

World Journal of *Gastroenterology*

World J Gastroenterol 2014 February 21; 20(7): 1635-1886





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2014-2017

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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 1348 experts in gastroenterology and hepatology from 68 countries.

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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).

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NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

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PUBLISHER
Baishideng Publishing Group Co., Limited
Flat C, 23/F, Lucky Plaza,
315-321 Lockhart Road, Wan Chai, Hong Kong, China
Fax: +852-65557188
Telephone: +852-31779906
E-mail: bpgoffice@wjgnet.com

<http://www.wjgnet.com>

PUBLICATION DATE
February 21, 2014

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

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WJG 20th Anniversary Special Issues (8): Gastric cancer

Treatment of gastric cancer

Michele Orditura, Gennaro Galizia, Vincenzo Sforza, Valentina Gambardella, Alessio Fabozzi, Maria Maddalena Laterza, Francesca Andreozzi, Jole Ventriglia, Beatrice Savastano, Andrea Mabilia, Eva Lieto, Fortunato Ciardiello, Ferdinando De Vita

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Received: September 23, 2013 Revised: October 29, 2013

Accepted: November 12, 2013

Published online: February 21, 2014

Abstract

The authors focused on the current surgical treatment of resectable gastric cancer, and significance of peri- and post-operative chemo or chemoradiation. Gastric cancer is the 4th most commonly diagnosed cancer and the second leading cause of cancer death worldwide. Surgery remains the only curative therapy, while perioperative and adjuvant chemotherapy, as well as chemoradiation, can improve outcome of resectable gastric cancer with extended lymph node dissection. More than half of radically resected gastric cancer patients relapse locally or with distant metastases, or receive the diagnosis of gastric cancer when tumor is

disseminated; therefore, median survival rarely exceeds 12 mo, and 5-years survival is less than 10%. Cisplatin and fluoropyrimidine-based chemotherapy, with addition of trastuzumab in human epidermal growth factor receptor 2 positive patients, is the widely used treatment in stage IV patients fit for chemotherapy. Recent evidence supports the use of second-line chemotherapy after progression in patients with good performance status

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Key words: Gastric cancer; Surgery; Radiotherapy; Adjuvant chemotherapy; Palliative chemotherapy; Chemoradiation

Core tip: Surgery remains the only curative therapy of localized gastric cancer, while perioperative and adjuvant chemotherapy, as well as chemoradiation, can improve outcome. Cisplatin and fluoropyrimidine-based chemotherapy, with addition of trastuzumab in human epidermal growth factor receptor 2 positive patients, is the widely used treatment in stage IV patients. Second-line chemotherapy after progression in patients with good performance status represents a good option.

Orditura M, Galizia G, Sforza V, Gambardella V, Fabozzi A, Laterza MM, Andreozzi F, Ventriglia J, Savastano B, Mabilia A, Lieto E, Ciardiello F, De Vita F. Treatment of gastric cancer. *World J Gastroenterol* 2014; 20(7): 1635-1649 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1635.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1635>

INTRODUCTION

Gastric cancer is the 4th most commonly diagnosed cancer

and the second leading cause of cancer death worldwide. Gastric cancer generally remains asymptomatic for a long time and is early detected more commonly in Japan and South Korea, due, at least in part, to active screening programs. Despite increased incidence, Asian gastric cancer patients have a better prognosis than Western patients, probably due to an active screening program or to a more aggressive therapeutic approach. Surgery remains the only curative therapy, while perioperative and adjuvant chemotherapy, as well as chemoradiation, can improve outcome of resectable gastric cancer with extended lymph node dissection. No clear superiority of one strategy over another has emerged, all contributing to a gain of 15% in survival over surgery alone; thus, head-to-head comparisons would be required. Unfortunately, more than half of radically resected gastric cancer patients relapse locally or with distant metastases, or receive the diagnosis of gastric cancer when tumor is disseminated; therefore, median survival rarely exceeds 12 mo, and in metastatic setting, 5-years survival is less than 10%. Cisplatin and fluoropyrimidine-based chemotherapy, with addition of trastuzumab in human epidermal growth factor receptor 2 (HER2) positive patients, is the widely used treatment in stage IV patients fit for chemotherapy. Recent evidence supports the use of second-line chemotherapy after progression in patients with good performance status. Biological therapies are among the new frontiers of research in the treatment of gastric cancer; increased survival with trastuzumab in patients with HER2-positive and with ramucirumab in second line has been indeed recorded.

SURGERY

Ever since surgery has played a crucial role in the treatment of gastric cancer^[1]. In the last decades, two new technical advances have revolutionized treatment methodology, namely endoscopic resection and minimally invasive access^[2,3].

In Eastern countries, detection of early gastric cancer, *i.e.*, tumors confined to mucosa (T1_a) or submucosa (T1_b) with a low rate of nodal metastasis, has become increasingly common due to extensive screening programs; thus, early gastric cancer currently represents a large percentage of newly diagnosed tumors in Japan and South Korea^[4]. Several years ago early gastric cancer was deemed to be radically treated with endoscopic resection, with no need for extensive abdominal manipulation. However, horizontal and vertical margin invasion, and particularly the risk of nodal involvement, had to be immediately considered to avoid true oncological disasters. Initially, endoscopic mucosal resection, or, even better, endoscopic submucosal dissection (ESD), were indicated as standard treatment for differentiated-type adenocarcinoma without ulcerative findings UL(-) (depth of invasion clinically diagnosed as T1_a and diameter \leq 2 cm). Accordingly, resection was judged as curative when all of the following conditions were fulfilled: en-bloc resection, size \leq 2 cm, differentiated-type on histology, PT1_a,

negative horizontal margin, negative vertical margin, and no lymphovascular infiltration [ly(-), v(-)]^[5]. The above rules (so called standard criteria) were followed for many years by endoscopists with excellent results^[6]. However, more recently, remarkable improvements in technical management allowed to extend such indications to more advanced forms of early gastric cancer (expanded criteria). Currently, ESD is also indicated in differentiated, \leq 3 cm, PT1_a, UL(+) tumors, or undifferentiated, \leq 2 cm, PT1_a, UL(-) tumors, or differentiated, \leq 3 cm, PT1_b (but with submucosal invasion \leq 500 μ m from the muscularis mucosae)^[5]. Although close follow-up surveillance remains essential, within these criteria ESD has recently been shown to be a feasible and effective method for treating early gastric cancer^[2,7,8].

Minimally invasive access, namely laparoscopic gastric surgery, was initially devised for benign esophago-gastric diseases and is currently standard option for hiatal hernia repair and achalasia^[9]. Due mainly to technical difficulties and oncological concerns, the laparoscopic access was initially confined to treatment of distal-sided early gastric cancer not requiring total gastrectomy and enlarged lymphadenectomy^[10,11]. Following reports of satisfactory oncological adequacy for laparoscopic surgical treatment of colorectal cancer, the laparoscopic approach has been gradually extended to also include advanced gastric cancer requiring total gastrectomy with radical lymphadenectomy. Although data are still controversial, a number of studies have shown laparoscopic approach in the treatment of advanced gastric cancer to be feasible, safe, and oncologically adequate^[3,12]. Recently, robot-assisted gastrectomy has been shown to offer potential advantages over conventional laparoscopy with regard to lymphadenectomy and digestive restoration^[13].

Whatever the approach (open or laparoscopic), there is no doubt that surgery remains the only potentially curative treatment for all T1_b to T4 gastric cancers, and after failure of endoscopic resection^[14]. The most important and still debated issues are represented by extent of resection and role and extension of lymphadenectomy.

In case of gastric cancer involving the fundus and/or the body of the stomach, the vast majority of surgeons perform total gastrectomy since proximal gastric resection is flawed with a significant rate of postoperative complications^[15]. On the contrary, controversy has long been in place about extension of resection and importance of histologic subtype (namely, intestinal or diffuse according to the Lauren's classification)^[16] in case of cancer of the antrum. Several years ago total gastrectomy was hypothesized to offer oncological advantages over subtotal distal resection in terms of wider lymphadenectomy and effective removal of multicenter neoplastic foci, particularly frequent in histologically proven undifferentiated or diffuse subtype carcinomas^[17]. However, at the end of the last century, two European trials showed no differences in overall survival rates between total and subtotal distal gastrectomy - provided extension of the proximal margin of the resection into healthy tissue, thus

ensuring adequate clearance of the margins - and correctly performed lymphadenectomy (see below)^[17,18] in the latter procedure. Currently, there is general agreement that subtotal distal resection should be considered the standard of care for cancer of the antrum. The Japanese Gastric Cancer Association (JGCA), formerly known as the Japanese Research Society for Gastric Cancer, has recently stated that “a proximal margin of at least 3 cm is recommended for T2 or deeper tumors with an expansive growth pattern (Types 1 and 2) and 5 cm is recommended for those with infiltrative growth pattern (Types 3 and 4)”, thus finally ending a long-standing debate^[5].

Controversy over the extent of lymphadenectomy in the treatment of gastric adenocarcinoma has persisted for decades. There has been very little disagreement that at least a D1 lymphadenectomy (namely lymph node stations from 1 to 7 according to the JGCA's classification) should be performed^[19]. However, in Japan, a D2 lymphadenectomy (namely D1 lymphadenectomy plus node stations 8a, 9, 10, 11d, 11p, and 12a) has been recommended as standard practice since the 1960s^[20]; since Eastern surgeons strongly believe that a D2 lymphadenectomy significantly improves long-term results and overall survival rates^[5]. On the contrary, in Western countries the majority of surgeons continue to perform a D1 (or even a D0, *i.e.*, a lymphadenectomy less than D1) resection. This is mainly due to the results of two European randomized trials carried out in the 1990s which failed to demonstrate a survival benefit for D2 over D1 lymphadenectomy^[21,22]. However, these two studies have been strongly criticized for significant differences between the two groups analyzed. Indeed, almost 50% of patients in the D2 group did not undergo resection of 12a node station. In addition, patients undergoing splenectomy and/or pancreatectomy as part of a D2 resection had high rates of post-operative morbidity and mortality, thus confounding the results and obscuring statistical differences between the two groups^[14,23]. However, a large number of subsequent retrospective studies has shown a correlation between better outcome and lymphadenectomy extended beyond the boundaries of a D1 resection, with dismal long-term survival rates when positive nodes are found beyond the boundaries of a D2 resection, thus suggesting progression of gastric adenocarcinoma to a systemic disease when spreading is beyond D2 nodes^[24]. Furthermore, the Dutch D1D2 trial^[25], after a median follow-up of 15 years, reported on a significant benefit of D2 lymphadenectomy over D1 lymphadenectomy in terms of locoregional recurrence and survival. D2 lymphadenectomy fulfills the AJCC Cancer Staging Manual, which recommends a minimum of 16 lymph nodes be examined, results in lower rates of loco-regional recurrence^[26], and, ultimately, improves overall survival^[23,27]. It is particularly evident when splenectomy and/or pancreatectomy are spared (so-called D1+ lymphadenectomy) thus decreasing post-operative complications rates^[5]. A recent meta-analysis including 12 randomized controlled trials involving 3573 patients,

with survival analyses from 1332 patients, has shown no significant differences in overall survival between D1 and D2. However, subgroup analysis of patients without splenectomy and/or pancreatectomy indicated a clear trend for longer overall survival and a significant better disease-free survival rate for D2 compared to D1 patients^[14]. These data strongly suggested that D2 lymphadenectomy with spleen and pancreas preservation should be recommended as the standard surgical approach to resectable gastric cancer.

In Western countries a substantial percentage of gastric cancer patients presents with unresectable disease. In such cases, the role of non-curative gastric resection, excluding the cases with signs of gastric outlet obstruction or uncontrolled bleeding, remains controversial^[28]. On the one hand, resection allows reduction of tumor burden and cancer-related complication rates, but it is associated with significant perioperative mortality and morbidity and may delay start of chemotherapy^[29]. On the other hand, simple operative exploration without resection may expose patients to severe tumor complications^[30]. Pending large, randomized, prospective studies, no definitive evidence supporting either one strategy exists. However, all single series provide evidence for chemotherapy to improve survival rates and to decrease the incidence of tumor-related complications^[31].

Peritoneal carcinosis is the most common type of recurrence in advanced gastric cancer, particularly in undifferentiated or with infiltrating growth pattern tumors^[32]. When possible, complete cytoreductive surgery and hyperthermic intraperitoneal chemotherapy (HIPEC) have been shown to be the best option for a disease that is otherwise incurable^[33]. Recently, in high risk gastric cancers, that is, tumors suspected to have serosal invasion and/or poor histologic differentiation, analysis of liquid from peritoneal lavage has been suggested to be crucial to individuate free tumor cells in the abdominal cavity in order to tailor more effective treatments^[34]. This is a new fascinating frontier in the management of gastric cancer^[35]. Preliminary results of potentially curative gastric resection of the primary tumor and HIPEC in patients without overt peritoneal carcinosis despite detection of free tumor cells in the peritoneal lavage are encouraging^[36,37].

The last challenge in the treatment of gastric cancer is represented by liver metastases. Until few years ago their detection was considered synonymous with generalized neoplastic disease, thus contraindicating curative treatment^[38,39]. There is no doubt that gastric cancer shows a very aggressive biology with high and early propensity to spread through lymphovascular vessels and peritoneal serosa, thus liver-only deposits are an uncommon event^[40]. However, even when this occurs, better survival rates have been demonstrated to be achievable with aggressive treatment. Curative hepatic resection of liver-limited metastases, particularly single liver metastases less than 5 cm in size, has been associated with significantly superior 5-year overall survival and median survival rates than those obtained with sys-

Table 1 Meta-analyses of adjuvant chemotherapy for resectable gastric cancer

Ref.	Year	Trials (n)	Patients (n)	OR/HR for death (95%CI)
Hermans <i>et al</i> ^[71]	1993	11	2096	0.88 (0.78-1.08)
Earle <i>et al</i> ^[57]	1999	13	1990	0.80 (0.66-0.97)
Mari <i>et al</i> ^[56]	2000	21	3658	0.82 (0.75-0.89)
Panzini <i>et al</i> ^[58]	2002	17	3118	0.72 (0.62-0.84)
Janunger <i>et al</i> ^[61]	2002	21	3962	0.84 (0.74-0.96)
Zhao <i>et al</i> ^[72]	2008	15	3212	0.90 (0.84-0.96)
Liu <i>et al</i> ^[73]	2008	19	4599	0.85 (0.80-0.90)
GASTRIC group ^[62]	2010	17	3838	0.82 (0.76-0.90)

temic chemotherapy alone^[41,42]. Radiofrequency ablation may represent a valid alternative to surgical resection in liver metastasis with ≤ 3 cm or for patients unfit for major hepatic surgery^[43]. Finally, delivery of high doses of cytotoxic agents to liver tumors through the hepatic artery with minimal systemic side effects may be an effective strategy for control of multiple liver metastases or in order to shrink liver deposits prior to subsequent surgical resection or radiofrequency ablation^[44].

ADJUVANT THERAPIES

Although complete resection of cancer (R0) and extended lymph node dissection (D2) are the only curative treatments for gastric cancer, a high rate of locoregional as well as distant recurrences has been reported. The site of recurrence is locoregional in 19%-42% of cases, peritoneal in 21%-72%, and distant in 18%-49%. A survival benefit has been observed from the addition of chemotherapy or chemoradiotherapy to surgery alone, while no benefit has been obtained with adjuvant radiotherapy alone^[28,45-47].

Adjuvant chemotherapy

In the last decades, several phase III trials have investigated the role of adjuvant chemotherapy *vs* surgery alone, but conflicting results have been obtained. These differences can be explained by the large heterogeneity of patients enrolled, the small number of series, the different surgical accuracy, and the different chemotherapy regimens used^[48-54]. We also investigated in a randomized, multicenter, phase III trial the efficacy and safety of epirubicin, leucovorin, 5-fluorouracil and etoposide combination (ELFE regimen) as adjuvant therapy for radically resected gastric cancer patients. After a 5-year follow-up, the ELFE regimen was not shown to improve overall survival when compared to surgery alone^[55].

In order to obtain more reliable results, several meta-analyses (Table 1) and two recent phase III trials have been carried out, conclusively establishing a statistically significant benefit for chemotherapy in terms of overall survival and recurrence rate^[56-62].

A recent meta-analysis performed by the GASTRIC group^[62], including 3838 patients from 17 different trials of adjuvant chemotherapy, concluded for a modest but statistically significant benefit with the use of adjuvant post-operative chemotherapy with respect to surgery

alone (HR = 0.82; 95%CI: 0.76-0.90, $P = 0.001$). The estimated median survival was 4.9 years (95%CI: 4.4-5.5) in the surgery-only group *vs* 7.8 years (95%CI: 6.5-8.7) in the group of treated patients. However, no standard CT regimen has been defined in this setting.

Mono-chemotherapy with fluoropyrimidines has been tested in the Asian ACTS-GC trial by Sakuramoto *et al*^[63] 1059 stage II-III gastric cancer patients were randomized to receive S-1, an oral fluoropyrimidine containing tegafur, gimeracil and oteracil potassium, as post-operative therapy (two oral doses of 40 mg per square meter per day for 4 wk followed by 2 wk of rest for 1 year), or surgery alone. A statistically significant advantage in terms of 3-year survival was observed in the chemotherapy arm (80.1%, 95%CI: 76.1-84.0) *vs* the surgery arm (70.1%, 95%CI: 65.5-74.6), with a good tolerability for S-1 and a low incidence of G3-4 toxicities (anorexia 6%, nausea 3.7%, diarrhea 3.1%). A similar advantage was also recorded in the following 5-year survival analysis (72.6% *vs* 61.4%, HR = 0.65; 95%CI: 0.53-0.81). However, these results were limited by patient selection, thus needing to be confirmed in a more heterogeneous population. Furthermore, the use of S-1 in Western countries could be limited by pharmacokinetic factors. Tegafur (5-fluorouracil pro-drug) pharmacokinetic is indeed limited by polymorphisms in cytochrome P-450 2A6, and, consequently, 5-fluorouracil plasma concentrations are more likely to be elevated in patients from Western countries^[64].

Furthermore, in the CLASSIC phase III trial led by Bang *et al*^[65], 1035 patients with stage II-III B gastric cancer were randomly assigned to receive adjuvant chemotherapy with 8 cycles of capecitabine (1000 mg per square meter twice daily for 2 wk in a cycle of 21 d) plus oxaliplatin (130 mg per square meter every 21 d), so called XELOX, or surgery alone. After a median follow-up of about 34 mo, 3-year disease-free survival rates were 74% and 59% in the surgery plus chemotherapy and surgery only group, respectively (HR = 0.56; 95%CI: 0.44-0.72, $P < 0.0001$). Grade 3 or 4 toxicities were recorded in 56% of patients in the chemotherapy arm (nausea 65.7%, neutropenia 60.5%, anorexia 59.3%). At the 15th ESMO World Congress in Gastrointestinal Cancer (July 2013), data from the 5-year follow-up of the CLASSIC trial demonstrated a 34% reduction in the risk of death in the XELOX arm, higher than the reduction previously reported after three years of follow-up^[66].

Adjuvant XELOX might represent a valid strategy in curable gastric cancer Asian patients. Currently, there is no doubt on the survival benefit derived from adjuvant chemotherapy in radically resected gastric cancer for stage \geq T2 or N+ according to United States, European, and Italian guidelines^[67-69], although further phase III trials are required to assess which regimen is optimal for both Western and Eastern populations.

The utilization of HIPEC as adjuvant setting in patients at high risk for carcinomatosis is very interesting.

The results of various clinical studies indicated that HIPEC could potentially allow for a better prognosis in patients who underwent resection for advanced gastric cancer playing a role in the prevention of peritoneal local-regional recurrence despite R0 resection. However because of small number of trials, further study about this matter are warranted^[70].

Adjuvant chemoradiation

Considering the high rate of local recurrence in gastric cancer, combined treatment with radiation therapy and sensitizing 5-fluorouracil or capecitabine has been compared with chemotherapy or surgery alone in several trials.

The addition of post-operative radiation to adjuvant chemotherapy has been firstly studied in a prospective randomized trials by Dent *et al.*^[74], Moertel *et al.*^[75], and the British Stomach Cancer Group^[45]. Data from this studies did not show a survival benefit for patients receiving adjuvant therapy, however, because of their small accrual, heterogeneous cohort, unstandardized surgery and radiotherapy, and 5-fluorouracil dosage, it is difficult to draw conclusions from these studies.

An important role was played by the Gastrointestinal Cancer Intergroup phase III Trial (INT 0116)^[26]: 566 patients were randomized to receive surgery alone or adjuvant chemoradiation consisting of 5-fluorouracil (425 mg per square meter daily) plus leucovorin (20 mg per square meter daily) for 5 d and radiation (4500 cGy of radiation, 180 cGy per day, given 5 d per week for 5 wk), followed by 2 cycles of 5-fluorouracil (425 mg per square meter daily for 5 d) plus leucovorin (20 mg per square meter daily for 5 d) for one month. After a median follow-up of 5 years, the chemoradiation group achieved a significant advantage in overall survival (36 mo *vs* 27 mo, $P < 0.005$) and in progression-free survival (HR = 1.52; 95%CI: 1.23-1.86, $P < 0.001$). The advantage in the chemoradiotherapy-treated group has been recently confirmed at the 10-year follow up (disease free survival HR = 1.51; $P < 0.001$; overall survival HR = 1.32; $P < 0.004$)^[76]. Local recurrence occurred in 29% of patients in the surgery alone group and in 19% in the chemoradiation group; regional relapse was reported in 72% of patients in the surgery alone group and in 65% of the patients in the chemoradiation group; distant metastases were observed in 18% of relapsing patients in the surgery alone group and in 33% of patients in the chemoradiation group. Of note, treatment was burdened by high toxicity, with the most common G3 toxicities being hematologic (54%) and gas-

tro-intestinal (33%). Although this treatment approach is considered to be standard therapy in the United States, it has not gained wide acceptance in Europe because of concerns about abdominal chemoradiation toxicity and the quality of surgery performed; indeed, 54% of enrolled patients received a sub-optimal lymph-node dissection (D0-D1). In order to clarify this issue, a subgroup analysis published in 2002 revealed that the survival benefit of adjuvant chemo-radiotherapy remained similar in the D0 and D1 lymph-node dissection groups, while survival benefits in the D2 dissection group were doubtful. Therefore, radiation therapy can be useful to compensate inadequate surgery, by improving local control of disease and reducing local relapses (19% *vs* 29%)^[77,78].

The results of the phase III ARTIST trial have been recently published^[79]. 458 patients with D2 resected gastric cancer were randomly assigned to receive adjuvant XP (capecitabine 2000 mg per square meter on days 1 to 14 and cisplatin 60 mg per square meter, repeated every 3 wk) or XP/XRT/XP (capecitabine 2000 mg per square meter on d 1 to 14 and cisplatin 60 mg per square meter, repeated every 3 wk followed by 45 Gy radiations plus capecitabine 1650 mg per square meter for 5 wk followed by 2 additional cycles of XP). With a median follow-up of 53.2 mo, the adjuvant chemoradiotherapy arm did not obtain a significant advantage over the chemotherapy alone arm, with 3-year disease-free survival rates of 78.2% and 74.2% in the XP/XRT/XP arm and in the XP arm ($P = 0.0862$), respectively. Of note, in a subgroup analysis of 396 patients with positive pathologic lymph nodes, a statistically significant prolonged disease-free survival was recorded in the chemoradiation arm (estimated 3-year disease-free survival rate of 77.5%) as opposed to the XP-alone arm (3-year disease-free survival: 72.3%, $P = 0.0365$). This improvement in disease-free survival was mainly due to radiation-induced decreased regional lymph node recurrence. Most common G3-G4 toxicities in chemo- and chemoradiation arms were respectively: neutropenia (40.7% and 48.4%), nausea (12.4% and 12.3%), and vomiting (3.5% and 3.1%). In the ARTIST-2 trial this promising role of chemoradiotherapy *vs* chemotherapy alone in patients with node positive gastric cancer is still being evaluated.

Recently, Zhu *et al.*^[80] have published data from a trial carried out in the Chinese population. Specifically, 380 patients with D2 resected gastric cancer were randomized to receive adjuvant chemotherapy alone *vs* adjuvant chemoradiation therapy with intensity-modulated radiotherapy (IMRT). A significant difference in DFS in patients with positive nodes and in the whole population as well was observed. The marked effect on disease-free survival in this trial as opposed to the ARTIST trial was probably due to inclusion of patients with more advanced disease, especially in terms of lymph nodes involvement, and to the use of IMRT.

These results still need to be reproduced in the Western population and will be defined by the ongoing CRITICS trial (see below)^[81].

The employment of a triplet in a chemoradiation regimen has also been recently investigated by the Intergroup Trial CALGB 80101 (presented as abstract at the 2011 ASCO Annual Meeting)^[82]. From April 2003 to May 2009, 546 patients with resected gastric or gastro-esophageal cancer patients were randomized to receive 1 cycle of 5-fluorouracil (425 mg per square meter daily) plus leucovorin (20 mg per square meter daily) for 5 d/mo, followed by 45 Gy (1.8 Gy/d) and concurrent 5-fluorouracil (200 mg per square meter daily CI throughout radiotherapy), followed by 2 additional cycles of 5-fluorouracil/leucovorin (arm A) or 1 additional cycle of ECF (epirubicin 50 mg per square meter on day 1, cisplatin 60 mg per square meter on day 1, and 5-FU 200 mg per square meter CI d 1-21) followed by 45 Gy (1.8 Gy/d) and concurrent 5-fluorouracil (200 mg per square meter daily CI throughout radiation therapy), followed by 2 cycles of reduced dose of ECF (epirubicin 40 mg per square meter on day 1, cisplatin 50 mg per square meter on day 1, and 5-FU 200 mg per square meter daily C.I. d 1-21) (arm B). Median survival was 37 mo in arm A and 38 mo in arm B (HR = 1.03, 95%CI: 0.80-1.34, $P = 0.80$). Three-year overall survival was 50% in arm A and 52% in arm B, respectively. 3 year-DFS was 46% in arm A and 47% in arm B. Grade 4 toxicities were: 40% arm A *vs* 26% arm B ($P < 0.001$). Specifically, neutropenia (53% *vs* 48%), diarrhea (15% *vs* 7%), and mucositis (15% *vs* 7%) for arms A and B, respectively, were the most frequent.

We also assessed, in a pilot study published three years ago, the safety of adjuvant chemoradiotherapy in patients with stage III or IV radically resected gastric cancer. Treatment with FOLFOX regimen plus radiotherapy was safe, and, after a 3-year follow-up, both disease-free and overall survival rates were shown to be substantially better than those observed in untreated patients^[83].

