, Bendtzen K. Genetic polymorphisms of tumour necrosis factor receptor superfamily 1b and fas ligand are associated with clinical efficacy and/or acute severe infusion reactions to infliximab in Crohn's disease. *Aliment Pharmacol Ther* 2012; **36**: 650-659 [PMID: 22860894 DOI: 10.1111/apt.12010]
26 **Medrano LM**, Taxonera C, Márquez A, Barreiro-de Acosta M, Gómez-García M, González-Artacho C, Pérez-Calle JL, Bermejo F, Lopez-Sanromán A, Martín Arranz MD, Gisbert JP, Mendoza JL, Martín J, Urcelay E, Núñez C. Role of TNFRSF1B polymorphisms in the response of Crohn's disease patients to infliximab. *Hum Immunol* 2014; **75**: 71-75 [PMID: 24121042 DOI: 10.1016/j.humimm.2013.09.017]

P- Reviewers: Coffey JC, Efthymiou A, Gara N, Marina IG, Maric I
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9178

World J Gastroenterol 2014 July 21; 20(27): 9178-9184
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

PROSPECTIVE STUDY

Hepatitis B surface antigen levels during natural history of chronic hepatitis B: A Chinese perspective study

Lin-Yan Zeng, Jiang-Shan Lian, Jian-Yang Chen, Hong-Yu Jia, Yi-Min Zhang, Dai-Rong Xiang, Liang Yu, Jian-Hua Hu , Ying-Feng Lu, Lin Zheng, Lan-Juan Li, Yi-Da Yang

Lin-Yan Zeng, Jiang-Shan Lian, Jian-Yang Chen, Hong-Yu Jia, Yi-Min Zhang, Dai-Rong Xiang, Liang Yu, Jian-Hua Hu, Ying-Feng Lu, Lin Zheng, Lan-Juan Li, Yi-Da Yang, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, the First Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 310003, Zhejiang Province, China

Author contributions: Zeng LY and Lian JS performed the majority of experiments; Chen JY, Jia HY, Zhang YM, Xiang DR, Yu L, Hu JH, Lu YF provided analytical tools and revised the manuscript; Zheng L, Li LJ and Yang YD designed the study; Lian JS and Yang YD wrote the manuscript.

Supported by National Key Program for Infectious Diseases of China to Yang YD, No. 2013ZX10002001; and the 12th Five-Year Significant New Drugs Creation Plan of the Ministry of Science and Technology of China to Li LJ, No. 2011ZX09302-003-03

Correspondence to: Yi-Da Yang, Professor, State Key Laboratory for Diagnosis and Treatment of Infectious Disease, the First Affiliated Hospital of Zhejiang University School of Medicine, 79 Qingchun Road, Hangzhou 310003, Zhejiang Province, China. yangyida65@163.com
Telephone: +86-571-87236755 Fax: +86-571-87236755

Received: November 17, 2013 Revised: March 12, 2014
Accepted: April 21, 2014
Published online: July 21, 2014

Abstract

AIM: To determine the baseline hepatitis B surface antigen (HBsAg) levels during the different phases of chronic hepatitis B (CHB) patients in China.

METHODS: Six hundred and twenty-three hepatitis B virus or un-infected patients not receiving antiviral therapy were analyzed in a cross-sectional study. The CHB patients were classified into five phases: immune-tolerant (IT, n = 108), immune-clearance (IC, n = 161), hepatitis B e antigen negative hepatitis (ENH, n = 149), low-replicative (LR, n = 135), and liver cirrhosis (LC, n = 70). HBsAg was quantified (Abbott ARCHITECT assay) and correlated with hepatitis B virus (HBV) DNA, and serum alanine aminotransferase/aspartate aminotransferase (ALT/AST) in each phase of CHB was also determined.

RESULTS: Median HBsAg titers were different in each phase of CHB (P < 0.001): IT (4.85 $\log_{10}$ IU/mL), IC (4.36 $\log_{10}$ IU/mL), ENH (2.95 $\log_{10}$ IU/mL), LR (3.18 $\log_{10}$ IU/mL) and LC (2.69 $\log_{10}$ IU/mL). HBsAg titers were highest in the IT phase and lowest in the LC phase. Serum HBsAg titers showed a strong correlation with HBV viral load in the IC phase (r = 0.683, P < 0.001). No correlation between serum HBsAg level and ALT/AST was observed.

CONCLUSION: The mean baseline HBsAg levels differ significantly during the five phases of CHB, providing evidence on the natural history of HBV infection. HBsAg quantification may predict the effects of immune-modulator or oral nucleos(t)ide analogue therapy.

Key words: Hepatitis B surface antigen quantification; Chronic hepatitis B; Natural history; Perspective

Core tip: The quantification of serum hepatitis B surface antigen (HBsAg) has been recently advocated as a favorable marker of disease activity in chronic hepatitis B (CHB). Knowledge of HBsAg in the natural history of chronic hepatitis B is important for the management of the disease, but there is a lack of corresponding data on the base level of HBsAg in the natural history of CHB in China. Hence, the aim of this cross-sectional study was to evaluate the levels of HBsAg in consecutive phases of the natural history of hepatitis B virus-infection without the influence of antiviral treatment before, including the patients' progression to liver cirrhosis.

Zeng LY, Lian JS, Chen JY, Jia HY, Zhang YM, Xiang DR, Yu L, Hu JH, Lu YF, Zheng L, Li LJ, Yang YD. Hepatitis B sur-

face antigen levels during natural history of chronic hepatitis B: A Chinese perspective study. *World J Gastroenterol* 2014; 20(27): 9178-9184 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9178.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9178

INTRODUCTION

The liver and peripheral blood of patients with chronic hepatitis B (CHB) contains large amounts of viral proteins, especially hepatitis B virus (HBV) surface antigens[1]. Detection of hepatitis B surface antigen (HBsAg) in serum is a fundamental diagnostic marker of HBV infection[2]. During the natural history of HBV infection and during antiviral therapy, the level of HBsAg in CHB changes over time, and the loss of HBsAg and the development of anti-HBs antibodies (HBsAg-seroconversion) are the ultimate goals of anti-HBV therapy, as they are believed to represent successful immunological control of active HBV replication[3-6].

The quantification of serum HBsAg has recently been advocated as a reliable marker of disease activity in CHB[7-11]. Knowledge of HBsAg in the natural history of CHB is important for disease management. The natural history of CHB has five phases: immune-tolerant (IT), immune-clearance (IC), non/low-replicative (LR), hepatitis B e antigen negative hepatitis (ENH) and liver cirrhosis (LC). These phases may not occur in all patients or sequentially[3]. As the natural course of HBV infection proceeds, liver histology may progressively worsen and some patients with CHB develop liver failure or hepatocellular carcinoma (HCC)[12,13].

Despite the usefulness of HBsAg quantification as a predictor for CHB, there is a lack of corresponding data on the baseline level of HBsAg during the natural history of CHB in China. Hence, the aim of this cross-sectional study was to evaluate the levels of HBsAg in the consecutive phases of HBV infection in patients not receiving antiviral therapy, and the patients' progress to liver cirrhosis.

MATERIALS AND METHODS

Patients

Six hundred and twenty-three patients with HBV infection, not receiving antiviral therapy, from the First Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China) were included in this cross-sectional study. Two hundred and seventy-nine patients were hepatitis B e antigen (HBeAg)(+) and 344 were HBeAg(-). There were 456 males and 167 females with a median age of 34 years (range: 18-65 years).

None of the patients were co-infected with hepatitis A virus, hepatitis C virus, hepatitis delta virus, hepatitis E virus or human immunodeficiency virus. Markers such as ceruloplasmin, anti-nuclear antibodies and anti-mitochondrial antibodies for possible co-existing autoimmune disorders and metabolic liver disease were negative. Patients with HCC and end-stage liver diseases were excluded. All useful indicators including patient age, biochemical parameters, and serum HBsAg and HBeAg levels were determined. HBV DNA levels in these patients were obtained when the serum samples were collected.

Table 1 Definitions of phases of persistent hepatitis B virus infection

Phase	HBV-DNA (IU/mL)	HBeAg status	ALT (U/L)	Liver imaging
IT	> 10^7	+	≤ ULN	No cirrhosis
IC	> 2000	+	≥ 2ULN	No cirrhosis
ENH	> 2000	-	≥ 2ULN	No cirrhosis
LR	< 2000	-	≤ ULN	No cirrhosis
LC	Any	+/-	Any	Cirrhosis

IT: Immune tolerance phase; IC: Immune clearance phase; ENH: HBeAg(-) hepatitis; LR: Low-replicative; LC: Liver cirrhosis induced by hepatitis B virus; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; ULN: Upper limit of normal; "+": Positive; "-": Negative.

The patients were categorized into the five phases of CHB: immune-tolerant (IT, n = 108), immune-clearance (IC, n = 161), HBeAg negative hepatitis (ENH, n = 149), low-replicative (LR, n = 135), and liver cirrhosis (LC, n = 70), which were diagnosed based on clinical manifestations or pathology. The classification of these patients was based on the 2012 European Association for the Study of the Liver (EASL), Clinical Practice Guidelines-management of chronic hepatitis B in Asia-Pacific and the 2010 Chinese Clinical Practice Guidelines of chronic hepatitis B[3,14,15] (Table 1).

The study protocol, which conformed to the guidelines of the Declaration of Helsinki, was approved by the Ethics Review Committee of the First Affiliated Hospital of Zhejiang University School of Medicine.

Serum HBsAg quantification

Serum HBsAg levels were quantified using the Abbott ARCHITECT assay (Abbott Diagnostics, Germany) following the manufacturers' instructions. The dynamic range was 0.05-250 IU/mL. Samples were diluted 1:500 or 1:1000 using ARCHITECT HBsAg Manual Diluent (Abbott Diagnostics) if > 250 IU/mL.

HBV DNA measurement

Serum HBV DNA was quantified by the Determination Kit for hepatitis B virus DNA (Life River, China) with a detection range of 5×10^2 IU/mL-10^8 IU/mL for samples with HBV DNA > 10^8 IU/mL, and the HBV DNA assay was repeated after a dilution of 1:1000.

HBV genotyping

The HBV genotypes were determined using sequence detection *via* PCR. All products were directly sequenced with a HBV Genotype Real Time PCR Kit (ZJ Bio-Tech, Shanghai, China) and run on a MegaBACE 500 according to the manufacturer's instructions. HBV genome sequence analysis software was used to analyze the results.

Table 2 Baseline population characteristics

	Immune tolerant	Immune clearance	HBeAg negative hepatitis	Low replicative	Liver cirrhosis	*P* value
HBeAg status	Positive	Positive	Negative	Negative	Positive/negative	
Sex M/F	60/48	127/34	124/25	95/40	50/20	< 0.001
Age (yr)	26 (18-44)	29 (18-45)	41 (22-60)	39 (22-65)	49 (26-65)	< 0.001
ALT (U/L)	25 (9-40)	188 (80-1322)	40 (80-3755)	23 (17-39)	45 (9-341)	< 0.001
AST (U/L)	20 (6-36)	100 (7-1063)	228 (20-2136)	14 (9-38)	49 (10-302)	< 0.001
HBsAg (log_{10} IU/mL)	4.85 (3.97-5.15)	4.36 (3.08-5.25)	2.95 (0.82-4.89)	3.18 (2.53-3.60)	2.69 (2.20-4.04)	< 0.001
HBV DNA (log_{10} IU/mL)	8.50 (7.12-9.49)	7.93 (3.93-9.23)	5.73 (3.3-9.28)	2.64 (2.18-3.30)	4.68 (3.28-8.75)	< 0.001
Genotype						
B/C	60/48	83/78	86/62	74/61	37/33	0.006

HBsAg: Hepatitis B s antigen; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; ALT: Alanine transaminase; AST: Aspartate aminotransferase.

Statistical analysis

Variables were compared between groups, using the Mann-Whitney *U* and Fisher's exact tests for univariate comparisons, and the Kruskall-Wallis test and ANOVA for multivariate comparisons. Pearson's correlation coefficient (*r*) was used to describe the correlation between two variables. Statistical analysis was performed using SPSS 16.0 software.

RESULTS

All patients with HBV infection were enrolled in the study from January 2012 to January 2013 and were classified into the five phases of CHB: IT (n = 108), IC (n = 161), ENH (n = 149), LR (n = 135) and LC (n = 70). More male patients (73.2%) than female patients were enrolled into this cohort study. HBeAg(+) patients were significantly younger than HBeAg(-) patients ($P < 0.001$), and the patients in the IT phase were younger than those in the IC phase ($P = 0.035$), while the patients in the LC phase were older than those in the LR and ENH phases ($P < 0.001$). There were no significant differences between the LR and ENH phase with regard to patient age ($P = 0.584$). A detailed description of the patients is shown in Table 2.

Distribution of serum HBsAg levels

Serum HBsAg levels were widely distributed, and there were significant differences in serum HBsAg levels between the HBV infected patients in the different phases. The highest HBsAg level among the five phases was found in the IT group (median 4.85 log_{10} IU/mL), followed by the IC group with a median HBsAg level of 4.36 log_{10} IU/mL, a moderate HBsAg level was found in the ENH (2.95 log_{10} IU/mL) and LR groups (3.18 log_{10} IU/mL), and the lowest level was observed in the LC group (2.69 log_{10} IU/mL) (Table 2 and Figure 1A). The median HBsAg level in HBeAg(+) patients was significantly higher than that in HBeAg(-) patients.

Correlation between HBsAg titers and HBV DNA

The correlations between serum HBsAg titers and serum HBV DNA in each phase of CHB are shown in Figure 1B-F. There was a strong correlation in the IC phase ($r = 0.683$, $P < 0.0001$), a modest correlation in the ENH ($r = 0.342$, $P = 0.001$) and IT ($r = 0.287$, $P = 0.003$) phases, but a poor correlation between serum HBsAg titers and serum HBV DNA in the LR ($r = 0.156$, $P = 0.071$) and LC ($r = 0.177$, $P = 0.143$) phases.

The ratio of HBsAg (log_{10} IU/mL) to HBV DNA in each phase of CHB was also determined (Figure 2). The HBsAg/HBV DNA ratio was significantly higher in the LR phase compared to the IT, IC, ENH and LC phases (1.17 *vs* 0.56, 0.56, 0.54 and 0.57, respectively, $P < 0.001$).

All patients were genotype B or C. In both viral genotypes, median HBsAg titers were also different among the five phases of CHB, however, there was no difference in the median HBsAg titers between genotype B and C in all five phases.

Correlation between HBsAg titers and clinical parameters

Serum HBsAg was not associated with ALT and AST levels in any of the phases of persistent CHB (Table 3). No significant correlations between serum HBsAg levels and gender, INR, serum bilirubin, albumin, sodium or cholinesterase levels were observed. Quantification of serum HBsAg was not associated with age (Table 3).

DISCUSSION

This study aimed to determine baseline HBsAg levels during the different phases of CHB infection in a Chinese cohort. Both serum HBsAg levels and HBsAg/HBV DNA ratios were obviously different during the five phases of CHB. This finding was previously clearly demonstrated by Jaroszewicz *et al*[16] in a European cohort, by Nguyen *et al*[17] in an Asian cohort and by Ramachandran *et al*[18] in an Indian cohort. However, the present study is the largest to characterize HBsAg levels in the different phases of CHB infection.

Our results showed that median HBsAg titer was highest in the IT phase (4.85 log_{10} IU/mL) followed by the IC phase (4.36 log_{10} IU/mL), and both of these phases were HBeAg positive. As indicated in Table 3, HBsAg titers were higher in the HBeAg positive phase compared with the HBeAg negative phase, including the ENH (2.95 log_{10} IU/mL) and LR (3.18 log_{10} IU/mL)

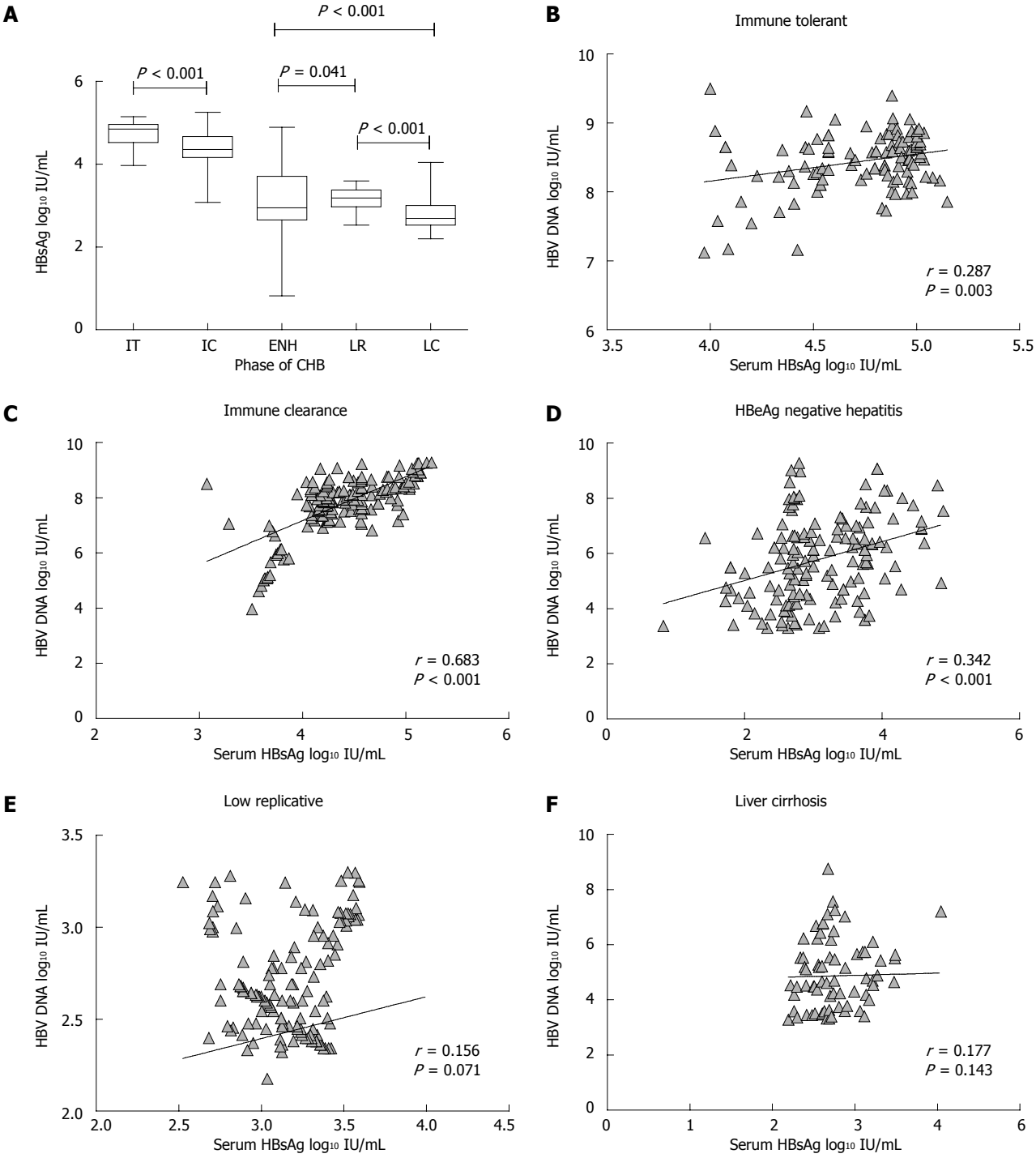


Figure 1 Distribution of hepatitis B surface antigen titers during natural history of chronic hepatitis B and correlation between serum hepatitis B surface antigen titers and hepatitis B virus DNA in different phases of chronic hepatitis B. A: Median values with 95% confidence interval; B-D: The ratio of serum hepatitis B surface antigen (HBsAg) to hepatitis B virus (HBV) DNA levels in the five phases of chronic hepatitis B (CHB). Dots represent individual values, bars 95%CI of median, and numbers are median values.

phases. The lowest HBsAg titers were observed in the LC phase (2.69 log10 IU/mL). All patients were genotype B or C. In both viral genotypes, the median HBsAg titers were also different among the five phases of CHB; however, there were no differences in median HBsAg titers between genotype B and C in all five phases.

Quantification of HBsAg was introduced more than 20 years ago, but has only recently been significantly improved by new automated quantitative assays. Several studies have suggested that quantitative HBsAg is an effective tool for identifying patients who are candidates for therapy, and for monitoring response to pegylated interferon (Peg-IFN), telbivudine and entecavir treatment, and HBV DNA levels[8,19-21]. Our results demonstrated that there was a positive correlation between serum HBsAg and serum HBV DNA levels in the IC

Table 3 Correlation between hepatitis B s antigen and laboratory parameters in different phases

	IT (n = 108)		IC (n = 161)		ENH (n = 149)		LR (n = 135)		LC (n = 70)	
	r	P value	r	P value	r	P value	r	P value	r	P value
Age	0.153	0.144	0.025	0.757	-0.053	0.519	-0.102	0.319	0.254	0.134
HBV-DNA ($\log_{10}$ IU/mL)	0.287	0.003	0.683	< 0.001	0.342	< 0.001	0.156	0.071	0.177	0.143
ALT (U/L)	-0.119	0.107	-0.102	0.198	0.102	0.215	-0.057	0.422	-0.093	0.445
AST (U/L)	0.060	0.535	-0.072	0.161	0.034	0.681	-0.011	0.880	-0.082	0.499

HBV: Hepatitis B virus; IT: Immune tolerance phase; IC: Immune clearance phase; ENH: Hepatitis B e antigen negative hepatitis; LR: Low-replicative; LC: Liver cirrhosis induced by HBV.

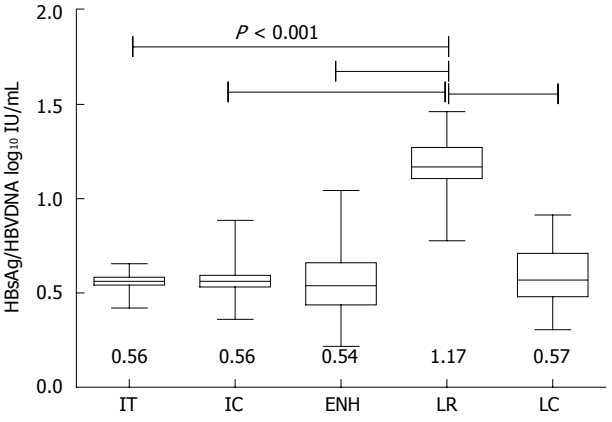


Figure 2 Ratio of hepatitis B surface antigen and hepatitis B virus DNA in each phase of chronic hepatitis B. Median values with 95% confidence interval (of median) are shown. IT: Immune-tolerant; IC: Immune-clearance; ENH: Hepatitis B e antigen negative hepatitis; LR: Low-replicative; LC: Liver cirrhosis; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus.

phase, a modest correlation in the ENH and IT phases, and a poor correlation between serum HBsAg titers and serum HBV DNA in the LR and LC phases. In a recent study by Nguyen *et al*[17], Asian patients with HBV infection also showed a strong correlation between HBsAg levels and HBV DNA during the IC phase. In agreement with the results of Nguyen *et al*[17], our study also indicated that the serum HBsAg/HBV DNA ratio was significantly higher in the LR phase compared with the IT, IC, ENH and LC phases (1.17 *vs* 0.56, 0.56, 0.54 and 0.57, respectively, $P < 0.001$). The reason why HBsAg levels and HBV DNA titers appear unrelated in different stages of HBV infection is unclear. A possible explanation may be that HBsAg production far exceeds that required for the production of virions and is secreted in the form of noninfectious particles. Commercially available HBsAg quantification methods may detect all forms of HBsAg; however, the clinical significance of the different HBsAg forms and their ratios is unknown. Another possible explanation is that the S gene of HBV may integrate into host genome in CHB patients.

Several studies have suggested that there is a relationship between serum HBsAg titers and intrahepatic markers of HBV infection, such as covalently closed circular DNA (cccDNA) and intrahepatic HBV DNA[22-24]. Compared to both serum HBV DNA and intrahepatic cccDNA, the costs for determining serum HBsAg are less. This is very important in a developing country, such as China. However, the usefulness of serum HBsAg levels as a substitute for both cccDNA and serum HBV DNA needs to be further elucidated, as studies have indicated a poor correlation with cccDNA[25], and only a positive correlation with HBV DNA in HBeAg positive CHB[26].

Our results also indicated that the levels of HBsAg were lowest in the LC phase, representing long-term immune clearance of HBV infection. A possible mechanism for this may be long-term vigorous immune action, but not complete clearance of HBV in the LC phase. Another possible explanation is a reduction in healthy hepatocytes which can synthesize HBsAg, due to liver fibrosis.

This was a cross-sectional study. A longitudinal study designed to follow the different phases of HBV infection is required. However, HBV infection is a progressive disease in most phases, such as the IC, ENH and LC phases. On the other hand, according to recent guidelines, CHB patients in the IC, ENH and LC phases should receive antiviral treatment. Therefore, it is very difficult to design a longitudinal follow-up study to evaluate HBsAg levels in different phases of HBV infection.

In conclusion, this Chinese cohort study demonstrated that (i) HBsAg titers are different in the five phases of HBV infection; HBsAg levels are highest in the IT phase and lowest in the LC phase; HBsAg shows a strong correlation with HBV DNA in the IC phase; and there were no differences in the median HBsAg titers between genotype B and C in all five phases. Our findings have provided evidence of the pathophysiology and natural history of hepatitis B infection. Future longitudinal studies should be conducted to confirm these results.