Finally, European and Italian guidelines encourage use of adjuvant chemoradiotherapy in patients with high risk of local relapse (stage T2 with histopathological risk factors, T3-4, N+) and in patients not receiving adequate lymphadenectomy (< D2) or are R1 after surgery^[68,69].

Necessarily, the planning of radiotherapy fields requires experience and a quality control system. Radiotherapy is influenced by its confirmation in 3D (3D-CRT) or IMRT, and these technologies have been shown to reduce toxicities. A total radiation dose of 45 Gy is set to run in 25 fractions of 1.8 Gy. The delimitation of volumes must meet the guidelines established by RTOG and EORTC and include tumor bed, celiac lymph nodes, and para-aortic nodes.

NEOADJUVANT (PERIOPERATIVE) TREATMENT

Neoadjuvant chemotherapy for gastric cancer aims at downstaging disease, increasing the rate of curative resection, and eradicating undetectable micrometastases. In addition, pre-surgical patients usually have better performance status and can tolerate treatments better.

This approach has been demonstrated to obtain downstaging of gastric cancer, increase in curative resections, and improvement of disease-free and overall survival in randomized clinical studies (MAGIC, FFCD 9703, and EORTC 40954). Currently, all guidelines recommend this approach for patients with locally advanced gastric cancer.

The use of radiation alone or in combination with chemotherapy in the preoperative setting is still controversial and more data from adequate powered randomized trial are needed.

Neoadjuvant chemotherapy

The role of neoadjuvant chemotherapy in gastric cancer, gastro-esophageal junction and lower esophageal adenocarcinoma has evolved in the past decade from disappointingly negative trials to a favorable one^[61]. Indeed, in the first Dutch randomized controlled trial of neoadjuvant chemotherapy, 56 patients with apparently operable gastric cancer were randomized to receive preoperatively 4 cycles of 5-fluorouracil, doxorubicin and methotrexate (FAMTX) followed by surgery or surgery alone. The rate of curative resection favored the surgery alone group, and in the latest update, the median survival since randomization was 18 mo in the FAMTX group *vs* 30 mo in the surgery alone group ($P = 0.17$); moreover, preoperative chemotherapy was associated with a negative effect^[84].

In Europe, perioperative chemotherapy has been promoted on the basis of the MAGIC^[85] and FFCD9703^[86] randomized trials. The former, performed in the United Kingdom, enrolled 503 patients with resectable adenocarcinoma of the stomach, esophagogastric junction, or lower esophagus cancer (25% had lower esophageal or gastro-esophageal junction cancer) to either perioperative chemotherapy and surgery (250 patients) or surgery alone (253 patients).

Chemotherapy consisted of 3 preoperative and 3 postoperative cycles of ECF: intravenous epirubicin (50 mg/m²) and cisplatin (60 mg/m²) on day 1 and a continuous intravenous infusion of 5-fluorouracil (200 mg/m² per day for 21 d). Curative resection rates were 69.3% and 66.4% in the perioperative and in the surgery group, respectively. There was a greater proportion of stage T1 and T2 tumors and less advanced nodal disease in the perioperative group.

The perioperative chemotherapy group had a higher likelihood of overall survival (HR for death = 0.75; 95%CI: 0.60-0.93, $P = 0.009$; 5-year survival rate: 36% *vs* 23%) and progression-free survival (HR for progression = 0.66; 95%CI: 0.53-0.81, $P < 0.001$). Although 90.7% of patients completed preoperative chemotherapy, only 103 of 208 (49.5%) who completed preoperative therapy and surgery also received postoperative treatment.

A similar benefit emerged from the French FFCD 9703 trial, in which 224 patients were randomly assigned to 2 or 3 cycles of preoperative chemotherapy with infusional 5-fluorouracil plus cisplatin (CF) followed by

surgery and adjuvant CF chemotherapy, or surgery alone. Of note, 75% of all patients had adenocarcinoma of the distal esophagus or of the gastro-esophageal junction. The R0 resection rate was significantly better in the perioperative arm compared to the surgical resection alone arm (84% *vs* 73%, $P = 0.04$). Differences in the 5-year disease-free survival and the 5-year overall survival rate were 13% (34% *vs* 21%, $P = 0.0033$) and 14% (38% *vs* 24%, $P = 0.021$), respectively, in favor of neoadjuvant therapy.

Recently, the European EORTC 40954 trial^[87] assessed the efficacy of preoperative cisplatin, 5-fluorouracil, and leucovorin in gastric and gastro-esophageal cancer patients. This study needed 282 events to detect with 80% power an improvement in median survival from 17 mo with surgery alone to 24 mo with neoadjuvant therapy. The trial was stopped early for poor accrual after 144 patients randomly assigned (72:72). The total of 52.8% patients had tumors located in the proximal third of the stomach, including AEG type II and III. The curative resection rate was 81.9% after neoadjuvant chemotherapy and 66.7% in the neoadjuvant and surgery alone arm ($P = 0.036$). The surgery-only group had more metastatic lymph nodes than the neoadjuvant group (76.5% *vs* 61.4%, $P = 0.018$). Postoperative complications were more frequent in the neoadjuvant arm (27.1% *vs* 16.2%, $P = 0.09$). After a median follow-up of 4.4 years and 67 deaths, a survival benefit could not be shown (HR = 0.84; 95%CI: 0.52-1.35, $P = 0.466$).

This trial showed a significantly increased R0 resection rate, but failed to demonstrate a survival benefit due to a low statistical power; there was a high rate of proximal gastric cancer including AEG and/or a better outcome than expected after radical surgery alone due to the high quality of surgery with resection of regional lymph nodes outside the perigastric area (celiac trunc, hepatic ligament, lymph node at *a. lienalis*; D2).

Radiation in perioperative therapy

The use of radiation alone as preoperative treatment remains unclear, due to limited numbers of randomized clinical trials evaluating the efficacy of radiotherapy alone.

Zhang *et al*^[88] randomized a large sample size (370 patients) of gastric adenocarcinomas of cardia to surgery alone or radiotherapy for a total dose of 40 Gy and surgery. Tumor resectability and T2 cancer were more frequently observed in the radiation arm with a 11.0% decrease in T4 tumors. Five- and 10-year survival rates for radiation plus surgery and surgery alone groups were 30.1%, 19.7%, and 20.2%, 13.3%, respectively, while no significant differences were observed between the two groups in terms of surgical complications.

In another randomized trial with a longer follow-up (20 years), 51 patients per arm were randomly assigned to 20 Gy in 5 daily fractions followed by surgery or surgery alone. The 5-year and 10-year survival rates were 39.0% and 32.0%, and 30.0% and 18.0%, for preoperative radiotherapy and surgery alone groups, respectively ($P > 0.05$);

however, after 20 years, the study failed to demonstrate a survival benefit for preoperative radiotherapy^[89].

Of note, these two studies were started in the 1970s, when radiation used to be delivered by telecobalt or 8-MV photon, now rarely used.

Finally, in the meta-analysis of Fiorica *et al*^[90], 9 randomized trials (4 preoperative and 5 postoperative trials) were evaluated. Preoperative radiotherapy was associated with a 3-year (HR = 0.57; 95%CI: 0.43-0.76, $P = 0.0001$) and 5-year (HR = 0.62; 95%CI: 0.46-0.84, $P = 0.002$) survival advantage. Although a trend in postoperative mortality in the preoperative treatment group was observed, this difference turned out not to be statistically significant (HR = 0.61; 95%CI: 0.24-1.57, $P = 0.31$). A recent meta-analysis confirmed a statistically significant benefit for resectable gastric cancer patients treated with radiation therapy, however, subgroup analyses for pre- and postoperative settings were not available^[91].

Perioperative chemoradiation

Recently, a phase III trial was carried out to investigate a possible survival benefit for preoperative chemoradiotherapy compared to chemotherapy alone in locally advanced gastroesophageal and gastric cancer patients.

In the German study PreOperative Chemotherapy or Radiochemotherapy in Esophagogastric Adenocarcinoma Trial^[92], 119 patients were randomized to receive 5-fluorouracil, leucovorin, and cisplatin (PLF) followed by surgery or PLF followed by chemoradiation with cisplatin and etoposide and then surgery. Unfortunately, the trial was stopped prematurely due to poor accrual, thus limiting result interpretation. Nevertheless, response rate and tumor-free lymph node status were higher in the chemoradiation arm (cPR = 15.6% *vs* 2%, $P = 0.03$; ypN0 = 64.4% *vs* 36.7%, $P = 0.01$), although the 3-year survival benefit for the two groups did not reach statistical significance (47.4% *vs* 27.7%, $P = 0.07$).

Finally, the ongoing CRITICS trial (NCT00407186), in which patients with resectable gastric cancer are being treated with 3 cycles of preoperative epirubicin, cisplatin, and capecitabine (ECC) followed by surgery and then either another 3 cycles of ECC or concurrent chemoradiation (45 Gy, cisplatin and capecitabine) will help clarify the role of postoperative chemoradiotherapy^[81].

METASTATIC DISEASE

In Western countries about two thirds of gastric cancer patients are diagnosed with locally advanced or metastatic disease. Median survival for these patients is around 10 mo, and less than 10% survive at 5 years. Furthermore, even after curative resection, about 50%-60% of patients relapse locally or with distant metastases. A PS > 2 , liver metastases, peritoneal metastases, and alkaline phosphatase > 100 are considered unfavorable prognostic factors^[93].

A meta-analysis by Wagener *et al*^[94] demonstrated efficacy of chemotherapy compared with best supportive

care. Specifically, data from three randomized clinical trials favored chemotherapy in terms of quality of life and survival of patients with a good performance status (HR = 0.39; 95%CI: 0.28-0.52). Several trials and a meta-analysis also confirmed an advantage with regard to quality of life and survival when advanced gastric cancer patients were treated with combination chemotherapy with respect to single agent^[95,96].

In the late 80's the FAM regimen (5-fluorouracil 600 mg/m² on days 1, 8, 29 and 36, adriamycin 30 mg/m² on days 1 and 29, and mitomycin C 10 mg/m² on day 1) became a widely used treatment^[97,98], only to be later replaced by FAMTX (methotrexate 1500 mg/m², followed after 1 h by 5-fluorouracil 1500 mg/m² on day 1. Leucovorin rescue at 15 mg/m² after 24 h, orally, every 6 h for 48 h, and adriamycin 30 mg/m²), according to the results of a randomized phase III trial including 213 patients. The response rate of FAMTX was 41% *vs* 9% ($P < 0.0001$); survival with FAMTX was also superior (42 wk *vs* 29 wk, $P = 0.004$). There were no major differences in toxicity^[99,100].

In Asian countries, cisplatin plus infusional 5-fluorouracil or capecitabine or S-1 is currently standard practice on the basis of a favorable Japanese trial^[101]. The combination of cisplatin plus S-1 was also tested in metastatic gastric cancer in Caucasian patients^[102,103] against cisplatin plus infusional 5-fluorouracil. Despite a slight better median survival for cisplatin/S-1, no statistical differences were found (8.6 mo *vs* 7.9 mo, HR = 0.92; 95%CI: 0.8-1.05, $P = 0.20$). Safety was significantly better in the cisplatin/S-1 group, however, the dose of cisplatin was lower (75 and 100 mg/m² in experimental and standard group, respectively).

The REAL II trial by Cunningham *et al*^[104] confirmed non-inferiority of capecitabine to infusional 5-fluorouracil (HR = 0.86; 95%CI: 0.80-0.99) and established non-inferiority of oxaliplatin to cisplatin (HR = 0.92; 95%CI: 0.80-1.10) in two-by-two comparisons. On day 1 of every 3-wk cycle, patients in all study groups received an intravenous bolus of epirubicin (50 mg/m²) and cisplatin (60 mg/m²) in both the ECF and ECX groups, while oxaliplatin (130 mg/m²) was administered intravenously in the EOF and EOX groups. 5-fluorouracil (daily dose of 200 mg/m²) and capecitabine (twice daily doses of 625 mg/m²) were given throughout treatment in the appropriate groups. Median survival times in the ECF, ECX, EOF, and EOX groups were 9.9, 9.9, 9.3 and 11.2 mo, respectively; 1 year-survival rates were 37.7%, 40.8%, 40.4%, and 46.8%, respectively. In a secondary analysis, overall survival was longer with EOX than with ECF, with a HR of 0.80 for death in the EOX group (95%CI: 0.66-0.97, $P = 0.02$). Progression-free survival and response rates did not differ significantly among the regimens. The EOX regimen was associated with the highest median survival. Response rates were 47.9% for EOX, 46.4% for EOF, 42.4% for ECX, and 40.7% for ECF (no significant differences among the four treatment arms). Oxaliplatin-based regimens were generally well tolerated, with inferior

incidence of severe neutropenia, alopecia, and nephrotoxicity, and higher incidence of severe peripheral neuropathy and diarrhea.

Furthermore, in a meta-analysis including the REAL II and MLI17032 trials, a longer survival and a higher response rate was observed with capecitabine (HR = 0.87) compared with infusional 5-fluorouracil-containing chemotherapy^[105].

In United States docetaxel is the drug of choice to add to cisplatin and 5-fluorouracil, based on V325 phase III trial results^[106], in which 445 advanced gastric cancer patients were randomized to receive docetaxel 75 mg/m² (day 1) plus cisplatin 75 mg/m² (day 1) and 5-fluorouracil 750 mg/m² per day continuous infusion (days 1 to 5; DCF), or once every 4 wk cisplatin 100 mg/m² (day 1) and 5-fluorouracil 1000 mg/m² per day continuous infusion (days 1 to 5; CF). The addition of docetaxel to CF significantly improved time to progression (5.6 mo *vs* 3.9 mo), survival (9.2 mo *vs* 8.6 mo), and overall response rate (37% *vs* 25%), despite the poor prognosis of the selected population, when compared with the CF-treated population. However, an increased rate of neutropenia (29% incidence of febrile neutropenia) was recorded. For this reason, the DCF regimen could be recommended for patients with good performance status^[107].

Conversely, epirubicin, cisplatin, 5-fluorouracil (ECF) is the favorite three-drug regimen in Europe on the basis of two randomized studies^[108,109] and a meta-analysis^[96]. ECF showed a higher overall response rate (45% *vs* 21%, $P = 0.0002$), a longer median time of survival (8.9 mo *vs* 5.7 mo, $P = 0.0009$) and a better median failure-free survival duration (7.4 mo *vs* 3.4 mo, $P = 0.00006$) when compared with FAMTX. A better quality of life with the ECF regimen was also recorded.

HER2 is overexpressed in 10%-25% of gastric cancer. Recently, the international phase III ToGA trial^[110] randomized 594 HER-2 positive metastatic gastric cancer to receive capecitabine (1000 mg/m² orally twice a day for 14 d followed by a 1-wk rest), or 5-fluorouracil (800 mg/m² per day by continuous intravenous infusion on d 1-5 of each cycle) plus cisplatin (80 mg/m² on day 1 by intravenous infusion) with or without trastuzumab (8 mg/kg intravenously on day 1 of the first cycle, followed by 6 mg/kg every 3 wk). The addition of trastuzumab to chemotherapy improved significantly overall survival compared with chemotherapy alone (13.8 mo *vs* 11.1 mo, HR = 0.74, $P = 0.0046$) as well as progression free survival (6.7 mo *vs* 5.5 mo, HR = 0.74, $P = 0.0002$). A greater survival benefit was detected in an exploratory subgroup analysis of patients HER2 2+ and FISH positive, and HER2 3+ and FISH positive (16.0 mo *vs* 11.8 mo, HR = 0.65). Also, response rate and time to progression were significantly improved by the addition of trastuzumab.

Thus, trastuzumab, in association with platinum and 5-fluorouracil or capecitabine, is now widely considered the standard of care for first line therapy of patients diagnosed with HER 2 positive gastro-esophageal junction

Table 2 Milestone phase III trials in metastatic gastric cancer

Ref.	Regimen	n	Response rate	Overall survival	DSF/PFS/TTP	G3-G4 toxicity
Cullinan <i>et al</i> ^[122] , 1985	FAM 5-FU	350	-	-	-	-
Wils <i>et al</i> ^[100] , 1991	FAMTX FAM	213	41% 9%	42 wk 29 wk		4% 3%
Kim <i>et al</i> ^[123] , 1993	FAM PF 5-FU	117	51% 26%	No difference	Median TTP: 12 21.8 9.1	-
Webb <i>et al</i> ^[108] , 1997	FAMTX ECF	256	21% 45%	5.7 8.9	PFS 3.4 7.4	-
Vanhoefer ^[124] , 2000	ELF PF FAMTX		9% 20% 12%	7.2 7.2 6.7		No toxicity G 3- 4
Van Cutsem <i>et al</i> ^[106] V325 trial, 2006	CF DCF	224 221	37% 25%	9.2 8.6	TTP: 3.9 5.6	69% 59%
Cunningham <i>et al</i> ^[104] REAL II, 2008	ECX EOX ECF EOF	250 244 263 245	42.4% 47.9% 40.7% 46.4%	11.2 9.9	ECF: 40.7 and similar in all groups	Neutropenia most frequent in ECX and ECF regimen 51.5% and 41.7% <i>vs</i> 29.9% and 27.6%
Koizumi <i>et al</i> ^[101] Spirit trial, 2008	S-1 CDDP + S-1	150 149		11 13	PFS: 4.0 6.0	Neutropenia: 59% <i>vs</i> 16% Anemia: 38% <i>vs</i> 6% Anorexia 45% <i>vs</i> 9%
Ajani <i>et al</i> ^[102] FLAGS, 2010	CDDP+ 5-FU CDDP + S-1	508 521	32% 29%	7.9 8.6	TTP 5.5 4.8	
Bang <i>et al</i> ^[110] ToGA, 2010	CDDP + 5-FU/Cap CDDP + 5-FU/Cap + Trastuzumab	290 294		11.1 13.8		Neutropenia 88% 79%

FAM: 5-fluorouracil, adriamycin, mitomycin; 5-FU: 5-fluorouracil; FAMTX: 5-fluorouracil, adriamycin, metrotexate; PF: Cisplatin, 5-fluorouracil; ECF: Epirubicin, cisplatin, 5-fluorouracil; ELF: Etoposide, leucovorin, 5-fluorouracil; CF: Cisplatin, 5-fluorouracil; DCF: Docetaxel, cisplatin, 5-fluorouracil; ECX: Epirubicin, cisplatin, capecitabine; EOX: Epirubicin, oxaliplatin, capecitabine; EOF: Epirubicin, oxaliplatin, 5-fluorouracil.

and gastric cancer. Table 2 summarizes the results of the main phase III trials of chemotherapy for advanced gastric cancer.

Despite the promising results obtained in phase II trials, addition of HER 1 inhibitors cetuximab and panitumumab to chemotherapy failed to increase overall and progression free survival of metastatic gastric cancer patients in the phase III randomized trials EXPAND^[111] and REAL III^[112]. Disappointing results were also obtained with the anti-angiogenetic antibody bevacizumab used in combination with platinum-based chemotherapy^[113,114].

Recently a phase III LoGic trial^[115] of first line capecitabine and oxaliplatin did not reach its primary endpoint, with a hazard ratio (HR) for OS of CapeOx + L compared to CapeOx + P of 0.91 (95%CI: 0.73-1.12, $P = 0.35$); median 12.2 mo *vs* 10.5 mo, respectively. Pre-specified subgroup analyses showed significant improvements in OS in Asian pts (HR = 0.68) and those under 60 years (HR = 0.69). There was no association between IHC and OS. though certain subgroups showed improvement. Further clinical and molecular analyses will be presented. The results of the phase III TYTAN^[116] trial

conducted in Asia indicate that HER2-targeted therapy, Lapatinib, has the potential to prolong patient survival when used in the second-line setting in HER2-positive advanced gastric cancer, but only in individuals who test HER2 positive by immunohistochemistry (IHC 3+).

The role of a second line has been recently clarified. Randomized clinical trials^[117-119] and a meta-analysis^[120] demonstrated improved overall survival and quality of life with irinotecan or docetaxel chemotherapy *vs* best supportive care.

Finally, at the latest ASCO Meeting, ramucirumab, a fully human immunoglobulin G1 monoclonal antibody highly specific for the extracellular VEGF-binding domain of VEGFR-2, was demonstrated to have a significant antitumor activity in a range of malignancies, according to results in clinical trials. The REGARD trial for gastro-esophageal and gastric adenocarcinoma demonstrated ramucirumab to significantly improve overall survival and progression-free survival *vs* BSC, with a median overall survival increasing from 3.8 to 5.2 mo ($P = 0.0473$)^[121]. This translated into a 22% reduction in the risk of death with ramucirumab.

Ramucirumab has also been evaluated in combination with paclitaxel in the phase III RAINBOW trial, but results are still pending.

CONCLUSION

Depending on the site and extent of cancer, surgery is the only potentially curative treatment for all T1b-T4 gastric cancers, and extended lymphadenectomy (D2) should be recommended as standard of care in resectable gastric cancer, while endoscopic submucosal resection followed by close surveillance is the preferred option for early stage cancer. Surgical treatment of liver-limited metastases and hyperthermic intraperitoneal chemotherapy for peritoneal carcinosis are fascinating frontiers.

Furthermore, a survival benefit for postoperative chemotherapy, chemoradiotherapy, and perioperative chemotherapy in case of pathologic T > 2 and/or node-positive gastric cancer patients has been established, and chemotherapy should contain 5-fluorouracil and cisplatin or their analogs capecitabine and oxaliplatin. Neoadjuvant chemoradiation should be implemented with caution.

Finally, in select metastatic gastric cancer patients, chemotherapy is better than best supportive care only, with cisplatin-5-fluorouracil or capecitabine as the most widely used drugs. Addition of anti-HER2 antibody trastuzumab to first-line chemotherapy for patients overexpressing HER2 receptor and addition of the anti VEGFR-2 antibody ramucirumab in second line improves overall survival and progression-free survival when compared to chemotherapy alone.

REFERENCES

- Nashimoto A, Akazawa K, Isobe Y, Miyashiro I, Katai H, Kodera Y, Tsujitani S, Seto Y, Furukawa H, Oda I, Ono H, Tanabe S, Kaminishi M. Gastric cancer treated in 2002 in Japan: 2009 annual report of the JGCA nationwide registry. *Gastric Cancer* 2013; **16**: 1-27 [PMID: 22729699 DOI: 10.1007/s10120-012-0163-4]
- Choi MK, Kim GH, Park do Y, Song GA, Kim DU, Ryu DY, Lee BE, Cheong JH, Cho M. Long-term outcomes of endoscopic submucosal dissection for early gastric cancer: a single-center experience. *Surg Endosc* 2013; **27**: 4250-4258 [PMID: 23765426]
- Cai J, Wei D, Gao CF, Zhang CS, Zhang H, Zhao T. A prospective randomized study comparing open versus laparoscopy-assisted D2 radical gastrectomy in advanced gastric cancer. *Dig Surg* 2011; **28**: 331-337 [PMID: 21934308 DOI: 10.1159/000330782]
- Ahn HS, Lee HJ, Yoo MW, Jeong SH, Park DJ, Kim HH, Kim WH, Lee KU, Yang HK. Changes in clinicopathological features and survival after gastrectomy for gastric cancer over a 20-year period. *Br J Surg* 2011; **98**: 255-260 [PMID: 21082693 DOI: 10.1002/bjs.7310]
- Japanese Gastric Cancer Association. Japanese gastric cancer treatment guidelines 2010 (ver. 3). *Gastric Cancer* 2011; **14**: 113-123 [PMID: 21573742 DOI: 10.1007/s10120-011-0042-4]
- Park YM, Cho E, Kang HY, Kim JM. The effectiveness and safety of endoscopic submucosal dissection compared with endoscopic mucosal resection for early gastric cancer: a systematic review and metaanalysis. *Surg Endosc* 2011; **25**: 2666-2677 [PMID: 21424201 DOI: 10.1007/s00464-011-1627-z]
- Sanomura Y, Oka S, Tanaka S, Noda I, Higashiyama M, Imagawa H, Shishido T, Yoshida S, Hiayama T, Arihiro K, Chayama K. Clinical validity of endoscopic submucosal dissection for submucosal invasive gastric cancer: a single-center study. *Gastric Cancer* 2012; **15**: 97-105 [PMID: 21785925 DOI: 10.1007/s10120-011-0076-7]
- Choi MH, Hong SJ, Han JP, Song JY, Kim DY, Seo SW, Ha JS, Lee YN, Ko BM, Lee MS. [Therapeutic outcomes of endoscopic submucosal dissection in undifferentiated-type early gastric cancer]. *Korean J Gastroenterol* 2013; **61**: 196-202 [PMID: 23624733]
- Katada N, Sakuramoto S, Yamashita K, Hosoda K, Shibata T, Moriya H, Kikuchi S, Watanabe M. Comparison of the Heller-Toupet procedure with the Heller-Dor procedure in patients who underwent laparoscopic surgery for achalasia. *Surg Today* 2013; Epub ahead of print [PMID: 23793852]
- Kitano S, Shiraishi N, Uyama I, Sugihara K, Tanigawa N. A multicenter study on oncologic outcome of laparoscopic gastrectomy for early cancer in Japan. *Ann Surg* 2007; **245**: 68-72 [PMID: 17197967]
- Memon MA, Butler N, Memon B. The issue of lymphadenectomy during laparoscopic gastrectomy for gastric carcinoma. *World J Gastrointest Oncol* 2010; **2**: 65-67 [PMID: 21160923 DOI: 10.4251/wjgo.v2.i2.65]
- Moisan F, Norero E, Slako M, Varas J, Palominos G, Crovari F, Ibañez L, Pérez G, Pimentel F, Guzmán S, Jarufe N, Boza C, Escalona A, Funke R. Completely laparoscopic versus open gastrectomy for early and advanced gastric cancer: a matched cohort study. *Surg Endosc* 2012; **26**: 661-672 [PMID: 22011940 DOI: 10.1007/s00464-011-1933-5]
- Coratti A, Anecchiarico M, Di Marino M, Gentile E, Coratti F, Giulianotti PC. Robot-assisted gastrectomy for gastric cancer: current status and technical considerations. *World J Surg* 2013; **37**: 2771-2781 [PMID: 23674257]
- Jiang L, Yang KH, Guan QL, Zhao P, Chen Y, Tian JH. Survival and recurrence free benefits with different lymphadenectomy for resectable gastric cancer: a meta-analysis. *J Surg Oncol* 2013; **107**: 807-814 [PMID: 23512524 DOI: 10.1002/jso.23325]
- Someya S, Shibata C, Tanaka N, Kudoh K, Naitoh T, Miura K, Unno M. Duodenal switch for intractable reflux gastroesophagitis after proximal gastrectomy. *Tohoku J Exp Med* 2013; **230**: 129-132 [PMID: 23803250]
- Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. *Acta Pathol Microbiol Scand* 1965; **64**: 31-49 [PMID: 14320675]
- Gouzi JL, Huguier M, Fagniez PL, Launois B, Flamant Y, Lacaine F, Paquet JC, Hay JM. Total versus subtotal gastrectomy for adenocarcinoma of the gastric antrum. A French prospective controlled study. *Ann Surg* 1989; **209**: 162-166 [PMID: 2644898]
- Bozzetti F, Marubini E, Bonfanti G, Miceli R, Piano C, Genari L. Subtotal versus total gastrectomy for gastric cancer: five-year survival rates in a multicenter randomized Italian trial. Italian Gastrointestinal Tumor Study Group. *Ann Surg* 1999; **230**: 170-178 [PMID: 10450730]
- Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma: 3rd English edition. *Gastric Cancer* 2011; **14**: 101-112 [PMID: 21573743 DOI: 10.1007/s10120-011-0041-5]
- Kajitani T. The general rules for the gastric cancer study in surgery and pathology. Part I. Clinical classification. *Jpn J Surg* 1981; **11**: 127-139 [PMID: 7300058]
- Bonenkamp JJ, Songun I, Hermans J, Sasako M, Welvaart K, Plukker JT, van Elk P, Obertop H, Gouma DJ, Taat CW. Randomised comparison of morbidity after D1 and D2 dissection for gastric cancer in 996 Dutch patients. *Lancet* 1995; **345**: 745-748 [PMID: 7891484]
- Cuschieri A, Fayers P, Fielding J, Craven J, Bancewicz J,

- Joypaul V, Cook P. Postoperative morbidity and mortality after D1 and D2 resections for gastric cancer: preliminary results of the MRC randomised controlled surgical trial. The Surgical Cooperative Group. *Lancet* 1996; **347**: 995-999 [PMID: 8606613]
- 23 **Schmidt B**, Yoon SS. D1 versus D2 lymphadenectomy for gastric cancer. *J Surg Oncol* 2013; **107**: 259-264 [PMID: 22513454 DOI: 10.1002/jso.23127]
 - 24 **Sasako M**, Sano T, Yamamoto S, Kurokawa Y, Nashimoto A, Kurita A, Hiratsuka M, Tsujinaka T, Kinoshita T, Arai K, Yamamura Y, Okajima K. D2 lymphadenectomy alone or with para-aortic nodal dissection for gastric cancer. *N Engl J Med* 2008; **359**: 453-462 [PMID: 18669424 DOI: 10.1056/NEJMoa0707035]
 - 25 **Songun I**, Putter H, Kranenbarg EM, Sasako M, van de Velde CJ. Surgical treatment of gastric cancer: 15-year follow-up results of the randomised nationwide Dutch D1D2 trial. *Lancet Oncol* 2010; **11**: 439-449 [PMID: 20409751 DOI: 10.1016/S1470-2045(10)70070-X]
 - 26 **Macdonald JS**, Smalley SR, Benedetti J, Hundahl SA, Estes NC, Stemmermann GN, Haller DG, Ajani JA, Gunderson LL, Jessup JM, Martenson JA. Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. *N Engl J Med* 2001; **345**: 725-730 [PMID: 11547741]
 - 27 **Lee HK**, Yang HK, Kim WH, Lee KU, Choe KJ, Kim JP. Influence of the number of lymph nodes examined on staging of gastric cancer. *Br J Surg* 2001; **88**: 1408-1412 [PMID: 11578301]
 - 28 **Ko KJ**, Shim JH, Yoo HM, O SI, Jeon HM, Park CH, Jeon DJ, Song KY. The clinical value of non-curative resection followed by chemotherapy for incurable gastric cancer. *World J Surg* 2012; **36**: 1800-1805 [PMID: 22450753 DOI: 10.1007/s00268-012-1566-4]
 - 29 **Schmidt B**, Look-Hong N, Maduekwe UN, Chang K, Hong TS, Kwak EL, Lauwers GY, Rattner DW, Mullen JT, Yoon SS. Noncurative gastrectomy for gastric adenocarcinoma should only be performed in highly selected patients. *Ann Surg Oncol* 2013; **20**: 3512-3518 [PMID: 23765416 DOI: 10.1245/s10434-013-3024-4]
 - 30 **Dittmar Y**, Rauchfuss F, Goetz M, Jandt K, Scheuerlein H, Heise M, Settmacher U. Non-curative gastric resection for patients with stage 4 gastric cancer—a single center experience and current review of literature. *Langenbecks Arch Surg* 2012; **397**: 745-753 [PMID: 22307547 DOI: 10.1007/s00423-012-0902-3]
 - 31 **Kokkola A**, Louhimo J, Puolakkainen P. Does non-curative gastrectomy improve survival in patients with metastatic gastric cancer? *J Surg Oncol* 2012; **106**: 193-196 [PMID: 22354864 DOI: 10.1002/jso.23066]
 - 32 **Huang B**, Sun Z, Wang Z, Lu C, Xing C, Zhao B, Xu H. Factors associated with peritoneal metastasis in non-serosa-invasive gastric cancer: a retrospective study of a prospectively-collected database. *BMC Cancer* 2013; **13**: 57 [PMID: 23379700 DOI: 10.1186/1471-2407-13-57]
 - 33 **Wu XJ**, Yuan P, Li ZY, Bu ZD, Zhang LH, Wu AW, Zong XL, Li SX, Shan F, Ji X, Ren H, Ji JF. Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy improves the survival of gastric cancer patients with ovarian metastasis and peritoneal dissemination. *Tumour Biol* 2013; **34**: 463-469 [PMID: 23108893 DOI: 10.1007/s13277-012-0571-4]
 - 34 **Wong J**, Coit D. Detection of gastric cancer peritoneal metastases by peritoneal lavage: Current limitations and future perspectives. *Surgery* 2012; **152**: 1-4 [PMID: 22703894 DOI: 10.1016/j.surg.2012.03.022]
 - 35 **Zhibing W**, Qinghua D, Shenglin M, Ke Z, Kan W, Xiadong L, Pengjun Z, Ruzhen Z. Clinical study of cisplatin hyperthermic intraperitoneal perfusion chemotherapy in combination with docetaxel, 5-fluorouracil and leucovorin intravenous chemotherapy for the treatment of advanced-stage gastric carcinoma. *Hepatogastroenterology* 2013; **60**: 989-994 [PMID: 23598741 DOI: 10.5754/hge13038]
 - 36 **Costa WL**, Coimbra FJ, Ribeiro HS, Diniz AL, de Godoy AL, Begnami M, Silva MJ, Fanelli MF, Mello CA. Safety and preliminary results of perioperative chemotherapy and hyperthermic intraperitoneal chemotherapy (HIPEC) for high-risk gastric cancer patients. *World J Surg Oncol* 2012; **10**: 195 [PMID: 22992263 DOI: 10.1186/1477-7819-10-195]
 - 37 **Kang LY**, Mok KT, Liu SI, Tsai CC, Wang BW, Chen IS, Chen YC, Chang BM, Chou NH. Intraoperative hyperthermic intraperitoneal chemotherapy as adjuvant chemotherapy for advanced gastric cancer patients with serosal invasion. *J Chin Med Assoc* 2013; **76**: 425-431 [PMID: 23796652]
 - 38 **Ajani JA**. Evolving chemotherapy for advanced gastric cancer. *Oncologist* 2005; **10** Suppl 3: 49-58 [PMID: 16368871]
 - 39 **Jerraya H**, Saidani A, Khalfallah M, Bouasker I, Nouria R, Dziri C. Management of liver metastases from gastric carcinoma: where is the evidence? *Tunis Med* 2013; **91**: 1-5 [PMID: 23404586]
 - 40 **Kumagai K**, Shimizu K, Yokoyama N, Aida S, Tanaka T, Yamagata K. Gastrointestinal cancer metastasis and lymphatic advancement. *Surg Today* 2010; **40**: 301-306 [PMID: 20339983 DOI: 10.1007/s00595-009-4142-2]
 - 41 **Kakeji Y**, Morita M, Maehara Y. Strategies for treating liver metastasis from gastric cancer. *Surg Today* 2010; **40**: 287-294 [PMID: 20339981 DOI: 10.1007/s00595-009-4152-0]
 - 42 **Liu J**, Chen L. Current status and progress in gastric cancer with liver metastasis. *Chin Med J (Engl)* 2011; **124**: 445-456 [PMID: 21362349]
 - 43 **Chen J**, Tang Z, Dong X, Gao S, Fang H, Wu D, Xiang D, Zhang S. Radiofrequency ablation for liver metastasis from gastric cancer. *Eur J Surg Oncol* 2013; **39**: 701-706 [PMID: 23597495 DOI: 10.1016/j.ejso.2013.03.023]
 - 44 **Ganeshan A**, Upponi S, Hon LQ, Warakaulle D, Uberoi R. Hepatic arterial infusion of chemotherapy: the role of diagnostic and interventional radiology. *Ann Oncol* 2008; **19**: 847-851 [PMID: 18029972]
 - 45 **Hallissey MT**, Dunn JA, Ward LC, Allum WH. The second British Stomach Cancer Group trial of adjuvant radiotherapy or chemotherapy in resectable gastric cancer: five-year follow-up. *Lancet* 1994; **343**: 1309-1312 [PMID: 7910321]
 - 46 A comparison of combination chemotherapy and combined modality therapy for locally advanced gastric carcinoma. Gastrointestinal Tumor Study Group. *Cancer* 1982; **49**: 1771-1777 [PMID: 6176313]
 - 47 **Schwarz RE**, Zagala-Nevarez K. Recurrence patterns after radical gastrectomy for gastric cancer: prognostic factors and implications for postoperative adjuvant therapy. *Ann Surg Oncol* 2002; **9**: 394-400 [PMID: 11986192]
 - 48 **Dixon WJ**, Longmire WP, Holden WD. Use of triethylene-thiophosphoramide as an adjuvant to the surgical treatment of gastric and colorectal carcinoma: ten-year follow-up. *Ann Surg* 1971; **173**: 26-39 [PMID: 4925450]
 - 49 **Serlin O**, Wolkoff JS, Amadeo JM, Keehn RJ. Use of 5-fluorodeoxyuridine (FUDR) as an adjuvant to the surgical management of carcinoma of the stomach. *Cancer* 1969; **24**: 223-228 [PMID: 4240353]
 - 50 **Schwarz RE**, Zagala-Nevarez K. Recurrence patterns after radical gastrectomy for gastric cancer: prognostic factors and implications for postoperative adjuvant therapy. *Ann Surg Oncol* 2002; **9**: 394-400 [PMID: 11986192]
 - 51 **Lise M**, Nitti D, Marchet A, Sahmoud T, Buyse M, Duez N, Fiorentino M, Dos Santos JG, Labianca R, Rougier P. Final results of a phase III clinical trial of adjuvant chemotherapy with the modified fluorouracil, doxorubicin, and mitomycin regimen in resectable gastric cancer. *J Clin Oncol* 1995; **13**: 2757-2763 [PMID: 7595735]
 - 52 **Krook JE**, O'Connell MJ, Wieand HS, Beart RW, Leigh JE, Kugler JW, Foley JF, Pfeifle DM, Twito DI. A prospective, randomized evaluation of intensive-course 5-fluorouracil

- plus doxorubicin as surgical adjuvant chemotherapy for resected gastric cancer. *Cancer* 1991; **67**: 2454-2458 [PMID: 2015545]
- 53 **Coombes RC**, Schein PS, Chilvers CE, Wils J, Beretta G, Bliss JM, Rutten A, Amadori D, Cortes-Funes H, Villar-Grimalt A. A randomized trial comparing adjuvant fluorouracil, doxorubicin, and mitomycin with no treatment in operable gastric cancer. International Collaborative Cancer Group. *J Clin Oncol* 1990; **8**: 1362-1369 [PMID: 2199622]
- 54 **Nakajima T**, Nashimoto A, Kitamura M, Kito T, Iwanaga T, Okabayashi K, Goto M. Adjuvant mitomycin and fluorouracil followed by oral uracil plus tegafur in serosa-negative gastric cancer: a randomised trial. Gastric Cancer Surgical Study Group. *Lancet* 1999; **354**: 273-277 [PMID: 10440302]
- 55 **De Vita F**, Giuliani F, Orditura M, Maiello E, Galizia G, Di Martino N, Montemurro F, Carteni G, Manzione L, Romito S, Gebbia V, Ciardiello F, Catalano G, Colucci G. Adjuvant chemotherapy with epirubicin, leucovorin, 5-fluorouracil and etoposide regimen in resected gastric cancer patients: a randomized phase III trial by the Gruppo Oncologico Italia Meridionale (GOIM 9602 Study). *Ann Oncol* 2007; **18**: 1354-1358 [PMID: 17525087 DOI: 10.1093/annonc/mdm128]
- 56 **Mari E**, Floriani I, Tinazzi A, Buda A, Belfiglio M, Valentini M, Cascinu S, Barni S, Labianca R, Torri V. Efficacy of adjuvant chemotherapy after curative resection for gastric cancer: a meta-analysis of published randomised trials. A study of the GISCAD (Gruppo Italiano per lo Studio dei Carcinomi dell'Apparato Digerente). *Ann Oncol* 2000; **11**: 837-843 [PMID: 10997811]
- 57 **Earle CC**, Maroun JA. Adjuvant chemotherapy after curative resection for gastric cancer in non-Asian patients: revisiting a meta-analysis of randomised trials. *Eur J Cancer* 1999; **35**: 1059-1064 [PMID: 10533448]
- 58 **Panzini I**, Gianni L, Fattori PP, Tassinari D, Imola M, Fabbri P, Arcangeli V, Drudi G, Canuti D, Fochessati F, Ravaioli A. Adjuvant chemotherapy in gastric cancer: a meta-analysis of randomized trials and a comparison with previous meta-analyses. *Tumori* 2002; **88**: 21-27 [PMID: 12004845]
- 59 **Neri B**, Cini G, Andreoli F, Boffi B, Francesconi D, Mazzanti R, Medi F, Mercatelli A, Romano S, Siliani L, Tarquini R, Moretti R. Randomized trial of adjuvant chemotherapy versus control after curative resection for gastric cancer: 5-year follow-up. *Br J Cancer* 2001; **84**: 878-880 [PMID: 11286464]
- 60 **Cirera L**, Balil A, Batiste-Alentorn E, Tusquets I, Cardona T, Arcusa A, Jolis L, Saigi E, Guasch I, Badia A, Boleda M. Randomized clinical trial of adjuvant mitomycin plus tegafur in patients with resected stage III gastric cancer. *J Clin Oncol* 1999; **17**: 3810-3815 [PMID: 10577853]
- 61 **Janunger KG**, Hafström L, Glimelius B. Chemotherapy in gastric cancer: a review and updated meta-analysis. *Eur J Surg* 2002; **168**: 597-608 [PMID: 12699095]
- 62 **Paoletti X**, Oba K, Burzykowski T, Michiels S, Ohashi Y, Pignon JP, Rougier P, Sakamoto J, Sargent D, Sasako M, Van Cutsem E, Buyse M. Benefit of adjuvant chemotherapy for resectable gastric cancer: a meta-analysis. *JAMA* 2010; **303**: 1729-1737 [PMID: 20442389 DOI: 10.1001/jama.2010.534]
- 63 **Sakuramoto S**, Sasako M, Yamaguchi T, Kinoshita T, Fujii M, Nashimoto A, Furukawa H, Nakajima T, Ohashi Y, Imamura H, Higashino M, Yamamura Y, Kurita A, Arai K. Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. *N Engl J Med* 2007; **357**: 1810-1820 [PMID: 17978289]
- 64 **Kobayakawa M**, Kojima Y. Tegafur/gimeracil/oteracil (S-1) approved for the treatment of advanced gastric cancer in adults when given in combination with cisplatin: a review comparing it with other fluoropyrimidine-based therapies. *Onco Targets Ther* 2011; **4**: 193-201 [PMID: 22162925 DOI: 10.2147/OTT.S19059]
- 65 **Bang YJ**, Kim YW, Yang HK, Chung HC, Park YK, Lee KH, Lee KW, Kim YH, Noh SI, Cho JY, Mok YJ, Kim YH, Ji J, Yeh TS, Button P, Sirzén F, Noh SH. Adjuvant capecitabine and oxaliplatin for gastric cancer after D2 gastrectomy (CLASSIC): a phase 3 open-label, randomised controlled trial. *Lancet* 2012; **379**: 315-321 [PMID: 22226517 DOI: 10.1016/S0140-6736(11)61873-4]
- 66 **Noh SH**, Park SR, Yang HK, Chung HC, Chung IJ, Lee HK, Kim HH, Ji J, Chen JS, Lim Y, Ha S, Bang YJ. Adjuvant capecitabine and oxaliplatin (XELOX) for gastric cancer after d2 gastrectomy: final results from the CLASSIC trial. *Ann Oncol* 2013; **24** suppl 4: iv14 [DOI: 10.1093/annonc/mdt201.7]
- 67 National Comprehensive Cancer Network: NCCN Clinical Practice Guidelines in Oncology: Gastric Cancer, Version 2. 2013. Available from: URL: http://www.nccn.org/professionals/physician_gls/f_guidelines.asp
- 68 **Okines A**, Verheij M, Allum W, Cunningham D, Cervantes A. Gastric cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2010; **21** Suppl 5: v50-v54 [PMID: 20555102 DOI: 10.1093/annonc/mdq164]
- 69 Linee guida dell'Oncologia italiana AIOM 2012: neoplasie dello stomaco. Available from: URL: http://www.aiom.it/area_pubblica/area_medica/prodotti_scientifici/linee_guida/Neoplasie_dello_stomaco/1,1992,0
- 70 **Yan TD**, Black D, Sugarbaker PH, Zhu J, Yonemura Y, Petrou G, Morris DL. A systematic review and meta-analysis of the randomized controlled trials on adjuvant intraperitoneal chemotherapy for resectable gastric cancer. *Ann Surg Oncol* 2007; **14**: 2702-2713 [PMID: 17653801]
- 71 **Hermans J**, Bonenkamp JJ, Boon MC, Bunt AM, Ohyama S, Sasako M, Van de Velde CJ. Adjuvant therapy after curative resection for gastric cancer: meta-analysis of randomized trials. *J Clin Oncol* 1993; **11**: 1441-1447 [PMID: 8336183]
- 72 **Zhao SL**, Fang JY. The role of postoperative adjuvant chemotherapy following curative resection for gastric cancer: a meta-analysis. *Cancer Invest* 2008; **26**: 317-325 [PMID: 18317973 DOI: 10.1080/07357900701834686]
- 73 **Liu TS**, Wang Y, Chen SY, Sun YH. An updated meta-analysis of adjuvant chemotherapy after curative resection for gastric cancer. *Eur J Surg Oncol* 2008; **34**: 1208-1216 [PMID: 18353606 DOI: 10.1016/j.ejso.2008.02.002]
- 74 **Dent DM**, Werner ID, Novis B, Cheverton P, Brice P. Prospective randomized trial of combined oncological therapy for gastric carcinoma. *Cancer* 1979; **44**: 385-391 [PMID: 113074]
- 75 **Moertel CG**, Childs DS, O'Fallon JR, Holbrook MA, Schutt AJ, Reitemeier RJ. Combined 5-fluorouracil and radiation therapy as a surgical adjuvant for poor prognosis gastric carcinoma. *J Clin Oncol* 1984; **2**: 1249-1254 [PMID: 6491703]
- 76 **Macdonald JS**, Benedetti J, Smalley S, Haller D, Hundahl S, Jessup J, Ajani J, Gunderson L, Goldman B, Martenson J. Chemoradiation of resected gastric cancer: a 10-year follow-up of the phase III trial INT0116 (SWOG 9008). *J Clin Oncol* 2009; **27**: 15s (abstract 4515)
- 77 **Macdonald JS**, Smalley S, Benedetti J, Estes N, Haller DG, Ajani JA, Gunderson LL, Jessup M, Martenson JA. Postoperative combined radiation and chemotherapy improves disease-free survival (DFS) and overall survival (OS) in resected adenocarcinoma of the stomach and gastroesophageal junction: Update of the results of Intergroup Study INT-0116 (SWOG 9008). Presented at the 2004 American Society of Clinical Oncology Gastrointestinal Cancers Symposium; January 22-24, 2004, San Francisco, CA; Abstract 6
- 78 **Viudez-Berral A**, Miranda-Murua C, Arias-de-la-Vega F, Hernández-García I, Artajona-Rosino A, Díaz-de-Liaño Á, Vera-García R. Current management of gastric cancer. *Rev Esp Enferm Dig* 2012; **104**: 134-141 [PMID: 22449155]
- 79 **Lee J**, Lim do H, Kim S, Park SH, Park JO, Park YS, Lim HY, Choi MG, Sohn TS, Noh JH, Bae JM, Ahn YC, Sohn I, Jung SH, Park CK, Kim KM, Kang WK. Phase III trial comparing capecitabine plus cisplatin versus capecitabine plus cisplatin

- with concurrent capecitabine radiotherapy in completely resected gastric cancer with D2 lymph node dissection: the ARTIST trial. *J Clin Oncol* 2012; **30**: 268-273 [PMID: 22184384 DOI: 10.1200/JCO.2011.39.1953]
- 80 **Zhu WG**, Xua DF, Pu J, Zong CD, Li T, Tao GZ, Ji FZ, Zhou XL, Han JH, Wang CS, Yu CH, Yi JG, Su XL, Ding JX. A randomized, controlled, multicenter study comparing intensity-modulated radiotherapy plus concurrent chemotherapy with chemotherapy alone in gastric cancer patients with D2 resection. *Radiother Oncol* 2012; **104**: 361-366 [PMID: 22985776 DOI: 10.1016/j.radonc.2012.08.024]
 - 81 **Dikken JL**, van Sandick JW, Maurits Swellengrebel HA, Lind PA, Putter H, Jansen EP, Boot H, van Grieken NC, van de Velde CJ, Verheij M, Cats A. Neo-adjuvant chemotherapy followed by surgery and chemotherapy or by surgery and chemoradiotherapy for patients with resectable gastric cancer (CRITICS). *BMC Cancer* 2011; **11**: 329 [PMID: 21810227 DOI: 10.1186/1471-2407-11-329]
 - 82 **Fuchs CS**, Tepper JE, Niedzwiecki D, Hollis D, Mamon HJ, Swanson R, Haller DG, Dragovich T, Alberts SR, Bjarnason GA, Willett CG, Enzinger PC, Goldberg RM, Venook AP, Mayer RJ. Postoperative adjuvant chemoradiation for gastric or gastroesophageal junction (GEJ) adenocarcinoma using epirubicin, cisplatin, and infusional (CI) 5-FU (ECF) before and after CI 5-FU and radiotherapy (CRT) compared with bolus 5-FU/LV before and after CRT: Intergroup trial CALGB 80101. *J Clin Oncol* 2011; **29**: Abstract 4003
 - 83 **Oditura M**, De Vita F, Muto P, Vitiello F, Murino P, Lieto E, Vecchione L, Romano A, Martinelli E, Renda A, Ferraraccio F, Del Genio A, Ciardiello F, Galizia G. Adjuvant chemoradiotherapy in patients with stage III or IV radically resected gastric cancer: a pilot study. *Arch Surg* 2010; **145**: 233-238 [PMID: 20231623 DOI: 10.1001/archsurg.2010.2]
 - 84 **Hartgrink HH**, van de Velde CJ, Putter H, Songun I, Tessaar ME, Kranenbarg EK, de Vries JE, Wils JA, van der Bijl J, van Krieken JH. Neo-adjuvant chemotherapy for operable gastric cancer: long term results of the Dutch randomised FAMTX trial. *Eur J Surg Oncol* 2004; **30**: 643-649 [PMID: 15256239]
 - 85 **Cunningham D**, Allum WH, Stenning SP, Thompson JN, Van de Velde CJ, Nicolson M, Scarffe JH, Lofts FJ, Falk SJ, Iveson TJ, Smith DB, Langley RE, Verma M, Weeden S, Chua YJ. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. *N Engl J Med* 2006; **355**: 11-20 [PMID: 16822992]
 - 86 **Boige V**, Pignon J, Saint-Aubert B, Lasser P, Conroy T, Bouche O, Segol P, Bedenne L, Rougier P, Ychou M. Final results of a randomized trial comparing preoperative 5-fluorouracil (f) cisplatin (P) to surgery alone in adeno-carcinoma of stomach and lower esophagus (aStE): FNLCC ACCORD 07-FFCD 9703 Trial. *J Clin Oncol* 2007; **25**: Abstract 4510
 - 87 **Schuhmacher C**, Gretschesel S, Lordick F, Reichardt P, Hohenberger W, Eisenberger CF, Haag C, Mauer ME, Hasan B, Welch J, Ott K, Hoelscher A, Schneider PM, Bechstein W, Wilke H, Lutz MP, Nordlinger B, Van Cutsem E, Siewert JR, Schlag PM. Neoadjuvant chemotherapy compared with surgery alone for locally advanced cancer of the stomach and cardia: European Organisation for Research and Treatment of Cancer randomized trial 40954. *J Clin Oncol* 2010; **28**: 5210-5218 [PMID: 21060024 DOI: 10.1200/JCO.2009.26.6114]
 - 88 **Zhang ZX**, Gu XZ, Yin WB, Huang GJ, Zhang DW, Zhang RG. Randomized clinical trial on the combination of preoperative irradiation and surgery in the treatment of adenocarcinoma of gastric cardia (AGC)--report on 370 patients. *Int J Radiat Oncol Biol Phys* 1998; **42**: 929-934 [PMID: 9869212]
 - 89 **Skoropad V**, Berdov B, Zagrebina V. Concentrated preoperative radiotherapy for resectable gastric cancer: 20-years follow-up of a randomized trial. *J Surg Oncol* 2002; **80**: 72-78 [PMID: 12173383]
 - 90 **Fiorica F**, Cartei F, Enea M, Licata A, Cabibbo G, Carau B, Liboni A, Ursino S, Cammà C. The impact of radiotherapy on survival in resectable gastric carcinoma: a meta-analysis of literature data. *Cancer Treat Rev* 2007; **33**: 729-740 [PMID: 17935888]
 - 91 **Valentini V**, Cellini F, Minsky BD, Mattiucci GC, Balducci M, D'Agostino G, D'Angelo E, Dinapoli N, Nicolotti N, Valentini C, La Torre G. Survival after radiotherapy in gastric cancer: systematic review and meta-analysis. *Radiother Oncol* 2009; **92**: 176-183 [PMID: 19586672 DOI: 10.1016/j.radonc.2009.06.014]
 - 92 **Stahl M**, Walz MK, Stuschke M, Lehmann N, Meyer HJ, Riera-Knorrenschild J, Langer P, Engenhart-Cabillie R, Bitzer M, Königsrainer A, Budach W, Wilke H. Phase III comparison of preoperative chemotherapy compared with chemoradiotherapy in patients with locally advanced adenocarcinoma of the esophagogastric junction. *J Clin Oncol* 2009; **27**: 851-856 [PMID: 19139439 DOI: 10.1200/JCO.2008.17.0506]
 - 93 **Chau I**, Norman AR, Cunningham D, Waters JS, Oates J, Ross PJ. Multivariate prognostic factor analysis in locally advanced and metastatic esophago-gastric cancer--pooled analysis from three multicenter, randomized, controlled trials using individual patient data. *J Clin Oncol* 2004; **22**: 2395-2403 [PMID: 15197201]
 - 94 **Wagner AD**, Grothe W, Haerting J, Kleber G, Grothey A, Fleig WE. Chemotherapy in advanced gastric cancer: a systematic review and meta-analysis based on aggregate data. *J Clin Oncol* 2006; **24**: 2903-2909 [PMID: 16782930]
 - 95 **Catalano V**, Labianca R, Beretta GD, Gatta G, de Braud F, Van Cutsem E. Gastric cancer. *Crit Rev Oncol Hematol* 2009; **71**: 127-164 [PMID: 19230702 DOI: 10.1016/j.critrevonc.2009.01.004]
 - 96 **Wagner AD**, Unverzagt S, Grothe W, Kleber G, Grothey A, Haerting J, Fleig WE. Chemotherapy for advanced gastric cancer. *Cochrane Database Syst Rev* 2010; **(3)**: CD004064 [PMID: 20238327 DOI: 10.1002/14651858.CD004064.pub3]
 - 97 **MacDonald JS**, Schein PS, Woolley PV, Smythe T, Ueno W, Hoth D, Smith F, Boiron M, Gisselbrecht C, Brunet R, Lagarde C. 5-Fluorouracil, doxorubicin, and mitomycin (FAM) combination chemotherapy for advanced gastric cancer. *Ann Intern Med* 1980; **93**: 533-536 [PMID: 7436184]
 - 98 **Douglass HO**, Lavin PT, Goudsmit A, Klaassen DJ, Paul AR. An Eastern Cooperative Oncology Group evaluation of combinations of methyl-CCNU, mitomycin C, Adriamycin, and 5-fluorouracil in advanced measurable gastric cancer (EST 2277). *J Clin Oncol* 1984; **2**: 1372-1381 [PMID: 6439836]
 - 99 **Klein HO**, Wickramanayake PD, Dieterle F, Mohr R, Oerker-mann H, Gross R. High-dose MTX/5-FU and adriamycin for gastric cancer. *Semin Oncol* 1983; **10**: 29-31 [PMID: 6603023]
 - 100 **Wils JA**, Klein HO, Wagener DJ, Bleiberg H, Reis H, Korsten F, Conroy T, Fickers M, Leyvraz S, Buyse M. Sequential high-dose methotrexate and fluorouracil combined with doxorubicin--a step ahead in the treatment of advanced gastric cancer: a trial of the European Organization for Research and Treatment of Cancer Gastrointestinal Tract Cooperative Group. *J Clin Oncol* 1991; **9**: 827-831 [PMID: 2016625]
 - 101 **Koizumi W**, Narahara H, Hara T, Takagane A, Akiya T, Takagi M, Miyashita K, Nishizaki T, Kobayashi O, Takiyama W, Toh Y, Nagaie T, Takagi S, Yamamura Y, Yanaoka K, Orita H, Takeuchi M. S-1 plus cisplatin versus S-1 alone for first-line treatment of advanced gastric cancer (SPIRITS trial): a phase III trial. *Lancet Oncol* 2008; **9**: 215-221 [PMID: 18282805 DOI: 10.1016/S1470-2045(08)70035-4]
 - 102 **Ajani JA**, Rodriguez W, Bodoky G, Moiseyenko V, Lichinitser M, Gorbunova V, Vynnychenko I, Garin A, Lang I, Falcon S. Multicenter phase III comparison of cisplatin/S-1 with cisplatin/infusional fluorouracil in advanced gastric or gastroesophageal adenocarcinoma study: the FLAAS trial. *J Clin Oncol* 2010; **28**: 1547-1553 [PMID: 20159816 DOI: 10.1200/JCO.2009.25.4706]
 - 103 **Ajani JA**, Buyse M, Lichinitser M, Gorbunova V, Bodoky G,