COMMENTS

Background

The quantification of serum hepatitis B surface antigen (HBsAg) has recently been advocated as a reliable marker of disease activity in chronic hepatitis B (CHB). Knowledge of HBsAg in the natural history of chronic hepatitis B is important for disease management. Despite the usefulness of HBsAg quantification as a predictor for CHB, there is a lack of corresponding data on the baseline level of HBsAg during the nature history of CHB in China.

Research frontiers

As the natural course of hepatitis B virus (HBV) infection proceeds, liver histology may progressively worsen and some patients with CHB may develop liver failure or hepatocellular carcinoma. Furthermore, serum HBsAg levels are

strongly correlated with intrahepatic HBV DNA and covalently closed circular DNA (cccDNA); cccDNA is superior to serum HBV DNA as a predictor of the sustained response to antiviral therapy.

Innovations and breakthroughs

Quantification of serum HBsAg helps in the management of patients with chronic HBV infection. Median HBsAg levels differ significantly during the natural history of HBV infection, progressively declining from immune tolerance to the inactive phase. The combination of HBsAg < 1000 IU/mL and HBV DNA < 2000 IU/mL at a single time point accurately identifies true inactive carriers. During antiviral treatment, HBsAg levels decline more rapidly in patients treated with pegylated interferon (Peg-IFN) than in those treated with nucleos(t)ide analogues, and in responders to Peg-IFN compared to non-responders, suggesting that a response-guided therapy in both HBeAg-positive and -negative patients treated with Peg-IFN could improve the cost-effectiveness of this therapeutic approach. This is the first study to determine the baseline HBsAg levels during each phase of the natural history of chronic hepatitis B.

Applications

As the population of hepatitis B carriers and CHB patients in China is approximately 1/3 to 1/2 of patients worldwide, baseline HBsAg quantification data could be used for further cohort studies in China.

Peer review

This is a good clinical study in which the authors, for the first time, determine the baseline HBsAg levels during each phase of the natural history of chronic hepatitis B. The results of this study help further understand the pathophysiology and the natural history of hepatitis B infection.

REFERENCES

1 **Seeger C**, Mason WS. Hepatitis B virus biology. *Microbiol Mol Biol Rev* 2000; **64**: 51-68 [PMID: 10704474]
2 **Nguyen DH**, Ludgate L, Hu J. Hepatitis B virus-cell interactions and pathogenesis. *J Cell Physiol* 2008; **216**: 289-294 [PMID: 18302164 DOI: 10.1002/jcp.21416]
3 **European Association For The Study Of The Liver**. EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. *J Hepatol* 2012; **57**: 167-185 [PMID: 22436845 DOI: 10.1016/j.jhep.2012.02.010]
4 **Martinot-Peignoux M**, Lapalus M, Asselah T, Marcellin P. The role of HBsAg quantification for monitoring natural history and treatment outcome. *Liver Int* 2013; **33** Suppl 1: 125-132 [PMID: 23286856 DOI: 10.1111/liv.12075]
5 **Martinot-Peignoux M**, Asselah T, Marcellin P. HBsAg quantification to predict natural history and treatment outcome in chronic hepatitis B patients. *Clin Liver Dis* 2013; **17**: 399-412 [PMID: 23905812 DOI: 10.1016/j.cld.2013.05.006]
6 **Chevaliez S**. Is HBsAg quantification ready, for prime time? *Clin Res Hepatol Gastroenterol* 2013; **37**: 559-563 [PMID: 23932705 DOI: 10.1016/j.clinre.2013.07.004]
7 **Brunetto MR**, Oliveri F, Coco B, Leandro G, Colombatto P, Gorin JM, Bonino F. Outcome of anti-HBe positive chronic hepatitis B in alpha-interferon treated and untreated patients: a long term cohort study. *J Hepatol* 2002; **36**: 263-270 [PMID: 11830339]
8 **Moucari R**, Mackiewicz V, Lada O, Ripault MP, Castelnau C, Martinot-Peignoux M, Dauvergne A, Asselah T, Boyer N, Bedossa P, Valla D, Vidaud M, Nicolas-Chanoine MH, Marcellin P. Early serum HBsAg drop: a strong predictor of sustained virological response to pegylated interferon alfa-2a in HBeAg-negative patients. *Hepatology* 2009; **49**: 1151-1157 [PMID: 19115222 DOI: 10.1002/hep.22744]
9 **Rijckborst V**, Hansen BE, Cakaloglu Y, Ferenci P, Tabak F, Akdogan M, Simon K, Akarca US, Flisiak R, Verhey E, Van Vuuren AJ, Boucher CA, ter Borg MJ, Janssen HL. Early on-treatment prediction of response to peginterferon alfa-2a for HBeAg-negative chronic hepatitis B using HBsAg and HBV DNA levels. *Hepatology* 2010; **52**: 454-461 [PMID: 20683945 DOI: 10.1002/hep.23722]
10 **Xun YH**, Zang GQ, Guo JC, Yu XL, Liu H, Xiang J, Liu J, Shi JP. Serum hepatitis B surface antigen quantification as a useful assessment for significant fibrosis in hepatitis B e antigen-positive hepatitis B virus carriers. *J Gastroenterol Hepatol* 2013; **28**: 1746-1755 [PMID: 23800140 DOI: 10.1111/jgh.12304]
11 **Kuo A**, Gish R. Chronic hepatitis B infection. *Clin Liver Dis* 2012; **16**: 347-369 [PMID: 22541703 DOI: 10.1016/j.cld.2012.03.003]
12 **Liu J**, Fan D. Hepatitis B in China. *Lancet* 2007; **369**: 1582-1583 [PMID: 17499584 DOI: 10.1016/S0140-6736(07)60723-5]
13 **Borgia G**, Gentile I. Treating chronic hepatitis B: today and tomorrow. *Curr Med Chem* 2006; **13**: 2839-2855 [PMID: 17073632]
14 **Chinese Society of Hepatology**, Chinese Medical Association; Chinese Society of Infectious Diseases, Chinese Medical Association. Guideline on prevention and treatment of chronic hepatitis B in China (2005). *Chin Med J* (Engl) 2007; **120**: 2159-2173 [PMID: 18167196]
15 **Liaw YF**, Leung N, Kao JH, Piratvisuth T, Gane E, Han KH, Guan R, Lau GK, Locarnini S. Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2008 update. *Hepatol Int* 2008; **2**: 263-283 [PMID: 19669255 DOI: 10.1007/s12072-008-9080-3]
16 **Jaroszewicz J**, Calle Serrano B, Wursthorn K, Deterding K, Schlue J, Raupach R, Flisiak R, Bock CT, Manns MP, Wedemeyer H, Cornberg M. Hepatitis B surface antigen (HBsAg) levels in the natural history of hepatitis B virus (HBV)-infection: a European perspective. *J Hepatol* 2010; **52**: 514-522 [PMID: 20207438 DOI: 10.1016/j.jhep.2010.01.014]
17 **Nguyen T**, Thompson AJ, Bowden S, Croagh C, Bell S, Desmond PV, Levy M, Locarnini SA. Hepatitis B surface antigen levels during the natural history of chronic hepatitis B: a perspective on Asia. *J Hepatol* 2010; **52**: 508-513 [PMID: 20206400 DOI: 10.1016/j.jhep.2010.01.007]
18 **Ramachandran J**, Ismail AM, Chawla G, Fletcher GJ, Goel A, Eapen CE, Abraham P. Serum HBsAg quantification in treatment-naïve Indian patients with chronic hepatitis B. *Indian J Gastroenterol* 2014; **33**: 131-135 [PMID: 24052375 DOI: 10.1007/s12664-013-0395-1]
19 **Chan HL**, Wong VW, Tse AM, Tse CH, Chim AM, Chan HY, Wong GL, Sung JJ. Serum hepatitis B surface antigen quantitation can reflect hepatitis B virus in the liver and predict treatment response. *Clin Gastroenterol Hepatol* 2007; **5**: 1462-1468 [PMID: 18054753 DOI: 10.1016/j.cgh.2007.09.005]
20 **Wursthorn K**, Jung M, Riva A, Goodman ZD, Lopez P, Bao W, Manns MP, Wedemeyer H, Naoumov NV. Kinetics of hepatitis B surface antigen decline during 3 years of telbivudine treatment in hepatitis B e antigen-positive patients. *Hepatology* 2010; **52**: 1611-1620 [PMID: 20931556 DOI: 10.1002/hep.23905]
21 **Lee JM**, Ahn SH, Kim HS, Park H, Chang HY, Kim do Y, Hwang SG, Rim KS, Chon CY, Han KH, Park JY. Quantitative hepatitis B surface antigen and hepatitis B e antigen titers in prediction of treatment response to entecavir. *Hepatology* 2011; **53**: 1486-1493 [PMID: 21520167 DOI: 10.1002/hep.24221]
22 **Thompson AJ**, Nguyen T, Iser D, Ayres A, Jackson K, Littlejohn M, Slavin J, Bowden S, Gane EJ, Abbott W, Lau GK, Lewin SR, Visvanathan K, Desmond PV, Locarnini SA. Serum hepatitis B surface antigen and hepatitis B e antigen titers: disease phase influences correlation with viral load and intrahepatic hepatitis B virus markers. *Hepatology* 2010; **51**: 1933-1944 [PMID: 20512987 DOI: 10.1002/hep.23571]
23 **Lee JM**, Ahn SH. Quantification of HBsAg: basic virology for clinical practice. *World J Gastroenterol* 2011; **17**: 283-289 [PMID: 21253386 DOI: 10.3748/wjg.v17.i3.283]
24 **Larsson SB**, Eilard A, Malmström S, Hannoun C, Dhillon AP, Norkrans G, Lindh M. HBsAg quantification for identification of liver disease in chronic hepatitis B virus carriers. *Liver Int* 2013; Epub ahead of print [PMID: 24118747 DOI:

10.1111/liv.12345]
25 **Papatheodoridis GV**, Manesis EK, Manolakopoulos S, Elefsiniotis IS, Goulis J, Giannousis J, Bilalis A, Kafiri G, Tzourmakliotis D, Archimandritis AJ. Is there a meaningful serum hepatitis B virus DNA cutoff level for therapeutic decisions in hepatitis B e antigen-negative chronic hepatitis B virus infection? *Hepatology* 2008; **48**: 1451-1459 [PMID: 18924246 DOI: 10.1002/hep.22518]
26 **Fried MW**, Piratvisuth T, Lau GK, Marcellin P, Chow WC, Cooksley G, Luo KX, Paik SW, Liaw YF, Button P, Popescu M. HBeAg and hepatitis B virus DNA as outcome predictors during therapy with peginterferon alfa-2a for HBeAg-positive chronic hepatitis B. *Hepatology* 2008; **47**: 428-434 [PMID: 18220290 DOI: 10.1002/hep.22065]

P- Reviewers: Borgia G, Choe BH, Filik L, Ozenirler S
S- Editor: Gou SX **L- Editor**: O'Neill M **E- Editor**: Zhang DN

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9185

World J Gastroenterol 2014 July 21; 20(27): 9185-9190
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RANDOMIZED CLINICAL TRIAL

Routine use of thiopurines in maintaining remission in pediatric Crohn's disease

Brendan M Boyle, Michael D Kappelman, Richard B Colletti, Robert N Baldassano, David E Milov, Wallace V Crandall

Brendan M Boyle, Wallace V Crandall, Nationwide Children's Hospital, Columbus, OH 43205, United States
Michael D Kappelman, Department of Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, United States
Richard B Colletti, University of Vermont, Burlington, VT 05405, United States
Robert N Baldassano, Children's Hospital of Philadelphia, Philadelphia, PA 19146, United States
David E Milov, Nemours Children's Clinic, Orlando, FL 32806, United States
Author contributions: Boyle BM, Crandall WV and Kappelman MD wrote the paper; Colletti RB, Baldassano RN, and Milov DE provided the manuscript review and designed the development of the PIBDNet cohort registry.
Correspondence to: Brendan M Boyle, MD, MPH, Nationwide Children's Hospital, 700 Children's Drive, Columbus, OH 43205, United States. brendan.boyle@nationwidechildrens.org
Telephone: +1-614-7223473 Fax: +1-614-7223454
Received: November 7, 2013 Revised: February 28, 2014
Accepted: April 15, 2014
Published online: July 21, 2014

Abstract

AIM: To evaluate the effectiveness of thiopurines in maintaining steroid-free remission in routine clinical practice.

METHODS: The multi-center Pediatric Inflammatory Bowel Disease Network (PIBDNet) cohort study prospectively collected data on thiopurine naïve patients initiating mercaptopurine (6MP) or azathioprine. Patients with a diagnosis of Crohn's disease (CD) were included in our study upon entering remission as determined by physician global assessment (PGA) within 365 d of initiation of thiopurines. The primary outcome of the study was maintenance of steroid-free remission (SFR) at each follow up visit. Patients were considered treatment failures if there had been a change in PGA from remission to mild, moderate or severe disease; disease relapse between visits; need for rescue therapy (biologic therapy, methotrexate, steroids); thiopurine discontinuation, hospitalization or surgical intervention. A secondary outcome defined treatment failure as a change from remission to moderate or severe (not mild) in addition to the previously defined criteria.

RESULTS: Sixty-five of 182 patients in the PIBDNet registry met criteria for inclusion in this study. Forty-five of 65 (69%) of included patients achieved remission within 180 d of thiopurine initiation. For the primary outcome, 47% and 23% of patients remained in SFR at 6 and 12 mo. The mean thiopurine dose at initiation for the 65 included patients was 0.89 ± 0.31 mg/kg per day. Metabolite levels were obtained in 48% (31/65) of the included patients with a mean 6TG level of 258 pmole/8 $\times$ 10^8 RBC ± 147. For the secondary outcome, 65% and 42% of patients remained in SFR at 6 and 12 mo.

CONCLUSION: Thiopurines were less effective in maintaining remission for pediatric CD in this "real world" cohort than has been previously described. Variation in thiopurine dosing and metabolite measurement was found among practitioners.

Key words: Crohn's disease; Pediatric; Remission; Thiopurines; Mercaptopurine

Core tip: This manuscript describes the real world effectiveness of thiopurines in maintaining remission for pediatric Crohn's disease. The outcomes differ in comparison to the initially published randomized controlled trial for pediatric Crohn's but are similar to more recently published studies evaluating the effectiveness of thiopurines. The study evaluates data from a pediatric inflammatory bowel disease registry and is representative of real world clinical care. Varitation in practitioner approach to thiopurine dosage and metabolite mea-

surement was found. Implementation of a more standardized approach to use of thiopurines could impact clinical outcomes.

Boyle BM, Kappelman MD, Colletti RB, Baldassano RN, Milov DE, Crandall WV. Routine use of thiopurines in maintaining remission in pediatric Crohn's disease. *World J Gastroenterol* 2014; 20(27): 9185-9190 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9185.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9185

INTRODUCTION

The incidence of pediatric Crohn's disease (CD) is increasing and nearly 25% of patients with inflammatory bowel disease (IBD) present during childhood[1-3]. Thiopurines have been shown to be effective in the treatment of children and adults with CD[4-6] and are associated with a steroid sparing effect[7]. However, studies describing the effectiveness in maintaining steroid-free remission (SFR) with thiopurines have been inconsistent[6-13]. The efficacy of thiopurines was previously evaluated by a randomized controlled trial (RCT) in pediatric patients with moderate to severe CD. This study found that greater than 90% of patients achieving clinical remission remained in clinical remission for 18 mo[7]. Subsequent retrospective pediatric studies have found thiopurines to be less effective than the initial RCT[14,15], as has the more recent SONIC trial performed in adults[16].

Variation in the delivery of chronic illness care and its negative impact upon outcomes has been previously described[17]. Evaluation of pediatric IBD registry data identified significant variation between care centers in the initial management of patients with CD including the frequency of thiopurine use, thiopurine dosing, and thiopurine methyl transferase (TPMT) measurement[18,19]. Efforts to reduce variation in pediatric IBD care using quality improvement methods have led to improved patient outcomes[20].

The aim of our study was to examine maintenance of SFR in patients who achieved clinical remission after initiating thiopurine therapy. The multi-center Pediatric Inflammatory Bowel Disease Network (PIBDNet) prospectively collected data on patients initiating thiopurine therapy, thus presenting an opportunity to investigate this research question using data from a large number of diverse clinical practices and care approaches representative of real world clinical care.

MATERIALS AND METHODS

Study subjects

The PIBDNet multi-center cohort study prospectively collected data from thiopurine naïve patients initiating mercaptopurine (6MP) or azathioprine (AZA) therapy. Forty-eight practice sites enrolled patients in the cohort study. Participating practitioners included pediatric gastroenterologists from both university and private practice settings of various sizes[19]. Evaluation of data from patients enrolled from 2004-2008 was performed. Enrolled patients were aged 1-17 years with a diagnosis of CD. Data was collected and recorded at initiation and each subsequent follow up visit. Disease activity was assessed by physician global assessment (PGA) at the time of the visit and categorized as inactive (remission), mild, moderate, or severe disease.

All patient management decisions were determined by each practitioner rather than a standardized protocol. Variations in practice approach among practitioners included thiopurine dosage, decisions about continuing thiopurines, timing or need to obtain 6MP metabolite levels, need for additional medications, and frequency of follow up visits.

Patients were included in this analysis if clinical remission by PGA had been achieved within 70-365 d from thiopurine initiation. This visit was considered time zero for this analysis. We restricted this analysis to patients achieving clinical remission between 70-365 d in order to isolate the effect of thiopurine monotherapy. Seventy days was chosen *a priori* with the expectation that the therapeutic effect of thiopurines would not have been achieved prior to 10 wk after initiation. Patients not achieving remission by 365 d after thiopurine initiation were considered non-responders and excluded.

Additional exclusion criteria for the primary analysis were: inadequate follow up data (< 70 d of follow up after thiopurine initiation or no follow up visit after achieving remission), current or previous treatment with infliximab, methotrexate (MTX), cyclosporine, or tacrolimus, heterozygote or homozygote mutant TPMT status, or patients who required surgical intervention prior to entering remission.

Study outcomes

After entering remission, each subsequent visit was evaluated for maintenance of SFR or treatment failure. In our primary analysis (analysis 1), treatment failure was defined as a change in PGA from remission to mild, moderate or severe; disease relapse recorded between visits; a need for rescue therapy [MTX, infliximab, cyclosporine, tacrolimus, corticosteroids (CS)]; thiopurine discontinuation; or hospitalization/surgical intervention after entering remission. Patients treated with CS upon entering remission who remained on CS for an additional 30 d were also considered treatment failures.

A secondary outcome considered a change in PGA from remission to moderate or severe disease (but not mild) as a treatment failure (analysis 2) in addition to the previously defined criteria for treatment failure in the primary analysis.

Exploratory analyses included patients with normal or heterozygote TPMT status that met inclusion criteria defined above. Primary and secondary outcomes for this combined cohort were performed using the above definitions for treatment failure to determine if TPMT

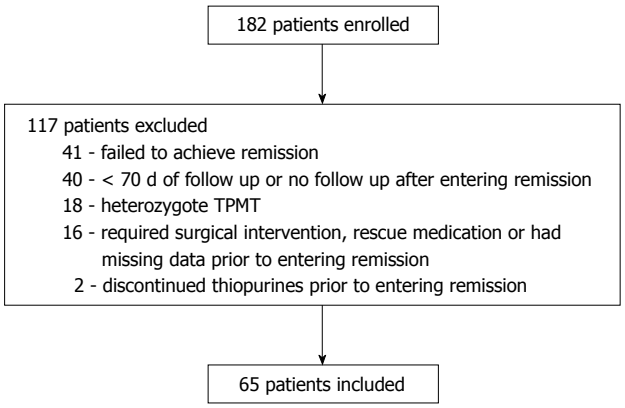


Figure 1 Flowchart of patient inclusion for primary analysis. TPMT: Thiopurine methyl transferase.

Table 1 Demographics-primary analysis *n* (%)

	Included (*n* = 65)	Excluded (*n* = 117)	*P* value
Age (yr)	12.98 ± 2.8	13.4 ± 2.9	NS
Gender			
Male	39 (60.0)	65 (55.6)	NS
Female	26 (40.0)	52 (44.4)	
Disease duration			
< 12 mo			
> 12 mo	59 (90.7)	100 (85.5)	NS
	6 (9.2)	17 (14.5)	
Race			
Caucasian	52 (80.0)	102 (87.2)	NS
Disease location			
Upper	37 (56.9)	79 (67.5)	NS
Ileal	49 (75.4)	91 (77.9)	NS
Colonic	54 (83.1)	97 (82.9)	NS
Thiopurine			
6MP	42 (64.6)	87 (77.0)	NS
AZA	23 (35.4)	26 (23.0)	
PGA			
Remission	7 (10.8)	5 (4.4)	NS
Mild	22 (33.8)	41 (36.3)	
Moderate	31 (47.7)	58 (51.3)	
Severe	5 (7.7)	9 (8.0)	
Mean initiation dosage (mg/kg)	0.89 ± 0.31	1.06 ± 0.37	0.002
6TG level (pmole/8 × 10^8 RBC)	258 ± 147 (*n* = 31)	231 ± 161 (*n* = 55)	NS

NS: No significant.

status impacted maintenance of remission.

Dose

Thiopurine dosage in mg/kg per day was determined at registry enrollment and upon entering remission and was expressed as dose of 6MP. For patients treated with AZA, a conversion factor of dose in mg/2.07[21] was used to express medication dose as a 6MP equivalent. Dosing and decisions to obtain 6MP metabolites levels was determined by each practitioner and varied according to their clinical practice.

Statistical analysis

Kaplan Meier survival curves evaluating maintenance of SFR were performed for each analysis described above. A comparison of demographic data for included and excluded patients was performed using chi-square testing for discrete variables and student *t*-test for continuous variables.

RESULTS

Demographics

One hundred eighty-two patients were enrolled in the PIBDNet cohort. Sixty-five patients met inclusion criteria for this analysis. Reasons for exclusion are shown in Figure 1. Demographic data including age, gender, disease duration, disease location, and disease activity at enrollment by PGA was similar for included and excluded patients (Table 1).

Study outcomes

For the primary outcome, 47% of patients remained in SFR 6 mo after achieving remission (Figure 2-analysis 1). By 12 mo, 23% of patients remained in SFR. Forty-five of the 65 patients achieved remission between 70-180 d after thiopurine initiation. For this cohort, 51% and 28% maintained remission at 6 and 12 mo.

Patients could have multiple reasons for treatment failure. Thirty-eight patients had a change in PGA from remission to active disease. Fourteen patients required steroid therapy. Sixteen patients were lost to follow up within 12 mo of study entry.

For the secondary outcome in which a PGA of mild was not considered a reason for treatment failure, we found that 65 % and 42 % of patients remained in SFR at 6 and 12 mo (Figure 2-analysis 2). For the 45 patients achieving remission between 70-180 d, 67% and 47% maintained remission for this secondary outcome at 6 and 12 mo.

The exploratory analysis included the 65 patients with normal TPMT status and 12 additional patients with heterozygote TPMT status (total *n* = 77) and found similar results: 45% and 26% of patients remained in SFR for the primary outcome at 6 and 12 mo; 61% and 42% of patients remained in SFR for the secondary outcome at 6 and 12 mo.

Dose

The mean dose in mg/kg per day (mean ± SD) at thiopurine initiation for the 65 included patients was 0.89 ± 0.31 mg/kg per day compared to 1.06 ± 0.38 mg/kg per day for the 117 excluded patients (P = 0.002). The mean dose upon entering remission for the 65 included patients was 1.09 ± 0.33 mg/kg per day.

Metabolite levels

In total, 47% (86/182) of patients had metabolite levels obtained. Thirty-one of the 65 included patients had 6TG levels [pmole/8 × 10^8 red blood cells (RBC)] obtained with an mean level of 258 pmole/8 × 10^8 RBC ± 147 [median 238 (range: 25-746)]. Of the 117 patients excluded from study entry, 55 patients had 6TG levels

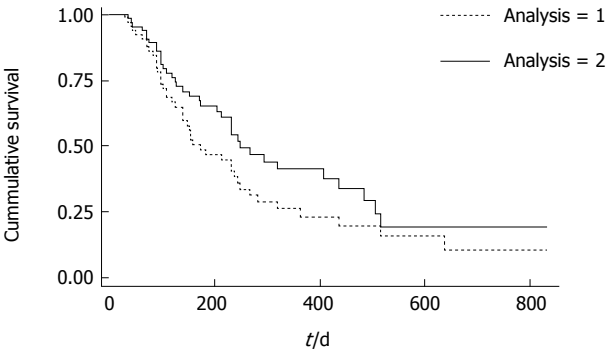


Figure 2 Kaplan Meier curves evaluating maintenance of remission.

with a mean of 231 pmole/8 × 10^8 RBC ± 161 [median 195 (range: 29-965)]. This difference between included and excluded patients was not significant ($P = 0.2$).

DISCUSSION

Thiopurines are commonly used therapies for adult and pediatric patients with CD, having been shown to be effective for maintaining remission and providing a steroid sparing effect. Our multi-center prospective study evaluating patients from diverse centers throughout the United States found that 47% and 23% of patients maintained SFR at 6 and 12 mo; 65% and 42 % of patients remained steroid free with a PGA of either remission or mild disease at 6 and 12 mo respectively.

The effectiveness of thiopurines in maintaining remission in previous pediatric studies in CD has been mixed. The RCT evaluating children with moderate to severe CD found greater than 90% of patients treated with steroid therapy and 6MP at diagnosis maintained disease remission through 18 mo[7]. Maintenance of remission in our study was comparatively reduced. Despite differences in study design (RCT *vs* prospective cohort study), methods of assessing disease activity (Harvey-Bradshaw index *vs* PGA), dosing variation and definition of treatment failures between the studies, the differences in study outcomes warrant further consideration.

Our findings expand upon a growing body of literature suggesting that the effectiveness of thiopurines in routine clinical practice for pediatric CD is lower than might have been predicted from the initial RCT. A single-center, retrospective study found 60% and 40% of pediatric patients with CD to maintain SFR at 6 and 12 mo. Another small pediatric study evaluating patients with CD or UC treated with thiopurines found 30% SFR at 6 mo[14,15].

The 2008 Cochrane review evaluating the maintenance of disease remission in adult patients with CD found thiopurines to be effective in maintaining remission with higher doses of AZA appearing more effective than lower dosages[22]. The SONIC trial found that steroid free clinical remission was achieved in only 30% and 24% at 26 and 52 wk respectively for patients receiving thiopurine monotherapy[16]. Furthermore, a trial of 63 patients aged 15-65 years found that 42% of patients receiving thiopurines *vs* 7% receiving placebo remained in remission 15 mo after thiopurine initiation[5].

For our study, variation in practitioner practice patterns could have impacted outcomes. The mean dose at thiopurine initiation for included patients was less than recommended dosing (< 1.0 mg/kg per day) suggesting the relative decreased SFR for this cohort could relate to inadequate dosing rather than medication ineffectiveness. The use of quality improvement methods in pediatric IBD to impact clinical outcomes by delivering more consistent care, including with regard to thiopurine dosing, has been previously described[18]. Metabolite measurement for this cohort was also variable with < 50% of included patients having metabolites obtained. This failure to optimize thiopurine dosing may also have contributed to the reduced maintenance of remission[23].

Additional limitations of our study common to all studies utilizing registry data include patients lost to follow-up and inability to account for missing data. Furthermore, the impact of medication adherence upon outcomes was not captured and therefore could not be evaluated. Previous studies evaluating adherence in pediatric IBD have found that nearly 40% of thiopurine doses may be missed[24,25]. The use of PGA as our study outcome also allowed for variation in practitioner assessment of clinical remission *vs* active disease. Finally, the number of patients who met inclusion criteria was somewhat limited.

However, despite the limitations described, it is recognized that variable thiopurine dosing, metabolite measurement by practitioners, and imperfect adherence by patients is a clinical reality. Thus, we believe that our results reflect clinical effectiveness of thiopurines in actual practice. A more structured approach to care, including more consistent thiopurine dosing and potentially metabolite testing may improve clinical outcomes.

In conclusion, the use of thiopurines by practicing clinicians was effective for some pediatric patients with CD in maintaining clinical remission. However, treatment failure within 12 mo of achieving remission was common for this cohort of patients. A more consistent approach to thiopurine dosing and metabolite measurement may improve clinical outcomes.

ACKNOWLEDGMENTS

We are thankful and appreciative of each of the practitioners who contributed data to the PIBDNet cohort study[19].

COMMENTS

Background

Thiopurines to maintain remission for pediatric Crohn's disease (CD) have had variable effectiveness. This manuscript describes the prospective observational outcomes for a cohort of patients in maintaining remission with thiopurines.

Research frontiers

The effectiveness of thiopurines in maintaining remission in pediatric CD may be less than might have been predicted by the results of the initial randomized control study. This real world cohort found variable approaches to the use of thiopurines among practitioners regarding dosing and metabolite measurement.

Innovations and breakthroughs

The results of this study are more similar to subsequent pediatric studies and to more recent adult studies evaluating the use of thiopurines in the treatment of CD. The variable approach to dosing and metabolite measurement could have impacted in the poorer outcomes that were found.

Applications

The study suggests thiopurines may be less effective in maintaining remission for pediatric CD than previously described. The impact of optimization of the thiopurines through more standardized dosing and metabolite measurement could be further explored.

Terminology

The term thiopurines refers to the medications mercaptopurine and azathiopurine that are commonly used therapies for treatment of pediatric CD.

Peer review

This multi-center, prospective observational study suggests the real world use of thiopurines may be less effective in maintaining remission for pediatric Crohn's disease than might have been predicted by the initial randomized controlled trial.

REFERENCES

1 **Benchimol EI**, Fortinsky KJ, Gozdyra P, Van den Heuvel M, Van Limbergen J, Griffiths AM. Epidemiology of pediatric inflammatory bowel disease: a systematic review of international trends. *Inflamm Bowel Dis* 2011; **17**: 423-439 [PMID: 20564651]

2 **Kelsen J**, Baldassano RN. Inflammatory bowel disease: the difference between children and adults. *Inflamm Bowel Dis* 2008; **14** Suppl 2: S9-S11 [PMID: 18816756]

3 **Molodecky NA**, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012; **142**: 46-54.e42; quiz e30 [PMID: 22001864 DOI: 10.1053/j.gastro.2011.10.001]

4 **Present DH**, Korelitz BI, Wisch N, Glass JL, Sachar DB, Pasternack BS. Treatment of Crohn's disease with 6-mercaptopurine. A long-term, randomized, double-blind study. *N Engl J Med* 1980; **302**: 981-987 [PMID: 6102739]

5 **Candy S**, Wright J, Gerber M, Adams G, Gerig M, Goodman R. A controlled double blind study of azathioprine in the management of Crohn's disease. *Gut* 1995; **37**: 674-678 [PMID: 8549944]

6 **Pearson DC**, May GR, Fick G, Sutherland LR. Azathioprine for maintaining remission of Crohn's disease. *Cochrane Database Syst Rev* 2000; (**2**): CD000067 [PMID: 10796482]

7 **Markowitz J**, Grancher K, Kohn N, Lesser M, Daum F. A multicenter trial of 6-mercaptopurine and prednisone in children with newly diagnosed Crohn's disease. *Gastroenterology* 2000; **119**: 895-902 [PMID: 11040176]

8 **Saibeni S**, Virgilio T, D'Incà R, Spina L, Bortoli A, Paccagnella M, Peli M, Sablich R, Meucci G, Colombo E, Benedetti G, Girelli CM, Casella G, Grasso G, de Franchis R, Vecchi M. The use of thiopurines for the treatment of inflammatory bowel diseases in clinical practice. *Dig Liver Dis* 2008; **40**: 814-820 [PMID: 18479986 DOI: 10.1016/j.dld.2008.03.016]

9 **Reinisch W**, Panés J, Lémann M, Schreiber S, Feagan B, Schmidt S, Sturniolo GC, Mikhailova T, Alexeeva O, Sanna L, Haas T, Korom S, Mayer H. A multicenter, randomized, double-blind trial of everolimus versus azathioprine and placebo to maintain steroid-induced remission in patients with moderate-to-severe active Crohn's disease. *Am J Gastroenterol* 2008; **103**: 2284-2292 [PMID: 18671816 DOI: 10.1111/j.1572-0241.2008.02024]

10 **Mantzaris GJ**, Christidou A, Sfakianakis M, Roussos A, Koilakou S, Petraki K, Polyzou P. Azathioprine is superior to budesonide in achieving and maintaining mucosal healing and histologic remission in steroid-dependent Crohn's disease. *Inflamm Bowel Dis* 2009; **15**: 375-382 [PMID: 19009634 DOI: 10.1002/ibd.20777]

11 **Jaspers GJ**, Verkade HJ, Escher JC, de Ridder L, Taminiau JA, Rings EH. Azathioprine maintains first remission in newly diagnosed pediatric Crohn's disease. *Inflamm Bowel Dis* 2006; **12**: 831-836 [PMID: 16954801]

12 **Punati J**, Markowitz J, Lerer T, Hyams J, Kugathasan S, Griffiths A, Otley A, Rosh J, Pfefferkorn M, Mack D, Evans J, Bousvaros A, Moyer MS, Wyllie R, Oliva-Hemker M, Mezoff A, Leleiko N, Keljo D, Crandall W. Effect of early immunomodulator use in moderate to severe pediatric Crohn disease. *Inflamm Bowel Dis* 2008; **14**: 949-954 [PMID: 18306311 DOI: 10.1002/ibd.20412]

13 **Lund JL**, Cook SF, Allen JK, Carroll CF, Kappelman MD. Patterns of 6-mercaptopurine and azathioprine maintenance therapy among a cohort of commercially insured individuals diagnosed with Crohn's disease in the United States. *Clin Epidemiol* 2013; **5**: 501-512 [PMID: 24348071 DOI: 10.2147/CLEP.S51625]

14 **Riello L**, Talbotec C, Garnier-Lengliné H, Pigneur B, Svahn J, Canioni D, Goulet O, Schmitz J, Ruemmele FM. Tolerance and efficacy of azathioprine in pediatric Crohn's disease. *Inflamm Bowel Dis* 2011; **17**: 2138-2143 [PMID: 21910176 DOI: 10.1002/ibd.21612]

15 **Goodhand JR**, Tshuma N, Rao A, Kotta S, Wahed M, Croft NM, Sanderson IR, Epstein J, Rampton DS. Do children with IBD really respond better than adults to thiopurines? *J Pediatr Gastroenterol Nutr* 2011; **52**: 702-707 [PMID: 21593643 DOI: 10.1097/MPG.0b013e31820ba46c]

16 **Colombel JF**, Sandborn WJ, Reinisch W, Mantzaris GJ, Kornbluth A, Rachmilewitz D, Lichtiger S, D'Haens G, Diamond RH, Broussard DL, Tang KL, van der Woude CJ, Rutgeerts P. Infliximab, azathioprine, or combination therapy for Crohn's disease. *N Engl J Med* 2010; **362**: 1383-1395 [PMID: 20393175 DOI: 10.1056/NEJMoa0904492]

17 **Wennberg JE**. Unwarranted variations in healthcare delivery: implications for academic medical centres. *BMJ* 2002; **325**: 961-964 [PMID: 12399352]

18 **Kappelman MD**, Bousvaros A, Hyams J, Markowitz J, Pfefferkorn M, Kugathasan S, Rosh J, Otley A, Mack D, Griffiths A, Evans J, Grand R, Langton C, Kleinman K, Finkelstein JA. Intercenter variation in initial management of children with Crohn's disease. *Inflamm Bowel Dis* 2007; **13**: 890-895 [PMID: 17286275]

19 **Colletti RB**, Baldassano RN, Milov DE, Margolis PA, Bousvaros A, Crandall WV, Crissinger KD, D'Amico MA, Day AS, Denson LA, Dubinsky M, Ebach DR, Hoffenberg EJ, Kader HA, Keljo DJ, Leibowitz IH, Mamula P, Pfefferkorn MD, Qureshi MA. Variation in care in pediatric Crohn disease. *J Pediatr Gastroenterol Nutr* 2009; **49**: 297-303 [PMID: 19590456 DOI: 10.1097/MPG.0b013e3181919695]

20 **Crandall WV**, Margolis PA, Kappelman MD, King EC, Pratt JM, Boyle BM, Duffy LF, Grunow JE, Kim SC, Leibowitz I, Schoen BT, Colletti RB. Improved outcomes in a quality improvement collaborative for pediatric inflammatory bowel disease. *Pediatrics* 2012; **129**: e1030-e1041 [PMID: 22412030 DOI: 10.1542/peds.2011-1700]

21 **Dubinsky MC**, Lamothe S, Yang HY, Targan SR, Sinnett D, Théorêt Y, Seidman EG. Pharmacogenomics and metabolite measurement for 6-mercaptopurine therapy in inflammatory bowel disease. *Gastroenterology* 2000; **118**: 705-713 [PMID: 10734022]

22 **Prefontaine E**, Sutherland LR, Macdonald JK, Cepoiu M. Azathioprine or 6-mercaptopurine for maintenance of remission in Crohn's disease. *Cochrane Database Syst Rev* 2009;

(**1**): CD000067 [PMID: 19160175]
23 **Dubinsky MC**. Azathioprine, 6-mercaptopurine in inflammatory bowel disease: pharmacology, efficacy, and safety. *Clin Gastroenterol Hepatol* 2004; **2**: 731-743 [PMID: 15354273]
24 **Hommel KA**, Greenley RN, Maddux MH, Gray WN, Mackner LM. Self-management in pediatric inflammatory bowel disease: A clinical report of the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition. *J Pediatr Gastroenterol Nutr* 2013; **57**: 250-257 [PMID: 23648790 DOI: 10.1097/MPG.0b013e3182999b21]
25 **Hommel KA**, Davis CM, Baldassano RN. Objective versus subjective assessment of oral medication adherence in pediatric inflammatory bowel disease. *Inflamm Bowel Dis* 2009; **15**: 589-593 [PMID: 18985746 DOI: 10.1002/ibd.20798]

P- Reviewers: Keljo D, Sorrentino D
S- Editor: Zhai HH **L- Editor**: A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9191

World J Gastroenterol 2014 July 21; 20(27): 9191-9199
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

META-ANALYSIS

Role of Notch signaling pathway in gastric cancer: A meta-analysis of the literature

Xiao Du, Zhong Cheng, Yi-Han Wang, Zi-Heng Guo, Si-Qin Zhang, Jian-Kun Hu, Zong-Guang Zhou

Xiao Du, Zhong Cheng, Yi-Han Wang, Zi-Heng Guo, Si-Qin Zhang, Jian-Kun Hu, Zong-Guang Zhou, Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Author contributions: Du X and Wang YH performed the majority of this work; Cheng Z performed the research; Guo ZH and Zhang SQ were involved in editing the manuscript; Hu JK and Zhou ZG designed the study and revised the manuscript.
Supported by National Natural Science Foundation of China, No. 81071777; and Sichuan Province Science and Technology Support Program, No. 2011SZ0293 and No. 2011SZ0087
Correspondence to: Jian-Kun Hu, Professor, Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, No. 37, Guo Xue Xiang, Chengdu 610041, Sichuan Province, China. hujkwch@126.com
Telephone: +86-28-85422872 Fax: +86-28-85422484
Received: January 17, 2014 Revised: February 27, 2014
Accepted: April 30, 2014
Published online: July 21, 2014

Abstract

AIM: To perform a meta-analysis to quantitatively summarize the evidence for the association between the Notch signaling pathway and gastric cancer (GC).

METHODS: An electronic search of the MEDLINE, EMBASE and Chinese National Knowledge Infrastructure, which contain articles published from 1966 onwards, was conducted to select studies for this meta-analysis.

RESULTS: Fifteen studies with a total of 1547 gastric cancer cases and 450 controls were included in this meta-analysis. Overall, the expression of Notch1, Notch2, Delta-like 4 and Hes1 was significantly higher in tumor tissues of GC compared to normal tissues. Specifically, stratified analyses showed that significantly increased expression of Notch1 was associated with non-cardia location, > 5 cm size, diffuse type, positive lymphovascular invasion and distal metastasis. Statistically significant higher expression of Notch3 was found in diffuse type GC. Jagged1 was also significantly over-expressed in diffuse type and poor differentiation type of GC. DLL4 was significantly over-expressed in advanced T stage, N stage and TNM stage in GC patients. However, the stratified analysis showed that there was no statistically significant difference in Hes1 expression between different subgroups. Sporadic reports showed that Notch1 and Jagged1 were independent poor prognostic predictors in GC.

CONCLUSION: The Notch signaling pathway plays an important role in tumor progression of gastric cancer.

Key words: Notch; Gastric cancer; Incidence; Prognosis; Meta-analysis

Core tip: This article quantitatively summarizes the evidence for the association between Notch signaling pathway and gastric cancer (GC) by meta-analysis, and finds that Notch1 and Notch2 signaling pathways have been activated in GC; increased expression of Notch1 is associated with non-cardia location, > 5 cm size, diffuse type, positive lymphovascular invasion and distal metastasis; Notch1 and Jagged1 may be independent poor prognostic predictors in GC. Notch signaling may participate in tumor formation and progression of GC.

Du X, Cheng Z, Wang YH, Guo ZH, Zhang SQ, Hu JK, Zhou ZG. Role of Notch signaling pathway in gastric cancer: A meta-analysis of the literature. *World J Gastroenterol* 2014; 20(27): 9191-9199 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9191.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9191

INTRODUCTION

Gastric cancer (GC) is one of the most fatal malignancies

and the fourth most common cancer worldwide, although its overall incidence is decreased in recent years[1]. 21600 new cases and 10990 new deaths of GC were estimated to occur in 2013 in United States[2] and these rates might double in Asian or Pacific Islanders due to the high rate of chronic infection with *Helicobacter pylori* (*H. pylori*). Some risk factors for this disease have been revealed, including diet, *H. pylori* infection and genetic alterations[3,4]. However, so far, less is known about how GC exactly occurs, although numerous investigations have been conducted.

The Notch signaling pathway plays a pivotal role in self-renewal of stem cells and cell-fate determination of progenitors[5]. In mammals, there are four Notch receptors (Notch 1-4) and five ligands, two of the Jagged family (Jagged1-2) and three of the Delta-like family (DLL1, DLL3, DLL4)[6]. After binding of the receptors to their ligands, the γ-secretase complex mediates the cleavage of the transmembrane domain of the Notch receptor to release the intracellular domain of the Notch receptor (NICD). Then, NICD translocates into the nucleus and works as a transcriptional coactivator, thus regulating the expression of target genes, including the hairy enhancer of split (Hes) and Hes-related (Hey) family[6].

Currently, a number of case-control studies have been conducted to investigate the association between the Notch signaling pathway and gastric cancer in humans. However, the function of components of the Notch pathway in GC is still controversial, because different even opposite effects were indicated. To date, no quantitative summary of the evidence has ever been performed. Therefore, we conduct this meta-analysis to quantitatively summarize the evidence for the roles which the Notch signaling pathway plays in GC.

MATERIALS AND METHODS

Literature search strategy

A search of the following electronic databases was performed: MEDLINE (1966 to December 2012), EMBASE (1980 to December 2012) and Chinese National Knowledge Infrastructure (CNKI) (1979 to December 2012). The following key words or text words were used: Notch or Notch intracellular domain or NICD or Delta or Delta-like or DLL or Jagged or HES or Herp or Hey, AND gastric or stomach or cardia or gastrointestinal, AND adenocarcinoma or carcinoma or cancer or neoplasm or tumor or tumour. Only studies conducted on human subjects were included, without restriction on language. The reference lists of reviews and retrieved articles were hand searched at the same time. We did not consider abstracts or unpublished reports. If more than one article was published by the same author using the same case series, we selected the study with higher sample size.

Inclusion and exclusion criteria

We reviewed abstracts of all citations and retrieved studies. For inclusion in the meta-analysis, the identified articles have to provide information on: (1) any study describing the association of at least one component of the Notch signaling pathway with gastric cancer; (2) any study reporting the numbers of both controls and gastric cancer cases; (3) results expressed as odds ratio (OR) with 95%CIs; and (4) case-control or nested case-control studies. Major reasons for exclusion of studies were (1) no control; (2) duplicate; or (3) no usable data reported.

Data extraction

All data were extracted independently by 2 investigators (Du X and Cheng Z) according to the pre-specified selection criteria. Disagreement was resolved by the investigator (Hu JK), who participated in the discussion with them and made an ultimate decision. The following data were extracted: study design and period, statistical methods, population, number of gastric cancer cases and controls studied and results of studies.

Statistical analysis

Statistical analyses were performed using Reviewer Manager Software (Version 5.1.7, The Nordic Cochrane Centre, Cochrane Collaboration), which was provided by Cochrane Collaboration. $P < 0.05$ was considered statistically significant. Meta-analysis was done using either the random effects model or fixed effects model. Heterogeneity was checked by the χ^2 test. If the results of the trials had heterogeneity, the random effects model was used for meta-analysis. The results were expressed as OR for the categorical variables and 95%CI. In addition, we observed whether there was any publication bias by use of the funnel plot, but tests for funnel plot asymmetry were only used when there are at least ten studies included in each meta-analysis.

RESULTS

Study characteristics

A total of 522 articles in English and 32 in Chinese were retrieved (Figure 1). After screening the title, reviewing the abstract and reading the full-text articles, 15 cohort studies were finally identified to match our inclusion criteria (shown in Supplementary data)[7-21]. Studies were carried out in China, Japan, South Korea and Italy. In those 15 studies which investigated the associations with gastric cancer regarding components of the Notch signaling pathway, 13 focused on acceptor Notch1[7-11,13-20], 4 on ligand Jagged1[9,16,17,20], 3 on target protein Hes1[9,13,14], and 2 on ligand DLL4[18,21]. Only 1 study focused on Notch2[13], Notch3[9], Jagged2[9] and DLL1[16], respectively. Characteristics of the studies included in this meta-analysis are presented in Table 1.

Quantitative data synthesis

Notch1: The combined results based on the included studies showed that there was a significant difference in Notch1 expression between GC tissue and normal tissue,

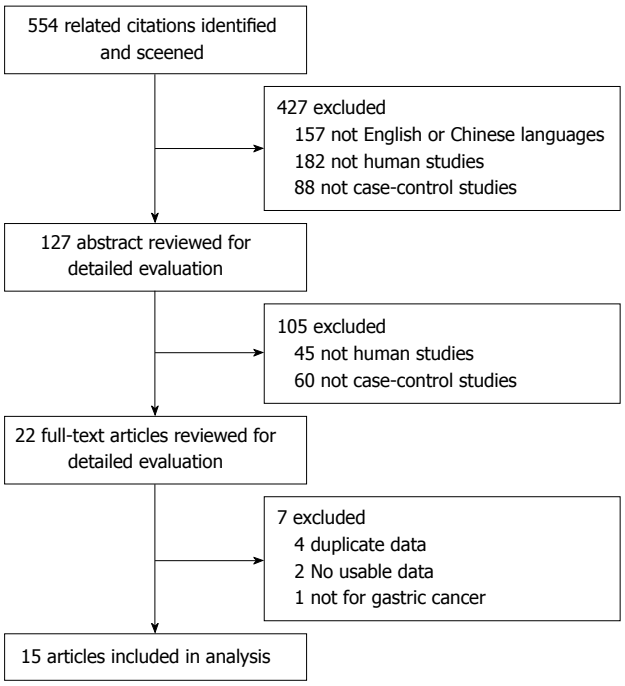


Figure 1 Flow diagram of study identification, inclusion, and exclusion.

and Notch1 expression was significantly higher in GC than in the control group (OR = 2.90, 95%CI: 2.07-4.07) (Figure 2A).

When stratifying for gender in GC, we found that there was not a statistically significant difference in Notch1 expression between males and females (OR = 1.21, 95%CI: 0.92-1.59) (Table 2). Similar results were also found in various stratified analyses of age (≤ 60 years *vs* > 60 years), histological differentiation (well/moderate *vs* poor/undifferentiated), T stage (T1-2 *vs* T3-4), N stage (N0 *vs* N1-3) and tumor node metastasis (TNM) stage (stages Ⅰ-Ⅱ *vs* Ⅲ-Ⅳ) (Table 2). When stratifying by the location, tumor size, Lauren's classification, lymphovascular invasion, and distal metastasis, we observed statistically significant differences in Notch1 expression between these subgroups (Table 2).

Statistically significant heterogeneity was observed among the included studies (χ^2 = 64.84, P < 0.00001, I^2 = 89%) (Figure 2A). Test of publication bias was shown by the funnel plot (Figure 2B). This is a scatter plot of the OR estimated from individual studies plotted on the horizontal axis (HOR), against the standard error of the estimate shown on the vertical axis (SE (log[OR])). Most of the studies analyzed lied within the 95% confidence interval (Figure 2B). Review of funnel plots could not rule out the potential for publication bias for the analysis.

Jagged1: The combined results based on all studies showed that there was no significant difference in Jagged1 expression between gastric cancer tissue and normal tissue (OR = 0.94, 95%CI: 0.00-254.96) (Figure 3A, Table 3). When stratifying for gender, age, location, T stage, N stage and TNM stage, no significant differences were among subgroups in patients with GC. When stratifying for Lauren's classification and histological differentiation, overall meta-analysis showed that Jagged1 expression was significantly different between intestinal type GC (i-GC) and diffuse type GC (d-GC) subgroups, well or moderate differentiation and poor or undifferentiated differentiation subgroups in GC patients (Table 3).

DLL4: The combined results based on all studies showed that the expression of DLL4 was significantly higher in cancer tissue of GC than in normal tissue (OR = 3.84, 95%CI: 2.52-5.83) (Figure 3B, Table 3). When stratifying for T stage, N stage and TNM stage, overall meta-analysis showed that DLL4 was significantly over-expressed in advanced stage in GC patients. There was no significant difference observed when stratifying for gender, age, differentiation and distal metastasis in patients with GC (Table 3).

Hes1: The combined results based on all studies showed that there was a significant difference in Hes1 expression between GC tissue and normal tissue (OR = 14.31, 95%CI: 4.11-49.87) (Figure 3C, Table 3). When stratifying for gender, age, Lauren's classification, histological differentiation, T stage, N stage, distal metastasis and TNM stage, we found no statistically significant difference between subgroups in patients with GC (Table 3).