- Douillard JY, Cascinu S, Heinemann V, Zaucha R, Carrato A, Ferry D, Moiseyenko V. Combination of cisplatin/S-1 in the treatment of patients with advanced gastric or gastroesophageal adenocarcinoma: Results of noninferiority and safety analyses compared with cisplatin/5-fluorouracil in the First-Line Advanced Gastric Cancer Study. *Eur J Cancer* 2013; **49**: 3616-3624 [PMID: 23899532 DOI: 10.1016/j.ejca.2013.07.003]
- 104 **Cunningham D**, Starling N, Rao S, Iveson T, Nicolson M, Coxon F, Middleton G, Daniel F, Oates J, Norman AR. Capecitabine and oxaliplatin for advanced esophagogastric cancer. *N Engl J Med* 2008; **358**: 36-46 [PMID: 18172173 DOI: 10.1056/NEJMoa073149]
- 105 **Okines AF**, Norman AR, McCloud P, Kang YK, Cunningham D. Meta-analysis of the REAL-2 and ML17032 trials: evaluating capecitabine-based combination chemotherapy and infused 5-fluorouracil-based combination chemotherapy for the treatment of advanced oesophago-gastric cancer. *Ann Oncol* 2009; **20**: 1529-1534 [PMID: 19474114 DOI: 10.1093/annonc/ndp047]
- 106 **Van Cutsem E**, Moiseyenko VM, Tjulandin S, Majlis A, Constenla M, Boni C, Rodrigues A, Fodor M, Chao Y, Voznyi E, Risse ML, Ajani JA. Phase III study of docetaxel and cisplatin plus fluorouracil compared with cisplatin and fluorouracil as first-line therapy for advanced gastric cancer: a report of the V325 Study Group. *J Clin Oncol* 2006; **24**: 4991-4997 [PMID: 17075117]
- 107 **Ajani JA**, Moiseyenko VM, Tjulandin S, Majlis A, Constenla M, Boni C, Rodrigues A, Fodor M, Chao Y, Voznyi E, Marabotti C, Van Cutsem E. Clinical benefit with docetaxel plus fluorouracil and cisplatin compared with cisplatin and fluorouracil in a phase III trial of advanced gastric or gastroesophageal cancer adenocarcinoma: the V-325 Study Group. *J Clin Oncol* 2007; **25**: 3205-3209 [PMID: 17664467]
- 108 **Webb A**, Cunningham D, Scarffe JH, Harper P, Norman A, Joffe JK, Hughes M, Mansi J, Findlay M, Hill A, Oates J, Nicolson M, Hickish T, O'Brien M, Iveson T, Watson M, Underhill C, Wardley A, Meehan M. Randomized trial comparing epirubicin, cisplatin, and fluorouracil versus fluorouracil, doxorubicin, and methotrexate in advanced esophagogastric cancer. *J Clin Oncol* 1997; **15**: 261-267 [PMID: 8996151]
- 109 **Yun J**, Lee J, Park SH, Park JO, Park YS, Lim HY, Kang WK. A randomised phase II study of combination chemotherapy with epirubicin, cisplatin and capecitabine (ECX) or cisplatin and capecitabine (CX) in advanced gastric cancer. *Eur J Cancer* 2010; **46**: 885-891 [PMID: 20060288]
- 110 **Bang YJ**, Van Cutsem E, Feyerislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Rüschhoff J, Kang YK. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet* 2010; **376**: 687-697 [PMID: 20728210 DOI: 10.1016/S0140-6736(10)61121-X]
- 111 **Lordick F**, Kang YK, Chung HC, Salman P, Oh SC, Bodoky G, Kurteva G, Volovat C, Moiseyenko VM, Gorbunova V, Park JO, Sawaki A, Celik I, Götte H, Melezínková H, Moehler M. Capecitabine and cisplatin with or without cetuximab for patients with previously untreated advanced gastric cancer (EXPAND): a randomised, open-label phase 3 trial. *Lancet Oncol* 2013; **14**: 490-499 [PMID: 23594786 DOI: 10.1016/S1470-2045(13)70102-5]
- 112 **Waddell TS**, Chau I, Barbachano Y, Gonzalez de Castro D, Wotherspoon A, Saffery C, Middleton GW, Wadsley J, Ferry DR, Mansoor W, Crosby TDL, Coxon FY, Smith D, Waters JS, Iveson T, Falk S, Slater S, Okines AFC, Cunningham D. A randomized multicenter trial of epirubicin, oxaliplatin, and capecitabine (EOC) plus panitumumab in advanced esophagogastric cancer (REAL3). *J Clin Oncol* 2012; **30**: 18 (Suppl LBA4000)
- 113 **Ohtsu A**, Shah MA, Van Cutsem E, Rha SY, Sawaki A, Park SR, Lim HY, Yamada Y, Wu J, Langer B, Starnawski M, Kang YK. Bevacizumab in combination with chemotherapy as first-line therapy in advanced gastric cancer: a randomized, double-blind, placebo-controlled phase III study. *J Clin Oncol* 2011; **29**: 3968-3976 [PMID: 21844504 DOI: 10.1200/JCO.2011.36.2236]
- 114 **Van Cutsem E**, de Haas S, Kang YK, Ohtsu A, Tebbutt NC, Ming Xu J, Peng Yong W, Langer B, Delmar P, Scherer SJ, Shah MA. Bevacizumab in combination with chemotherapy as first-line therapy in advanced gastric cancer: a biomarker evaluation from the AVAGAST randomized phase III trial. *J Clin Oncol* 2012; **30**: 2119-2127 [PMID: 22565005 DOI: 10.1200/JCO.2011.39.9824]
- 115 **Hecht JR**, Bang YJ, Qin S, Chung HC, Xu JM, Park JO, Jeziorski K, Shparyk Y, Hoff PM, Sobrero AF, Salman P, Li J, Protzenko S, Buyse ME, Afenjar K, Kaneko T, Kemner A, Santillana S, Press MF, Slamon DJ. Lapatinib in combination with capecitabine plus oxaliplatin (CapeOx) in HER2-positive advanced or metastatic gastric, esophageal, or gastroesophageal adenocarcinoma (AC): The TRIO-013/LOGiC Trial. *J Clin Oncol* 2013; **31**: Suppl Abstr LBA4001
- 116 **Satoh T**, Bang Y, Wang J, Xu J, Chung HJ, Yeh K, Chen J, Mukaiyama A, Yoshida P, Ohtsu A. Interim safety analysis from TYTAN: a phase III Asian study of lapatinib in combination with paclitaxel as second-line therapy in gastric cancer. *J Clin Oncol* 2010; **28**: 15s Abstract 4057
- 117 **Thuss-Patience PC**, Kretzschmar A, Bichev D, Deist T, Hinke A, Breithaupt K, Dogan Y, Gebauer B, Schumacher G, Reichardt P. Survival advantage for irinotecan versus best supportive care as second-line chemotherapy in gastric cancer—a randomised phase III study of the Arbeitsgemeinschaft Internistische Onkologie (AIO). *Eur J Cancer* 2011; **47**: 2306-2314 [PMID: 21742485 DOI: 10.1016/j.ejca.2011.06.002]
- 118 **Kang JH**, Lee SI, Lim do H, Park KW, Oh SY, Kwon HC, Hwang IG, Lee SC, Nam E, Shin DB, Lee J, Park JO, Park YS, Lim HY, Kang WK, Park SH. Salvage chemotherapy for pretreated gastric cancer: a randomized phase III trial comparing chemotherapy plus best supportive care with best supportive care alone. *J Clin Oncol* 2012; **30**: 1513-1518 [PMID: 22412140 DOI: 10.1200/JCO.2011.39.4585]
- 119 **Ueda S**, Hironaka S, Yasui H, Nishina T, Tsuda M, Tsumura T, Sugimoto N, Shimodaira H, Tokunaga S, Moriwaki T, Esaki T, Nagase M, Fujitani K, Yamaguchi K, Ura T, Hamamoto Y, Morita S, Okamoto I, Boku N, Hyodo I, West Japan Oncology Group. Randomized phase III study of irinotecan (CPT-11) vs weekly paclitaxel (wPTX) for advanced gastric cancer (AGC) refractory to combination chemotherapy (CT) of fluoropyrimidine plus platinum (FP): WJOG4007. *J Clin Oncol* 2012; **30**: Abstr 4002
- 120 **Kim HS**, Kim HJ, Kim SY, Kim TY, Lee KW, Baek SK, Kim TY, Ryu MH, Nam BH, Zang DY. Second-line chemotherapy versus supportive cancer treatment in advanced gastric cancer: a meta-analysis. *Ann Oncol* 2013; **24**: 2850-2854 [PMID: 23942775]
- 121 **Fuchs CS**, Tomasek J, Cho JY, Dumitru F, Passalacqua R, Goswami C, Safran H, Vieira dos Santos L, Aprile G, Ferry DR, Melichar B, Tehfe M, Topuzov E, Tabernero J, Zalcberg JR, Chau I, Koshiji M, Hsu Y, Schwartz JD, Ajani JA. REGARD: A phase III, randomized, double-blinded trial of ramucirumab and best supportive care (BSC) vs placebo and BSC in the treatment of metastatic gastric or gastroesophageal junction (GEJ) adenocarcinoma following disease progression on first-line platinum- and/or fluoropyrimidine-containing combination therapy. *J Clin Oncol* 2012; **30**: Abstract LBA5
- 122 **Cullinan SA**, Moertel CG, Fleming TR, Rubin JR, Krook JE, Everson LK, Windschitl HE, Twito DI, Marschke RF, Foley JF. A comparison of three chemotherapeutic regimens in the treatment of advanced pancreatic and gastric carcinoma. Fluorouracil vs fluorouracil and doxorubicin vs fluorouracil, doxorubicin, and mitomycin. *JAMA* 1985; **253**: 2061-2067

- [PMID: 2579257]
- 123 **Kim NK**, Park YS, Heo DS, Suh C, Kim SY, Park KC, Kang YK, Shin DB, Kim HT, Kim HJ. A phase III randomized study of 5-fluorouracil and cisplatin versus 5-fluorouracil, doxorubicin, and mitomycin C versus 5-fluorouracil alone in the treatment of advanced gastric cancer. *Cancer* 1993; **71**: 3813-3818 [PMID: 8508349]
 - 124 **Vanhoefer U**, Rougier P, Wilke H, Ducreux MP, Lacave AJ, Van Cutsem E, Planker M, Santos JG, Piedbois P, Paillot B,

Bodenstein H, Schmoll HJ, Bleiberg H, Nordlinger B, Couvreur ML, Baron B, Wils JA. Final results of a randomized phase III trial of sequential high-dose methotrexate, fluorouracil, and doxorubicin versus etoposide, leucovorin, and fluorouracil versus infusional fluorouracil and cisplatin in advanced gastric cancer: A trial of the European Organization for Research and Treatment of Cancer Gastrointestinal Tract Cancer Cooperative Group. *J Clin Oncol* 2000; **18**: 2648-2657 [PMID: 10894863]

P- Reviewers: Chetty R, Mura B, Sperti C **S- Editor:** Gou SX
L- Editor: A **E- Editor:** Zhang DN



WJG 20th Anniversary Special Issues (8): Gastric cancer

Role of imaging in predicting response to neoadjuvant chemotherapy in gastric cancer

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Received: October 29, 2013 Revised: December 19, 2013

Accepted: January 3, 2014

Published online: February 21, 2014

performing early response assessment with use of ¹⁸F-fluoro-2-deoxy-D-glucose positron-emission tomography demonstrate controversial results. The usefulness of other molecular imaging modalities, among which diffusion-weighted-magnetic resonance imaging, remains to be investigated.

Kwee RM, Kwee TC. Role of imaging in predicting response to neoadjuvant chemotherapy in gastric cancer. *World J Gastroenterol* 2014; 20(7): 1650-1656 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1650.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1650>

Abstract

With the proven overall benefit of neoadjuvant chemotherapy in patients with locally advanced gastric cancer, there has come a need to discriminate responders from non-responders. In this article, the current role of anatomical and molecular imaging in the prediction of response to neoadjuvant therapy in gastric cancer is outlined and future prospects are discussed.

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Key words: Gastric cancer; Neoadjuvant therapy; Chemotherapy; Response; Imaging

Core tip: Studies have shown that there is an association between tumor response at anatomical imaging evaluation and histopathological response and survival in patients with gastric cancer who are treated with neoadjuvant chemotherapy. However, as it takes time for gross tumor changes to become apparent, anatomical imaging may be of limited value in the early assessment of neoadjuvant chemotherapy efficacy. Studies

INTRODUCTION

Gastric cancer is the fourth most common cancer^[1]. In 2008, 988602 new cases were diagnosed and 737419 people died of the disease worldwide^[1]. In Japan and South Korea, gastric cancer is usually detected at an early stage owing to mass screening programmes^[2]. In Western countries, however, gastric cancer is mostly detected at a more advanced stage, which incurs a poorer prognosis. Clinical trials have shown that neoadjuvant chemotherapy improves overall and disease-free survival of patients with advanced gastric cancer^[3]. Perioperative chemotherapy based on the medical research council adjuvant gastric infusional chemotherapy-trial approach is currently an acceptable standard of care^[4]. This approach consists of continuous intravenous infusion of epirubicin, cisplatin, and 5-fluorouracil in three 21-d cycles preoperatively and three 21-d cycles postoperatively^[5]. However, not all patients benefit. The results of a meta-analysis indicated that the numbers needed to treat (NNT) with neoadjuvant chemotherapy to prevent one death in three years was as high as 84, whereas for a 3-year disease-free survival, the NNT was 8^[3]. Chemotherapy-

induced adverse effects, among which gastrointestinal problems and leukopenia, have been reported to occur in 8.8% and 18.1% of patients, respectively^[3]. In patients who will not respond sufficiently, costly but ineffective neoadjuvant chemotherapy should not be continued or, preferably, even not be started. There is therefore a need for a method to discriminate patients who will benefit from those who will not. In this article, the current role of imaging in the early prediction of response to neoadjuvant therapy in gastric cancer is outlined and future prospects are discussed.

SEARCH STRATEGY AND SELECTION CRITERIA

Original publications concerning the value of imaging in predicting histopathological response, survival, and/or improvement in quality of life to neoadjuvant therapy in patients with resectable gastric cancer were retrieved. Data for this review were identified by a computer-aided search in the PubMed/MEDLINE database. The terms (gastric cancer or stomach cancer) and (neoadjuvant or chemotherapy) and [magnetic resonance (MR), MR imaging (MRI), NMR, fluorodeoxyglucose, or 2-fluoro-2-deoxy-D-glucose (FDG), positron emission tomography or positron-emission tomography (PET), computed tomography (CT) or ultrasound or ultrasonography (US)] were used. Bibliographies of relevant articles were screened for other relevant articles. Abstracts and reports from meetings were excluded. Only papers published up to September 2013 were included. The literature search resulted in 9 original articles on anatomical imaging^[6-14] and 5 original articles on molecular imaging^[8,15-18] in the assessment of response to neoadjuvant chemotherapy in patients with gastric cancer.

ANATOMICAL IMAGING

At present, the majority of clinical trials evaluating cancer treatments for objective response in solid tumors are using the response evaluation criteria in solid tumors (RECIST)^[19]. Using these criteria, an assessment is made whether cancer patients improve ("respond"), stay the same ("stable") or worsen ("progression") during treatments^[19]. However, RECIST requires the presence of a measurable lesion, which is often not the case in gastric cancer^[6]. The Japanese classification of gastric carcinoma (JCGC) evaluation criteria were developed to evaluate response even for tumors without measurable lesions^[20]. Kurokawa *et al.*^[6] assessed the value of both the RECIST and JCGC criteria in patients who were enrolled in two phase II trials. In both trials, the safety and efficacy of S-1 plus cisplatin were evaluated. After completion of neoadjuvant chemotherapy, response evaluation using RECIST in the JCOG0405 trial was based only on CT findings, whereas tumor response evaluated with JCGC criteria was based on CT, barium X-ray, and endoscopic findings in the JCOG0210 trial. The hazard ratios (HRs) for death

of responders to non-responders using RECIST and JCGC criteria and were found to be nonsignificant (HR = 0.67, $P = 0.35$; and HR = 0.54, $P = 0.06$, respectively). In contrast, histological response was found to be a significant predictor of overall survival (HRs = 0.40, $P = 0.005$).

Liu *et al.*^[7] investigated 48 patients with gastric cancer who were treated with 3 cycles of oxaliplatin and 5-fluorouracil-based neoadjuvant chemotherapy. All patients underwent radical resection performed within 2 wk after ending chemotherapy. Pre- and post-chemotherapy short-axis diameter and volumetric mean tumor attenuation of target lymph nodes on contrast-enhanced CT images were measured. Tumor response was assessed by using both RECIST^[19] and adapted Choi criteria^[21]. According to the adapted Choi criteria^[21], tumor response was defined as at least a 10% decrease in the sum of short diameters or at least a 15% decrease in mean volumetric attenuation of target lymph nodes. The investigators found that both the RECIST and adapted Choi criteria had a significant predictive value for progression-free survival ($P = 0.037$ and $P < 0.001$, respectively) and overall survival ($P = 0.012$ and $P < 0.001$, respectively). However, the investigators found that RECIST might underestimate tumor response; post-therapy decreased tumor attenuation correlated with improved clinical outcome. They concluded that the adapted Choi criteria could be valuable to predict survival of these patients^[7].

Lee *et al.*^[8] used their own CT criteria to evaluate tumor response in 33 patients with advanced gastric cancer who were prospectively enrolled. All these patients underwent CT before and after four cycles (8 wk) of neoadjuvant chemotherapy, including oxaliplatin, 5-fluorouracil, and leucovorin. Patients underwent radical resection within 2 wk after the completion of neoadjuvant chemotherapy. The percentage diameter or volume reduction rate of the primary tumor and the largest lymph node at CT were compared to histopathological response. Histopathological tumor response was assessed using the histopathologic criteria by Mandard *et al.*^[22]. Patients with tumor regression grade 1-3 were defined as responders, whereas patients with tumor regression grade 4-5 were defined as non-responders. Lee *et al.*^[8] found that only the volume reduction rate of the primary gastric cancer at CT was found to be significantly correlated to histopathological tumor response. When the optimal cutoff level of the percentage volume reduction rate of the primary gastric tumor was determined to be 35.6%, a sensitivity of 100% and a specificity of 58.8% were achieved. When the optimal cutoff level of the percentage volume reduction rate was determined to be 64.5%, a sensitivity of 56.3% and a specificity of 88.2% were obtained^[8].

Guo *et al.*^[9] assessed the value of endoscopic ultrasonography (EUS), in 48 patients with advanced gastric cancer who underwent neoadjuvant chemotherapy for three cycles. The chemotherapy regimen consisted of leucovorin, 5-fluorouracil and oxaliplatin simultaneously. Radical gastric resection was performed 3 to 4 wk after

the third cycle of chemotherapy. EUS was performed before neoadjuvant chemotherapy and before R0 resection. T and/or N downstaging at EUS was used as criterion for tumor response. Using a cut-off point of more than two-thirds affected regressive and necrotic tumor cells within the tumor bed at histopathological analysis, EUS yielded a sensitivity of 72.2% and specificity of 90.0%. In the study of Guo *et al.*^[9], no correlation to survival was performed.

Ang *et al.*^[10] assessed the value of contrast-enhanced ultrasonography (CE-US) in 43 patients with advanced gastric cancer. US contrast agents are gas-filled microbubbles which behave as pure intravascular tracers, enabling assessment of the dynamic features of tumor vascularity. In Ang *et al.*^[10] study, patients randomly received either 5-fluorouracil plus oxaliplatin, or S-1 plus oxaliplatin as neoadjuvant chemotherapy regimen. Surgery was performed 3 to 5 wk after completion of neoadjuvant chemotherapy. All patients underwent CE-US before and after two courses of pre-operative neoadjuvant chemotherapy. The investigators stated that they assessed tumor response at CE-US according to the static change of ultrasonic echo, and the dynamic assessment of tumor vascularity and lymph nodes. Histopathological response was evaluated according to the criteria of Mandard's tumor regression grade^[22] and served as standard of reference. Patients with tumor regression grade 1-2 were defined as responders, whereas patients with tumor regression grade 3-5 were defined as non-responders. Ang *et al.*^[10] found a moderate sensitivity of 62.9% and specificity of 56.3%. Furthermore, they found that the overall accuracy of CE-US was not significantly better than that of CT using RECIST criteria ($P = 0.663$)^[10].

Other included studies used a combination of anatomical imaging modalities to assess tumor response^[11-14]. Park *et al.*^[11] prospectively investigated 40 patients with locally advanced gastric cancer who underwent neoadjuvant chemotherapy, consisting of 3 cycles of intravenous docetaxel and cisplatin on days 1 and 8 of a 3-wk cycle. Surgery was performed within 6 wk after the start of the third cycle. TNM-staging^[23] using EUS and CT was performed before and after neoadjuvant chemotherapy. The investigators found that the 3-year overall survival rate for patients downstaged with EUS for T and/or N-stage was greater than that for nondownstaged patients (69% *vs* 41%; $P = 0.05$). The 2-year recurrence-free survival rate was also better for the EUS-downstaged patients (77% *vs* 47%; $P = 0.04$). However, the differences in overall survival and recurrence-free survival between the patients downstaged with CT and those not downstaged were not found to be statistically significant. Park *et al.*^[11] did not give an explanation for the different prognostic values of EUS and CT.

D'Ugo *et al.*^[12] investigated 30 patients with resectable locally advanced gastric cancer who were treated with neoadjuvant polychemotherapy consisting of either a combination of etoposide, epirubicin, plus cisplatin, or of epirubicin, cisplatin, plus 5-fluorouracil. All patients underwent restaging with use of chest X-ray, CT, abdom-

inal ultrasonography after completion of preoperative chemotherapy. The investigators found that T-downstaging was significantly associated with survival.

In two studies a combination of endoscopic and CT findings was used to evaluate response^[13,14]. Lorenzen *et al.*^[13] retrospectively evaluated a cohort of 410 patients with locally advanced gastric cancer who were treated with neoadjuvant chemotherapy. Neoadjuvant chemotherapy consisted of at least 6 wk of oxaliplatin or cisplatin, plus 5-fluorouracil. Patients aged 60 years or younger and those with a good health status were additionally treated with paclitaxel. A minority of patients (15%) received chemotherapy with doxorubicin, etoposide, and cisplatin. EUS and CT were performed before treatment and in the last 3 d of every cycle of chemotherapy. Assessment of response to neoadjuvant therapy was based on reduction of primary tumor size, as measured by upper endoscopy and CT scan. Clinical response was predefined as a reduction in bidimensional tumor diameter of > 50% compared to the pretherapeutic findings. The researchers found an association between clinical response and overall survival: patients who had a response had an estimated 2- and 5-year survival rate of 86.4% and 72.5%, respectively, whereas patients who did not clinically respond to therapy had an estimated 2- and 5-year survival rate of 56.3% and 34.3%, respectively^[13]. Heger *et al.*^[14] retrospectively investigated 47 patients, of which most received two cycles of platinum, 5-fluorouracil, and leucovorin-based chemotherapy with or without the addition of paclitaxel lasting 36 d each with a 2-wk interval between the two cycles. All patients received baseline endoscopy and CT scans, and after 50% (6 wk) of their chemotherapy. Clinical response was defined as a reduction of > 75% of the tumor mass at endoscopy and > 50% in tumor wall diameter at CT. Patients with less than 10% residual tumor were classified as histopathological responders^[24]. Endoscopic and CT response were found to be significantly associated with histopathological response and overall survival^[14].

MOLECULAR IMAGING

Metabolic PET imaging using the glucose analog ¹⁸F-DG is widely used in clinical oncology. The prospective study by Ott *et al.*^[15], published in 2003, was one of the first to show its potential value in early response monitoring to neoadjuvant chemotherapy in gastric cancer. The investigators performed FDG PET at baseline and 14 d after initiation of polychemotherapy consisting of cisplatin, leucovorin, and 5-fluorouracil, in 44 patients with locally advanced gastric cancer. A reduction of tumor FDG standardized uptake value (SUV) by more than 35% between the two scans was used as a predefined criterion for response. Response at PET was correlated to histopathologic response after completion of therapy (defined as < 10% viable tumor cells in the resected specimen^[24]) and patient survival. In 20% of patients, the primary tumor was visualized with insufficient contrast for quantitative analysis. In the remaining patients, the authors found that

response at PET predicted histopathologic response in 77% of responders and 86% of nonresponders. Median overall survival for patients with response at PET had not been reached (2-year survival rate, 90%), whereas for patients without a response at PET, median survival was only 18.9 mo (2-year survival rate, 25%; $P = 0.002$). Aforementioned study by Ott *et al.*^[15] was expanded with another 27 patients (total: 71 patients) with longer follow-up^[16]. Again, responders at PET showed a high histopathologic response rate (69%) and a favorable prognosis (median survival not reached), whereas metabolic nonresponders showed a histopathologic response in only 17% and had a poor prognosis (median survival of 24.1 mo). However, later studies by researchers from other institutions^[8,17] could not confirm Ott *et al.*^[15,16] findings: Lee *et al.*^[8] investigated the value of FDG PET in 33 patients with advanced gastric cancer. FDG PET was performed before and after four cycles (8 wk) of neoadjuvant chemotherapy, including oxaliplatin, 5-fluorouracil, and leucovorin. Using FDG PET, the reduction rate of the maximum SUV of the primary gastric tumor was assessed. Histopathological tumor response was defined as dominant fibrotic changes with a few tumor cells or groups or more regression. The percentage change in maximum SUV did not significantly correlate to the histopathologic grade of tumor regression^[8]. Vallböhmer *et al.*^[17] investigated 42 patients with advanced gastric cancer who received two cycles of neoadjuvant chemotherapy consisting of a combination of cisplatin, leucovorin, and 5-fluorouracil. They found no significant correlation between pretreatment maximum tumor SUV, maximum SUV 2 wk after completion of neoadjuvant chemotherapy, and change in maximum SUV between the two scans, and histopathological tumor response (defined as less than 10% vital residual tumor cells). Moreover, they did not find any significant correlation either between the aforementioned FDG PET parameters and overall survival. Thus, the results concerning the use of FDG PET in predicting response to neoadjuvant chemotherapy in gastric cancer seem controversial. Moreover, not all tumors show FDG uptake, which may limit its utility in many patients. Especially diffusely growing and mucus containing tumors may exhibit low FDG uptake^[25]. PET imaging with the proliferation marker ¹⁸F-fluorothymidine (FLT) may be an attractive alternative. In the study by Herrmann *et al.*^[26], all primary tumors showed focal FLT uptake, whereas as much as 31% did not show FDG uptake. Another study by Ott *et al.*^[18] tested the predictive value of FLT and compared it to that of FDG. They prospectively included 45 patients who underwent PET imaging before and 2 wk after initiation of preoperative chemotherapy, consisting of a combination cisplatin, leucovorin, and 5-fluorouracil. Tumor FLT and FDG uptake were assessed at both time points. Imaging findings were compared to histopathological response (patients with less than 10% residual tumor cells were classified as responders^[24]) and survival. FLT uptake value 2 wk after start of chemotherapy was the only imaging parameter with significant prognostic impact on overall survival. FDG uptake was found to be a surro-

gate parameter for neither histopathological response nor overall survival prognosis^[18].

DISCUSSION

In this review, results of anatomical and molecular imaging modalities to predict tumor response to neoadjuvant chemotherapy in gastric cancer are outlined. Most of the included studies using anatomical imaging performed CT and/or EUS. Overall, these studies demonstrated that there is an association between anatomical tumor response and histopathological response and/or survival. Two studies evaluated the established RECIST criteria: one study found no association to survival^[6], whereas the study did find a significant association^[7]. Two studies demonstrated the usefulness of a combination of CT and endoscopic response evaluation^[13,14]. One of the included studies performed CE-US to evaluate tumor response^[10], but the sensitivity and specificity values they found seem too low to be used for response assessment in clinical practice. A clear disadvantage of anatomical imaging is that it takes time before gross tumor changes become apparent. Accordingly, the studies included in the review usually performed anatomical imaging late in the course of neoadjuvant treatment. Metabolic changes precede anatomical changes. Therefore, molecular imaging may predict tumor response to neoadjuvant chemotherapy much earlier in the course of treatment. Almost all of the included studies used FDG PET imaging. These studies^[8,15-18] yielded controversial results. These interstudy differences may be explained by different methods to evaluate tumor FDG uptake and by differences in patient populations and tumor heterogeneity. Instead of measuring FDG PET SUVmax, future studies may aim at assessing the value of (partial volume corrected) total lesion glycolysis (also known as metabolic tumor volume) as a new quantitative FDG PET/CT approach to provide both better pretreatment risk stratification and early therapy response assessment in gastric cancer^[27]. Only one of the studies assessed the value of FLT PET and found that FLT uptake value 2 wk after start of chemotherapy was an independent significant predictor of overall survival. Whether FLT PET is clinically useful to determine response to neoadjuvant chemotherapy in gastric cancer still remains to be further investigated.

The included studies used different study endpoints. Almost half of the included studies correlated imaging response to disease-free survival and/or overall survival, which are accepted study endpoints^[28,29]. Other studies (also) used histopathological tumor regression as surrogate marker for survival, which has the advantage that no patient follow-up is needed. Histopathologic tumor regression to cytotoxic therapy is considered to be a prognostic marker for long-term survival in gastric carcinoma, as has been shown by several studies^[24,30]. However, the included studies in this review used different criteria and cut-off points to define histopathologic tumor regression. In addition, the results of a recent study indicated that a multifactorial histopathological score, including the

UICC/AJCC ypT-category, ypN-category, and the degree of histopathological tumor regression, results in the most accurate prediction of survival for patients with gastric carcinoma after neoadjuvant chemotherapy followed by surgery^[31]. Future studies using histopathological analysis as study endpoint may adhere to this multifactorial scoring system^[31].

No studies using MRI to evaluate tumor response to neoadjuvant chemotherapy in gastric cancer were identified. Diffusion-weighted (DW)-MRI, in particular, is a promising MRI method^[32]. DW-MRI is based on the principle that treatment with chemotherapy causes necrosis or cellular lysis which will lead to increases in tissue water diffusivity, thus lowering signal intensity on high-b value images with corresponding increases in apparent diffusion coefficient values. Since cell death in response to treatment precedes changes in lesion size, changes in DW-MRI may be an effective early marker of response to therapies that induce apoptosis^[32]. DW-MRI for monitoring neoadjuvant therapy has already been applied in a wide variety of cancer types and organ sites, including the liver, breast, bone, soft tissue tumors, cervical tumors, head and neck tumors, as well as rectal cancer^[32]. DW-MRI of gastric cancer is feasible^[33], but technically challenging due to movement related to respiration, peristalsis and cardiac motion, and the presence of local field inhomogeneities. Other molecular imaging techniques are currently still under investigation. For instance, it has been shown that ⁸⁹Zr-trastuzumab PET can be used to delineate human epidermal growth factor receptor 2 (HER2)-positive gastric cancer and to monitor the pharmacodynamic effects of the epidermal growth factor receptor/HER2 tyrosine kinase inhibitor afatinib in mice^[34]. Future studies using molecular imaging techniques should investigate the optimal timing of imaging; if imaging is performed too early, no significant effect of neoadjuvant treatment may be demonstrated. On the other hand, imaging should also not be performed too late, in order to allow for a timely modification of therapy.

Several studies have investigated the value of clinicopathologic features to predict outcome in gastric cancer patients who are preoperatively treated with chemotherapy^[13,35,36]. One of the largest of these studies, by Lorenzen *et al.*^[13], retrospectively evaluated a cohort of 410 patients. Multivariate analysis showed that age, gender, body mass index, hemoglobin level, clinical staging and tumor location did not predict histopathological tumor response (defined as < 10% residual tumor cells^[24]) and overall survival. Yet, tumor location in the middle third of the stomach, well-differentiated tumors, and intestinal tumor type according to the Lauren classification were significantly and independently associated with both histopathologic response and better survival. These findings need confirmation by future independent studies.

The molecular genetic basis of carcinogenesis, cancer progression and drug resistance is complex. Like antibiotic-resistant bacteria, tumors often show resistance to anti-cancer drugs, leading to inefficient chemotherapy. Several

studies have shown promising results of predictive value of tumor biomarkers which can be obtained by biopsy. A possible advantage of this approach is that no “test period” of neoadjuvant chemotherapy may be needed before an assessment can be made whether the patient will benefit or not. For instance, it has been shown that expressions of certain chemotherapy-related genes are related to worse survival in gastric cancer patients who are treated with neoadjuvant chemotherapy^[37,38]. However, there is still a lack of an established (set of) biomarker(s) for chemotherapeutic response prediction of gastric cancer. Many other biomarkers are still under investigation^[39,40], but it is beyond the scope of this review to discuss these in full detail. For more information, the reader may refer to the excellent review article by Fareed *et al.*^[41].

In conclusion, studies have shown that there is an association between tumor response at anatomical imaging evaluation and histopathological response and survival in patients with gastric cancer who are treated with neoadjuvant chemotherapy. However, as it takes time for gross tumor changes to become apparent, anatomical imaging may be of limited value in the early assessment of neoadjuvant chemotherapy efficacy. Studies performing early response assessment with use of FDG PET demonstrate controversial results. The usefulness of other molecular imaging modalities, among which DW-MRI, remains to be investigated.

REFERENCES

- 1 **World Health Organization.** GLOBOCAN 2008. Available from: URL: <http://globocan.iarc.fr/factsheets/populations/factsheet.asp?uno=900>
- 2 **Leung WK,** Wu MS, Kakugawa Y, Kim JJ, Yeoh KG, Goh KL, Wu KC, Wu DC, Sollano J, Kachintorn U, Gotoda T, Lin JT, You WC, Ng EK, Sung JJ. Screening for gastric cancer in Asia: current evidence and practice. *Lancet Oncol* 2008; **9**: 279-287 [PMID: 18308253 DOI: 10.1016/S1470-2045(08)70072-X]
- 3 **Li W,** Qin J, Sun YH, Liu TS. Neoadjuvant chemotherapy for advanced gastric cancer: a meta-analysis. *World J Gastroenterol* 2010; **16**: 5621-5628 [PMID: 21105197 DOI: 10.3748/wjg.v17.i40.4542]
- 4 **Knight G,** Earle CC, Cosby R, Coburn N, Youssef Y, Malthaner R, Wong RK. Neoadjuvant or adjuvant therapy for resectable gastric cancer: a systematic review and practice guideline for North America. *Gastric Cancer* 2013; **16**: 28-40 [PMID: 22467061 DOI: 10.1007/s10120-012-0148-3]
- 5 **Cunningham D,** Allum WH, Stenning SP, Thompson JN, Van de Velde CJ, Nicolson M, Scarffe JH, Lofts FJ, Falk SJ, Iveson TJ, Smith DB, Langley RE, Verma M, Weeden S, Chua YJ. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. *N Engl J Med* 2006; **355**: 11-20 [PMID: 16822992 DOI: 10.1056/NEJMoa055531]
- 6 **Kurokawa Y,** Shibata T, Sasako M, Sano T, Tsuburaya A, Iwasaki Y, Fukuda H. Validity of response assessment criteria in neoadjuvant chemotherapy for gastric cancer (JCOG0507-A). *Gastric Cancer* 2013 Sep 3; Epub ahead of print [PMID: 23999869 DOI: 10.1007/s10120-013-0294-2]
- 7 **Liu K,** Li G, Fan C, Zhou C, Li J. Adapted Choi response criteria for prediction of clinical outcome in locally advanced gastric cancer patients following preoperative chemotherapy. *Acta Radiol* 2012; **53**: 127-134 [PMID: 22156007 DOI: 10.1258/ar.2011.110273]

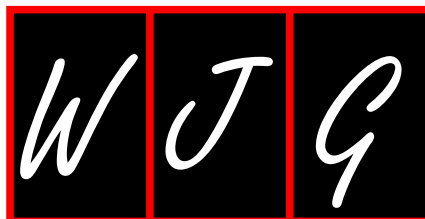
- 8 Lee SM, Kim SH, Lee JM, Im SA, Bang YJ, Kim WH, Kim MA, Yang HK, Lee HJ, Kang WJ, Han JK, Choi BI. Usefulness of CT volumetry for primary gastric lesions in predicting pathologic response to neoadjuvant chemotherapy in advanced gastric cancer. *Abdom Imaging* 2009; **34**: 430-440 [PMID: 18546037 DOI: 10.1007/s00261-008-9420-8]
- 9 Guo T, Yao F, Yang AM, Li XY, Zhong DR, Wu DS, Wu X, Lu XH. Endoscopic ultrasound in restaging and predicting pathological response for advanced gastric cancer patients after neoadjuvant chemotherapy. *Asia Pac J Clin Oncol* 2012 Dec 21; Epub ahead of print [PMID: 23279745 DOI: 10.1111/ajco.12045]
- 10 Ang J, Hu L, Huang PT, Wu JX, Huang LN, Cao CH, Zheng YX, Chen L. Contrast-enhanced ultrasonography assessment of gastric cancer response to neoadjuvant chemotherapy. *World J Gastroenterol* 2012; **18**: 7026-7032 [PMID: 23323004 DOI: 10.3748/wjg.v18.i47.7026]
- 11 Park SR, Lee JS, Kim CG, Kim HK, Kook MC, Kim YW, Ryu KW, Lee JH, Bae JM, Choi IJ. Endoscopic ultrasound and computed tomography in restaging and predicting prognosis after neoadjuvant chemotherapy in patients with locally advanced gastric cancer. *Cancer* 2008; **112**: 2368-2376 [PMID: 18404697 DOI: 10.1002/cncr.23483]
- 12 D'Ugo D, Persiani R, Rausei S, Biondi A, Vigorita V, Boccia S, Ricci R. Response to neoadjuvant chemotherapy and effects of tumor regression in gastric cancer. *Eur J Surg Oncol* 2006; **32**: 1105-1109 [PMID: 16930932 DOI: 10.1016/j.ejso.2006.07.009]
- 13 Lorenzen S, Blank S, Lordick F, Siewert JR, Ott K. Prediction of response and prognosis by a score including only pretherapeutic parameters in 410 neoadjuvant treated gastric cancer patients. *Ann Surg Oncol* 2012; **19**: 2119-2127 [PMID: 22395980 DOI: 10.1245/s10434-012-2254-1]
- 14 Heger U, Bader F, Lordick F, Burian M, Langer R, Dobritz M, Blank S, Bruckner T, Becker K, Herrmann K, Siewert JR, Ott K. Interim endoscopy results during neoadjuvant therapy for gastric cancer correlate with histopathological response and prognosis. *Gastric Cancer* 2013 Sep 1; Epub ahead of print [PMID: 23996162 DOI: 10.1007/s10120-013-0296-0]
- 15 Ott K, Fink U, Becker K, Stahl A, Dittler HJ, Busch R, Stein H, Lordick F, Link T, Schwaiger M, Siewert JR, Weber WA. Prediction of response to preoperative chemotherapy in gastric carcinoma by metabolic imaging: results of a prospective trial. *J Clin Oncol* 2003; **21**: 4604-4610 [PMID: 14673049 DOI: 10.1200/JCO.2003.06.574]
- 16 Ott K, Herrmann K, Lordick F, Wiedner H, Weber WA, Becker K, Buck AK, Dobritz M, Fink U, Ulm K, Schuster T, Schwaiger M, Siewert JR, Krause BJ. Early metabolic response evaluation by fluorine-18 fluorodeoxyglucose positron emission tomography allows in vivo testing of chemosensitivity in gastric cancer: long-term results of a prospective study. *Clin Cancer Res* 2008; **14**: 2012-2018 [PMID: 18381939 DOI: 10.1158/1078-0432.CCR-07-0934]
- 17 Vallböhmer D, Hölscher AH, Schneider PM, Schmidt M, Dietlein M, Bollschweiler E, Baldus S, Alakus H, Brabender J, Metzger R, Mönig SP. [18F]-fluorodeoxyglucose-positron emission tomography for the assessment of histopathologic response and prognosis after completion of neoadjuvant chemotherapy in gastric cancer. *J Surg Oncol* 2010; **102**: 135-140 [PMID: 20648583 DOI: 10.1002/jso.21592]
- 18 Ott K, Herrmann K, Schuster T, Langer R, Becker K, Wiedner HA, Wester HJ, Siewert JR, zum Büschenfelde CM, Buck AK, Wilhelm D, Ebert MP, Peschel C, Schwaiger M, Lordick F, Krause BJ. Molecular imaging of proliferation and glucose utilization: utility for monitoring response and prognosis after neoadjuvant therapy in locally advanced gastric cancer. *Ann Surg Oncol* 2011; **18**: 3316-3323 [PMID: 21537865 DOI: 10.1245/s10434-011-1743-y]
- 19 Response evaluation criteria in solid tumors. Available from: URL: <http://www.recist.com/>
- 20 Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma--2nd English edition--response assessment of chemotherapy and radiotherapy for gastric carcinoma: clinical criteria. *Gastric Cancer* 2001; **4**: 1-8 [PMID: 11706621 DOI: 10.1007/s101200100009]
- 21 Stacchiotti S, Verderio P, Messina A, Morosi C, Collini P, Lombart-Bosch A, Martin J, Comandone A, Cruz J, Ferraro A, Grignani G, Pizzamiglio S, Quagliuolo V, Picci P, Frustaci S, Dei Tos AP, Casali PG, Gronchi A. Tumor response assessment by modified Choi criteria in localized high-risk soft tissue sarcoma treated with chemotherapy. *Cancer* 2012; **118**: 5857-5866 [PMID: 22605504 DOI: 10.1002/cncr.27624]
- 22 Mandard AM, Dalibard F, Mandard JC, Marnay J, Henry-Amar M, Petiot JF, Roussel A, Jacob JH, Segol P, Samama G. Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma. Clinicopathologic correlations. *Cancer* 1994; **73**: 2680-2686 [PMID: 8194005 DOI: 10.1002/1097-0142]
- 23 American Joint Committee on Cancer. Greene FL, Page DL, Fleming ID, editors. *AJCC Cancer Staging Manual*. 6th edition. New York: Springer, 2002: 99-103
- 24 Becker K, Mueller JD, Schulmacher C, Ott K, Fink U, Busch R, Böttcher K, Siewert JR, Höfler H. Histomorphology and grading of regression in gastric carcinoma treated with neoadjuvant chemotherapy. *Cancer* 2003; **98**: 1521-1530 [PMID: 14508841 DOI: 10.1002/cncr.11660]
- 25 Stahl A, Ott K, Weber WA, Becker K, Link T, Siewert JR, Schwaiger M, Fink U. FDG PET imaging of locally advanced gastric carcinomas: correlation with endoscopic and histopathological findings. *Eur J Nucl Med Mol Imaging* 2003; **30**: 288-295 [PMID: 12552348 DOI: 10.1007/s00259-002-1029-5]
- 26 Herrmann K, Ott K, Buck AK, Lordick F, Wilhelm D, Souvatoglou M, Becker K, Schuster T, Wester HJ, Siewert JR, Schwaiger M, Krause BJ. Imaging gastric cancer with PET and the radiotracers 18F-FLT and 18F-FDG: a comparative analysis. *J Nucl Med* 2007; **48**: 1945-1950 [PMID: 18006614 DOI: 10.2967/jnumed.107.044867]
- 27 Van de Wiele C, Kruse V, Smeets P, Sathekge M, Maes A. Predictive and prognostic value of metabolic tumour volume and total lesion glycolysis in solid tumours. *Eur J Nucl Med Mol Imaging* 2013; **40**: 290-301 [PMID: 23151913 DOI: 10.1007/s00259-012-2280-z]
- 28 Pazdur R. Endpoints for assessing drug activity in clinical trials. *Oncologist* 2008; **13** Suppl 2: 19-21 [PMID: 18434634 DOI: 10.1634/theoncologist.13-S2-19]
- 29 Zhuang SH, Xiu L, Elsayed YA. Overall survival: a gold standard in search of a surrogate: the value of progression-free survival and time to progression as end points of drug efficacy. *Cancer J* 2009; **15**: 395-400 [PMID: 19826359 DOI: 10.1097/PPO.0b013e3181be231d]
- 30 Lowy AM, Mansfield PF, Leach SD, Pazdur R, Dumas P, Ajani JA. Response to neoadjuvant chemotherapy best predicts survival after curative resection of gastric cancer. *Ann Surg* 1999; **229**: 303-308 [PMID: 10077040]
- 31 Becker K, Reim D, Novotny A, Zum Büschenfelde CM, Engel J, Friess H, Höfler H, Langer R. Proposal for a multifactorial prognostic score that accurately classifies 3 groups of gastric carcinoma patients with different outcomes after neoadjuvant chemotherapy and surgery. *Ann Surg* 2012; **256**: 1002-1007 [PMID: 22968067 DOI: 10.1097/SLA.0b013e318262a591]
- 32 Thoeny HC, Ross BD. Predicting and monitoring cancer treatment response with diffusion-weighted MRI. *J Magn Reson Imaging* 2010; **32**: 2-16 [PMID: 20575076 DOI: 10.1002/jmri.22167]
- 33 Shinya S, Sasaki T, Nakagawa Y, Guiking Z, Yamamoto F, Yamashita Y. The usefulness of diffusion-weighted imaging (DWI) for the detection of gastric cancer. *Hepatogastroenterology* 2007; **54**: 1378-1381 [PMID: 17708258]
- 34 Janjigian YY, Viola-Villegas N, Holland JP, Divilov V,

- Carlin SD, Gomes-DaGama EM, Chiosis G, Carbonetti G, de Stanchina E, Lewis JS. Monitoring afatinib treatment in HER2-positive gastric cancer with 18F-FDG and 89Zr-trastuzumab PET. *J Nucl Med* 2013; **54**: 936-943 [PMID: 23578997 DOI: 10.2967/jnumed.112.110239]
- 35 **An JY**, Kim HI, Cheong JH, Hyung WJ, Kim CB, Noh SH. Pathologic and oncologic outcomes in locally advanced gastric cancer with neoadjuvant chemotherapy or chemoradiotherapy. *Yonsei Med J* 2013; **54**: 888-894 [PMID: 23709422 DOI: 10.3349/ymj.2013.54.4.888]
- 36 **Wang LB**, Teng RY, Jiang ZN, Hu WX, Dong MJ, Yuan XM, Chen WJ, Jin M, Shen JG. Clinicopathologic variables predicting tumor response to neoadjuvant chemotherapy in patients with locally advanced gastric cancer. *J Surg Oncol* 2012; **105**: 293-296 [PMID: 21882201 DOI: 10.1002/jso.22085]
- 37 **Napieralski R**, Ott K, Kremer M, Specht K, Vogelsang H, Becker K, Müller M, Lordick F, Fink U, Rüdiger Siewert J, Höfler H, Keller G. Combined GADD45A and thymidine phosphorylase expression levels predict response and survival of neoadjuvant-treated gastric cancer patients. *Clin Cancer Res* 2005; **11**: 3025-3031 [PMID: 15837757 DOI: 10.1158/1078-0432.CCR-04-1605]
- 38 **Liu K**, Qian T, Tang L, Wang J, Yang H, Ren J. Decreased expression of microRNA let-7i and its association with chemotherapeutic response in human gastric cancer. *World J Surg Oncol* 2012; **10**: 225 [PMID: 23107361 DOI: 10.1186/1477-7819-10-225]
- 39 **Tan IB**, Ivanova T, Lim KH, Ong CW, Deng N, Lee J, Tan SH, Wu J, Lee MH, Ooi CH, Rha SY, Wong WK, Boussioutas A, Yeoh KG, So J, Yong WP, Tsuburaya A, Grabsch H, Toh HC, Rozen S, Cheong JH, Noh SH, Wan WK, Ajani JA, Lee JS, Tellez MS, Tan P. Intrinsic subtypes of gastric cancer, based on gene expression pattern, predict survival and respond differently to chemotherapy. *Gastroenterology* 2011; **141**: 476-85, 485.e1-11 [PMID: 21684283 DOI: 10.1053/j.gastro.2011.04.042]
- 40 **Han Y**, Cai H, Ma L, Ding Y, Tan X, Chang W, Guan W, Liu Y, Shen Q, Yu Y, Zhang H, Cao G. Expression of orphan nuclear receptor NR4A2 in gastric cancer cells confers chemoresistance and predicts an unfavorable postoperative survival of gastric cancer patients with chemotherapy. *Cancer* 2013; **119**: 3436-3445 [PMID: 23821160 DOI: 10.1002/cncr.28228]
- 41 **Fareed KR**, Kaye P, Soomro IN, Ilyas M, Martin S, Parsons SL, Madhusudan S. Biomarkers of response to therapy in oesophago-gastric cancer. *Gut* 2009; **58**: 127-143 [PMID: 19091831 DOI: 10.1111/j.1365-2559.2009.03404.x]

P- Reviewers: Fang WL, Nunobe S **S- Editor:** Cui XM

L- Editor: A **E- Editor:** Wu HL





WJG 20th Anniversary Special Issues (8): Gastric cancer

Immunotherapy in gastric cancer

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Supported by Research Center for Innovative Cancer Therapy, Cancer Vaccine Development Division, Kurume University to Matsueda S; in part by the Office of Research and Development Medical Research Service Department of Veterans Affairs, Public Health Service grants DK067366 and DK56338 which funds the Texas Medical Center Digestive Diseases Center to Graham DY

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Received: September 27, 2013 Revised: November 15, 2013

Accepted: December 12, 2013

Published online: February 21, 2014

Abstract

Gastric cancer is the second most common of cancer-related deaths worldwide. In the majority of cases gastric cancer is advanced at diagnosis and although medical and surgical treatments have improved, survival rates remain poor. Cancer immunotherapy has emerged as a powerful and promising clinical approach for treatment of cancer and has shown major success in breast cancer, prostate cancer and melanoma. Here, we provide an overview of concepts of modern cancer immunotherapy including the theory, current approaches, remaining hurdles to be overcome, and the future prospect of cancer immunotherapy in the treatment of gastric cancer. Adaptive cell therapies, cancer vaccines, gene therapies, monoclonal antibody therapies have all been used with some initial successes in gastric cancer. However, to date the results in gastric cancer have been disappointing as current approaches often do not stimulate immunity efficiently allowing tumors continue

to grow despite the presence of a measurable immune response. Here, we discuss the identification of targets for immunotherapy and the role of biomarkers in prospectively identifying appropriate subjects or immunotherapy. We also discuss the molecular mechanisms by which tumor cells escape host immunosurveillance and produce an immunosuppressive tumor microenvironment. We show how advances have provided tools for overcoming the mechanisms of immunosuppression including the use of monoclonal antibodies to block negative regulators normally expressed on the surface of T cells which limit activation and proliferation of cytotoxic T cells. Immunotherapy has greatly improved and is becoming an important factor in such fields as medical care and welfare for human being. Progress has been rapid ensuring that the future of immunotherapy for gastric cancer is bright.