Other components of Notch signaling pathway: There were significant differences between gastric cancer tissue and normal tissue in Notch2 expression (OR = 292.00, 95%CI: 23.75-3589.39) (Table 3). No difference in Notch2 expression was found between i-GC and d-GC, whereas Notch3 expression was significantly higher in i-GC compared to d-GC. Jagged2 expression was also significantly different among subgroups by Lauren's classification and T stage (Table 3).

Prognostic impact of Notch signaling pathway: A small number of articles reported the prognostic significance of the Notch signaling pathway in GC. Positive expression of Notch1 or Jagged1 protein has been proven to be associated with poor prognosis, respectively[10,15,17], and both were independent prognostic predictors in GC[10,17]. Kang *et al*[9] showed that high mRNA expression of Notch3 and Jagged2 was related to better survival outcome on univariate analysis, and only Notch3 expression was an independent marker of prognosis when using multivariate Cox's proportional hazard regression analysis.

DISCUSSION

Notch signaling is a key pathway in the self-renewal of stem cells, cell fate determination and differentiation during embryonic and postnatal development and adult cell homeostasis. So far, the role of each Notch component, as an oncogene or a tumor suppressor, is still controversial. Clearly, the function of Notch signaling is context-dependent and could act both as an oncogene

Table 1 Characteristics of studies included in the meta-analysis

Ref.	Country	Ethnicity	Study design	Detected target	No. of cases detected for Notch1	No. of controls detected for Notch1	No. of cases detected for Hes1	No. of controls detected for Hes1	No. of cases detected for Jagged1	No. of controls detected for Jagged1
Gou *et al*[7]	China	Asians	HCC	Notch1	108	NC	-	-	-	-
Huang *et al*[8]	China	Asians	HCC	Notch1	68	28	-	-	-	-
Kang *et al*[9]	South Korea	Asians	HCC	Notch1, Notch3, Jagged1, Jagged2, Hes1	103	NC	103	NC	103	NC
Li *et al*[10]	China	Asians	HCC	Notch1	168	27	-	-	-	-
Liu *et al*[11]	China	Asians	HCC	Notch1	317	NC	-	-	-	-
Piazzi *et al*[12]	Italy	Caucasians	HCC	DLL1	-	-	-	-	-	-
Sun *et al*[13]	China	Asians	HCC	Notch1, Notch2, Hes1	74	10	74	10	-	-
Wang *et al*[14]	China	Asians	HCC	Notch1, Hes1, NICD	72	16	72	16	-	-
Yang *et al*[15]	China	Asians	HCC	Notch1	135	27	-	-	-	-
Yang *et al*[16]	China	Asians	HCC	Notch1, Jagged1, DLL1	63	63	-	-	63	63
Yeh *et al*[17]	Taiwan, China	Asians	HCC	Notch1, Jagged1	90	NC	-	-	96	NC
Zhang *et al*[18]	China	Asians	HCC	Notch1, DLL4	45	25	-	-	-	-
Zhang *et al*[19]	China	Asians	HCC	Notch1	54	54	-	-	-	-
Zhou *et al*[20]	China	Asians	HCC	Jagged1, Notch1	60	NC	-	-	60	20
Ishigami *et al*[21]	Japan	Asians	HCC	DLL4	-	-	-	-	-	-

HCC: Hospital-based case-control; NC: No control; DLL: Delta-like; NICD: Intracellular domain of Notch.

Table 2 Meta-analysis of Notch1 and gastric cancer

Stratification of gastric cancer	No. of participants	OR (95%CI)	Statistical method	*P* value	Ref.
Gender: Male *vs* female	1040	1.21 (0.92-1.59)	Fixed	0.18	9[8-11,15,17-20]
Age (yr): ≤ 60 *vs* > 60	607	1.36 (0.96-1.91)	Fixed	0.08	4[8,10,11,19]
Location: Cardia *vs* noncardia	589	0.62 (0.43-0.91)	Fixed	0.01[a]	5[8,11,17,19,20]
Tumor size: ≤ 5 cm *vs* > 5 cm	634	0.68 (0.49-0.94)	Fixed	0.02[a]	5[8,11,15,19,20]
Lauren's classification: Intestinal *vs* diffuse	680	1.45 (1.03-2.03)	Fixed	0.03[a]	5[9,11,13,15,17]
Histological differentiation: Well/moderate *vs* poor/undifferentiated	1220	1.42 (0.85-2.37)	Random	0.17	11[7-11,14,15,17-20]
Lymphovascular invasion: Negative *vs* positive	258	0.49 (0.28-0.87)	Fixed	0.01[a]	2[10,17]
T stage: T1-2 *vs* T3-4	453	0.71 (0.24-2.09)	Random	0.54	5[7,10,14,18,20]
N stage: N0 *vs* N1-3	1220	1.07 (0.57-2.00)	Random	0.83	11[7-11,14,15,17-20]
Distal metastasis: Negative *vs* positive	285	0.33 (0.14-0.78)	Fixed	0.01[a]	3[10,14,18]
TNM stage: Stages Ⅰ-Ⅱ *vs* Ⅲ-Ⅳ	567	0.91 (0.53-1.57)	Random	0.74	7[8,9,14,15,17-19]

[a]*P* < 0.05. TNM: Tumor node metastasis.

and as a tumor suppressor gene in tumorigenesis of different types of cancer[22,23]. For instance, Notch has an oncogenic role in colorectal cancer[24], breast cancer[25], lung cancer[26], and neuroblastoma[27]. On the contrary, Notch acts as a tumor suppressor in squamous cell carcinoma of the skin[28] and cervical uterus[29], hepatocellular carcinoma and neuroendocrine tumors of the lung and gastrointestinal tract[30]. The multifaceted features of Notch family members suggest the necessity to check the activation patterns and potential roles of Notch signaling in different tumor types without any initial impression. Researchers also focused on the relationship between the Notch signaling pathway and gastric cancer, and a rapidly growing number of related outcomes has

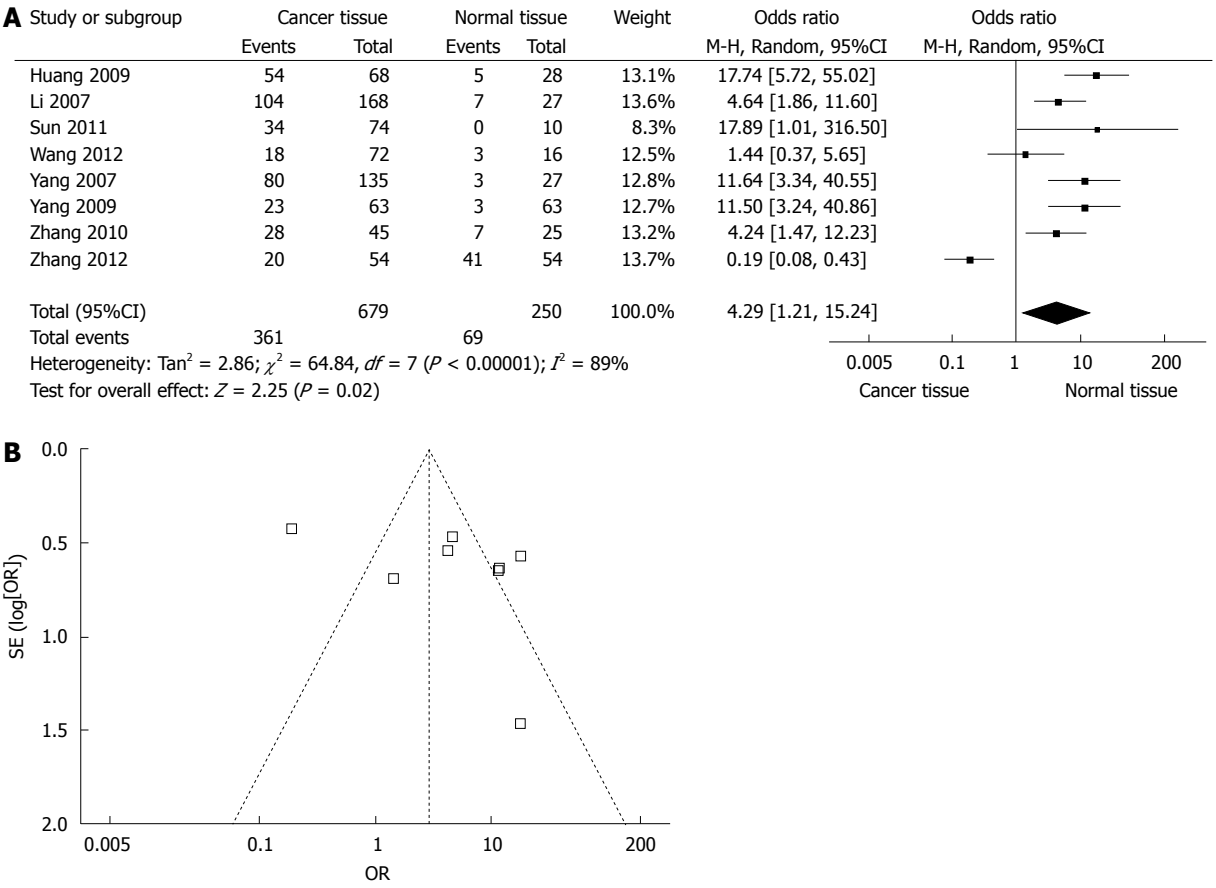


Figure 2 Forest plot for the association between Notch1 and gastric cancer (A) and funnel plot for all studies included in this meta-analysis of Notch1 and gastric cancer (B).

created but conclusions remain controversial[8,13,19]. For example, Zhang *et al*[19] found that Notch1 expression was decreased in gastric tumors compared to normal tissue, which was contrary to the traditional concept. Meta-analysis provides a quantitative approach for combining the results of various studies on the same topic, and for estimating and explaining their diversity.

In this meta-analysis, we searched all English and Chinese articles focused on the role of Notch signaling in GC. Surprisingly, only 1 paper was conducted in Caucasians, and others were all investigated in Asian countries. Geographical distribution imbalance of GC due to the diverse infection rate of *H. pylori* may be one of major reasons. Notch1 has been found to be expressed in most GC cell lines as well as normal gastric mucosa[31], but other data showed that no expression was detected in normal gastric mucosa[13]. According to the results of the current study, we found that Notch1 was expressed in both gastric cancer tissues and normal mucosa, but significantly higher expression was seen in cancer tissues than in normal tissues (OR = 2.90, $P = 0.02$), suggesting that Notch1 is activated in GC. This is consistent with the role of Notch1 as an oncogene in many solid malignancies. Thus far, mutated Notch1 has only been detected in T-cell acute lymphoblastic leukemia, but not in other common human cancers including GC[32]. More interestingly, Notch1 was found to be more preferably expressed in intestinal metaplasia tissues and well-differentiated intestinal type GC (i-GC), whereas four poorly or undifferentiated GC cell lines were negative for its expression. Our meta-analysis also found that higher Notch1 expression was seen in i-GC, but no differences existed in relation to histological differentiation. Therefore, it is speculated that Notch1 itself, not mutated type, may play a role in promoting metaplastic transition of gastric epithelial cells to tumor cells. Moreover, GC patients with larger tumor size (> 5 cm), positive lymphovascular invasion and distal metastasis had significantly higher expression rates of Notch1 (Table 2), suggesting that Notch1 may also participate in tumor progression and metastasis of GC.

Only one study considering Notch2 function was included in this review. Notch2 has also been proven to act as an oncogene in some types of cancers, and in GC, Notch2 expression was rare in normal or inflammatory tissues, whereas in both i-GC and d-GC tissues the positive rate could reach as high as 98.6% and 97.3%, respectively[13]. Although some authors found that inhibition of the Notch2 pathway with γ-secretase antagonists may not cause either growth arrest or death of GC cells, this phenomenon may be a result of other signaling pathways' compensation in response to the suppressed Notch signaling activity[13]. Conversely, other studies showed that activation of Notch2 signaling would promote both cell proliferation and xenografted tumor

Table 3 Meta-analysis of other components of Notch signaling pathway and gastric cancer

Stratification of gastric cancer	Notch2		Notch3		Jagged1		Jagged2		DLL4		Hes1	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Tumor tissue *vs* normal tissue	292.00 (23.75-3589.39)	< 0.01[a]	-	-	0.94 (0.00-254.96)	0.98	-	-	3.84 (2.52-5.83)	< 0.01[a]	14.31 (4.11-49.87)	< 0.01[a]
Gender: Male *vs* female	-	-	1.01 (0.46-2.26)	0.97	1.26 (0.36-4.39)	0.36	2.90 (0.76-11.02)	0.12	1.03 (0.57-1.86)	0.93	1.05 (0.47-2.35)	0.90
Age (yr): ≤ 55 *vs* > 55	-	-	0.75 (0.30-1.88)	0.54	1.19 (0.48-2.94)	0.70	0.17 (0.02-1.40)	0.10	0.52 (0.13-2.16)	0.37	1.13 (0.45-2.81)	0.80
Location: Cardia *vs* noncardia	-	-	-	-	0.63 (0.26-1.50)	0.30	-	-	-	-	-	-
Lauren's classification: Intestinal *vs* diffuse	0.60 (0.02-15.19)	0.76	2.61 (1.17-5.84)	0.02[a]	2.02 (1.11-3.69)	0.02[a]	3.97 (1.17-13.46)	0.03[a]	-	-	1.71 (0.88-3.30)	0.11
Histological differentiation: Well/moderate *vs* poor/undifferentiated	-	-	2.04 (0.89-4.67)	0.09	2.18 (1.23-3.86)	< 0.0[a]	1.36 (0.44-4.18)	0.60	0.89 (0.52-1.54)	0.69	1.78 (0.95-3.34)	0.07
T stage: T1-2 *vs* T3-4	-	-	2.40 (0.73-7.92)	0.15	0.50 (0.03-8.54)	0.63	3.70 (1.05-13.08)	0.04[a] (T1 *vs* T2-4)	0.29 (0.16-0.54)	< 0.01[a]	1.72 (0.49-6.00)	0.39 (T1 *vs* T2-4)
N stage: N0 *vs* N1-3	-	-	1.84 (0.71-4.75)	0.21	0.82 (0.44-1.50)	0.51	0.91 (0.23- 3.55)	0.89	0.12 (0.06-0.23)	< 0.01[a]	0.39 (0.04-4.38)	0.45
Distal metastasis: Negative *vs* positive	-	-	-	-	-	-	-	-	0.55 (0.10-3.00)	0.49	1.76 (0.36-8.51)	0.48
TNM stage: Stages Ⅰ-Ⅱ *vs* Ⅲ-Ⅳ	-	-	1.19 (0.54-2.63)	0.67	0.78 (0.42-1.42)	0.41	1.32 (0.44-3.98)	0.62	0.15 (0.03-0.80)	0.03[a]	0.70 (0.15-3.36)	0.66

[a]$P < 0.05$. DLL: Delta-like; TNM: Tumor node metastasis.

growth of GC cells[33]. More importantly, co-expression and nuclear co-translocation of Notch2 and target protein Hes1 were found to be more frequent than Notch1 both *in vivo* and *in vitro* in GC[13], suggesting that Notch2 mediated signaling would be more important in GC carcinogenesis and progression.

Using qRT-PCR tests, Notch3, Jagged1 and Jagged2 expression were found to be increased significantly in tumors compared to normal tissue[9]. Our meta-analysis results showed that overexpression of Notch3, Jagged1 and Jagged2 was associated with intestinal-type carcinomas ($P < 0.05$, Table 3). Moreover, Jagged1 expression has been correlated with aggressiveness of GC and poor survival rate[34]. Jagged2 was also found to be expressed significantly higher in early T stage, implying a possibility that Jagged2 may participate in the initiation of tumorigenesis. Other two Notch ligands DLL1 and DLL4 have been found to be able to control Notch1 signaling activation in GC[35], and expression of DLL4 was associated with advanced T and TNM stages. However, the exact roles of Notch3, Jagged and DLL in gastric carcinogenesis remain unclear.

Hes family members are major downstream target genes in the network of Notch signaling pathway, thus, high expression of Hes partially reflects activation of Notch signaling. Hes1/4/6 were expressed in most GC cell lines as well as normal gastric mucosa, while Hes2/3 were expressed in neither these cell lines nor the normal stomach[31,36]. From our meta-analysis, Hes1 expression was significantly higher in tumor tissues than in normal tissues (OR = 14.31, $P < 0.01$). Further investigation found that Hes1 could repress transcription of the ATOH1 gene, which encodes a transcription factor implicated in the gastrointestinal epithelial differentiation. These facts indicate that the canonical Notch signaling pathway might play a role in maintenance of stem or progenitor cells through inhibition of epithelial cell differentiation in gastrocarcinogenesis[37].

Few studies focused on the prognositic significance of Notch signaling in GC was found, therefore we could not perform a collective meta-analysis. Sporadic reports showed that positive expression of Notch1 and Jagged1 was independent makers for poorer prognosis. This is consistent with our meta-analysis results that Notch1 and Jagged1 were more frequently expressed in advanced GC. Kang *et al*[9] showed that high mRNA expression of Notch3 and Jagged2 was associated with prolonged survival, whereas our meta-analysis failed to show significant clinicopathological value of Notch3 and Jagged2. The real mechanism of these components acted in GC needs to be further investigated.

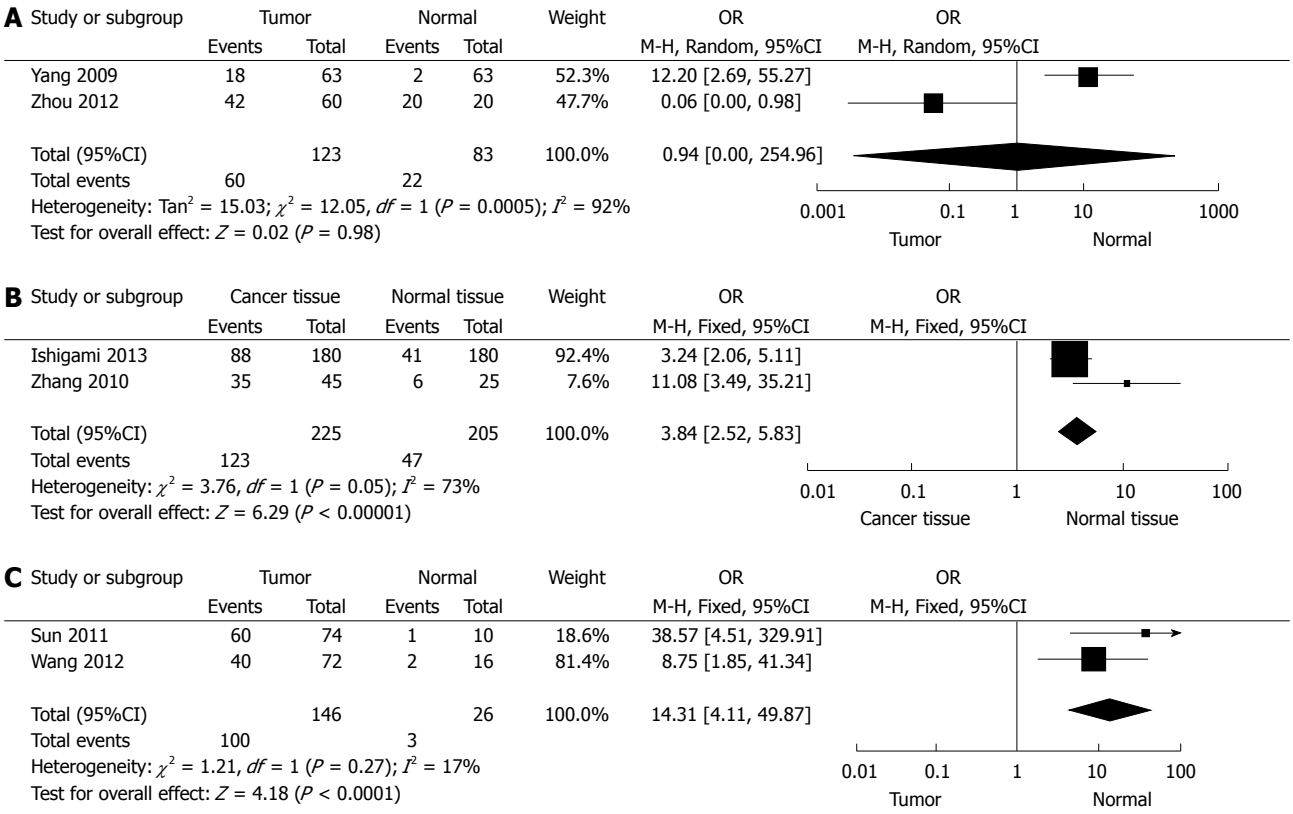


Figure 3 Forest plot. A: For the association between Jagged1 and gastric cancer; B: For the association between Delta-like 4 and gastric cancer; C: For the association between Hes1 and gastric cancer.

There are several limitations in this meta-analysis. First, only published studies were included in the meta-analysis; therefore, publication bias may have occurred as shown in Figure 2B. Second, as in most meta-analyses, these results should be interpreted with caution because the populations were from different countries and controls were not uniform. Third, no information on the association between infection with *H. pylori*, a strong risk factor for GC, and Notch signaling was obtained from most studies. Fourth, the conclusions drawn from subgroup analyses may be limited because of the small sample size. To minimize the potential bias, we designed a rigorous protocol before conducting meta-analysis, and performed a scrupulous search for published studies using explicit methods for study selection, data extraction and statistical analysis.

In summary, this meta-analysis suggests that Notch1 and Notch2 signaling pathways have been activated in gastric cancer and Notch signaling may participate in tumor formation and progression. Better designed studies based on larger cases both *in vivo* and *in vitro* are needed to further evaluate the role that the Notch signaling pathway plays in gastric carcinogenesis.

ACKNOWLEDGMENTS

The study was an internal teamwork from the Gastrointestinal Tract Reconstruction and Metabolic Surgery Association, West China Hospital, Sichuan University.

COMMENTS

Background

The role of the Notch signaling pathway in gastric cancer has been widely researched; however, the real relationship remained unclear, and studies investigating the association between Notch signaling pathway and gastric cancer still reported conflicting results.

Research frontiers

The Notch signaling pathway plays a pivotal role in self-renewal of stem cells and cell-fate determination of progenitors, and its function is context-dependent and could act both as an oncogene and as a tumor suppressor gene in tumorigenesis of different types of cancer. The objective of this systematic review is to quantitatively summarize the evidence for the relationship between the Notch pathway and gastric cancer.

Innovations and breakthroughs

This article quantitatively summarizes the evidence for the association between the Notch signaling pathway and gastric cancer by meta-analysis, and finds that Notch1 and Notch2 signaling pathways have been activated in gastric cancer; increased expression of Notch1 is associated with non-cardia location, > 5 cm size, diffuse type, positive lymphovascular invasion and distal metastasis; Notch1 and Jagged1 may be independent poor prognostic predictors in gastric cancer.

Applications

Notch signaling may participate in tumor formation and progression of gastric cancer.

Peer review

This is a good descriptive paper that provides cutting edge information on the Notch signaling pathway in gastric cancer, which will provide great interest to readers. It is a rigorous and thorough report which clearly states and adds important information.