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Key words: Gastric cancer; Immune checkpoint; Immune escape; Adoptive cell therapy; Cancer vaccine; Antibody therapy; Predictive biomarker

Core tip: In the majority of cases gastric cancer is advanced at diagnosis and although medical and surgical treatments have improved, survival rates remain poor. Cancer immunotherapy has emerged as a powerful tool for cancer therapy and has recently shown major success in breast cancer, prostate cancer and melanoma. The field of cancer immunotherapy is in the midst of a huge transition due to the discovery of immunological networks and better understanding of the molecular mechanisms of immunosuppression in the cancer microenvironment. We discuss how immunotherapy will most likely play a major role in the cure of cancer.

Matsueda S, Graham DY. Immunotherapy in gastric cancer. *World J Gastroenterol* 2014; 20(7): 1657-1666 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1657.htm>

INTRODUCTION

Gastric cancer an inflammation-associated cancer etiologically related to infection with the human gastric bacterial pathogen, *Helicobacter pylori* (*H. pylori*)^[1]; gastric cancer is also the second leading cause of cancer-related deaths worldwide. *H. pylori* infection is typically acquired in childhood and can then be life-long. The infection is associated with infiltration of the gastric mucosa with both acute and chronic inflammatory cells. This inflammatory process results in progressive damage to the gastric mucosa and to transformation of the normal acid secreting mucosa into metaplastic epithelia consisting of combinations of pyloric (spasmolytic polypeptide-expressing) and intestinal metaplasia and ultimately to gastric cancer. Chronic atrophic gastritis is thus the soil from which gastric cancer arises.

Ultimately worldwide eradication of *H. pylori*, the fundamental cause of gastric cancer, will prevent the entire process and gastric cancer will become a rare disease. Until then, we must deal with the innumerable people now living with active *H. pylori* infection who will develop gastric cancer. Treatment choices for gastric cancer depend on tumor type and stage. Currently, the only hope for cure rests on removal of the malignant tissue either endoscopically or by surgical resection. For advanced disease, treatment is largely palliative and consists of a combination of surgery, chemotherapy, and radiation. Overall, the results of current therapy for advanced disease are poor with low 5 years survivals. Immunotherapy provides another dimension to the prevention and management of gastric cancer and offers hope of breaking through current constraints.

HUMAN IMMUNE SYSTEM AGAINST TUMORS

The immune system is designed to discriminate “self” from “non-self” such that when something is recognized as non-self, the immune system attempts to eliminate it. The immune system can be thought of as patrolling the body to recognize and destroy pathogens as well as nascent transformed cells. Cancers are caused by the progressive growth and spread of the progeny of single transformed cell. It is likely that tumor cells appear daily in healthy individuals but in the vast majority of instances they are removed by the immune system and do not develop into clinical malignancies. This ability of the immune system to detect tumor cells as non-self and destroy them is called “immunosurveillance”^[2]. It is currently thought that immunosurveillance primarily functions by immunoediting. “Cancer immunoediting” has been described as both the host protective and as promoting the ability of the tumor to resist the immune response. Immunoediting goes through three main

phases: elimination, equilibrium and escape. Tumors are recognized by innate and adaptive immune cells which recognize the local tissue damaged caused when the growing tumors begins to remodel the stroma. Innate and adaptive immune cell, natural killer (NK) cells, NK T cells, CD8⁺ T cells, CD4⁺ T cells, secrete interferon (IFN)- γ which inhibits angiogenesis and proliferation of tumor cells. Macrophages and dendritic cells are also recruited and secrete cytokines to activate immune cells to phagocytize and remove dead tumor cells. If successful progression to clinical cancer is prevented (Figure 1A). Tumor cells killed in the process are digested by dendritic cells for presentation to T cells. If some tumor cells survive the elimination phase, immunoediting enters the equilibrium phase during which the residual tumor cells remain in equilibrium under pressure from the immune system. This phase it typically the longest of the three phases of cancer immunoediting. CD8⁺ T cells and dendritic cells which secrete IFN- γ and interleukin (IL)-12, respectively maintain the tumor cells in a state of functional dormancy. During this time, because the tumor cells are highly heterogeneous and genetically unstable, they may change their characteristics/populations in response to immune system editing and escape suppression (Figure 1B). In an immunosuppressed state within the tumor microenvironment allowing the tumor cells to escape from the immune system and begin to grow. The proliferation of immune cells is also reduced and tumor-specific effector cells experience apoptosis such that regulatory T cells (Tregs) associated immunosuppression occurs (Figure 1C). Cancer immunotherapy is designed to prevent the immunoediting process by enhancing the ability of the immune system to destroy the tumor.

The ultimate goal of immunotherapy is to achieve cancer cures by inducing an effective immune response against the tumor cells. Immune therapy of cancer is based on using the normal immune system to eliminate or control a malignancy. The presence of a tumor means the tumor cells are either not recognized as non-self or possess mechanisms to evade or overcome immunosurveillance. Research in cancer immunology is currently focused how overcome these blocks and to train the immune system to identify and target cancers for elimination. Further advances are predicated on better understanding of how to overcome the ability of tumor cells to evade being eliminated.

Theoretically, if specific antigens can be identified in the precursor lesions leading to cancer, the immune responses could also be utilized eliminate them which would prevent tumors from ever developing. Gastric cancer is potentially an ideal target for such preventive immunotherapy as it has a long latent period and clearly recognizable premalignant lesions which if successfully targeted could prevent progression to frank cancer.

WHAT IS IMMUNOTHERAPY?

Traditionally, immunotherapy considered to have begun in 1798 when Edward Jenner showed that inoculation

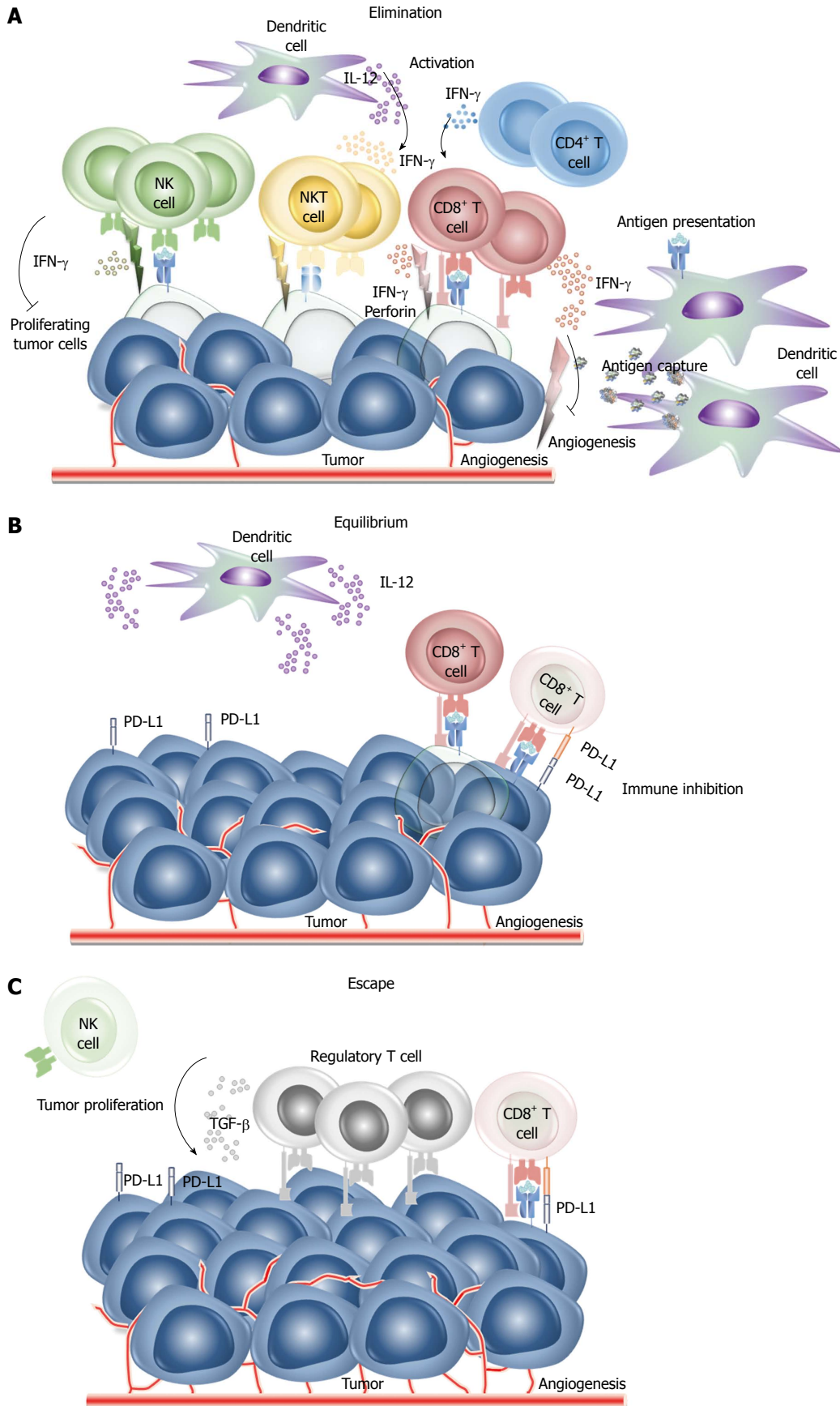


Figure 1 Cancer immunoediting phases. A: Phase 1: Elimination. Tumor cells are recognized by innate and adaptive immune cell and are destroyed before they can become a clinical malignancy. Modified after Dunn GP^[69]; B: Phase 2: Equilibrium. If tumor cells are not destroyed in the elimination phase, the tumor may enter an equilibrium phase. Genetic change and/or resistant to immune detection occur in the equilibrium phase and tumor cells are maintained chronically. Modified after Dunn GP^[69]; C: Phase 3: Escape. The tumor microenvironment allows that tumor cells to grow and change to become poorly immunogenic. The tumor microenvironment becomes immunosuppressive. Modified after Dunn GP^[69]. NK: Natural killer; PD: Programmed death; IFN: Interferon; IL: Interleukin.

with cowpox could prevent smallpox in humans. Since that time, a variety of different immunotherapies have been utilized to control diseases. Initially the focus was on vaccination and serum therapies. The history of cancer immunotherapy began in 1891 when William B Coley injected streptococcal organisms into a patient with inoperable cancer^[3] resulting in shrinkage of the malignant tumor. That experiment suggested that it might also be possible to utilize natural defense mechanisms to rid the body of a malignancy. Success with tumor immunotherapy has been slow as the immune system is exquisitely regulated with multiple checkpoints and feedbacks to prevent damage to the host. Further success requires a detailed understanding of the immune system which is only now beginning to be achieved.

Current immunotherapies are often based on use of monoclonal antibodies, cytotoxic immunocytes, or gene transferred vaccines. Monoclonal antibodies can also be used as alternatives to the traditional approach of administering small molecules (*i.e.*, drugs) to inhibit factors critical for tumor growth and survival. The human monoclonal antibody SC-1 was isolated from a patient with ring cell carcinoma and SC-1 antibody inhibited tumor cell growth by inducing apoptosis of tumor cells^[4]. Solid tumors require growth of blood vessels to survive and grow. Vascular endothelial growth factor (VEGF) plays an important role in this process by stimulating new blood vessel formation (*i.e.*, angiogenesis) and anti-VEGF antibody has been used as immunotherapy to bind the growth factor thus inhibit angiogenesis. While, the search for targets that when inhibited by monoclonal antibodies reduce tumor growth is a major effort in cancer research, this review focuses on cellular immunotherapy. Monoclonal antibodies may still play a role because antibodies directed against tumor-specific antigens can be used to target the cellular immune system to destroy tumors. For example, the receptor human epidermal growth factor receptor 2 (HER-2/neu) is often overexpressed in breast cancer. Administration of anti-HER-2/neu monoclonal antibodies results in the antibodies binding to tumor cells which is followed by the attraction and activation of effector cells, such as NK cells and monocytes (*via* their Fc receptors) and ultimately, lysis of the tumor cells.

CELLULAR IMMUNOTHERAPY

The cellular immune response can employ the innate (*e.g.*, NK cells, macrophages, and eosinophils) or adaptive (CD8⁺ and CD4⁺ cells) immune response, or both. The response is mounted when specialized cytotoxic cells are induced to recognize and directly attack tumor cells based on expression of antigens on the tumor cell surface called tumor rejection antigens. Tumor rejection antigens are peptides of tumor cell proteins that are recognized by the immune system when presented to T cells by major histocompatibility complex (MHC) molecules. These peptides then become the targets of a tumor-spe-

cific T cells response. The actual strategy requires both choice of the target peptide and the immunocytes for therapy (*i.e.*, success requires both identifying a target and a strategy to attack that target). Because tumors primarily consist of self the possibility remains that any attempt at immunotherapy against a tumor would also attack normal non-tumor tissues. Clinically successful immunotherapy must therefore be able to tread the delicate line between attacking the tumor while doing minimal damage to normal tissues.

STRATEGIES AND CURRENT APPROACHES FOR IMMUNOTHERAPY IN GASTRIC CANCER

Current cellular immune strategies rely on the use of immunocytes designed to either activate tumor specific cytotoxic T cells to lyse tumor cells, or to specifically bind to target molecules or proteins expressed on the malignant tumor cells. A number of tumor rejection antigens have been identified. Experimental vaccination strategies have included use of whole protein and peptide vaccines and are based on identification of peptides recognized by cytotoxic T lymphocytes and helper T lymphocytes. Tumor rejection antigens melanoma-associated antigen 3 (MAGE-3) and HER-2/neu are examples of antigens selectively expressed in human tumors including gastric cancer which can be recognized by cytotoxic T cells.

ADOPTIVE CELL THERAPY

The transfusion of tumor-specific T cells into a cancer patient is called "adoptive cell therapy". A number of different cell types can be used such as killer cells, lymphokine-activated killer cells^[5], tumor infiltration lymphocytes (TILs)^[6], anti-CD3 monoclonal antibody-induced killer cells^[7], and cytokine induced killer cells^[8] (Figure 2). The first trial of adoptive cell therapy in humans utilized lymphokine-activated killer cells. In that study patients with metastatic melanoma were treated with the combination of lymphokine-activated killer cells plus IL-2^[9]. IL-2 was used to ensure the survival and sustained activation of the infused lymphokine-activated killer cells. IL-2 is a cytokine produced by human T lymphocytes that is necessary for the growth, proliferation, and differentiation of T cells to become effector T cells and was approved for the treatment of metastatic melanoma in 1998^[10]. This approach has resulted in marked tumor regression in up to or approximately 30% of the patients with renal-cell, melanoma, colorectal, non-Hodgkin's lymphoma, and lung cancer showing proof of principle^[11].

TILs are lymphocytes isolated from the patient's tumor. TILs have been used for immunotherapy of gastric cancer^[12]. TILs are potentially especially useful because they already recognize some tumor-specific antigens in that tumor. Adoptive immunotherapy with TILs has provided promising results in preclinical studies in sarcoma

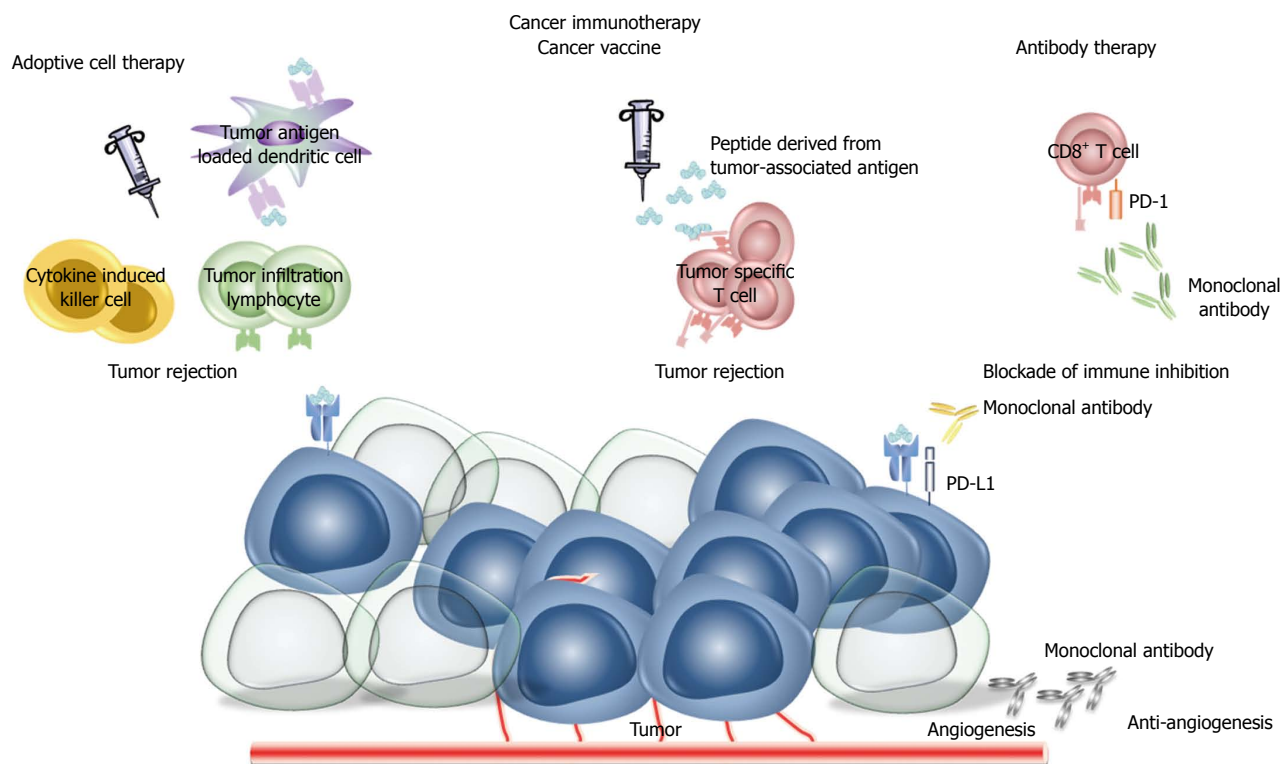


Figure 2 Cancer immunotherapy. Immunological approaches to cancer therapy are based on use of cytotoxic immunocytes (cytokine induced killer cells, tumor infiltration cells, tumor antigen loaded dendritic cells), cancer vaccines, monoclonal antibodies. Therapeutic vaccines enhance pre-existing immunity and lead to a more robust antitumor immune response whereas monoclonal antibodies are used to inhibit critical molecules for tumor growth and survival. PD: Programmed death.

and colonic adenocarcinoma^[13]. A clinical study of adoptive immunotherapy with tumor-associated lymphocytes in combination with chemotherapy in gastric cancer resulted in a longer 50% survival with the combination of adoptive immunotherapy and chemotherapy than with chemotherapy alone^[14].

Cytokine induced killer cells are rapidly proliferating lymphocytes with strong anti-tumor activity. These cells are generated by the *in vitro* expansion of peripheral blood lymphocytes using the combination of anti-CD3 antibodies and IL-2. The antigen receptor molecules on T cells are non-covalently associated on the cell surface with the CD3 molecular complex and perturbation of the complex with anti-CD3 monoclonal antibodies induces T cell activation^[15]. Clinical studies have confirmed a survival benefit in gastric cancer patients treated with chemotherapy combined with cytokine induced killer cells compared to chemotherapy alone^[16,17].

CANCER VACCINES

Cancer vaccines are designed to activate and expand tumor-specific T cells as effector T cells. Therapeutic vaccines can enhance pre-existing immunity, induce novel immunity, or lead to a more robust anti-tumor immune response (Figure 2). In order to induce tumor-specific T cells, peptides derived from tumor-associated antigens must be presented to T cells by professional antigen-presenting cells, such as dendritic cells, which are the most powerful and efficient antigen-presenting cells able to

activate naïve and memory T cells^[18]. Immature dendritic cells with high phagocytic capacity are localized to sites where tumor cells grow. They take up antigens which are digested into small oligopeptides which are then loaded onto the MHC class I molecule for presentation to CD8⁺ cytotoxic T cells or to MHC class II molecules for presentation to CD4⁺ helper T cells. The process can also be done *in vitro*. For this, monocytes are obtained by apheresis and are induced to form immature dendritic cells with cytokines (GM-CSF, IL-4). The immature dendritic cells are then cultured *in vitro* with tumor lysates or peptides derived from tumor-associated antigens and the cytokine tumor necrosis factor (TNF)- α , IL-1 or IFN- γ . The mature dendritic cells that develop are then injected to patients by the intradermal or intravenous routes where they present antigens to T cells to induce a robust anti-tumor immune response.

Tumor-associated antigens are defined as antigens expressed on tumor cells that can elicit an immune response in the host. Thousands of potential tumor associated antigens have been identified and many studies have confirmed that cytotoxic T cells activated by immunogenic peptides derived from tumor-associated antigens presented on the surface of tumor cells with MHC-I are capable of lysing tumor cells^[19-22]. Both protein and peptide targets have been used to attempt to stimulate a specific immune response in gastric cancer. Those experiments have been based on peptides derived from the tumor associated antigen HER2/neu-derived peptide^[19] and MAGE^[23-27] which are restricted to MHC class I have

been shown to induce cytotoxic T cells against tumors. Gastric cancers typically overexpress HER-2/neu and vaccination using dendritic cells pulsed with HER-2/neu peptide has resulted in tumor regression. MAGE-3 peptide/chitosan-deoxycholic acid vaccine-loaded nanoparticles have also been used to simulate an antitumor immune response and successfully produced regression of tumor growth in a mouse model of gastric cancer^[28].

Peptides derived from human vascular endothelial growth factor (VEGF) receptor 1 and vascular endothelial growth factor receptor 2 combined with chemotherapy (S-1 plus cisplatin) have been shown to induce a VEGF-specific cytotoxic lymphocyte response in patients with advanced gastric cancer resulting in a partial response in 55% of patients as well as prolonged overall survival^[29] suggesting that cancer vaccines combined with standard chemotherapy may be a promising strategy for the treatment of advanced cancer.

RNA-BASED VACCINES

Dendritic cells incubated with mRNA are capable of presenting the encoded antigen^[30] making mRNA-based gene transfer vaccine an attractive possibility for immunotherapy^[31,32] (Figure 2). Dendritic cells transfected with mRNA coding for a tumor-associated antigen or whole tumor RNA have been able to induce potent antigen- and tumor-specific T cell responses. The generation of immune responses with naked but stabilized mRNA has also been accomplished in mouse models^[33-37] and clinical trials have been encouraging in melanoma^[38,39] and renal cell carcinoma^[40].

There are a number of potential advantages of RNA-based vaccines. For example, naturally transient and cytosolic active mRNA molecules are considered to be a possibly safer pharmaceutical because of expression is transient and the absence of genomic integration. The mRNA application also allows targeting multiple tumor-associated antigens simultaneously^[39]. RNA vaccination does not cause severe side effects such as the generation of autoimmune disease or anti-DNA antibodies and finally, unlike peptide-based vaccinations it is not MHC-restricted.

HOW CANCERS EVADE THE IMMUNE RESPONSE

Immune escape and immunosuppressive tumor network

The key cells of the immune system for tumor surveillance are T cells and NK cells. However, despite the theoretical advantages of immunotherapy, current approaches often do not stimulate immunity efficiently and the tumors continue to grow despite the presence of an immune response^[41-43]. Multiple mechanisms have been identified allowing tumors to escape rejection by the immune system^[44-46]. Theoretically, downregulation or loss of HLA class I antigen in cancers would be an important evasion mechanism and has been reported. However,

expression of HLA class I antigens has not been shown to correlate with any important clinical or pathologic parameters of gastric cancers^[47]. Other mechanisms are downregulation of antigen expression on tumor cells and production of immunosuppressive cytokines [transforming growth factor (TGF)- β 1, IL-10, IL-6, VEGF, prostaglandin] by the tumor.