REFERENCES

1 **Guggenheim DE**, Shah MA. Gastric cancer epidemiology and risk factors. *J Surg Oncol* 2013; **107**: 230-236 [PMID: 23129495 DOI: 10.1002/jso.23262]
2 **Siegel R**, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin* 2013; **63**: 11-30 [PMID: 23335087 DOI: 10.3322/caac.21166]
3 **Correa P**, Piazuelo MB, Camargo MC. Etiopathogenesis of gastric cancer. *Scand J Surg* 2006; **95**: 218-224 [PMID: 17249268]
4 **González CA**, Sala N, Capellá G. Genetic susceptibility and gastric cancer risk. *Int J Cancer* 2002; **100**: 249-260 [PMID: 12115538 DOI: 10.1002/ijc.10466]
5 **Tien AC**, Rajan A, Bellen HJ. A Notch updated. *J Cell Biol* 2009; **184**: 621-629 [PMID: 19255248 DOI: 10.1083/jcb.200811141]
6 **Ranganathan P**, Weaver KL, Capobianco AJ. Notch signalling in solid tumours: a little bit of everything but not all the time. *Nat Rev Cancer* 2011; **11**: 338-351 [PMID: 21508972 DOI: 10.1038/nrc3035]
7 **Gou CY**, Ha MW, Li H. Notch1 expression and its significance in human gastric Ccancer. *Zhongguo Yike Daxue Xuebao* 2010; **39**: 356-358
8 **Huang PX**, Wang JS, Tao CH, Huang ZG, Liu ZJ. Expression of iNOS, MMP-2 and Notch1 in gastric cancer and their correlation with metastasis. *Tongji Daxue Xuebao* 2009; **30**: 53-57
9 **Kang H**, An HJ, Song JY, Kim TH, Heo JH, Ahn DH, Kim G. Notch3 and Jagged2 contribute to gastric cancer development and to glandular differentiation associated with MUC2 and MUC5AC expression. *Histopathology* 2012; **61**: 576-586 [PMID: 22691042]
10 **Li DW**, Wu Q, Peng ZH, Yang ZR, Wang Y. Expressions and clinical significance of Notchl and NF-KB in gastric cancer. *Tumor* 2007; **27**: 458-461
11 **Liu B**, Sun LB, Chen WQ, Zhou JD, Wu JC. Expression and clinical significance of Notch1 and HER2 in gastric carcinoma. *Najing Yike Daxue Xuebao* 2012; **32**: 236-240
12 **Piazzi G**, Fini L, Selgrad M, Garcia M, Daoud Y, Wex T, Malfertheiner P, Gasbarrini A, Romano M, Meyer RL, Genta RM, Fox JG, Boland CR, Bazzoli F, Ricciardiello L. Epigenetic regulation of Delta-Like1 controls Notch1 activation in gastric cancer. *Oncotarget* 2011; **2**: 1291-1301 [PMID: 22249198]
13 **Sun Y**, Gao X, Liu J, Kong QY, Wang XW, Chen XY, Wang Q, Cheng YF, Qu XX, Li H. Differential Notch1 and Notch2 expression and frequent activation of Notch signaling in gastric cancers. *Arch Pathol Lab Med* 2011; **135**: 451-458 [PMID: 21466361]
14 **Wang YM**, Yang JJ, Quan MM, Zhang HY, Wang GX, Liang Y. The expressions and significance of HES1, ICN1 and Notch1 in gastric cancer. *Zhongguo Weisheng Jianyan Zazhi* 2012; **22**: 87-89
15 **Yang ZM**, Wu Q, Li DW, Chen JW. Expression and clinical significance of Notchl and HIF-1α proteins in human gastric carcinoma. *Shanxi Yike Daxue Xuebao* 2007; **38**: 498-502
16 **Yang XD**, Zhang H, Bai ZG, Jiang KW, Ye YJ, Wang S. Notch signaling pathway and clinicopathology in human gastric cancer. *Zhonghua Putong Waike Zazhi* 2009; **24**: 146-148
17 **Yeh TS**, Wu CW, Hsu KW, Liao WJ, Yang MC, Li AF, Wang AM, Kuo ML, Chi CW. The activated Notch1 signal pathway is associated with gastric cancer progression through cyclooxygenase-2. *Cancer Res* 2009; **69**: 5039-5048 [PMID: 19491270 DOI: 10.1158/0008-5472.CAN-08-4021]
18 **Zhang AH**, Sun HW, Su JS, Cui ZH. The expression of Notchl and DLL4 in gastric carcinoma and its clinical significance. *Linchuang Waike Zazhi* 2010; **18**: 257-259
19 **Zhang SF**, Wang YL. Expression of Notch1 in gastric carcinoma tissues. *Xinxiang Yixueyuan Zazhi* 2012; **29**: 754-756
20 **Zhou MK**, Zhao N, Ye YW, Chen ZF, Li Q, Zhou YN. Expression and relationship of Notch1, Jagged1 and COX-2 in gastric cancer. *Zhongguo Zhongliu Linchuang Zazhi* 2012; **39**: 1082-1086
21 **Ishigami S**, Arigami T, Uenosono Y, Okumura H, Kurahara H, Uchikado Y, Setoyama T, Kita Y, Kijima Y, Nishizono Y, Nakajo A, Owaki T, Ueno S, Natsugoe S. Clinical implications of DLL4 expression in gastric cancer. *J Exp Clin Cancer Res* 2013; **32**: 46 [PMID: 23898884 DOI: 10.1186/1756-9966-32-46]
22 **Dotto GP**. Notch tumor suppressor function. *Oncogene* 2008; **27**: 5115-5123 [PMID: 18758480 DOI: 10.1038/onc.2008.225]
23 **South AP**, Cho RJ, Aster JC. The double-edged sword of Notch signaling in cancer. *Semin Cell Dev Biol* 2012; **23**: 458-464 [PMID: 22309843 DOI: 10.1016/j.semcdb.2012.01.017]
24 **Chu D**, Zhang Z, Zhou Y, Wang W, Li Y, Zhang H, Dong G, Zhao Q, Ji G. Notch1 and Notch2 have opposite prognostic effects on patients with colorectal cancer. *Ann Oncol* 2011; **22**: 2440-2447 [PMID: 21378202 DOI: 10.1093/annonc/mdq776]
25 **Reedijk M**, Odorcic S, Chang L, Zhang H, Miller N, McCready DR, Lockwood G, Egan SE. High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. *Cancer Res* 2005; **65**: 8530-8537 [PMID: 16166334 DOI: 10.1158/0008-5472.CAN-05-1069]
26 **Donnem T**, Andersen S, Al-Shibli K, Al-Saad S, Busund LT, Bremnes RM. Prognostic impact of Notch ligands and receptors in nonsmall cell lung cancer: coexpression of Notch-1 and vascular endothelial growth factor-A predicts poor survival. *Cancer* 2010; **116**: 5676-5685 [PMID: 20737536 DOI: 10.1002/cncr.25551]
27 **Chang HH**, Lee H, Hu MK, Tsao PN, Juan HF, Huang MC, Shih YY, Wang BJ, Jeng YM, Chang CL, Huang SF, Tsay YG, Hsieh FJ, Lin KH, Hsu WM, Liao YF. Notch1 expression predicts an unfavorable prognosis and serves as a therapeutic target of patients with neuroblastoma. *Clin Cancer Res* 2010; **16**: 4411-4420 [PMID: 20736329 DOI: 10.1158/1078-0432.CCR-09-3360]
28 **Thélu J**, Rossio P, Favier B. Notch signalling is linked to epidermal cell differentiation level in basal cell carcinoma, psoriasis and wound healing. *BMC Dermatol* 2002; **2**: 7 [PMID: 11978185 DOI: 10.1186/1471-5945-2-7]
29 **Proweller A**, Tu L, Lepore JJ, Cheng L, Lu MM, Seykora J, Millar SE, Pear WS, Parmacek MS. Impaired notch signaling promotes de novo squamous cell carcinoma formation. *Cancer Res* 2006; **66**: 7438-7444 [PMID: 16885339 DOI: 10.1158/0008-5472.CAN-06-0793]
30 **Panelos J**, Batistatou A, Paglierani M, Zioga A, Maio V, Santi R, Pimpinelli N, De Giorgi V, Santucci M, Massi D. Expression of Notch-1 and alteration of the E-cadherin/beta-catenin cell adhesion complex are observed in primary cutaneous neuroendocrine carcinoma (Merkel cell carcinoma). *Mod Pathol* 2009; **22**: 959-968 [PMID: 19396152 DOI: 10.1038/modpathol.2009.55]
31 **Sekine A**, Akiyama Y, Yanagihara K, Yuasa Y. Hath1 upregulates gastric mucin gene expression in gastric cells. *Biochem Biophys Res Commun* 2006; **344**: 1166-1171 [PMID: 16647036 DOI: 10.1016/j.bbrc.2006.03.238]
32 **Lee SH**, Jeong EG, Yoo NJ, Lee SH. Mutational analysis of NOTCH1, 2, 3 and 4 genes in common solid cancers and acute leukemias. *APMIS* 2007; **115**: 1357-1363 [PMID: 18184405 DOI: 10.1111/j.1600-0463.2007.00751.x]
33 **Tseng YC**, Tsai YH, Tseng MJ, Hsu KW, Yang MC, Huang KH, Li AF, Chi CW, Hsieh RH, Ku HH, Yeh TS. Notch2-induced COX-2 expression enhancing gastric cancer progression. *Mol Carcinog* 2012; **51**: 939-951 [PMID: 21976141 DOI: 10.1002/mc.20865]
34 **Wu WK**, Cho CH, Lee CW, Fan D, Wu K, Yu J, Sung JJ. Dysregulation of cellular signaling in gastric cancer. *Cancer Lett* 2010; **295**: 144-153 [PMID: 20488613 DOI: 10.1016/j.canlet.2010.04.025]
35 **Piazzi G**, Bazzoli F, Ricciardiello L. Epigenetic silencing of Notch signaling in gastrointestinal cancers. *Cell Cycle* 2012;

11: 4323-4327 [PMID: 23085543 DOI: 10.4161/cc.22388]
36 **Katoh M**, Katoh M. Integrative genomic analyses on HES/HEY family: Notch-independent HES1, HES3 transcription in undifferentiated ES cells, and Notch-dependent HES1, HES5, HEY1, HEY2, HEYL transcription in fetal tissues, adult tissues, or cancer. *Int J Oncol* 2007; **31**: 461-466 [PMID: 17611704]
37 **Katoh M**. Dysregulation of stem cell signaling network due to germline mutation, SNP, Helicobacter pylori infection, epigenetic change and genetic alteration in gastric cancer. *Cancer Biol Ther* 2007; **6**: 832-839 [PMID: 17568183 DOI: 10.4161/cbt.6.6.4196]

P- Reviewers: Bentrem D, Kim HH, Yamamoto H
S- Editor: Ma YJ **L- Editor**: Wang TQ **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9200

World J Gastroenterol 2014 July 21; 20(27): 9200-9204
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Concomitant pancreatic adenocarcinoma in a patient with branch-duct intraductal papillary mucinous neoplasm

Joanna K Law, Christopher L Wolfgang, Matthew J Weiss, Anne Marie Lennon

Joanna K Law, Christopher L Wolfgang, Matthew J Weiss, Anne Marie Lennon, Division of Gastroenterology, Johns Hopkins Hospital, Baltimore, MD 21287, United States

Author contributions: Law JK was involved in the concept, data acquisition and writing of this manuscript, and approval of the final manuscript; Wolfgang CL and Weiss MJ were involved in the editing of the manuscript and final approval of the submitted version; Lennon AM developed the concept, wrote, and edited the manuscript.

Supported by The Full Moon Full Life fund and, in part, the Open Access Promotion Fund of Johns Hopkins University Libraries; Dr Law is the Volker Dolch Fellow in Gastroenterology at Johns Hopkins Hospital

Correspondence to: Anne Marie Lennon, MB, PhD, Division of Gastroenterology, Johns Hopkins Hospital, Sheikh Zayed Tower, Suite 7125J, Baltimore, MD 21287, United States. amlennon@jhmi.edu
Telephone: +1-410-9555800 Fax: +1-410-6148337

Received: October 14, 2013 Revised: December 31, 2013
Accepted: February 26, 2014
Published online: July 21, 2014

Abstract

Branch duct intraductal papillary mucinous neoplasms (BD-IPMN) are pre-malignant pancreatic cystic lesions which carry a small risk of malignant transformation within the cyst. Guidelines exist with respect to surveillance of the cysts using computed tomography, magnetic resonance imaging, and/or endoscopic ultrasound (EUS). There are reports that patients with IPMNs are at increased risk of developing pancreatic adenocarcinoma, which arises in an area separate to the IPMNs. We present two cases of pancreatic adenocarcinoma arising within the parenchyma, distinct from the IPMN-associated cyst, identified with EUS. This case report highlights that patients with BD-IPMN are at increased risk for pancreatic adenocarcinoma separate from the cyst and also the importance for endosonographers to carefully survey the rest of the pancreatic parenchyma separate from the cyst in order to identify small pancreatic adenocarcinomas.

Key words: Pancreatic adenocarcinoma; Intraductal papillary mucinous neoplasm; Endoscopic ultrasound; Surveillance

Core tip: Patients with intraductal papillary mucinous neoplasm are not only at risk for malignant degeneration within the cyst, but some reports have indicated an increased risk for the development of pancreatic adenocarcinoma separate from the cyst. The current international guidelines emphasize surveillance of the cyst but this case report highlights the importance for endosonographers to carefully evaluate parenchyma not involved with the cyst to identify small pancreatic adenocarcinomas.

Law JK, Wolfgang CL, Weiss MJ, Lennon AM. Concomitant pancreatic adenocarcinoma in a patient with branch-duct intraductal papillary mucinous neoplasm. *World J Gastroenterol* 2014; 20(27): 9200-9204 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9200.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9200

INTRODUCTION

Incidental pancreatic cysts are detected frequently due to the widespread availability and use of cross-sectional imaging. Over the past decade, 2.6% of patients undergoing multidetector computed tomography (CT) scans and up to 13.5% of patients undergoing magnetic resonance imaging (MRI) were found to have incidentally detected pancreatic cysts[1,2].

Intraductal papillary mucinous neoplasm (IPMN) account for almost 50% of surgically resected cystic pancreatic neoplasms[3] and can progress from benign

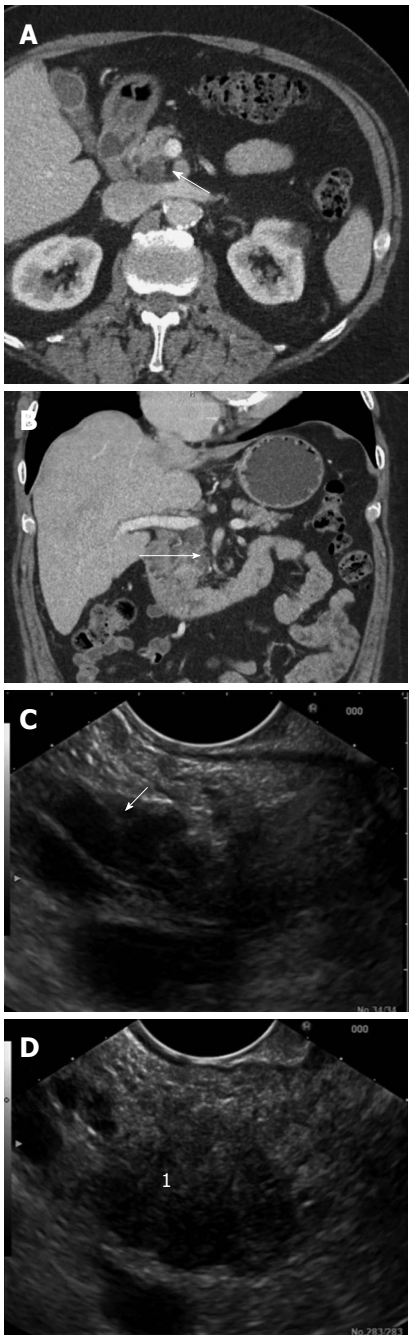


Figure 1 Abdominal imaging and endoscopic ultrasound of a patient 1 undergoing routine surveillance for a pancreatic cyst. (A) and (B) demonstrate the cystic lesion in the head/uncinate process of the pancreas as seen on computed tomography scan (arrow); and seen on endoscopic ultrasound (C). Image (D) shows a mass[1] within the head of the pancreas which was separate to the cyst.

neoplastic epithelium to invasive carcinoma through increasing severity of dysplasia. The risk of malignant transformation is dependent on whether or not there is involvement of the main pancreatic duct. Main, or mixed duct IPMN is associated with a risk of high-grade dysplasia or invasive adenocarcinoma of 44% to 62% in surgical series[4]. In contrast, only 16% to 25% of surgically resected branch-duct IPMN contain either high-grade dysplasia or invasive adenocarcinoma[4].

We present two cases of patients with BD-IPMN who had no high-risk or worrisome features within their cysts but were found to have a pancreatic adenocarcinoma unrelated to a cyst while undergoing routine surveillance with EUS.

CASE REPORT

Case 1

A 67-year-old man with a history of resected prostate cancer and no significant family history underwent an abdominal CT scan for surveillance of his prostate cancer. An incidental 1.4 cm cyst in the uncinate process of the pancreas was found. EUS with fine needle aspiration (EUS-FNA) was performed at an outside hospital. The findings were consistent with a branch duct IPMN (BD-IPMN), with no worrisome features. He underwent surveillance with a pancreatic protocol CT scan six months later, which revealed a 2 cm cystic lesion in the uncinate process (Figures 1A, B) with multiple smaller cysts in the body, none of which had any worrisome or high-risk features. Six months later the patient was seen in our multidisciplinary pancreatic cyst clinic; he underwent a routine EUS and was found to have a 2.2 cm cyst (Figure 1C). In addition, a 2.6 cm × 1.9 cm mass was identified within the head of the pancreas, separate from the cyst (Figure 1D). EUS-FNA of the lesion was performed, which confirmed an adenocarcinoma on cytopathology. The patient underwent a pancreaticoduodenectomy and was found to have a moderately differentiated adenocarcinoma, with invasion through the muscularis propria and into the submucosa of the small intestine, with 1 of 34 lymph nodes involved by metastatic adenocarcinoma. In addition, an IPMN with low to intermediate grade dysplasia was found.

Case 2

A 55-year-old man had an incidental finding of a 3 cm × 3 cm multi-lobulated cyst in the uncinate process and a 2 cm × 2 cm cyst in the distal tail of the pancreas, when he underwent an abdominal CT scan for evaluation of flank pain. He was found to have markedly atypical cells worrisome for malignancy in the cyst in the uncinate process, and underwent a pylorus-preserving pancreaticoduodenectomy. The pathology demonstrated an IPMN with high-grade dysplasia with no evidence of involvement of the surgical resection margin. He underwent routine transabdominal surveillance of the remnant pancreas with a combination of CT and MR imaging at 6-monthly intervals. Five years after initial detection, the lesion in the tail was noted to have increased minimally in size to 2.4 cm on a MRI scan. In addition, multiple sub-centimeter cystic lesions were present throughout the remnant body and tail (Figure 2A), none of which contained any suspicious features. The patient underwent a routine EUS which demonstrating a 2.9 cm cystic lesion in the tail of the pancreas (Figure 2B), with additional smaller cysts in the body and tail of the pancreas

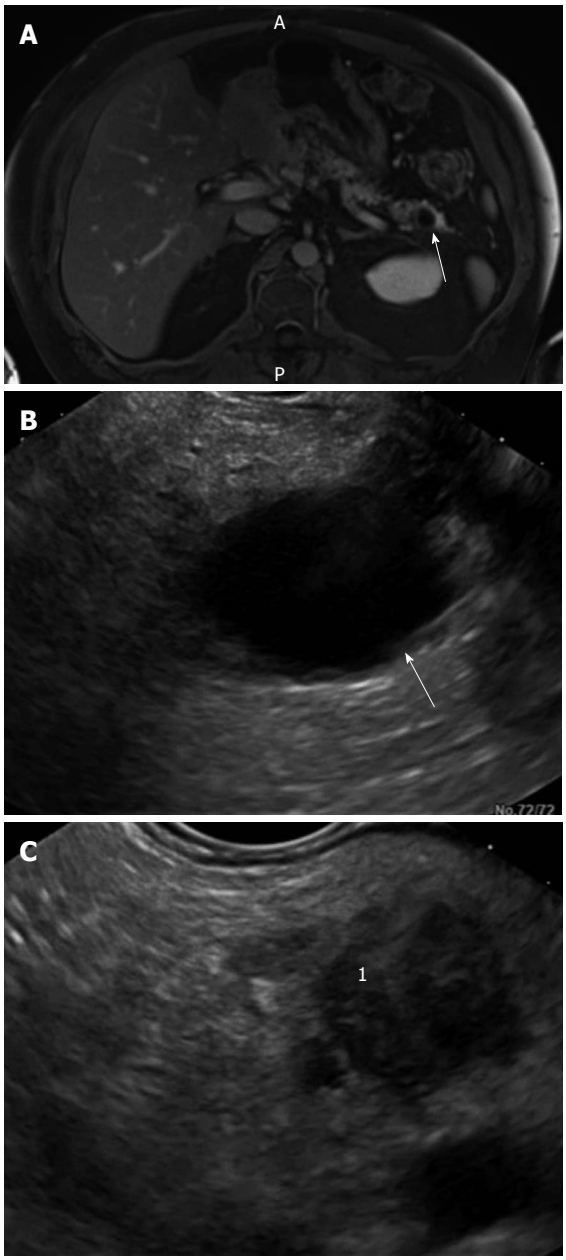


Figure 2 Abdominal imaging and endoscopic ultrasound of the pancreatic remnant in patient 2 who was status post pancreaticoduodenctomy for intraductal papillary mucinous neoplasm. A: Magnetic resonance imaging of the remnant pancreas performed 6 mo before the endoscopic ultrasound (EUS). The arrow demonstrates the cyst in the tail of the pancreas; B: EUS image of the cyst in the tail of the pancreas (arrow) corresponding to the MRI image seen in (A); C: Demonstrates an ill-defined hypoechoic area[1] in the body of the pancreas.

none of which contained any worrisome features. However in the body of the pancreas, separate to any cyst, a 1.4 cm mass was visualized (Figure 2C). EUS-FNA was performed, and cytopathology confirmed a pancreatic adenocarcinoma. The patient underwent a staging CT scan, which found multiple spiculated nodules in the lung, which were confirmed as metastatic pancreatic adenocarcinoma on biopsy.

Table 1 High-risk and worrisome criteria

High-risk stigmata	Worrisome features
Jaundice secondary to a pancreatic cyst	Acute pancreatitis
Enhancing solid component	Cyst ≥ 3 cm
Main pancreatic duct ≥ 10 mm	Thickened or enhancing cyst walls on cross-sectional imaging
	Main pancreatic duct 5-9 mm
	Non-enhancing mural nodule
	Abrupt change in the caliber of the main pancreatic duct with distal pancreatic atrophy

DISCUSSION

Patients with IPMN are known to be at increased risk of developing pancreatic ductal adenocarcinoma, the majority of which arise from an IPMN related cyst. Patients with IPMN are followed using the International Consensus Criteria guidelines, which recommend surveillance of patients with BD-IPMN and no worrisome features at 3-6 mo intervals alternating with MRI and EUS for cysts > 2 cm and for patients with smaller cysts, CT and/or MRI are recommended every one to three years depending on the cyst size[4]. Both the EUS literature[5,6], and these guidelines, stress the importance of looking for high-risk or worrisome features in the IPMN related cysts (Table 1). However, there have been a number of retrospective studies reporting cases of patients with IPMN developing adenocarcinoma in areas unrelated to pancreatic cysts with incidences of between 4% to 11%[7-14]. In a single prospective study, which followed 89 patients with IPMN over a 17 years period, 4 developed concomitant pancreatic adenocarcinoma[15].

The current guidelines recommend that BD-IPMN measuring < 2 cm are followed with MRI or CT, with EUS being used for larger cysts or those with worrisome features[4]. However, very few studies have compared the sensitivity and specificity of these three imaging modalities. One prospective, multicenter study by Canto *et al*[16], compared CT, MRI and EUS in high-risk patients, and found that EUS was the most sensitive test for identifying cysts. Several studies have shown that EUS is superior to CT for identifying small solid pancreatic neoplasms, but there are no studies to date examining its role in detecting concomitant pancreatic adenocarcinoma in patients with IPMN. In this series, both patients underwent high quality imaging with either CT or MRI with a mass only detected on EUS. A recent study by He *et al*[17], found that up to 17% of patients who underwent resection of IPMN, will develop lesions in the remnant pancreas which fulfill the criteria for surgical resection. It may be that high risk groups such as this may benefit from increased use of EUS. Large, prospective studies are necessary to compare these three imaging modalities and determine their optimum combination and timing.

In conclusion, patients with IPMN are at risk of developing concomitant pancreatic adenocarcinoma. Small concomitant pancreatic adenocarcinomas may not be seen on CT or MRI. Endosonographers should examine not only the cysts, but also the entire pancreatic parenchyma carefully.

COMMENTS

Case characteristics

Two patients undergoing surveillance for branch duct intraductal papillary mucinous neoplasm are found to have concomitant adenocarcinoma (*i.e.*, separate from the cystic lesion) found on endoscopic ultrasound.

Clinical diagnosis

Pancreatic adenocarcinoma separate and distinct from branch duct intraductal papillary mucinous neoplasm (BD-IPMN) was detected in two patients undergoing surveillance by endoscopic ultrasound (EUS).

Differential diagnosis

In both cases, the adenocarcinoma was an incidental finding that was not detected on cross sectional imaging; the differential for a solid-appearing lesion in the pancreas includes pancreatic neuroendocrine tumor, lymphoma, and metastases.

Imaging diagnosis

Both patients were undergoing surveillance of BD-IPMN with cross-sectional imaging as recommended by the international consensus guidelines and included either computed tomography (CT), magnetic resonance imaging (MRI), and/or EUS.

Pathological diagnosis

Through EUS-guided fine needle aspiration, a cytopathologic diagnosis confirmed pancreatic adenocarcinoma.

Treatment

In the first case, the patient underwent surgical resection in the form of a pancreaticoduodenectomy; the second patient presented with lung metastases and was treated with palliative chemotherapy

Term explanation

Concomitant pancreatic adenocarcinoma is the term used to describe a lesion that is unrelated to the cystic lesion.

Experiences and lessons

These two cases are meant to emphasize the need for endosonographers to survey the pancreatic parenchyma not involved with cyst in the evaluation of patients with BD-IPMN.

Peer review

In the two cases, endoscopic ultrasound were preferable to CT and MRI in surveillance of pancreatic adenocarcinoma from BD-IPMN. However, it is still hard for us to choose which cyst to take a FNA if EUS finds some cysts which are < 2 cm with no worrisome features in clinical practice.