There is considerable current interest in Tregs and MSCs as major components of the immune suppressive tumor microenvironment. Tregs cells inhibit cytotoxic lymphocytes and/or helper T activity as well as NK cells. Tregs are characterized by the CD4⁺CD25⁺FOXP3⁺ phenotype and normally play an indispensable role in maintaining immunological tolerance to self-antigens and in suppressing excessive immune responses that would be deleterious to the host. Regulatory immune cells, mostly Tregs, have been identified as the major regulatory component of the adaptive immune response and are also involved in *H. pylori*-related inflammation and bacterial persistence^[48]. For example, *H. pylori*-induced gastritis is regulated by Tregs. Tregs play an important role in the equilibrium between *H. pylori* and immune system and a better understanding of the role of these cells in immunosuppression in the tumor environment should lead to approaches to blunt or eliminate Treg-associated immunosuppression.

Recently, the role of mesenchymal- or bone marrow-derived stem cells (BM-MSCs) for the malignant transformation has been studied^[49,50] and are known to migrate to tumor issues^[51]. BM-MSCs into a chronic *H. pylori*-infected mouse model showed the generation of an immunosuppressive environment. The local and systemic immunosuppression mediated by BM-MSCs likely contributed to an environment that is compatible with the development of *H. pylori*-induced gastric cancer^[52]. It has been demonstrated that this cell population can serve as a “seeding point” for gastric carcinogenesis in animal models but the relevance with respect to human disease still remains unclear^[50]. Development of immunotherapies targeted to Tregs and BM-MSCs is an attractive new strategy to activate antitumor immunity in patients with cancer.

Eliminating both tumor and lymphocyte-mediated immune suppressive mechanisms without damaging normal cells also holds promise. Specifically, the blockade of secreted immunosuppressive molecules, (*e.g.*, TGF- β 1, IL-10 or prostaglandins) may be required in addition to eliminating Tregs.

Immune checkpoint

Immune checkpoints are inhibitory pathways hardwired into the immune system that are crucial for maintaining self-tolerance and modulating the duration and amplitude of physiological immune responses in order to minimize collateral tissue damage. Thus, immune checkpoints play critical roles for physiological homeostasis especially in protection of tissues from damage when the immune system responds to infections. These checkpoints may also allow immune escape in cancer.

Checkpoint pathways are regulated by ligand/receptor interactions. For example, programmed death-1 receptor (PD-1) and CTL-associated antigen 4 (CTLA-4) are inhibitory molecules whose presence on lymphocytes signifies a blunted immune response. PD-1 negatively regulates T cell responses and downregulation and eventually apoptosis is initiated following binding of a PD-1 ligand with PD-1. PD-1 ligands, PD-L1 or PD-L2, are frequently expressed on tumor cells and can thus thwart the immune response. One approach to overcome this inhibition of the immune response has been to target immune checkpoints with blocking monoclonal antibodies (mAb) (Figure 2). For example, PD-1 mAb binds to the PD-1 receptors on T cells and inhibits their binding to the ligands on tumor cells thus preventing the tumors from down regulating the cytotoxic lymphocyte response. This approach has been successful clinically. For example, anti-CTLA-4 mAb is the basis for immunotherapy producing a survival benefit in advanced melanoma^[53,54]. Phase I clinical trials of anti-PD-L1 mAb are under investigation for gastric cancer. Other mechanisms focus on the T-cell immunoglobulin domain and mucin domain 3 for promoting inflammation or to restrain a T helper 1 cell response. Inducible T-cell co-stimulator is a CD28-superfamily co-stimulatory molecule expressed on activated T cells and considered important for Th2 cell, B and T lymphocyte attenuator whose activation inhibits the function of tumor-specific cytotoxic T cells^[55]. These co-stimulatory and co-inhibitory receptors modulate the function of both antigen-presenting cells and T cells. The available immunostimulatory monoclonal antibodies have not proved sufficient suggesting that there must be other target molecules that are extracellularly accessible and are candidates for manipulation with monoclonal antibodies in cancer therapy. We expect more will be discovered and developed in the future.

FUTURE PROSPECTS FOR IMMUNOTHERAPY

Biomarkers are needed to predict good candidates for immunotherapy

The use of immunotherapy would be enhanced if one could identify biomarkers predictive of response and thus allow matching of treatments with suitable patients. HER2 has proven to be an excellent biomarker in breast cancer. Trastuzumab (herceptin), a humanized anti-HER2 receptor monoclonal antibody, has been shown to improve the outcome in patients with HER2-positive metastatic breast cancer^[56]. The combination of pertuzumab (HER2-targeted humanized monoclonal antibody) plus trastuzumab plus docetaxel has been compared with placebo plus trastuzumab plus docetaxel as first-line treatment for HER2-positive metastatic breast cancer and significantly prolonged progression-free survival^[57]. HER2 is also expressed in gastric cancer suggesting that this approach may show therapeutic efficacy. There is significant current interest in identifying predictive biomarkers

in cancer in general and in gastric cancer in particular.

Recently, DNA microarray technology have been developed and extensively used to search for new biomarkers for individualized therapies^[58-62]. Gene expression profiles in tumor tissues, TILs and peripheral blood have been reported to clearly reflect clinical outcomes and/or responses to treatments in cancer patients. Furthermore, expression array data of peripheral blood and TILs have also shown an association with survival and immune response^[60,62]. Gene expression profiling is developing into a mainstream tool for the assessment of immune system and monitoring immune responses to drugs or therapies.

Engineered cellular immunotherapy

Rapid advances in understanding of the details of the molecular events and regulatory pathways involved in effective use of cytotoxic cells as anti-tumor therapy have prompted work on developing customized or engineered cells. The ideal regimen would be one that targeted antigens that are specific to a particular type of malignancy and then engineer cells programmed to evade the tumors repertoire of anti-immune defenses and to respond to those antigens. Challenges include (1) identification of one or preferably several antigens specific to the tumor; (2) programming the appropriate cytotoxic cells to respond to only those antigens; (3) ensuring that the cytotoxic cells were capable of avoiding the tumor's defenses; and (4) engineering signals to initiate the process as well as signals to end the attack if or when this becomes desirable.

Genetic tools have been developed to engineer T-cell specificity and enhance T cell function. Chimeric antigen receptors are receiving increasing attention and becoming a promising new therapeutic method. Chimeric antigen receptors lead to enhanced proliferation, cytotoxicity, and persistence *in vivo*. Apheresed T cells from a patient are stimulated with CD3 antibody and IL-2. Activated cells are then transduced with the chimeric antigen receptors using a retro- or lentiviral platform. Because the chimeric antigen receptor is integrated into the T-cell genome, all daughter cells that are generated during this expansion also express the chimeric antigen receptor. Chimeric antigen receptor-transduced T cells are then infused into patients^[63]. Early clinical studies have revealed a very encouraging therapeutic efficacy of chimeric antigen receptor-mediated immunotherapy in a variety of cancers including lymphoma, chronic lymphocytic leukemia, melanoma, and neuroblastoma^[64]. Despite the promising results obtained from clinical trials with infusion of chimeric antigen receptor-modified T cells, some severe adverse events have been reported^[65-67]. Recent reports have highlighted key issues and future directions to avoid these adverse events^[68]. Selection of candidate target antigens is essential for improved efficacy and safety of the chimeric antigen receptor-based therapy.

Although one can also theoretically strip the effector cells of all checkpoints, it is important that such cells have as much tumor specific ability as possible to pre-

vent the cells from becoming indiscriminate killers. The fundamental problem is to unleash an attack capable of elimination of the target but one that is restrained and does not do irreversible harm to the host. Ideally, one would like to be able to control all of the elements of the process from choosing the most tumor specific antigens and then be able to engineer the cells to respond only to those antigens and at the same time be able to control when and where the attack is focused. Thus, the cells might be engineered with a safety switch that can be switched to on and initiate the process and then switched off if the cells move out of the area or the process is completed. The “off” switch might consist of implementation of a suicide program that completely eliminated the cells.

Identification of one or preferably several antigens specific to the tumor

Because the primary tumor or metastasis may be difficult to sample, the challenges to identification of the appropriate antigens include obtaining access to the tumor or tumor cells to identify critical antigens specific that tumor (*e.g.*, cancer testis antigens) or identification of antigens that are expressed on the majority of similar tumors (*e.g.*, universal antigens). Advances in obtaining circulating tumor cells and analyzing them would help not only monitoring efficacy of therapies, but also useful to identify appropriate, effective and specific tumor antigen or molecules for metastasis. It also expected to provide selection of appropriate therapy for individual patients by characterization of circulation tumor cells.

CONCLUSION

The practice and theory of cancer immunotherapy has seen major advancements during the past 20 years. However, many hurdles to remain to be overcome before cancer immunotherapy becomes the first line and most reliable and effective cancer treatment. In view of the complexity and diversity of tumors and immune cell repertoires, it would be of critical importance to identify new target molecules or develop new ways to utilize already known targets, and expand knowledge of the effectiveness of combinations of immunotherapies with conventional therapies. In addition, it is essential to identify reliable biomarkers to identify candidates that would most benefit from immunotherapy, and/or early diagnosis to prevent cancer progression as a vital part of that therapy.

REFERENCES

- 1 Chiba T, Marusawa H, Ushijima T. Inflammation-associated cancer development in digestive organs: mechanisms and roles for genetic and epigenetic modulation. *Gastroenterology* 2012; **143**: 550-563 [PMID: 22796521 DOI: 10.1053/j.gastro.2012.07.009.]
- 2 Burnet M. Cancer; a biological approach. I. The processes of control. *Br Med J* 1957; **1**: 779-786 [PMID: 13404306]
- 3 McCarthy EF. The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas. *Iowa Orthop J* 2006; **26**: 154-158 [PMID: 16789469]
- 4 Vollmers HP, Dämmrich J, Ribbert H, Wozniak E, Müller-Hermelink HK. Apoptosis of stomach carcinoma cells induced by a human monoclonal antibody. *Cancer* 1995; **76**: 550-558 [PMID: 8625146]
- 5 Rosenberg S. Lymphokine-activated killer cells: a new approach to immunotherapy of cancer. *J Natl Cancer Inst* 1985; **75**: 595-603 [PMID: 3876465]
- 6 Rosenberg SA, Spiess P, Lafreniere R. A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. *Science* 1986; **233**: 1318-1321 [PMID: 3489291]
- 7 Yun YS, Hargrove ME, Ting CC. In vivo antitumor activity of anti-CD3-induced activated killer cells. *Cancer Res* 1989; **49**: 4770-4774 [PMID: 2527087]
- 8 Rutella S, Iudicone P, Bonanno G, Fioravanti D, Procoli A, Lavorino C, Foddai ML, Lorusso D, Martinelli E, Vacca M, Ipsevich F, Nuti M, Scambia G, Pierelli L. Adoptive immunotherapy with cytokine-induced killer cells generated with a new good manufacturing practice-grade protocol. *Cytotherapy* 2012; **14**: 841-850 [PMID: 22563888 DOI: 10.3109/14653249.2012.681038]
- 9 Rosenberg SA, Lotze MT, Muul LM, Leitman S, Chang AE, Ettinghausen SE, Matory YL, Skibber JM, Shiloni E, Vetto JT. Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *N Engl J Med* 1985; **313**: 1485-1492 [PMID: 3903508]
- 10 Bhatia S, Tykodi SS, Thompson JA. Treatment of metastatic melanoma: an overview. *Oncology (Williston Park)* 2009; **23**: 488-496 [PMID: 19544689]
- 11 Rosenberg SA, Lotze MT, Muul LM, Chang AE, Avis FP, Leitman S, Linehan WM, Robertson CN, Lee RE, Rubin JT. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N Engl J Med* 1987; **316**: 889-897 [PMID: 3493432]
- 12 Yamaue H, Tanimura H, Tsunoda T, Iwahashi M, Tani M, Inoue M, Tamai M. [Clinical application of adoptive immunotherapy by cytotoxic T lymphocytes induced from tumor-infiltrating lymphocytes]. *Nihon Gan Chiryo Gakkai Shi* 1990; **25**: 978-989 [PMID: 2391445]
- 13 Alexander RB, Rosenberg SA. Long-term survival of adoptively transferred tumor-infiltrating lymphocytes in mice. *J Immunol* 1990; **145**: 1615-1620 [PMID: 1974569]
- 14 Kono K, Takahashi A, Ichihara F, Amemiya H, Iizuka H, Fujii H, Sekikawa T, Matsumoto Y. Prognostic significance of adoptive immunotherapy with tumor-associated lymphocytes in patients with advanced gastric cancer: a randomized trial. *Clin Cancer Res* 2002; **8**: 1767-1771 [PMID: 12060615]
- 15 Tsoukas CD, Landgraf B, Bontin J, Valentine M, Lotz M, Vaughan JH, Carson DA. Activation of resting T lymphocytes by anti-CD3 (T3) antibodies in the absence of monocytes. *J Immunol* 1985; **135**: 1719-1723 [PMID: 3926881]
- 16 Jiang J, Xu N, Wu C, Deng H, Lu M, Li M, Xu B, Wu J, Wang R, Xu J, Nilsson-Ehle P. Treatment of advanced gastric cancer by chemotherapy combined with autologous cytokine-induced killer cells. *Anticancer Res* 2006; **26**: 2237-2242 [PMID: 16821594]
- 17 Jiang JT, Shen YP, Wu CP, Zhu YB, Wei WX, Chen LJ, Zheng X, Sun J, Lu BF, Zhang XG. Increasing the frequency of CIK cells adoptive immunotherapy may decrease risk of death in gastric cancer patients. *World J Gastroenterol* 2010; **16**: 6155-6162 [PMID: 21182234]
- 18 Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 1991; **9**: 271-296 [PMID: 1910679]
- 19 Kono K, Rongcun Y, Charo J, Ichihara F, Celis E, Sette A, Appella E, Sekikawa T, Matsumoto Y, Kiessling R. Identification of HER2/neu-derived peptide epitopes recognized by

- gastric cancer-specific cytotoxic T lymphocytes. *Int J Cancer* 1998; **78**: 202-208 [PMID: 9754653]
- 20 **Nguyen T**, Naziruddin B, Dintzis S, Doherty GM, Mohanakumar T. Recognition of breast cancer-associated peptides by tumor-reactive, HLA-class I restricted allogeneic cytotoxic T lymphocytes. *Int J Cancer* 1999; **81**: 607-615 [PMID: 10225452]
 - 21 **Dudley ME**, Ngo LT, Westwood J, Wunderlich JR, Rosenberg SA. T-cell clones from melanoma patients immunized against an anchor-modified gp100 peptide display discordant effector phenotypes. *Cancer J* 2000; **6**: 69-77 [PMID: 11069222]
 - 22 **Dudley ME**, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, Topalian SL, Sherry R, Restifo NP, Hubicki AM, Robinson MR, Raffeld M, Duray P, Seipp CA, Rogers-Freezer L, Morton KE, Mavroukakis SA, White DE, Rosenberg SA. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002; **298**: 850-854 [PMID: 12242449]
 - 23 **Traversari C**, van der Bruggen P, Luescher IF, Lurquin C, Chomez P, Van Pel A, De Plaen E, Amar-Costesec A, Boon T. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. *J Exp Med* 1992; **176**: 1453-1457 [PMID: 1402688]
 - 24 **Gaugler B**, Van den Eynde B, van der Bruggen P, Romero P, Gaforio JJ, De Plaen E, Lethé B, Brasseur F, Boon T. Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. *J Exp Med* 1994; **179**: 921-930 [PMID: 8113684]
 - 25 **van der Bruggen P**, Bastin J, Gajewski T, Coulie PG, Boël P, De Smet C, Traversari C, Townsend A, Boon T. A peptide encoded by human gene MAGE-3 and presented by HLA-A2 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE-3. *Eur J Immunol* 1994; **24**: 3038-3043 [PMID: 7805731]
 - 26 **Herman J**, van der Bruggen P, Luescher IF, Mandruzzato S, Romero P, Thonnard J, Fleischhauer K, Boon T, Coulie PG. A peptide encoded by the human MAGE3 gene and presented by HLA-B44 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE3. *Immunogenetics* 1996; **43**: 377-383 [PMID: 8606058]
 - 27 **Tanaka F**, Fujie T, Tahara K, Mori M, Takesako K, Sette A, Celis E, Akiyoshi T. Induction of antitumor cytotoxic T lymphocytes with a MAGE-3-encoded synthetic peptide presented by human leukocytes antigen-A24. *Cancer Res* 1997; **57**: 4465-4468 [PMID: 9377553]
 - 28 **Yang J**, Li ZH, Zhou JJ, Chen RF, Cheng LZ, Zhou QB, Yang LQ. Preparation and antitumor effects of nanovaccines with MAGE-3 peptides in transplanted gastric cancer in mice. *Chin J Cancer* 2010; **29**: 359-364 [PMID: 20346208]
 - 29 **Masuzawa T**, Fujiwara Y, Okada K, Nakamura A, Takiguchi S, Nakajima K, Miyata H, Yamasaki M, Kurokawa Y, Osawa R, Takeda K, Yoshida K, Tsunoda T, Nakamura Y, Mori M, Doki Y. Phase I/II study of S-1 plus cisplatin combined with peptide vaccines for human vascular endothelial growth factor receptor 1 and 2 in patients with advanced gastric cancer. *Int J Oncol* 2012; **41**: 1297-1304 [PMID: 22842485 DOI: 10.3892/ijo.2012.1573]
 - 30 **Boczkowski D**, Nair SK, Snyder D, Gilboa E. Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. *J Exp Med* 1996; **184**: 465-472 [PMID: 8760800]
 - 31 **Kyte JA**, Gaudernack G. Immuno-gene therapy of cancer with tumour-mRNA transfected dendritic cells. *Cancer Immunol Immunother* 2006; **55**: 1432-1442 [PMID: 16612595]
 - 32 **Weide B**, Garbe C, Rammensee HG, Pascolo S. Plasmid DNA- and messenger RNA-based anti-cancer vaccination. *Immunol Lett* 2008; **115**: 33-42 [PMID: 18006079]
 - 33 **Hoerr I**, Obst R, Rammensee HG, Jung G. In vivo application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies. *Eur J Immunol* 2000; **30**: 1-7 [PMID: 10602021]
 - 34 **Carralot JP**, Probst J, Hoerr I, Scheel B, Teufel R, Jung G, Rammensee HG, Pascolo S. Polarization of immunity induced by direct injection of naked sequence-stabilized mRNA vaccines. *Cell Mol Life Sci* 2004; **61**: 2418-2424 [PMID: 15378210]
 - 35 **Granstein RD**, Ding W, Ozawa H. Induction of anti-tumor immunity with epidermal cells pulsed with tumor-derived RNA or intradermal administration of RNA. *J Invest Dermatol* 2000; **114**: 632-636 [PMID: 10733665]
 - 36 **Kreiter S**, Selmi A, Diken M, Koslowski M, Britten CM, Huber C, Türeci O, Sahin U. Intranodal vaccination with naked antigen-encoding RNA elicits potent prophylactic and therapeutic antitumoral immunity. *Cancer Res* 2010; **70**: 9031-9040 [PMID: 21045153 DOI: 10.1158/0008-5472.CAN-10-0699]
 - 37 **Fotin-Mlecsek M**, Duchardt KM, Lorenz C, Pfeiffer R, Ojkić-Zrna S, Probst J, Kallen KJ. Messenger RNA-based vaccines with dual activity induce balanced TLR-7 dependent adaptive immune responses and provide antitumor activity. *J Immunother* 2011; **34**: 1-15 [PMID: 21150709 DOI: 10.1097/CJI.0b013e3181f7dbe8]
 - 38 **Weide B**, Carralot JP, Reese A, Scheel B, Eigentler TK, Hoerr I, Rammensee HG, Garbe C, Pascolo S. Results of the first phase I/II clinical vaccination trial with direct injection of mRNA. *J Immunother* 2008; **31**: 180-188 [PMID: 18481387 DOI: 10.1097/CJI.0b013e31815ce501]
 - 39 **Weide B**, Pascolo S, Scheel B, Derhovanessian E, Pflugfelder A, Eigentler TK, Pawelec G, Hoerr I, Rammensee HG, Garbe C. Direct injection of protamine-protected mRNA: results of a phase 1/2 vaccination trial in metastatic melanoma patients. *J Immunother* 2009; **32**: 498-507 [PMID: 19609242 DOI: 10.1097/CJI.0b013e3181a00068]
 - 40 **Rittig SM**, Haentschel M, Weimer KJ, Heine A, Muller MR, Brugger W, Horger MS, Maksimovic O, Stenzl A, Hoerr I, Rammensee HG, Holderried TA, Kanz L, Pascolo S, Brossart P. Intradermal vaccinations with RNA coding for TAA generate CD8+ and CD4+ immune responses and induce clinical benefit in vaccinated patients. *Mol Ther* 2011; **19**: 990-999 [PMID: 21189474 DOI: 10.1038/mt.2010.289]
 - 41 **Smyth MJ**, Godfrey DI, Trapani JA. A fresh look at tumor immunosurveillance and immunotherapy. *Nat Immunol* 2001; **2**: 293-299 [PMID: 11276199]
 - 42 **Rosenberg SA**. Progress in human tumour immunology and immunotherapy. *Nature* 2001; **411**: 380-384 [PMID: 11357146]
 - 43 **Wick M**, Dubey P, Koeppen H, Siegel CT, Fields PE, Chen L, Bluestone JA, Schreiber H. Antigenic cancer cells grow progressively in immune hosts without evidence for T cell exhaustion or systemic anergy. *J Exp Med* 1997; **186**: 229-238 [PMID: 9221752]
 - 44 **Antonia SJ**, Extermann M, Flavel RA. Immunologic nonresponsiveness to tumors. *Crit Rev Oncog* 1998; **9**: 35-41 [PMID: 9754446]
 - 45 **Villunger A**, Strasser A. The great escape: is immune evasion required for tumor progression? *Nat Med* 1999; **5**: 874-875 [PMID: 10426306]
 - 46 **Gilboa E**. How tumors escape immune destruction and what we can do about it. *Cancer Immunol Immunother* 1999; **48**: 382-385 [PMID: 10501851]
 - 47 **Lee HW**, Min SK, Ju YS, Sung J, Lim MS, Yang DH, Lee BH. Prognostic significance of HLA class I expressing in gastric carcinoma defined by monoclonal anti-pan HLA class I antibody, EMR8-5. *J Gastrointest Surg* 2011; **15**: 1336-1343 [PMID: 21512844 DOI: 10.1007/s11605-011-1545-3]
 - 48 **Kandulski A**, Malfertheiner P, Wex T. Role of regulatory T-cells in H. pylori-induced gastritis and gastric cancer. *Anticancer Res* 2010; **30**: 1093-1103 [PMID: 20530414]
 - 49 **Haraguchi N**, Inoue H, Tanaka F, Mimori K, Utsunomiya T, Sasaki A, Mori M. Cancer stem cells in human gastrointestinal cancers. *Hum Cell* 2006; **19**: 24-29 [PMID: 16643604]
 - 50 **Houghton J**, Wang TC. Helicobacter pylori and gastric