REFERENCES

1 **Laffan TA**, Horton KM, Klein AP, Berlanstein B, Siegelman SS, Kawamoto S, Johnson PT, Fishman EK, Hruban RH. Prevalence of unsuspected pancreatic cysts on MDCT. *AJR Am J Roentgenol* 2008; **191**: 802-807 [PMID: 18716113 DOI: 10.2214/AJR.07.3340]

2 **Lee KS**, Sekhar A, Rofsky NM, Pedrosa I. Prevalence of incidental pancreatic cysts in the adult population on MR imaging. *Am J Gastroenterol* 2010; **105**: 2079-2084 [PMID: 20354507 DOI: 10.1038/ajg.2010.122]

3 **Valsangkar NP**, Morales-Oyarvide V, Thayer SP, Ferrone CR, Wargo JA, Warshaw AL, Fernández-del Castillo C. 851 resected cystic tumors of the pancreas: a 33-year experience at the Massachusetts General Hospital. *Surgery* 2012; **152**: S4-S12 [PMID: 22770958 DOI: 10.1016/j.surg.2012.05.033]

4 **Tanaka M**, Fernández-del Castillo C, Adsay V, Chari S, Falconi M, Jang JY, Kimura W, Levy P, Pitman MB, Schmidt CM, Shimizu M, Wolfgang CL, Yamaguchi K, Yamao K. International consensus guidelines 2012 for the management of IPMN and MCN of the pancreas. *Pancreatology* 2012; **12**: 183-197 [PMID: 22687371 DOI: 10.1016/j.pan.2012.04.004]

5 **Ohno E**, Hirooka Y, Itoh A, Ishigami M, Katano Y, Ohmiya N, Niwa Y, Goto H. Intraductal papillary mucinous neoplasms of the pancreas: differentiation of malignant and benign tumors by endoscopic ultrasound findings of mural nodules. *Ann Surg* 2009; **249**: 628-634 [PMID: 19300203 DOI: 10.1097/SLA.0b013e31819edle5]

6 **Zhong N**, Zhang L, Takahashi N, Shalmiyev V, Canto MI, Clain JE, Deutsch JC, DeWitt J, Eloubeidi MA, Gleeson FC, Levy MJ, Mallery S, Raimondo M, Rajan E, Stevens T, Topazian M. Histologic and imaging features of mural nodules in mucinous pancreatic cysts. *Clin Gastroenterol Hepatol* 2012; **10**: 192-198, 198.e1-2 [PMID: 21982970 DOI: 10.1016/j.cgh.2011.09.029]

7 **Kamisawa T**, Tu Y, Egawa N, Nakajima H, Tsuruta K, Okamoto A. Malignancies associated with intraductal papillary mucinous neoplasm of the pancreas. *World J Gastroenterol* 2005; **11**: 5688-5690 [PMID: 16237766]

8 **Yamaguchi K**, Kanemitsu S, Hatori T, Maguchi H, Shimizu Y, Tada M, Nakagohri T, Hanada K, Osanai M, Noda Y, Nakaizumi A, Furukawa T, Ban S, Nobukawa B, Kato Y, Tanaka M. Pancreatic ductal adenocarcinoma derived from IPMN and pancreatic ductal adenocarcinoma concomitant with IPMN. *Pancreas* 2011; **40**: 571-580 [PMID: 21499212 DOI: 10.1097/MPA.0b013e318215010c]

9 **Yamaguchi K**, Ohuchida J, Ohtsuka T, Nakano K, Tanaka M. Intraductal papillary-mucinous tumor of the pancreas concomitant with ductal carcinoma of the pancreas. *Pancreatology* 2002; **2**: 484-490 [PMID: 12378117 DOI: 10.1159/000064716]

10 **Tamura K**, Ohtsuka T, Ideno N, Aso T, Kono H, Nagayoshi Y, Shindo K, Ushijima Y, Ueda J, Takahata S, Ito T, Oda Y, Mizumoto K, Tanaka M. Unresectable pancreatic ductal adenocarcinoma in the remnant pancreas diagnosed during every-6-month surveillance after resection of branch duct intraductal papillary mucinous neoplasm: a case report. *JOP* 2013; **14**: 450-453 [PMID: 23846946 DOI: 10.6092/1590-8577/1494]

11 **Sawai Y**, Yamao K, Bhatia V, Chiba T, Mizuno N, Sawaki A, Takahashi K, Tajika M, Shimizu Y, Yatabe Y, Yanagisawa A. Development of pancreatic cancers during long-term follow-up of side-branch intraductal papillary mucinous neoplasms. *Endoscopy* 2010; **42**: 1077-1084 [PMID: 21120776 DOI: 10.1055/s-0030-1255971]

12 **Tada M**, Kawabe T, Arizumi M, Togawa O, Matsubara S, Yamamoto N, Nakai Y, Sasahira N, Hirano K, Tsujino T, Tateishi K, Isayama H, Toda N, Yoshida H, Omata M. Pancreatic cancer in patients with pancreatic cystic lesions: a prospective study in 197 patients. *Clin Gastroenterol Hepatol* 2006; **4**: 1265-1270 [PMID: 16979953 DOI: 10.1016/j.cgh.2006.07.013]

13 **Yamaguchi K**, Nakamura K, Yokohata K, Shimizu S, Chijiiwa K, Tanaka M. Pancreatic cyst as a sentinel of in situ carcinoma of the pancreas. Report of two cases. *Int J Pancreatol* 1997; **22**: 227-231 [PMID: 9444555 DOI: 10.1007/BF02788389]

14 **Uehara H**, Nakaizumi A, Ishikawa O, Iishi H, Tatsumi K, Takakura R, Ishida T, Takano Y, Tanaka S, Takenaka A. Development of ductal carcinoma of the pancreas during follow-up of branch duct intraductal papillary mucinous neoplasm of the pancreas. *Gut* 2008; **57**: 1561-1565 [PMID: 18477671 DOI: 10.1136/gut.2007.145631]

15 **Tanno S**, Nakano Y, Koizumi K, Sugiyama Y, Nakamura K, Sasajima J, Nishikawa T, Mizukami Y, Yanagawa N, Fujii T, Okumura T, Obara T, Kohgo Y. Pancreatic ductal adenocarcinomas in long-term follow-up patients with branch duct intraductal papillary mucinous neoplasms. *Pancreas* 2010; **39**: 36-40 [PMID: 19745777 DOI: 10.1097/MPA.0b013e3181b91cd0]

16 **Canto MI**, Hruban RH, Fishman EK, Kamel IR, Schulick R, Zhang Z, Topazian M, Takahashi N, Fletcher J, Petersen G, Klein AP, Axilbund J, Griffin C, Syngal S, Saltzman JR, Mortele KJ, Lee J, Tamm E, Vikram R, Bhosale P, Margolis

D, Farrell J, Goggins M. Frequent detection of pancreatic lesions in asymptomatic high-risk individuals. *Gastroenterology* 2012; **142**: 796-804; quiz e14-15 [PMID: 22245846 DOI: 10.1053/j.gastro.2012.01.005]

17 **He J**, Cameron JL, Ahuja N, Makary MA, Hirose K, Choti MA, Schulick RD, Hruban RH, Pawlik TM, Wolfgang CL. Is it necessary to follow patients after resection of a benign pancreatic intraductal papillary mucinous neoplasm? *J Am Coll Surg* 2013; **216**: 657-665; discussion 665-667 [PMID: 23395158 DOI: 10.1016/j.jamcollsurg.2012.12.026]

P- Reviewers: Chen JQ, Liang H
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9205

World J Gastroenterol 2014 July 21; 20(27): 9205-9209
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Benign esophageal stricture after thermal injury treated with esophagectomy and ileocolon interposition

Toshihiro Kitajima, Kota Momose, Seigi Lee, Shusuke Haruta, Hisashi Shinohara, Masaki Ueno, Takeshi Fujii, Harushi Udagawa

Toshihiro Kitajima, Kota Momose, Seigi Lee, Shusuke Haruta, Hisashi Shinohara, Masaki Ueno, Harushi Udagawa, Department of Digestive Surgery, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan
Takeshi Fujii, Department of Pathology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan
Author contributions: Kitajima T and Udagawa H designed the report; Momose K, Lee S, Haruta S, Shinohara H and Ueno M acted as the attending doctors for the patient and performed the operation; Fujii T performed the pathological examination; and Kitajima T wrote the manuscript.
Correspondence to: Toshihiro Kitajima, MD, Department of Digestive Surgery, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan. t.kitajima0407@gmail.com
Telephone: +81-3-35881111 Fax: +81-3-35827068
Received: November 27, 2013 Revised: February 14, 2014
Accepted: March 4, 2014
Published online: July 21, 2014

Abstract

Thermal injuries of the esophagus are rare causes of benign esophageal stricture, and all published cases were successfully treated with conservative management. A 28-year-old Japanese man with a thermal esophageal injury caused by drinking a cup of hot coffee six months earlier was referred to our hospital. The hot coffee was consumed in a single gulp at a party. Although the patient had been treated conservatively at another hospital, his symptoms of dysphagia gradually worsened after discharge. An upper gastrointestinal endoscopy and computed tomography revealed a pinhole like area of stricture located 19 cm distally from the incisors to the esophagogastric junction, as well as circumferential stenosis with notable wall thickness at the same site. The patient underwent a thoracoscopic esophageal resection with reconstruction using ileocolon interposition. The pathological findings revealed wall thickening along the entire length of the esophagus, with massive fibrosis extending to the muscularis propria and adventitia at almost all levels. Treatment with balloon dilation for long areas of stricture is generally difficult, and stent placement in patients with benign esophageal stricture, particularly young patients, is not yet widely accepted due to the incidence of late adverse events. Considering the curability and quality-of-life associated with a long expected prognosis, we determined that surgery was the best treatment option for this young patient. In this case, we decided to perform an esophagectomy and reconstruction with ileocolon interposition in order to preserve the reservoir function of the stomach and to avoid any problems related to the reflux of gastric contents. In conclusion, resection of the esophagus is a treatment option in patients with benign esophageal injury, especially in cases involving young patients with refractory esophageal stricture. In addition, ileocolon interposition may help to improve the quality-of-life of patients.

Key words: Thermal injury; Benign esophageal stricture; Esophageal resection; Ileocolon interposition; Video-assisted thoracic surgery

Core tip: This is a very rare case of the refractory esophageal thermal injury which required the esophagectomy. In general, there are several non-surgical options available to treat benign esophageal stricture. However, considering the curability and quality-of-life associated with a long expected prognosis, we determined that esophagectomy with ileocolon interposition was the most proper treatment option for this young patient.

Kitajima T, Momose K, Lee S, Haruta S, Shinohara H, Ueno M, Fujii T, Udagawa H. Benign esophageal stricture after thermal injury treated with esophagectomy and ileocolon interposition. *World J Gastroenterol* 2014; 20(27): 9205-9209 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9205.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9205

INTRODUCTION

Although benign esophageal stricture can be induced by several causes, thermal injuries of the esophagus caused by swallowing extremely hot foreign bodies are uncommon[1,2]. There are only a few case reports of thermal injury to the esophagus, all of which were successfully treated with conservative management[2-8]. In patients who receive non-surgical treatments that ultimately prove ineffective, such as balloon dilation and stent placement, surgery is typically the only remaining therapeutic option; however, the procedure is highly invasive. Moreover, when treating young patients for benign esophageal stricture, it is important that surgeons preserve digestive function and improve the quality-of-life of the patient. A previous report indicated that ileocolon interposition after gastrectomy or distal esophagectomy is superior to the placement of a gastric conduit as an esophageal substitute with respect to quality-of-life[9,10]; in addition, a favorable outcome of ileocolon interposition following esophagectomy for esophageal cancer was described in our previous report[11]. We herein report a case of benign esophageal stricture caused by thermal injury that was successfully treated with esophageal resection and ileocolon interposition, additionally providing a review of the pertinent literature.

CASE REPORT

A 28-year-old Japanese young man with a history of a thermal esophageal injury six months earlier that had been treated conservatively at another hospital was referred to our hospital. He had consumed a cup of coffee in a single gulp at a party and was later examined at the medical department of another hospital with complaints of odynophagia and dyspnea. He underwent an emergency tracheotomy because his respiratory status rapidly worsened and tracheal intubation was impossible due to the laryngo-pharyngeal mucosal swelling. Upon admission to the hospital, a computed tomography (CT) scan revealed edematous changes in the mucosa between the oral cavity and esophagus, although perforation was not confirmed, and an upper gastrointestinal endoscope was not able to pass through the esophagus due to the severe mucosal swelling. After 40 d of conservative management with parenteral nutrition and the administration of a proton pump inhibitor, upper gastrointestinal endoscopy revealed healing of the mucosal edematous changes, and the patient was able to resume the oral intake of food. He was discharged from the hospital 53 d after admission. After discharge, the symptoms of dysphagia gradually worsened to the point that he was only able to consume liquid and jelly-like foods. He was referred to our hospital five months after the onset of dysphagia. The patient had no past medical history and no history of smoking or drinking. A laboratory analysis revealed no abnormalities in any of the parameters examined. An upper gastrointestinal endoscopy revealed a pin-hole like stricture located 19 cm distally from the incisors (Figure 1A). An esophagography revealed circumferential stenosis of the esophagus extending distally 19 cm from the incisors to the esophagogastric junction (Figure 1B). A CT scan detected circumferential stenosis and wall thickening at all levels that showed stenosis on the esophagogram (Figure 1C).

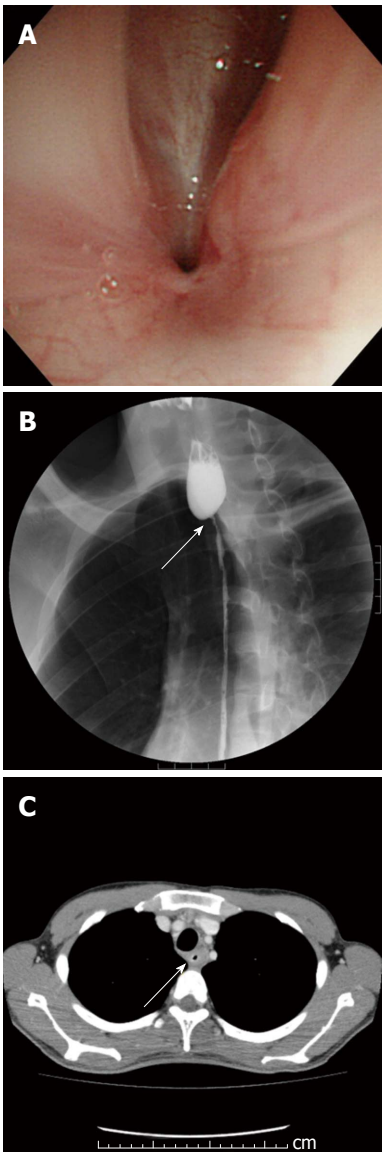


Figure 1 Preoperative findings. A: An upper gastrointestinal endoscopy revealed a pin-hole like stricture that was located 19 cm distally from the incisors; B: An esophagography revealed circumferential stenosis of the esophagus extending distally 19 cm from the incisors to the esophagogastric junction (arrow); C: A Computed tomography detected circumferential stenosis and wall thickness at the same site (arrow).

The patient was diagnosed with a benign esophageal stricture caused by thermal injury. He underwent a thoracoscopic esophageal resection with preservation of the bilateral vagus nerves, and a surgical reconstruction was performed *via* the retrosternal route using ileocolon interposition (Figure 2), as reported in our previous paper[11]. The intraoperative findings revealed that the esophagus was firm throughout its entire length and tightly adhered to the surrounding organs. Grossly, it was noted that the

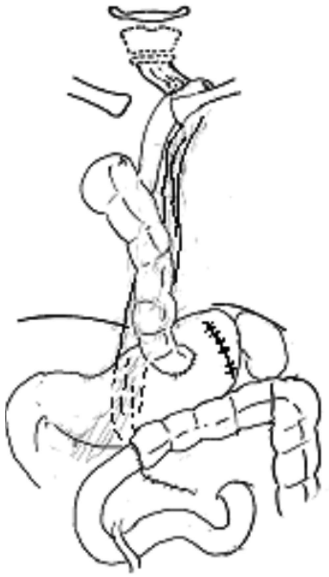


Figure 2 Design of the surgical reconstruction performed *via* the retrosternal route using ileocolon interposition.

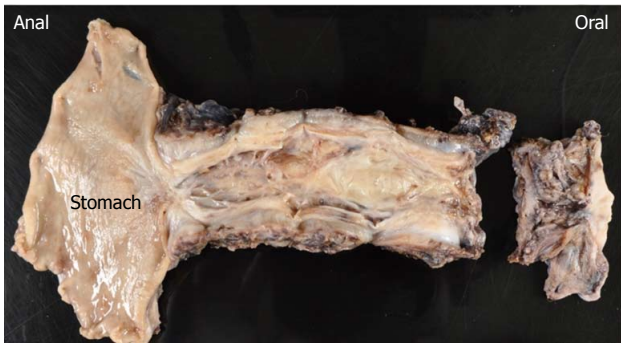


Figure 3 Gross findings of the resected specimen. Remarkable wall thickness was observed throughout the entire length of the esophagus, and the luminal area was distinctively stenosed, particularly at the esophagogastric junction. The oral side of the specimen was additionally resected because of the pin-hole like stricture detected at the proximal margin.

wall was thickened and had become trabeculated throughout the entire length of the resected esophagus, and the luminal area was distinctly stenosed, particularly at the esophagogastric junction (Figure 3). Histopathologically, massive fibrosis with focal infiltration of plasmacytes and lymphocytes was primarily observed in the submucosa throughout the esophagus (Figure 4A). Furthermore, the massive fibrosis extended to the muscularis propria and adventitia at almost all levels along the esophagus. The lumen was largely covered by a regenerative squamous epithelium with scattered erosion (Figure 4B).

Anastomotic leakage was not observed in the esophagogram on postoperative day (POD) 7 and the oral intake of food was resumed. However, the patient suffered from aspiration pneumonia due to transient unilateral recurrent laryngeal nerve paralysis. He restarted the oral intake of food on POD 16 and was discharged from our hospital on POD 22. After discharge, no regurgitation of gastric

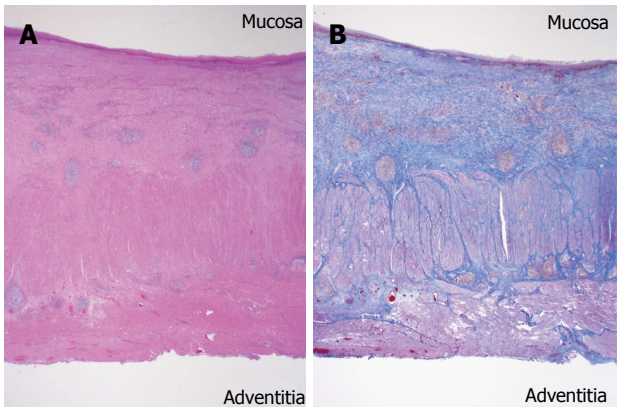


Figure 4 Histopathological findings. A: Massive fibrosis and infiltration of plasmacytes and lymphocytes were primarily detected in the submucosa throughout the esophagus (hematoxylin and eosin stain (H and E); original magnification, × 11); B: Massive fibrosis extended to the muscularis propria and adventitia along nearly all of the esophagus. The lumen was largely covered by regenerative squamous epithelium with scattered erosion (Masson`s trichrome stain; original magnification, × 11).

contents or repetitive aspiration occurred. His dysphagia significantly improved after the procedure. The patient has been able to maintain a well-balanced dietary life style with his school colleagues and has gained back the 10 kg of body weight that he had lost before the operation. As of the time of writing, the patient has been doing well for 10 months, with no evidence of complications.

DISCUSSION

Benign esophageal stricture is the result of deep esophageal injuries and is known to be induced by chemical, infectious, and physical factors, as well as other causes[1,2]. Chemical factors include gastroesophageal reflux diseases (GERD) and caustic ingestion, and infectious factors include Candida, herpes, cytomegalovirus, tuberculosis and syphilis. Physical factors involve surgery, radiation therapy and a long retention time for a nasogastric tube. Other causes include prior anastomosis and a heterogeneous group of inflammatory conditions, such as Crohn's disease[1,2]. Thermal injury is a rare cause of benign esophageal stricture, and there have been only seven reports of esophageal acute thermal injury published in the English-language literature[2-8], according to a search of the PubMed. To perform this search, we used the following key words: "esophageal thermal injury/burn" and "thermal injury/burn of the esophagus." Although all reported cases improved with conservative management and antisecretory treatment, such as proton pump inhibitors or histamine 2-receptor antagonists, to prevent further injury from gastric acid, the present case is the first case report of a severe esophageal stricture caused by thermal injury that required esophageal resection.

Several non-surgical options are available to treat benign esophageal stricture, including balloon dilation and stent placement. Benign esophageal stricture can be categorized as simple or complex based on the length,

shape and diameter of the stricture. The present case can be classified as a complex stricture, which is defined as an area of stricture that is long (> 2 cm) or tortuous or that has a diameter that prevents the passage of an endoscope with a normal diameter[12,13]. Treatment with balloon dilation for complex strictures is generally difficult, and permanent or temporary stenting in patients with benign esophageal stricture, particularly young patients, is not widely accepted due to the incidence of late adverse events, including the development of new areas of stricture due to stent-induced granulation tissue formation, esophageal ulceration or stent migration[14-16]. Therefore, in this case, we considered surgery to be the best treatment option with respect to curability in this young patient.

In the present case, we judged resection of the esophagus to be necessary because the patient was young and there was a possibility of esophageal cancer as a late complication of esophageal thermal injury[17]. We performed an esophageal resection *via* video-assisted thoracic surgery rather than transhiatal esophagectomy because the wall thickness of the thoracic esophagus was circumferentially and extensively confirmed on the preoperative CT scan, and we expected that it would be technically difficult to dissect the areas of severe adhesion between the esophagus and surrounding structure.

In cases of esophageal cancer requiring reconstruction after esophagectomy, the stomach is the first choice as an esophageal substitute due to its plasticity, facility and rich submucosal vascular network[18]. Interposition with a long jejunal segment is another option; however, it can be difficult to obtain a sufficiently long jejunal segment, and the antethoracic subcutaneous route with microvascular anastomoses of intermammary vessels is often adopted, which seemed unsuitable for this young patient with benign disease. When a patient has a history of gastrectomy, concurrent gastric disease or involvement of cancer in the stomach, we use the colon instead of the stomach or jejunum. In terms of the choice of graft site in the colon, our facility prefers to use the ileocolon for several reasons[11]. Our previous report demonstrated the feasibility and favorable outcomes of colonic interposition after esophagectomy with extended lymphadenectomy in cases of esophageal cancer[11]; we therefore extended our application of ileocolon interposition to cases involving a relatively long expected prognosis due to the detection of early-stage esophageal cancer, a lack of comorbid disease or the presence of benign esophageal disease. Similarly, some studies have shown that this method is superior to gastric pull-up as an esophageal substitute in terms of the quality-of-life of esophageal cancer patients[9,10]. Long-segment colon interposition that does not include the ileal segment has been reported to provide acceptable long-term functional results in patients with benign acquired esophageal disease, such as caustic injury and gastroesophageal reflux[19]. On the other hand, Uhl *et al*[20] have also reported fundus rotation gastroplasty (FRG) technique after esophageal resection. Although the FRG technique allows for an increase in the remaining gastric reservoir, the reported rate of anastomotic leakage was 9.2%, which is greater than the 5.4% described in our previous report[11]. In addition, it is unlikely that the FRG technique is more effective than ileocolon interposition at preventing the regurgitation of gastric contents. Therefore, in this case, we chose ileocolon interposition reconstruction in order to preserve gastric function and improve the quality-of-life of the patient, and we preserved the bilateral vagus nerves to preserve postoperative gastric motility and pyloric function. Furthermore, the ileocolic segment, including the ileocecal valve, can prevent the reflux of gastric contents, which may cause long-standing postoperative reflux problems.

However, the long-term problems of patients who undergo ileocolon interposition remain unclear. Wain *et al*[19] reported that the frequency of patients with stenosed pharyngeal anastomosis due to corrosive injuries was higher than that caused by other acquired esophageal disease. Functional failure of the colonic graft has been found to be related to the severity of the initial insult, and most failures were recognized in patients who recovered nutritional autonomy more than 1 year after jejunostomy removal[21]. In addition to late failures of grafts, we should also acknowledge the possibility of certain specific types of malabsorption, such as vitamin B12 deficiency, because the terminal ileum is responsible for the selective assimilation of vitamin B12 and bile acids[22], especially in young patients. Therefore, long-term follow-up is necessary for success after ileocolon interposition in this patient.

In conclusion, this is a rare case of thermal esophageal injury that resulted in refractory esophageal stricture. To the best of our knowledge, this is the first case report of thermal esophageal injury that required esophageal resection. We conclude that resection of the esophagus in patients with benign esophageal injury is a treatment option, especially in cases involving young patients with refractory esophageal stricture. In addition, performing ileocolon interposition may help to improve the quality-of-life of the patient.

COMMENTS

Case characteristics

A 28-year-old man with a thermal esophageal injury caused by drinking a cup of hot coffee six months earlier.

Differential diagnosis

This is the first case report of thermal esophageal injury that required esophageal resection.

Imaging diagnosis

An upper gastrointestinal endoscopy and computed tomography revealed a pin-hole like area of stricture located 19 cm distally from the incisors to the esophagogastric junction, as well as circumferential stenosis with notable wall thickness at the same site.

Pathological diagnosis

The pathological findings revealed wall thickening along the entire length of the esophagus, with massive fibrosis extending to the muscularis propria and adventitia at almost all levels.

Peer review

This paper illustrates is an interesting application of oesophagectomy in a relatively rare presentation of refractory oesophageal stricture. The case report is well written; outlines the necessity for the surgery; and adequately describes the

choice of surgical technique and it's appropriateness for this particular patient

REFERENCES

1 **Lucktong TA**, Morton JM, Shaheen NJ, Farrell TM. Resection of benign esophageal stricture through a minimally invasive endoscopic and transgastric approach. *Am Surg* 2002; **68**: 720-723 [PMID: 12206608]

2 **Choi EK**, Lee GH, Jung HY, Hong WS, Kim JH, Kim JS, Min YI. The healing course of thermal esophageal injury: a case report. *Gastrointest Endosc* 2005; **62**: 158-160 [PMID: 15990841 DOI: 10.1016/S0016-5107(05)00370-6]

3 **Javors BR**, Panzer DE, Goldman IS. Acute thermal injury of the esophagus. *Dysphagia* 1996; **11**: 72-74 [PMID: 8556881]

4 **Eliakim R**. Thermal injury from a hamburger: a rare cause of odynophagia. *Gastrointest Endosc* 1999; **50**: 282-283 [PMID: 10425431]

5 **Go H**, Yang HW, Jung SH, Park YA, Lee JY, Kim SH, Lim SH. Esophageal thermal injury by hot adlay tea. *Korean J Intern Med* 2007; **22**: 59-62 [PMID: 17427650]

6 **Chung WC**, Paik CN, Jung JH, Kim JD, Lee KM, Yang JM. Acute thermal injury of the esophagus from solid food: clinical course and endoscopic findings. *J Korean Med Sci* 2010; **25**: 489-491 [PMID: 20191054 DOI: 10.3346/jkms.2010.25.3.489]

7 **Dutta SK**, Chung KY, Bhagavan BS. Thermal injury of the esophagus. *N Engl J Med* 1998; **339**: 480-481 [PMID: 9705693]

8 **Cohen ME**, Kegel JG. Candy cocaine esophagus. *Chest* 2002; **121**: 1701-1703 [PMID: 12006465]

9 **Metzger J**, Degen L, Beglinger C, von Flüe M, Harder F. Clinical outcome and quality of life after gastric and distal esophagus replacement with an ileocolon interposition. *J Gastrointest Surg* 1999; **3**: 383-388 [PMID: 10482690]

10 **Metzger J**, Degen L, Harder F, Von Flüe M. Subjective and functional results after replacement of the stomach with an ileocecal segment: a prospective study of 20 patients. *Int J Colorectal Dis* 2002; **17**: 268-274 [PMID: 12073076]

11 **Mine S**, Udagawa H, Tsutsumi K, Kinoshita Y, Ueno M, Ehara K, Haruta S. Colon interposition after esophagectomy with extended lymphadenectomy for esophageal cancer. *Ann Thorac Surg* 2009; **88**: 1647-1653 [PMID: 19853126 DOI: 10.1016/j.athoracsur.2009.05.081]

12 **Kim JH**, Shin JH, Song HY. Benign strictures of the esophagus and gastric outlet: interventional management. *Korean J Radiol* 2010; **11**: 497-506 [PMID: 20808692 DOI: 10.3348/kjr.2010.11.5.497]

13 **Siersema PD**. Stenting for benign esophageal strictures. *Endoscopy* 2009; **41**: 363-373 [PMID: 19340743 DOI: 10.1055/s-0029-1214532]

14 **Cwikiel W**, Willén R, Stridbeck H, Lillo-Gil R, von Holstein CS. Self-expanding stent in the treatment of benign esophageal strictures: experimental study in pigs and presentation of clinical cases. *Radiology* 1993; **187**: 667-671 [PMID: 8497612]

15 **Tan BS**, Kennedy C, Morgan R, Owen W, Adam A. Using uncovered metallic endoprostheses to treat recurrent benign esophageal strictures. *AJR Am J Roentgenol* 1997; **169**: 1281-1284 [PMID: 9353442]

16 **Kim JH**, Song HY, Choi EK, Kim KR, Shin JH, Lim JO. Temporary metallic stent placement in the treatment of refractory benign esophageal strictures: results and factors associated with outcome in 55 patients. *Eur Radiol* 2009; **19**: 384-390 [PMID: 18726598 DOI: 10.1007/s00330-008-1151-2]

17 **Muñoz N**, Victora CG, Crespi M, Saul C, Braga NM, Correa P. Hot maté drinking and precancerous lesions of the oesophagus: an endoscopic survey in southern Brazil. *Int J Cancer* 1987; **39**: 708-709 [PMID: 3583450]

18 **Akiyama H**, Miyazono H, Tsurumaru M, Hashimoto C, Kawamura T. Use of the stomach as an esophageal substitute. *Ann Surg* 1978; **188**: 606-610 [PMID: 718285]

19 **Wain JC**, Wright CD, Kuo EY, Moncure AC, Wilkins EW, Grillo HC, Mathisen DJ. Long-segment colon interposition for acquired esophageal disease. *Ann Thorac Surg* 1999; **67**: 313-317; discussion 317-318 [PMID: 10197646]

20 **Uhl W**, Strobel O, Friess H, Schilling M, Büchler MW. Fundus rotation gastroplasty: rationale, technique and results. *Dis Esophagus* 2002; **15**: 101-105 [PMID: 12220414]

21 **Chirica M**, Veyrie N, Munoz-Bongrand N, Zohar S, Halimi B, Celerier M, Cattan P, Sarfati E. Late morbidity after colon interposition for corrosive esophageal injury: risk factors, management, and outcome. A 20-years experience. *Ann Surg* 2010; **252**: 271-280 [PMID: 20622655 DOI: 10.1097/SLA.0b013e3181e8fd40]

22 **Rampazzo A**, Salgado CJ, Gharb BB, Chung TT, Mardini S, Chen HC. Necessity of Vitamin B12 replacement following free ileocolon flap transfer. *Plast Reconstr Surg* 2008; **122**: 153e-154e [PMID: 18971697 DOI: 10.1097/PRS.0b013e318186cda7]

P- Reviewers: Nickel F, Uttley L **S- Editor**: Wen LL
L- Editor: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9210

World J Gastroenterol 2014 July 21; 20(27): 9210-9214
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Endoscopic submucosal tunnel dissection salvage technique for ulcerative early gastric cancer

Hyuk Soon Choi, Hoon Jai Chun, Min Ho Seo, Eun Sun Kim, Bora Keum, Yeon Seok Seo, Yoon Tae Jeen, Hong Sik Lee, Soon Ho Um, Chang Duck Kim, Ho Sang Ryu

Hyuk Soon Choi, Hoon Jai Chun, Min Ho Seo, Eun Sun Kim, Bora Keum, Yeon Seok Seo, Yoon Tae Jeen, Hong Sik Lee, Soon Ho Um, Chang Duck Kim, Ho Sang Ryu, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Institute of Digestive Disease and Nutrition, Korea University College of Medicine, Seoul 136-705, South Korea

Author contributions: Choi HS wrote the manuscript; Chun HJ designed the study and performed the ESD procedure; Seo MH, Kim ES, Keum B and Seo YS provided the illustration and figure images; Jeen YT, Lee HS and Um SH provided medical treatment and advice; Kim CD and Ryu HS conceptualized this work and provided advice.

Supported by Korea Healthcare Technology R and D Project, Ministry of Health and Welfare, South Korea, No. A111182; A grant from Korea University

Correspondence to: Hoon Jai Chun, MD, PhD, AGAF, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Institute of Digestive Disease and Nutrition, Korea University College of Medicine, Korea University Medical Center, Inchon-ro 73, Seongbuk-gu, Seoul 136-705, South Korea. drchunhj@chol.com
Telephone: +82-2-9205699 Fax: + 82-2-9531943

Received: December 21, 2013 Revised: March 2, 2014
Accepted: March 19, 2014
Published online: July 21, 2014

Abstract

Endoscopic submucosal dissection is an effective treatment modality for early gastric cancer (EGC), though the submucosal fibrosis found in ulcerative EGC is an obstacle for successful treatment. This report presents two cases of ulcerative EGC in two males, 73- and 80-year-old, with severe fibrosis. As endoscopic ultrasonography suggested that the EGCs had invaded the submucosal layer, the endoscopic submucosal tunnel dissection salvage technique was utilized for complete resection of the lesions. Although surgical gastrectomy was originally scheduled, the two patients had severe coronary heart disease, and surgeries were refused because of the risks associated with their heart conditions. The endoscopic submucosal tunnel dissection salvage technique procedures described in these cases were performed under conscious sedation, and were completed within 30 min. The complete *en bloc* resection of EGC using endoscopic submucosal tunnel dissection salvage technique was possible with a free resection margin, and no other complications were noted during the procedure. This is the first known report concerning the use of the endoscopic submucosal tunnel dissection salvage technique salvage technique for treatment of ulcerative EGC. We demonstrate that endoscopic submucosal tunnel dissection salvage technique it is a feasible method showing several advantages over endoscopic submucosal dissection for cases of EGC with fibrosis.

Key words: Endoscopy; Early gastric cancer; Endoscopic submucosal dissection; Endoscopic submucosal tunnel dissection; Fibrosis

Core tip: The fibrosis found in ulcerative early gastric cancer (EGC) has been an obstacle to successful endoscopic submucosal dissection (ESD). This report demonstrates the first use of the endoscopic submucosal tunnel dissection (ESTD) salvage technique for treatment of ulcerative EGC with fibrosis. ESTD involves the use of an endoscopic cap under the tunnel flap, which enables a clear view of the submucosal dissection line, making it easier than conventional ESD to resect an EGC lesion without complication. Therefore, the ESTD salvage technique has several advantages for resection of EGC with severe fibrosis due to previous ESD or severe ulceration.

Choi HS, Chun HJ, Seo MH, Kim ES, Keum B, Seo YS, Jeen YT, Lee HS, Um SH, Kim CD, Ryu HS. Endoscopic submucosal

tunnel dissection salvage technique for ulcerative early gastric cancer. *World J Gastroenterol* 2014; 20(27): 9210-9214 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9210.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9210

INTRODUCTION

Early gastric cancer (EGC) is defined by gastric carcinomas confined to the mucosa or submucosa regardless of the presence of regional lymph node metastases[1]. Endoscopic submucosal dissection (ESD) is an effective treatment modality for EGC, though the fibrosis found in ulcerative EGC has been an obstacle to successful treatment[2,3]. A recent study demonstrated that the severity of endoscopic submucosal fibrosis correlated with lower *en bloc* resection rates and incomplete resection[2]. Following the publication of a report detailing the use of peroral endoscopic myotomy to treat achalasia[4], an endoscopic submucosal tunnel dissection (ESTD) method has been described for the treatment of submucosal tumors in the esophagus and gastric cardia[4-6]. In two cases reported here, the ESTD salvage technique was used to overcome the procedural limitations associated with fibrosis. These cases represent the first report in which ESTD was used to treat ulcerative EGC with fibrosis.

CASE REPORT

Case 1

A 73-year-old asymptomatic man was referred to our hospital for the treatment of EGC detected by esophagogastroduodenoscopy in a local clinic. Upper endoscopy revealed an ulcerative lesion, 30 mm in diameter, covered with a white coat and partial erythema on the anterior wall of the proximal antrum. The surrounding mucosa was irregular and slightly elevated (Figure 1A). The patient was diagnosed with type Ⅲ EGC that included a depressed lesion in the center with swollen surrounding mucosa. Radial endoscopic ultrasonography (EUS) (GF-UCT 240; Olympus Co., Tokyo, Japan) revealed an irregular heterogeneous hypoechoic lesion with smooth tapering in the third layer of the gastric wall and indicated that the EGC had invaded the submucosal layer (Figure 1B). No lymph node enlargement was observed around the stomach by EUS or abdominal computed tomography. ESD was not indicated due to invasion of the EGC above the submucosal 1 layer, as determined by esophagogastro-duodenoscopy and EUS. As the patient also suffered from severe coronary heart disease, the scheduled surgical gastrectomy was refused because of the risks associated with his condition. Therefore, an endoscopic resection was proposed as an alternative and performed following the patient's informed consent.

For the endoscopic procedure, propofol was administered intravenously to induce sedation, and supplemental oxygen (2 L/min) was administered nasally throughout sedation. Additional propofol was used to maintain anesthesia, and cardiorespiratory function was monitored during the 30-min procedure. ESTD was performed using carbon dioxide insufflation. Severe fibrosis was revealed with an ESD endoscope (GIF-Q260J; Olympus Co.). A submucosal injection with saline and sodium hyaluronate into the EGC lesion failed to induce mucosal elevation. Therefore, the ESTD salvage technique (Figure 2) was used for resection of the EGC. For this procedure, marking dots were placed on the surrounding EGC using argon plasma coagulation (APC-300; ERBE Elektromedizin, Tübingen, Germany), and markings for the tunnel gate were placed 4 cm from the proximal side of the lesion. A solution of saline and sodium hyaluronate with diluted epinephrine and indigo carmine was injected into the submucosal layer of the tunnel area and surrounding the EGC lesion to raise the mucosal layer. The tunnel gate was incised with a hook knife (Olympus Co.) and a circumferential incision around the lesion, excluding the tunnel side, was performed. The hook knife was inserted through the tunnel gate for direct dissection of the EGC following confirmation of the lesion margin. A standard water-jet endoscope cap (D-201-11804; Olympus Co.) was used to facilitate hook knife dissection below the tunnel flap and EGC lesion. The resected lesion was removed completely from the muscle layer, and the overlying tunnel mucosal flap from the tunnel gate to the lesion was also removed (Figure 3). A complete *en bloc* resection was successfully performed with no adverse events.

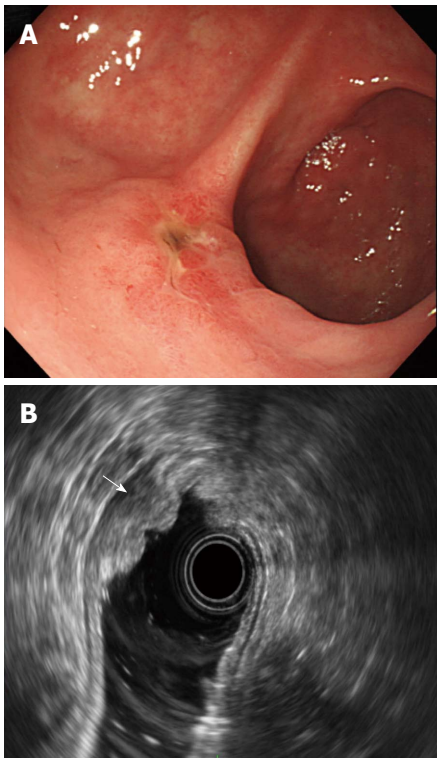


Figure 1 Endoscopic images. A: Ulcerative early gastric cancer on the anterior wall of the proximal antrum; B: Radial endoscopic ultrasonography revealed an irregular heterogeneous hypoechoic lesion with smooth tapering in the third layer of the gastric wall with indication that the cancer had invaded the superficial submucosal layer (arrow).

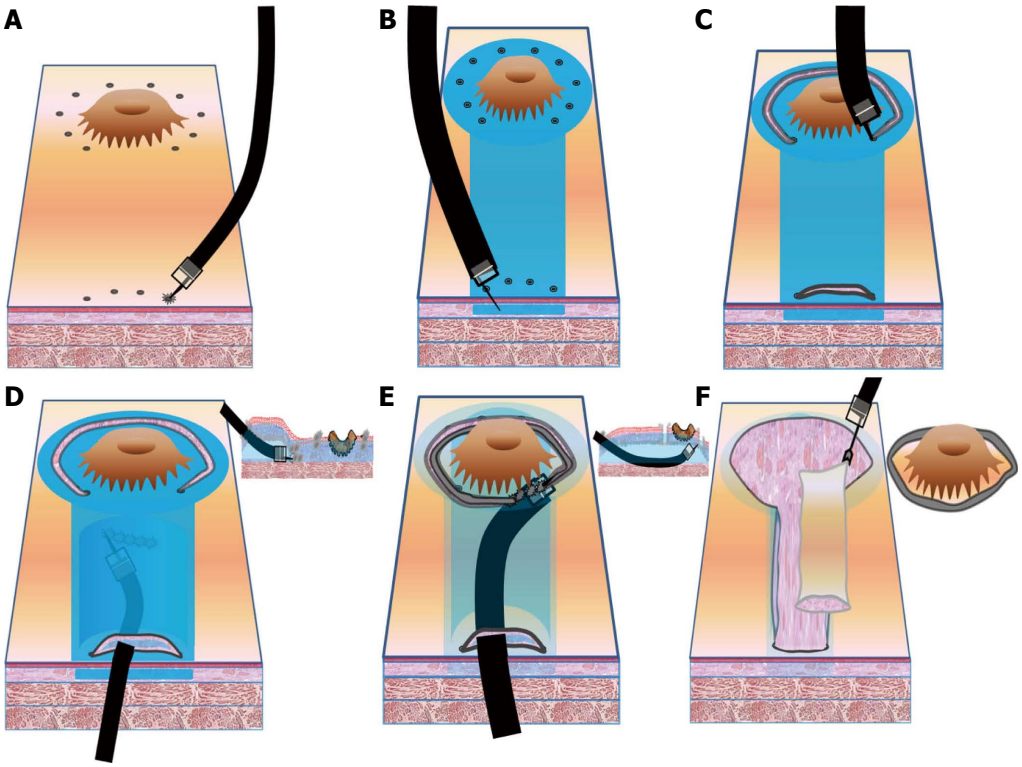


Figure 2 Key procedures of the endoscopic submucosal tunnel dissection salvage technique. A: An area surrounding the early gastric cancer (EGC) lesion and tunnel gate is marked with argon plasma coagulation; B: A solution containing saline and sodium hyaluronate with diluted epinephrine and indigo carmine is injected into the submucosal layer around the EGC lesion and tunnel area; C: A hook knife is used to make an incision at the tunnel gate and a circumferential incision surrounding the lesion; D: A tunnel dissection of the EGC lesion is made with a hook knife and an endoscopic submucosal dissection cap; E: The EGC margin is confirmed and the lesion is directly dissected; F: The resected lesion and the overlying tunnel mucosae are removed without clipping.

The histopathology of the excised tissue indicated a well-differentiated tubular carcinoma that was defined as an intestinal type according to Lauren's classification. The tumor measured 30 mm × 27 mm, and invasion was determined to be up to the muscularis mucosae (pT1a) (Figure 4). Lymphatic, venous, and perineural invasion was not evident, and the removed tissue had a free resection margin with no lateral or deep invasion. An esophagogastroduodenoscopy performed three months after the operation showed no significant findings other than some scarring as a result of the ESTD. Examination of a biopsy specimen of the resection scar showed no remnants of the tumor. The patient had no evidence of cancer recurrence after one year and is scheduled for periodic checkups.

Case 2

An 80-year-old man was admitted to our hospital for the treatment of EGC that was detected in a local clinic. Esophagogastroduodenoscopy showed an ulcerative EGC lesion 30 mm in diameter on the anterior wall of the distal antrum. The patient underwent radial EUS, which revealed an irregular hypoechoic lesion that appeared to invade the submucosal layer. This patient also refused surgical gastrectomy due to a coronary heart problem. Upper endoscopy showed ulcerative EGC with severe fibrosis. After explaining the risks associated with endoscopic resection, the patient provided informed consent for an ESTD procedure. A complete *en bloc* resection was completed by the same endoscopist using a similar method as described in case 1, and there were no adverse events. The histopathology of the resected tissue indicated a well-differentiated tubular carcinoma of an intestinal type according to Lauren's classification. The EGC measured 27 mm × 15 mm in size with invasion to the muscularis mucosae (pT1a). There was a free resection margin with no lymphatic, venous, or perineural invasion. Esophagogastroduodenoscopy performed six months after the procedure showed no significant findings other than the scar from ESTD. The patient is scheduled for follow-up in an outpatient clinic.

DISCUSSION

ESD is gaining recognition as the preferred treatment option for EGC[7]. Nevertheless, fibrosis accompanying large ulcerative EGC remains an obstacle to successful ESD, resulting in a difficulty in achieving complete and *en bloc* resection[2,3,8]. To overcome these limitations, we used an ESTD salvage technique to treat EGC with features that exceeded the expanded indications for ESD.

When performing an ESTD, the endoscopist must create the tunnel gate on the proximal side of the lesion. Hence, ESTD can be performed on the body or antrum along the greater curvature side of the stomach. There is currently no consensus concerning the distance of the

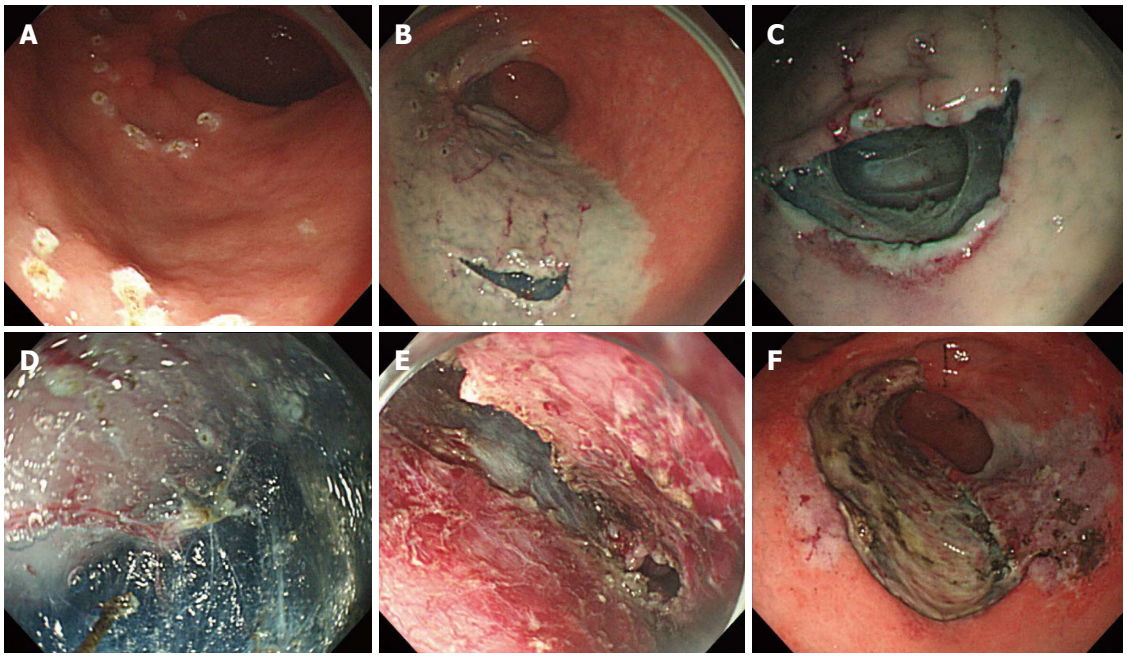


Figure 3 Endoscopic submucosal tunnel dissection salvage technique. Endoscopic images showing; A: Marking of the surrounding the early gastric cancer (EGC) lesion and tunnel gate; B: Submucosal injection and incision of the tunnel gate; C: Initiation of tunnel dissection from tunnel gate; D: Tunnel dissection with hook knife and an endoscopic submucosal dissection cap; E: Direct dissection of EGC; F: *En bloc* resection.

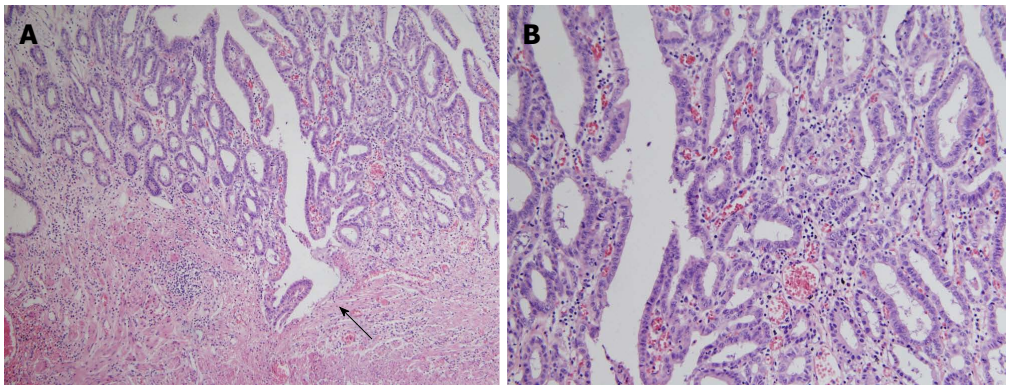


Figure 4 Histopathology findings. Hematoxylin and eosin staining of a biopsy sample (case 1) revealed the presence of a well-differentiated tubular carcinoma invading the muscularis mucosa (arrow) (magnification × 100) (A) and lesion was composed of predominantly discrete glands with pseudostratified hyperchromatic nuclei and some fused glands (magnification × 200) (B).

tunnel gate from the proximal side of the lesion. In the cases reported here, the tunnel gate was placed 4-5 cm from the proximal side of the EGC, as previously described[5]. However, we suggest that a distance of 2-3 cm from the proximal side may produce a sufficient submucosal tunnel to treat EGC.

The use of ESTD to remove EGC has limited indications, such as EGC with severe fibrosis due to previous ESD or severe ulceration, and achievement of a sufficient resection margin because of submucosal invasion. In the present cases, ulcerative EGC with severe fibrosis was confirmed, and submucosal invasion was suspected from preoperative EUS. Current indications for ESD based on the criteria of Gotoda *et al*[9] include an ulcerative mucosal EGC < 3 cm in diameter or a submucosal 1 invasion depth of the EGC ⩽ 3 cm. As the EGC in our patients could exceed these criteria before the procedure due to invasion above the submucosal 1 layer, gastrectomy was recommended as the preferred treatment option. However, this treatment was refused by both patients, and the amount of observed fibrosis indicated that a complete *en bloc* resection was unlikely using the standard ESD method. Therefore, the ESTD salvage technique was performed, with favorable results. Although the EUS indicated invasion of the submucosal layer, histopathology revealed the invasion was only to the muscularis mucosae (pT1a). It is possible that previous biopsies may have induced ulceration and fibrosis, which appeared as an invasion of the submucosal layer on EUS.

The use of ESTD to treat EGC has several advantages over ESD. First, it is easier to establish a safety margin when complete *en bloc* resection is difficult to perform

due to severe fibrosis or when submucosal invasion is suspected. Second, the higher risk of bleeding that is associated with severe fibrosis can be prevented. As the dissection in ESTD is parallel to the lesion, the vessels are exposed, allowing easier control of bleeding. In addition, ESD can be a time-consuming and technically difficult procedure if the resected mucosa cannot be pulled up and the resection line cannot be fully seen. In ESTD, the endoscopic cap under the tunnel flap enables a clear view of the submucosal dissection line, making it easier to resect an EGC lesion and shortening the overall procedure time. Despite the severe fibrosis in the present cases, the ESTD procedures were performed within 30 min. Although general anesthesia has been used to perform ESTD, conscious sedation sufficed in these cases. However, further research is required to assess the safety and effectiveness of the ESTD salvage technique for resection of EGC due to the limited number of patients and short follow-up time of cases using this procedure.

COMMENTS

Case characteristics

Two males, 73- and 80-year-old, with histories of early gastric cancer with fibrosis were resected by endoscopic submucosal tunnel dissection.

Clinical diagnosis

Both patients were asymptomatic, with no specific physical exams.

Laboratory diagnosis

Complete blood counts, electrolytes, metabolic panels, and liver function tests were within normal limits.

Imaging diagnosis

Abdominal computed tomography scans showed no metastases to lymph nodes.

Pathological diagnosis

Histopathology indicated intestinal tumors as defined by Lauren's classification, and revealed well-differentiated tubular carcinomas, measuring 30 mm × 27 mm (case 1) and 27 mm × 15 mm (case 2), with invasion depths up to muscularis mucosae (pT1a); lymphatic, venous, and perineural invasion were not evident in either case, and the removed tissues had free resection margins with no lateral or deep margin invasion.

Treatment

The patients were treated by complete *en bloc* resection by endoscopic submucosal tunnel dissection.

Term explanation

Endoscopic submucosal tunnel dissection is an endoscopic technique that creates a submucosal tunnel to provide working space for direct dissection of a submucosal area.

Experiences and lessons

Complete resection by endoscopic submucosal dissection is often difficult to perform in large ulcerative early gastric cancer due to fibrosis and this report describes the first known use of endoscopic submucosal tunnel dissection to complete *en bloc* resection for ulcerative early gastric cancer with fibrosis.

Peer review

In this case report, endoscopic submucosal tunnel dissection was used to treat two cases of ulcerative early gastric cancer with submucosal fibrosis that were not suited for treatment by conventional endoscopic submucosal dissection.

REFERENCES

1 **Chun HJ**, Keum B, Kim JH, Seol SY. Current status of endoscopic submucosal dissection for the management of early gastric cancer: a Korean perspective. *World J Gastroenterol* 2011; **17**: 2592-2596 [PMID: 21677825 DOI: 10.3748/wjg.v17.i21.2592]

2 **Jeong JY**, Oh YH, Yu YH, Park HS, Lee HL, Eun CS, Han DS. Does submucosal fibrosis affect the results of endoscopic submucosal dissection of early gastric tumors? *Gastrointest Endosc* 2012; **76**: 59-66 [PMID: 22726467 DOI: 10.1016/j.gie.2012.03.172]

3 **Higuchi K**, Tanabe S, Azuma M, Sasaki T, Katada C, Ishido K, Naruke A, Mikami T, Koizumi W. Double-endoscope endoscopic submucosal dissection for the treatment of early gastric cancer accompanied by an ulcer scar (with video). *Gastrointest Endosc* 2013; **78**: 266-273 [PMID: 23472995 DOI: 10.1016/j.gie.2013.01.010]

4 **Inoue H**, Minami H, Kobayashi Y, Sato Y, Kaga M, Suzuki M, Satodate H, Odaka N, Itoh H, Kudo S. Peroral endoscopic myotomy (POEM) for esophageal achalasia. *Endoscopy* 2010; **42**: 265-271 [PMID: 20354937 DOI: 10.1055/s-0029-1244080]

5 **Inoue H**, Ikeda H, Hosoya T, Onimaru M, Yoshida A, Eleftheriadis N, Maselli R, Kudo S. Submucosal endoscopic tumor resection for subepithelial tumors in the esophagus and cardia. *Endoscopy* 2012; **44**: 225-230 [PMID: 22354822 DOI: 10.1055/s-0031-1291659]

6 **Gong W**, Xiong Y, Zhi F, Liu S, Wang A, Jiang B. Preliminary experience of endoscopic submucosal tunnel dissection for upper gastrointestinal submucosal tumors. *Endoscopy* 2012; **44**: 231-235 [PMID: 22354823 DOI: 10.1055/s-0031-1291720]

7 **Choi HS**, Kim KO, Chun HJ, Keum B, Seo YS, Kim YS, Jeen YT, Um SH, Lee HS, Kim CD, Ryu HS. The efficacy of transdermal fentanyl for pain relief after endoscopic submucosal dissection: a prospective, randomised controlled trial. *Dig Liver Dis* 2012; **44**: 925-929 [PMID: 22824834 DOI: 10.1016/j.dld.2012.06.015]

8 **Lee H**, Yun WK, Min BH, Lee JH, Rhee PL, Kim KM, Rhee JC, Kim JJ. A feasibility study on the expanded indication for endoscopic submucosal dissection of early gastric cancer. *Surg Endosc* 2011; **25**: 1985-1993 [PMID: 21136092 DOI: 10.1007/s00464-010-1499-7]

9 **Gotoda T**, Yamamoto H, Soetikno RM. Endoscopic submucosal dissection of early gastric cancer. *J Gastroenterol* 2006; **41**: 929-942 [PMID: 17096062 DOI: 10.1007/s00535-006-1954-3]

P- Reviewers: Chen JQ, Hamashima C, Ihara E
S- Editor: Zhai HH **L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i27.9215

World J Gastroenterol 2014 July 21; 20(27): 9215-9216
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Analysis of risk factors associated with complications of colonic stenting for malignant obstruction

Gokhan Dindar, Yucel Ustundag, Tarkan Karakan

Gokhan Dindar, Yucel Ustundag, Department of Gastroenterology, Bulent Ecevit University School of Medicine, 67100 Zonguldak, Turkey
Tarkan Karakan, Department of Gastroenterology, Gazi University School of Medicine, 0600 Ankara, Turkey
Author contributions: Dindar G wrote the paper; Ustundag Y performed research; Karakan T performed research.
Correspondence to: Gokhan Dindar, MD, Department of Gastroenterology, Bulent Ecevit University School of Medicine, İncivez Mh., 67100 Zonguldak, Turkey. mirsadkaan@mynet.com
Telephone: +90-3722-612226 Fax: +90-3726-660090
Received: November 12, 2013 Revised: December 19, 2013
Accepted: February 20, 2014
Published online: July 21, 2014

Abstract

Self expanding metalic stent (SEMS) application can cause serious problems up to one third of the patients and some studies reported negative effect of SEMSs on survival in patients with malignancy. The SEMS type especially the rigid one like Wall-stent rather than more flexible type Ultraflex was also reported to have bad impact on the risk of perforation we believe that stent based management protocol for patients with non-perforating left sided obstructing colorectal cancer is a complex method that needs qualified medical and technical team.

Key words: Self expanding metalic stent; Colon tumor; Colonic obstruction; Complications; Perforation

Core tip: Self expanding metalic stent (SEMS) application can cause serious problems up to one third of the patients and some studies reported negative effect of SEMSs on survival in patients with malignancy. We believe that stent based management protocol for patients with non-perforating left sided obstructing colorectal cancer is a complex method that needs qualified medical and technical team.

Dindar G, Ustundag Y, Karakan T. Analysis of risk factors associated with complications of colonic stenting for malignant obstruction. *World J Gastroenterol* 2014; 20(27): 9215-9216 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i27/9215.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i27.9215

TO THE EDITOR

We read with a great interest the article by Almadi *et al*[1] about the complications and survival in 73 patients undergoing colonic stenting for malignant reasons leading to colorectal luminal obstruction. The authors mainly investigated the predictors for the stent related complications such as perforation, bleeding and obstruction and for the long term survival. They noted that many parameters such as age, sex, length and location of stenosis, neoadjuvant therapy, time between the onset of symptoms and self expanding metal stent (SEMS) insertion, and time between SEMS insertion and surgery had no significant association with the risk of developing complications and long term survival.

Although there is ongoing debate, SEMS placement has been accepted as effective and relatively safe procedure for either palliation or bridge to surgery for the left sided colonic or proximal rectum obstruction due to underlying malignancy[2]. Nevertheless, SEMS application can cause serious problems up to one third of the patients and some studies reported negative effect of SEMSs on survival in patients with malignancy[3]. One of the most important life threatening complications of SEMS insertion is perforation. It has been already reported that the classical risk factors for SEMS associated perforation in the colorectum are the existence of acute angular or very curved stenotic segments,

right sided colonic obstruction and use of dual-design stents[4,5]. The SEMS type especially the rigid one like Wall-stent rather than more flexible type Ultraflex was also reported to have bad impact on the risk of perforation[6]. In the present study, the authors reported 4.1% rate of perforation associated with the use of Wall-flex type SEMSs and they stated that none of the patient or tumor characteristics were found to be a predictor for complications. However, they did not analyze the role of these risk factors mentioned above. The authors also did not give any data about if co-axial stent application in this study which was reported to be around 12.6% had resulted in any case of perforation. In our daily practice, we principally never put SEMS into curved stenotic colonic segments with acute angles to avoid SEMS induced perforation and always use flexible stents for the same purpose as well. We also avoid using multiple stents and practically do not use the co-axial application of metallic stents since this does not provide greater luminal space at all. Furthermore, like multiple stents, co-axial technique can increase the possibility of pressure injury due to excessive tensile force to the outer corner wall by the long stent ends. Another flaw with this report is that the authors did not seem to analyze the relation with the severity of obstruction (complete or incomplete) and the rate of stent associated complications.

Thus, we believe that stent based management protocol for patients with non-perforating left sided obstructing colorectal cancer is a complex method that needs qualified medical and technical team. In every case, it is necessary to pay attention to the real risk factors for SEMS associated complications like bowel perforation.

REFERENCES

1 **Almadi MA**, Azzam N, Alharbi O, Mohammed AH, Sadaf N, Aljebreen AM. Complications and survival in patients undergoing colonic stenting for malignant obstruction. *World J Gastroenterol* 2013; **19**: 7138-7145 [PMID: 24222958 DOI: 10.3748/wjg.v19.i41.7138]

2 **Zhang Y**, Shi J, Shi B, Song CY, Xie WF, Chen YX. Self-expanding metallic stent as a bridge to surgery versus emergency surgery for obstructive colorectal cancer: a meta-analysis. *Surg Endosc* 2012; **26**: 110-119 [PMID: 21789642 DOI: 10.1007/s00464-011-1835-6]

3 **Choi JH**, Lee YJ, Kim ES, Choi JH, Cho KB, Park KS, Jang BK, Chung WJ, Hwang JS. Covered self-expandable metal stents are more associated with complications in the management of malignant colorectal obstruction. *Surg Endosc* 2013; **27**: 3220-3227 [PMID: 23494513 DOI: 10.1007/s00464-013-2897-4]

4 **Lee JG**, Yoo KH, Kwon CI, Ko KH, Hong SP. Angular positioning of stent increases bowel perforation after self-expandable metal stent placement for malignant colorectal obstruction. *Clin Endosc* 2013; **46**: 384-389 [PMID: 23964336 DOI: 10.5946/ce.2013.46.4.384]

5 **Cho YK**, Kim SW, Lee BI, Lee KM, Lim CH, Kim JS, Chang JH, Park JM, Lee IS, Choi MG, Choi KY, Chung IS. Clinical outcome of self-expandable metal stent placement in the management of malignant proximal colon obstruction. *Gut Liver* 2011; **5**: 165-170 [PMID: 21814596 DOI: 10.5009/gnl.2011.5.2.165]

6 **Inquirer M**. Wealth and benefactions. *Science* 1923; **57**: 445 [PMID: 17757928 DOI: 10.1016/j.gie.2007.08.043]

P- Reviewers: Sira MM, Tang WF, Vettoretto N
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Liu XM

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
www.wjgnet.com

World J Gastroenterol 2014 July 21; 20(27): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access (OA) journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1353 experts in gastroenterology and hepatology from 68 countries.

Aims and scope

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

WJG is published by Baishideng Publishing Group (BPG) in both electronic and online forms. All *WJG* articles are published in *WJG* website and PubMed Central. The major advantages of OA journals are faster release and delivery, no page or graph restrictions, and increased visibility, usage and impact. Full-text PDF articles and electronic/online versions are freely available to global readers. After the paper is published, the author(s) can obtain high-quality PDF files, which contain the journal cover, a list of editorial board members, table of contents, text, and back cover of the journal. BPG has a strong professional editorial team composed of editorial board members, editors-in-chief, science editors, language editors, and electronic editors. BPG currently publishes 43 OA clinical medical journals, including 42 in English, has a total of 15471 editorial board members or peer reviewers, and is a world first-class publisher.

Columns

The columns in the issues of *WJG* will include: (1) Editorial: The editorial board members are invited to make comments on an important topic in their field in terms of its current research status and future directions to lead the development of this discipline; (2) Frontier: The editorial board members are invited to select a highly cited cutting-edge original paper of his/her own to summarize major findings, the problems that have been resolved and remain to be resolved, and future research directions to help readers understand his/her important academic point of view and future research directions in the field; (3) Diagnostic Advances: The editorial board members are invited to write high-quality diagnostic advances in their field to improve the diagnostic skills of readers. The topic covers general clinical diagnosis, differential diagnosis, pathological diagnosis, laboratory diagnosis, imaging diagnosis, endoscopic diagnosis, biotechnological diagnosis, functional diagnosis, and physical diagnosis; (4) Therapeutics Advances: The editorial board members are invited to write high-quality therapeutic advances in their field to help improve the therapeutic skills of readers. The topic covers medication therapy, psychotherapy, physical therapy, replacement therapy, interventional therapy, minimally invasive therapy, endoscopic therapy, transplantation therapy, and surgical therapy; (5) Field of Vision: The editorial board members are invited to write commentaries on classic articles, hot topic articles, or latest articles to keep readers at the forefront of research and increase their levels of clinical research. Classic articles refer to papers that are included in Web of Knowledge and have received a large number of citations (ranking in the top 1%) after being published for more than years, reflecting the quality and impact of papers. Hot topic articles refer to papers that are included in Web of Knowledge and have received a large number of citations after being published for no more than 2 years, reflecting cutting-edge trends in scientific research. Latest articles refer to the latest published high-quality papers that are included in PubMed, reflecting the latest research trends. These commentary articles should focus on the status quo of research, the most important research topics, the problems that have now been resolved and remain to be resolved, and future research directions. Basic information about the article to be commented (including authors, article title, journal name, year, volume, and inclusive page numbers; (6) Minireviews: The editorial board members are invited to write short reviews on recent advances and trends in research of molecular biology, genomics, and related cutting-edge technologies to provide readers with the latest knowledge and help improve their diagnostic and therapeutic skills; (7) Review: To make a systematic review to focus on the status quo of research, the most important research topics, the problems that have now been resolved and remain to be resolved, and future research directions; (8) Topic Highlight: The editorial board members are invited to write a series of articles (7-10 articles) to comment and discuss a hot topic to help improve the diagnostic and therapeutic skills of readers; (9) Medical Ethics: The editorial board members are invited to write articles about medical ethics to increase readers' knowledge of medical ethics. The topic covers international ethics guidelines, animal studies, clinical trials, organ transplantation, *etc.*; (10) Clinical Case Conference or Clinicopathological Conference: The editorial board members are invited to contribute high-quality clinical case conference; (11) Original Articles: To report innovative and original findings in gastroenterology and hepatology; (12) Brief Articles: To briefly report the novel

and innovative findings in gastroenterology and hepatology; (13) Meta-Analysis: Covers the systematic review, mixed treatment comparison, meta-regression, and overview of reviews, in order to summarize a given quantitative effect, *e.g.*, the clinical effectiveness and safety of clinical treatments by combining data from two or more randomized controlled trials, thereby providing more precise and externally valid estimates than those which would stem from each individual dataset if analyzed separately from the others; (14) Case Report: To report a rare or typical case; (15) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (16) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (17) Autobiography: The editorial board members are invited to write their autobiography to provide readers with stories of success or failure in their scientific research career. The topic covers their basic personal information and information about when they started doing research work, where and how they did research work, what they have achieved, and their lessons from success or failure.

Name of journal
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

Launch date
October 1, 1995

Frequency
Weekly

Editorial office
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: bpgoffice@wjgnet.com
Help desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

Publisher
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

Instructions to authors
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm

Indexed and abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. ISI, Thomson Reuters, 2011 Impact Factor: 2.471 (32/74 Gastroenterology and Hepatology).

SPECIAL STATEMENT

Biostatistical editing
Statistical review is performed after peer review. We invite an expert in Biomedical Statistics to evaluate the statistical method used in the paper, including *t* test (group or paired comparisons), chi-squared test, ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc.* The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word "significantly" should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

Conflict-of-interest statement
In the interests of transparency and to help reviewers assess any potential bias, *WJG* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: http://www.icmje.org/ethical_4conflicts.html.

Statement of informed consent
Manuscripts should contain a statement to the effect that all human studies have been reviewed by the appropriate ethics com-

mittee or it should be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. Authors should also draw attention to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964, as revised in 2004).

Sample wording: [Name of individual] has received fees for serving as a speaker, a consultant and an advisory board member for [names of organizations], and has received research funding from [names of organization]. [Name of individual] is an employee of [name of organization]. [Name of individual] owns stocks and shares in [name of organization]. [Name of individual] owns patent [patent identification and brief description].

Statement of human and animal rights

When reporting the results from experiments, authors should follow the highest standards and the trial should conform to Good Clinical Practice (for example, US Food and Drug Administration Good Clinical Practice in FDA-Regulated Clinical Trials; UK Medicines Research Council Guidelines for Good Clinical Practice in Clinical Trials) and/or the World Medical Association Declaration of Helsinki. Generally, we suggest authors follow the lead investigator's national standard. If doubt exists whether the research was conducted in accordance with the above standards, the authors must explain the rationale for their approach and demonstrate that the institutional review body explicitly approved the doubtful aspects of the study.

Before submitting, authors should make their study approved by the relevant research ethics committee or institutional review board. If human participants were involved, manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and appropriate informed consent of each. Any personal item or information will not be published without explicit consents from the involved patients. If experimental animals were used, the materials and methods (experimental procedures) section must clearly indicate that appropriate measures were taken to minimize pain or discomfort, and details of animal care should be provided.

SUBMISSION OF MANUSCRIPTS

Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of Baishideng Publishing Group Co., Limited, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the ICMJE to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www.clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

Online submissions

Manuscripts should be submitted through the Online Submission System at: http://www.wjgnet.com/esps/. Authors are highly recommended to consult the ONLINE INSTRUCTIONS TO AUTHORS (http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm) before attempting to submit online. For assistance, authors encountering problems with the Online Submission System may send an email describing the problem to bpgoffice@wjgnet.com, or by telephone: +86-10-5908-0039. If you submit your manuscript online, do not make a postal contribution. Repeated online submission for the same manuscript is strictly prohibited.

MANUSCRIPT PREPARATION

All contributions should be written in English. All articles must be submitted using word-processing software. All submissions must be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Style should conform to our house format. Required information for each of the manuscript sections is as follows:

Title page

Title: Title should be less than 12 words.

Running title: A short running title of less than 6 words should be provided.

Authorship: Authorship credit should be in accordance with the standard proposed by ICMJE, based on (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published. Authors should meet conditions 1, 2, and 3.

Institution: Author names should be given first, then the complete name of institution, city, province and postcode. For example, Xu-Chen Zhang, Li-Xin Mei, Department of Pathology, Chengde Medical College, Chengde 067000, Hebei Province, China. One author may be represented from two institutions, for example, George Sgourakis, Department of General, Visceral, and Transplantation Surgery, Essen 45122, Germany; George Sgourakis, 2nd Surgical Department, Korgialenio-Benakio Red Cross Hospital, Athens 15451, Greece.

Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

Supportive foundations: The complete name and number of supportive foundations should be provided, *e.g.* Supported by National Natural Science Foundation of China, No. 30224801

Correspondence to: Only one corresponding address should be

provided. Author names should be given first, then author title, affiliation, the complete name of institution, city, postcode, province, country, and email. All the letters in the email should be in lower case. A space interval should be inserted between country name and email address. For example, Montgomery Bissell, MD, Professor of Medicine, Chief, Liver Center, Gastroenterology Division, University of California, Box 0538, San Francisco, CA 94143, United States. montgomery.bissell@ucsf.edu

Telephone and fax: Telephone and fax should consist of +, country number, district number and telephone or fax number, *e.g.*, Telephone: +86-10-59080039 Fax: +86-10-85381893

Peer reviewers: All articles received are subject to peer review. Normally, three experts are invited for each article. Decision on acceptance is made only when at least two experts recommend publication of an article. All peer-reviewers are acknowledged on Express Submission and Peer-review System website.

Abstract

There are unstructured abstracts (no less than 200 words) and structured abstracts. The specific requirements for structured abstracts are as follows:

An informative, structured abstract should accompany each manuscript. Abstracts of original contributions should be structured into the following sections: AIM (no more than 20 words; Only the purpose of the study should be included. Please write the Aim in the form of "To investigate/study/…"), METHODS (no less than 140 words for Original Articles; and no less than 80 words for Brief Articles), RESULTS (no less than 150 words for Original Articles and no less than 120 words for Brief Articles; You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, *e.g.*, 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$), and CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Core tip

Please write a summary of less than 100 words to outline the most innovative and important arguments and core contents in your paper to attract readers.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A:...; B:...; C:...; D:...; E:...; F:...; G: …*etc.* It is our principle to publish high resolution-figures for the E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of *P* values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of *P* values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

Coding system

The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that...".

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

PMID and DOI

Pleased provide PubMed citation numbers to the reference list, *e.g.*, PMID and DOI, which can be found at http://www.ncbi.nlm.nihgov/sites/entrez?db=pubmed and http://www.crossref.org/SimpleTextQuery/, respectively. The numbers will be used in E-version of this journal.

Style for journal references

Authors: the name of the first author should be typed in boldfaced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID:

11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23243641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315223018.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*

Biology: *H. pylori*, *E coli*, *etc.*

Examples for paper writing

All types of articles' writing style and requirement will be found in the link: http://www.wjgnet.com/esps/NavigationInfo.aspx?id=15.

RESUBMISSION OF THE REVISED MANUSCRIPTS

Authors must revise their manuscript carefully according to the revision policies of Baishideng Publishing Group Co., Limited. The revised version, along with the signed copyright transfer agreement, responses to the reviewers, and English language Grade A certificate (for non-native speakers of English), should be submitted to the online system *via* the link contained in the e-mail sent by the editor. If you have any questions about the revision, please send e-mail to esps@wjgnet.com.

Language evaluation

The language of a manuscript will be graded before it is sent for revision. (1) Grade A: priority publishing; (2) Grade B: minor language polishing; (3) Grade C: a great deal of language polishing needed; and (4) Grade D: rejected. Revised articles should reach Grade A.

Copyright assignment form

Please download a Copyright assignment form from http://www.wjgnet.com/1007-9327/g_info_20100315222818.htm.

Responses to reviewers

Please revise your article according to the comments/suggestions provided by the reviewers. The format for responses to the reviewers' comments can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315222607.htm

Proof of financial support

For papers supported by a foundation, authors should provide a copy of the approval document and serial number of the foundation.

STATEMENT ABOUT ANONYMOUS PUBLICATION OF THE PEER REVIEWERS' COMMENTS

In order to increase the quality of peer review, push authors to carefully revise their manuscripts based on the peer reviewers' comments, and promote academic interactions among peer reviewers, authors and readers, we decide to anonymously publish the reviewers' comments and author's responses at the same time the manuscript is published online.

PUBLICATION FEE

WJG is an international, peer-reviewed, open access, online journal. Articles published by this journal are distributed under the terms of the Creative Commons Attribution Non-commercial License, which permits use, distribution, and reproduction in any medium, provided the original work is properly cited, the use is non commercial and is otherwise in compliance with the license. Authors of accepted articles must pay a publication fee. Publication fee: 1398 USD per article. All invited articles are published free of charge.

Published by **Baishideng Publishing Group Inc**
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

ISSN 1007-9327