- cancer: a new paradigm for inflammation-associated epithelial cancers. *Gastroenterology* 2005; **128**: 1567-1578 [PMID: 15887152]
- 51 **Zhang T**, Lee YW, Rui YF, Cheng TY, Jiang XH, Li G. Bone marrow-derived mesenchymal stem cells promote growth and angiogenesis of breast and prostate tumors. *Stem Cell Res Ther* 2013; **4**: 70 [PMID: 23763837 DOI: 10.1186/scrt221]
 - 52 **Lin R**, Ma H, Ding Z, Shi W, Qian W, Song J, Hou X. Bone marrow-derived mesenchymal stem cells favor the immunosuppressive T cells skewing in a *Helicobacter pylori* model of gastric cancer. *Stem Cells Dev* 2013; **22**: 2836-2848 [PMID: 23777268]
 - 53 **Robert C**, Thomas L, Bondarenko I, O'Day S, M D JW, Garbe C, Lebbe C, Baurain JF, Testori A, Grob JJ, Davidson N, Richards J, Maio M, Hauschild A, Miller WH, Gascon P, Lotem M, Harmankaya K, Ibrahim R, Francis S, Chen TT, Humphrey R, Hoos A, Wolchok JD. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* 2011; **364**: 2517-2526 [PMID: 21639810 DOI: 10.1056/NEJMoa1104621]
 - 54 **Prieto PA**, Yang JC, Sherry RM, Hughes MS, Kammula US, White DE, Levy CL, Rosenberg SA, Phan GQ. CTLA-4 blockade with ipilimumab: long-term follow-up of 177 patients with metastatic melanoma. *Clin Cancer Res* 2012; **18**: 2039-2047 [PMID: 22271879 DOI: 10.1158/1078-0432.CCR-11-1823]
 - 55 **Derré L**, Rivals JP, Jandus C, Pastor S, Rimoldi D, Romero P, Michielin O, Olive D, Speiser DE. BTLA mediates inhibition of human tumor-specific CD8⁺ T cells that can be partially reversed by vaccination. *J Clin Invest* 2010; **120**: 157-167 [PMID: 20038811 DOI: 10.1172/JCI40070]
 - 56 **Krop IE**, LoRusso P, Miller KD, Modi S, Yardley D, Rodriguez G, Guardino E, Lu M, Zheng M, Girish S, Amler L, Winer EP, Rugo HS. A phase II study of trastuzumab emtansine in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer who were previously treated with trastuzumab, lapatinib, an anthracycline, a taxane, and capecitabine. *J Clin Oncol* 2012; **30**: 3234-3241 [PMID: 22649126 DOI: 10.1200/JCO.2011.40.5902]
 - 57 **Baselga J**, Cortés J, Kim SB, Im SA, Hegg R, Im YH, Roman L, Pedrini JL, Pienkowski T, Knott A, Clark E, Benyunes MC, Ross G, Swain SM. Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. *N Engl J Med* 2012; **366**: 109-119 [PMID: 22149875 DOI: 10.1056/NEJMoa1113216]
 - 58 **van de Vijver MJ**, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, van der Velde T, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH, Bernards R. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 2002; **347**: 1999-2009 [PMID: 12490681]
 - 59 **Bedognetti D**, Wang E, Sertoli MR, Marincola FM. Gene-expression profiling in vaccine therapy and immunotherapy for cancer. *Expert Rev Vaccines* 2010; **9**: 555-565 [PMID: 20518712 DOI: 10.1586/erv.10.55]
 - 60 **Bogunovic D**, O'Neill DW, Belitskaya-Levy I, Vacic V, Yu YL, Adams S, Darvishian F, Berman R, Shapiro R, Pavlick AC, Lonardi S, Zavadil J, Osman I, Bhardwaj N. Immune profile and mitotic index of metastatic melanoma lesions enhance clinical staging in predicting patient survival. *Proc Natl Acad Sci USA* 2009; **106**: 20429-20434 [PMID: 19915147 DOI: 10.1073/pnas.0905139106]
 - 61 **Pham MX**, Teuteberg JJ, Kfoury AG, Starling RC, Deng MC, Cappola TP, Kao A, Anderson AS, Cotts WG, Ewald GA, Baran DA, Bogaev RC, Elashoff B, Baron H, Yee J, Valentine HA. Gene-expression profiling for rejection surveillance after cardiac transplantation. *N Engl J Med* 2010; **362**: 1890-1900 [PMID: 20413602 DOI: 10.1056/NEJMoa0912965]
 - 62 **Chaussabel D**, Pascual V, Banchereau J. Assessing the human immune system through blood transcriptomics. *BMC Biol* 2010; **8**: 84 [PMID: 20619006 DOI: 10.1186/1741-7007-8-84]
 - 63 **Lee DW**, Barrett DM, Mackall C, Orentas R, Grupp SA. The future is now: chimeric antigen receptors as new targeted therapies for childhood cancer. *Clin Cancer Res* 2012; **18**: 2780-2790 [PMID: 22589486 DOI: 10.1158/1078-0432.CCR-11-1920]
 - 64 **Cheadle EJ**, Sheard V, Hombach AA, Chmielewski M, Riet T, Berrevoets C, Schooten E, Lamers C, Abken H, Debets R, Gilham DE. Chimeric antigen receptors for T-cell based therapy. *Methods Mol Biol* 2012; **907**: 645-666 [PMID: 22907378 DOI: 10.1007/978-1-61779-974-7_36]
 - 65 **Morgan RA**, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther* 2010; **18**: 843-851 [PMID: 20179677 DOI: 10.1038/mt.2010.24]
 - 66 **Brentjens R**, Yeh R, Bernal Y, Riviere I, Sadelain M. Treatment of chronic lymphocytic leukemia with genetically targeted autologous T cells: case report of an unforeseen adverse event in a phase I clinical trial. *Mol Ther* 2010; **18**: 666-668 [PMID: 20357779 DOI: 10.1038/mt.2010.31]
 - 67 **Kochenderfer JN**, Dudley ME, Feldman SA, Wilson WH, Spaner DE, Maric I, Stetler-Stevenson M, Phan GQ, Hughes MS, Sherry RM, Yang JC, Kammula US, Devillier L, Carpenter R, Nathan DA, Morgan RA, Laurencot C, Rosenberg SA. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. *Blood* 2012; **119**: 2709-2720 [PMID: 22160384 DOI: 10.1182/blood-2011-10-384388]
 - 68 **Han EQ**, Li XL, Wang CR, Li TF, Han SY. Chimeric antigen receptor-engineered T cells for cancer immunotherapy: progress and challenges. *J Hematol Oncol* 2013; **6**: 47 [PMID: 23829929 DOI: 10.1186/1756-8722-6-47]
 - 69 **Dunn GP**, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* 2002; **3**: 991-998 [PMID: 12407406 DOI: 10.1038/ni1102-991]

P- Reviewers: Aoyagi K, Baba H, Vieth M **S- Editor:** Gou SX
L- Editor: A **E- Editor:** Zhang DN



WJG 20th Anniversary Special Issues (8): Gastric cancer

Role of the tumor microenvironment in the pathogenesis of gastric carcinoma

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Received: October 28, 2013 Revised: November 22, 2013

Accepted: December 5, 2013

Published online: February 21, 2014

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Key words: Tumor microenvironment; Gastric carcinoma; Inflammation; *Helicobacter pylori*; Cytokine

Core tip: The intensive interplay that exists between tumor cells and the tumor microenvironment can play an important role in tumor initiation, growth and metastasis. A better understanding of the molecular pathogenesis of the tumor microenvironment of Gastric carcinoma would be crucial for the design of novel molecular targets. In this review, we have provided an overview of the currently available knowledge of the role of the TME in gastric cancer and have highlighted the potential prognostic and therapeutic implications.

Abstract

Gastric carcinoma (GC) is the 4th most prevalent cancer and has the 2nd highest cancer-related mortality rate worldwide. Despite the incidence of GC has decreased over the past few decades, it is still a serious health problem. Chronic inflammatory status of the stomach, caused by the infection of *Helicobacter pylori* (*H. pylori*) and through the production of inflammatory mediators within the parenchyma is suspected to play an important role in the initiation and progression of GC. In this review, the correlation between chronic inflammation and *H. pylori* infection as an important factor for the development of GC will be discussed. Major components, including tumor-associated macrophages, lymphocytes, cancer-associated fibroblasts, angiogenic factors, cytokines, and chemokines of GC microenvironment and their mechanism of action on signaling pathways will also be discussed. Increasing our understanding of how the components of the tumor microenvironment interact with GC cells and the signaling pathways involved could help identify new therapeutic and chemopreventive targets.

Chung HW, Lim JB. Role of the tumor microenvironment in the pathogenesis of gastric carcinoma. *World J Gastroenterol* 2014; 20(7): 1667-1680 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1667.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1667>

INTRODUCTION

Gastric carcinoma (GC) is the 4th most prevalent cancer and has the 2nd highest cancer-related mortality rate worldwide. The risk of developing GC is 1 in 115, with a 5-year survival rate of only 20%-30%^[1]. Despite that the incidence of GC has decreased over the past few decades, it is still a serious health problem^[2]. The prognosis of advanced GC (AGC) with extensive node invasion and metastasis remains poor, while early GC is associated with excellent long-term survival^[3].

Ever since Rudolf Virchow, the founder of modern pathology, observed the connection between tumor cells and their surrounding tumor microenvironment (TME),

TME has long been suspected to play an important role in the initiation and progression of tumors^[4,5]. TME is thought to determine the behavior of cancers not only through genetic or epigenetic makeups of the tumor cells, but also through the surrounding milieu that the tumor cells interact with for survival, growth, proliferation and metastasis. The TME is composed of many different kinds of cells such as endothelial cells, fibroblasts, lymphocytes and macrophages. It also consists of numerous soluble molecules such as growth factors, cytokines, chemokines, antibodies, proteases, various types of enzymes, and metabolites as well as an extracellular matrix. As the tumor progresses, states of hypoxia and acidosis develop in the TME^[6-8], and the intensive relationship that exists between tumor cells and the TME plays a major role in tumor initiation, growth and metastasis.

Among the numerous factors in the TME, inflammatory mediators have received attention recently, and an estimated 15%-20% of cancer deaths are associated with chronic infection and inflammation. Population-based studies have shown that individuals who are prone to chronic inflammatory disorders have an increased risk of cancer development^[9]. Accordingly, treatment with non-steroidal anti-inflammatory agents decreases the incidence and mortality of several tumor types^[10,11]. In the case of GC, the chronic inflammatory state of the stomach, caused by *Helicobacter pylori* (*H. pylori*) infection, as well as the production of inflammatory mediators, such as cytokines and chemokines within gastric tissues, is suspected to play an important role in the initiation and progression of GC.

Better understanding of the special interplay between GC cells and the surrounding microenvironment may be useful for recognizing the mechanism underlying tumor development and progression as well as the discovery of novel molecular therapeutic targets^[12,13]. In this review, we have provided an overview of the currently available knowledge of the role of the TME in GC and have highlighted the potential prognostic and therapeutic implications.

CHRONIC INFLAMMATION AND

H. PYLORI IN GC

H. pylori, a microaerophilic, spiral gram-negative bacterium, colonizes the human stomach and, is a major cause of chronic gastritis, peptic ulcers, and gastric malignancies, including gastric non-cardia adenocarcinoma and mucosal-associated lymphoid tissue lymphoma^[14]. *H. pylori* infects over 50% of the world's population, with 1% of those infected going on to develop GC. An estimated 75% of all GC cases are associated with *H. pylori* infection^[15].

The carcinogenic potential of *H. pylori* is driven by the interplay between bacterial virulence factors and the host's immune responses resulting in chronic inflammation, which in turn leads to tumorigenesis^[16]. Four major virulence factors have been identified from *H. pylori*, that is cytotoxin-associated antigen A (CagA), cag-pathogenicity island (cagPAI), vacuolating cytotoxin, and outer mem-

brane proteins. *H. pylori* cagPAI encodes approximately, 30 genes, including type four secretion system genes, which are essential for pathogenesis and are responsible for the delivery of CagA protein and peptidoglycan into host cells^[17,18]. It has recently reported that CagA binds an Src homology 2-containing tyrosine phosphatase (SHP-2) in a tyrosine phosphorylation-dependent manner and activates the phosphatase activity of SHP-2^[19]. Deregulation of SHP-2 by CagA is an important mechanism by which CagA-positive *H. pylori* promotes gastric carcinogenesis. *H. pylori* is a potent activator of nuclear factor- κ B (NF- κ B) in gastric epithelial cells^[20,21] causing the production of tumor necrosis factor- α , TNF-inducing protein (Tip), which in turn activates NF- κ B in gastric epithelial cells using an independent pathway involving virulence factors such as CagA^[18].

Activation of NF- κ B by *H. pylori* infection induces the expression of a variety of genes, including those encoding the cytokines interleukin (IL)-1, IL-6, IL-8, TNF- α , vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), cell-cycle regulators, the matrix metalloproteinases (MMP)-2, MMP-7, MMP-9 and adhesion molecules^[22,23]. High level of COX2 mRNA and protein expression and enzymatic activity are detected in GC cells^[24], and COX-2 activity is induced by a variety of mediators including inflammatory cytokines such as TNF- α , interferon (IFN)- γ and IL-1^[25]. COX-2 facilitates tumor growth by inhibiting apoptosis, promoting cell proliferation and stimulating angiogenesis within cancer cells^[26]. *H. pylori* infection produces reactive oxygen and nitrogen species that cause DNA damage, followed by chronic gastritis and intestinal metaplasia. Nitric oxide generated by iNOS is converted to reactive nitrogen species that bring about direct DNA mutation such as those in p53, causing protein damage, inhibition of apoptosis, and promotion of angiogenesis^[27,28]. CagA also activates the nuclear factor of activated T-cells signaling pathway, and interacts with E-cadherin to deregulate β -catenin signaling, which induces the expression of genes downstream of β -catenin, such as Caudal type homeobox gene-1 and promotes the transdifferentiation of intestinal cells^[29].

SIGNALING PATHWAY OF GC-RELATED INFLAMMATION

Multiple steps and multiple factors are involved in the development of GC. More than 90% of GCs are adenocarcinomas, which are divided into two histological types, intestinal and diffuse, based on the Lauren's classification^[30]. *H. pylori* infection and chronic inflammation are important factors, particularly in the intestinal type of GC. The Correa's hypothesis postulates that there is a progression from chronic gastritis to gastric atrophy, intestinal metaplasia, dysplasia, and finally to cancer ("gastritis-dysplasia-carcinoma" sequence)^[31]. In each step of GC progression, many cytokines and intracellular sig-

naling pathways are involved.

GC-related inflammation activate transcription factors, mainly NF- κ B, hypoxia-inducible factor (HIF)-1 α , and signal transducer and activator of transcription (STAT)-3, which are the key inducers of inflammatory mediators such as cytokines, chemokines, prostaglandins, nitric oxide^[32].

The transcription factor NF- κ B is a key orchestrator of innate immunity and inflammation and recent evidence suggests that it play an important role in development and maintenance of cancer-related inflammation^[33]. In cancer and epithelial cells exposed to carcinogens, NF- κ B promotes cell survival and proliferation through the activation of genes encoding proteins that are important for cell cycle progression such as cyclin D1, and c-Myc and the anti-apoptotic pathway (cIAPs, A1/BFL1, BCL-2, c-FLIP)^[34,35]. In GC, NF- κ B potentiates inflammation in response to *H. pylori* infection. Some studies reported that *H. pylori* induces expression of the pro-inflammatory cytokine IL-8 through activation of NF- κ B^[20,36]. Moreover, NF- κ B amplifies the inflammatory signals of other cytokines, such as tumor necrosis factor and interferon^[37]. A previous study reported that the positive rate of NF- κ B/RelA is 42.6% in South Korea and NF- κ B/RelA expression in tumor tissues was also related to serum levels of IL-6 ($P = 0.044$) and C-reactive protein ($P = 0.010$)^[38]. Interestingly, several microRNAs (miRNA) which target NF- κ B have been shown to be involved in development and progression of GC. miR-146a expression is up-regulated in a majority of gastric cancers where it targets Caspase recruitment domain-containing protein 10 and COP9 signalosome complex subunit, inhibiting G protein coupled receptor-mediated activation of NF- κ B, thus reducing expression of NF-B-regulated tumor-promoting cytokines and growth factors^[39].

HIF-1 α is centrally involved in multiple aspects of tumorigenesis including tumor angiogenesis, proliferation, metabolism, metastasis, differentiation, as well as responses to radiation and chemotherapy^[40]. HIF-1 α is up-regulated in inflammatory conditions and there is accumulating evidence indicating the presence of interconnections and compensatory pathways between the NF- κ B and HIF-1 α systems^[41]. The expression of HIF-1 α commonly increases in a variety of human solid tumors and elevated HIF-1 α expression is associated with poor patient outcome in pancreatic cancer, glioblastoma, GC and other cancers^[40,42]. Furthermore, the contribution of HIF-1 α to chemoresistance has been observed in several solid tumors, including GC^[43,44]. Interestingly, inhibition of HIF-1 α *via* RNA interference or pharmacological compounds has improved their anti-tumor efficacy in murine cancer models^[45] through modulation of the p53 and NF- κ B signaling pathway. In this regards, a recent study has demonstrated that HIF-1 α expression correlates with the metastatic phenotype of human GC^[46].

STAT-3 is constitutively activated in several human cancer cells and tumor associated leukocytes and it represents a point of convergence for several oncogenic sig-

naling pathways^[47]. In approximately 50% of human GC, STAT3 is overactivated^[48,49], and its high activation and/or expression status has been shown to correlate with a lower survival rate for GC patients^[50]. This transcription factor supports oncogenesis through different mechanisms, ranging from the activation of genes crucial for proliferation and survival to the enhancement of angiogenesis and metastasis. In GC, IL-11 produced from tumor cells and TME activate the common signal-transducing gp130 β -receptor subunit to activate the JAK/STAT-3, Ras/Mitogen-activated protein kinase and phosphoinositide-3-kinase (PI3K)/Akt signaling pathways^[51-53]. Activated STAT-3 signaling pathway directly induces the transcription of the *T/r2* gene in the gastric epithelium, which upon overexpression promotes proliferation and inhibits apoptosis of gastric epithelial cells^[48]. The activation of STAT-3 in tumor cells also has been shown to increase the capacity of tumors to evade the immune system by inhibiting the maturation of dendritic cells (DCs)^[54], thereby suppressing the immune response^[55]. A recent study showed that STAT-3 plays a divergent role in the modulation of IL-23 and IL-12, two related cytokines, which play opposite roles in tumour development. In particular, STAT-3 inhibits anti-tumor IL-12p35 expression in DCs while promoting the expression of the pro-carcinogenic IL-23 cytokine in TAMs^[56].

Epidemiological studies have highlighted that treatment with non-steroidal anti-inflammatory agents, such as COX-2 inhibitors, decrease the risk of developing certain cancers, such as colon cancer, breast cancer, and GC^[10,11,57,58]. The frequency of COX-2 expression did not differ between gastric adenomas and early intestinal carcinomas, indicating that COX-2 expression might act as one of the factors related to early tumorigenesis in the stomach. Interestingly, the frequency of COX-2 expression was significantly higher in advanced carcinomas than in early carcinomas and was higher in intestinal-type carcinomas than in diffuse-type carcinomas. COX-2 expression may be more important for the progression of intestinal-type carcinomas than that of diffuse-type carcinoma^[59].

Transforming growth factor (TGF)- β 1 mRNA and protein are highly expressed in GC cells^[60-62]. TGF- β 1 is closely related to invasion and metastasis and the TME; it alters the biologic behavior of malignant gastric lesion^[63]. TGF- β 1 produced by carcinoma cells stimulates collagen synthesis in both fibroblasts and cancer cells, which leads to diffuse fibrosis in the case scirrhous GC^[63].

The receptor tyrosine-protein kinase (HER) family consists of four members: HER-1 [epidermal growth factor receptor (EGFR)], HER-2, HER-3 and HER-4. Activation of these receptors leads to homo- or hetero-dimerization that in turn initiates phosphorylation cascades and subsequent activation of the PI3K-Akt-mammalian target of rapamycin (mTOR) and Ras-Raf-mitogenactivated mitogenactivated protein kinase/extracellular signal-related kinase (ERK) kinase (MEK)-ERK pathways, which are important in cancer cell proliferation and survival^[64,65].

EGFR overexpression, observed in 27%-44% of gas-

tric cancer cases, is generally reported to be a poor prognostic factor, despite contradictory evidence^[66]. HER-2 overexpression is observed in 10%-38% of gastric cancer tumor samples^[67,68], with a higher prevalence in intestinal-type and gastroesophageal junction tumors than that in diffuse-type and gastric tumors^[68,69]. The prognostic value of HER-2 overexpression in gastric cancer remains controversial; it is generally associated with a poorer outcome^[70,71], although contradictory evidence exists^[72,73]. In fact, *PIK3CA* activating mutation was reported in 4%-36% of gastric cancer cases^[74,75] and phosphatase and tensin homolog loss was reported in 20%-36% of cases^[74,76]. For gastric cancer, the *KRAS* mutation was observed in 2%-20% of cases^[77,78], and the *BRAF* mutation was observed in 0%-2.7% of cases^[77,79].

The overexpression/activation of c-Met, a receptor for hepatocyte growth factor, leads to proliferation and antiapoptotic signals^[80]. It was found to be activated both in vitro in human gastric cancer cell lines and in vivo in human gastric cancer tissue^[81], and this may result from the infection of gastric cells by *H. pylori*^[82].

The Hedgehogs (Hh) protein family includes Sonic (Shh), Indian (Ihh) and Desert (Dhh). In gastric cancer, the aberrant activation of Shh, through binding Patched 1 receptor and subsequent disinhibition of Smoothened in turn activates the transcription factor Gli-1^[83].

COMPONENT OF MICROENVIRONMENT OF GC TAM

Macrophages recruited to the tumor stroma are called TAMs. The role of TAMs in tumor progression is complicated and wide ranging. Although activated macrophages may have anti-tumor activity, tumor cells have been reported to evade the anti-tumor activity of TAMs^[84,85]. Indeed, removal of macrophages by genetic mutation reduces tumor progression and metastasis^[86]. TAMs are recruited from circulating monocytes into tissues in response to chemoattractants, and interact with tumor cells to make up the cancer stroma. Macrophage infiltration into tumor tissue correlates significantly with tumor vascularity in human esophageal cancer and GC^[87,88]. There is a direct association between the degree of TAM infiltration and depth of tumor invasion, nodal status, and clinical stage of GC^[88]. Macrophage recruitment is mediated by a variety of chemoattractants, including the following; monocyte chemoattractant protein-1 (MCP-1/CCL2), macrophage inflammatory protein-1 α (MIP-1 α /CCL3); and regulated upon activation, normal T cell expressed and secreted (RANTES/CCL5)^[87,88].

Lymphocytes

Regulatory T cells (Tregs) are functionally immune-suppressive subsets of T cells that are reported to play important roles in immunological self-tolerance^[89-92]. Tregs are defined more strictly as CD4⁺CD25⁺Foxp3⁺ cells. The frequency of Tregs among tumor infiltrating lymphocytes (TILs), lymphocytes derived from tumor-

draining regional lymph nodes, and peripheral blood lymphocytes is higher in GC and esophageal cancer patients than their normal counterparts^[93,94]. In addition, patients with a higher proportion of Tregs showed poorer survival rates than those with a lower proportion. Interestingly, after patients underwent curative resection for GC, the proportion of Tregs decreased and was restored to levels comparable to those for normal healthy donors^[95]. These results strongly suggest that tumor-related factors induce the expansion and the accumulation of Tregs in GC. Furthermore, the frequencies of CCL17⁺ cells and of CCL22⁺ cells, both of which induce in vitro migration of Tregs, within tumors were significantly higher than those in normal gastric mucosa. Increased levels of TGF-1 in GC patients have been correlated with the frequency of Tregs, and, conversely, numerous studies have reported a correlation between an increased frequency of circulating Treg and increased levels of TGF-1 during GC progression^[96-98]. On the other hand, some reports have indicated that activated effector T cells are converted into Treg cells, capable of suppressing autologous effector T cells^[99-101]. Thus, it is likely that naturally occurring Foxp3⁺ Tregs in peripheral sites faintly perceive tumor-related signals such as CCL17 or CCL22, migrate to the tumor site, and create a favorable environment for tumor growth.

Recently, a subset of IL-17 producing T cells that are distinct from Th1 and Th2 cells have been described as key players in inflammation and autoimmune diseases as well as cancer development. Interestingly, IL-17 also has been reported to be up-regulated in *H. pylori* infected gastric mucosa. IL-17 positively regulates the synthesis of IL-8 by gastric mononuclear cells and epithelial cells, which thus emphasizes the role of IL-17 in *H. pylori*-driven inflammation^[102]. When the ratio of Th17/Treg cells of TILs was evaluated in GC patients, it was found to be markedly higher in early disease than in advanced disease. The accumulation of Th17 cells as well as of Tregs in the TME of GC occurs in early disease following which the infiltration of Th17 cells gradually decrease as the disease progresses, in contrast to the increased accumulation of Tregs.

Cancer associated fibroblasts

Cancer associated fibroblasts (CAFs) are a central elements of TME. They are the most prominent cell type within the tumor stroma of many cancers and play a critical role in tumor-stromal interactions^[103,104]. CAFs demonstrate differential gene expression profiles compared to normal fibroblasts^[105], and they acquire a modified phenotype, similar to fibroblasts associated with wound healing. Although the mechanisms that regulate activation of fibroblasts and their accumulation in tumors are not fully understood, platelet-derived growth factor, TGF- β 1, and fibroblast growth factor-2 (FGF-2) are known to be partly involved in this process^[106]. There are some candidates for the origins of CAFs, such as following; fibroblasts residing in local tissues^[105], periaxillary cells including

pericytes and vascular smooth muscle cells^[107], endothelial cells^[108], and bone marrow-derived cells including various stem cells^[109]. Worthley *et al.*^[110] recently reported that bone marrow-derived cells can differentiate into CAFs in human GC that developed in female recipients of male allogeneic stem cell transplantation. A previous study showed that direct interaction between scirrhous-type GC cells and gastric fibroblasts could promote fibrosis of the gastric wall and increasing the malignant behavior of cancer cells through vascular cell adhesion molecule-1 and induced Snail expression, and through the resultant E-cadherin suppression and vimentin induction in HSC-39 cells^[111].

Angiogenetic factors

Angiogenesis which is necessary for tumor progression, is also influenced by the tumor microenvironment. Stromal reaction (desmoplasia) is observed in GC, but not in non-invasive neoplasms^[112]. The generation of tumor stroma is triggered by tumor cells and induces the ingrowth of new blood vessels and mesenchymal cells from the adjacent normal tissue^[113]. However, recent studies have shown that bone marrow-derived stem cells are integrated into the tumor stroma and differentiate into myofibroblasts and vascular endothelial cells^[109,114]. A recent study reported that the density of blood vessels directly correlates with the incidence of metastasis in GC^[114-117]. Angiogenesis of tumor is mediated by various molecules released by tumor cells and TME^[118,119] and GC cells produce various angiogenic factors, including VEGF^[120], IL-8^[121], FGF-2^[122], and platelet-derived endothelial cell growth factor (PD-ECGF)^[123]. VEGF-A promotes the angiogenesis and progression of human GC, especially those of the intestinal type. A significant correlation between lymph node metastasis and VEGF-C expression has been reported in human GC^[124,125]. However, no association was found between VEGF-D immunoreactivity and clinicopathologic features in submucosally invasive GC^[126]. These results suggest that VEGF-C is a dominant regulator of lymphangiogenesis in early-stage human GC.

Stem cells

The stem cell niche or microenvironment is composed of different populations of cells, including not only stem cells, but also differentiated cells, soluble factors, and extracellular matrix, all of which are critical for stem cell fate and differentiation^[127]. Important signaling pathways such as the Wnt, Notch, Hedgehog, PI3K, NF- κ B, endothelial growth factor (EGF), TGF- β and STAT-3 pathways have been shown to regulate stem cell renewal and maintenance, and their effects overlap in both normal and cancer stem cells^[128]. The Interactions of stem cells with their surroundings are currently under intensive investigation. The inflammatory mediators and oncogenic pathways also regulate stem cell differentiation either directly or indirectly and are frequently deregulated in tumors^[129-131]. Given the fact that gastric stem cells are such a rare population of cells and can be affected by so many

intrinsic and extrinsic factors, it is very complicated to identify the specific role of a signaling factor in regulating their differentiation and migration. It has been noted that NF- κ B, IL-6, VEGF, HIF-1 α , angiogenesis, reactive oxygen species and tissue factors are all involved in the maintenance of stem cell and cancer stem cells^[127] and that *H. pylori* infection can alter most of their expression. This suggests that *H. pylori* might impact the local microenvironment and affect stem/progenitor cell differentiation, and also cause genetic or epigenetic damages in these cells, leading to carcinogenesis. However, further studies addressing these pathways and mediators of gastric stem cells and progenitors during infection are awaited.

CYTOKINES/CHEMOKINES

Infection by *H. pylori* also disrupts gastric homeostasis and induces the production of multiple inflammatory cytokine within the local mucosa. Expression of IL-1 β , TNF- α , and IL-10 is associated with an increased risk for developing GC^[132,133].

IL-1 β is a proinflammatory cytokine involved in inflammation and immunity. IL-1 β polymorphisms are associated with enhanced IL-1 β production and increased risk of GC^[133], IL-1 β also inhibits gastric acid secretion. In transgenic mice, stomach specific overexpression of IL-1 β induces stepwise spontaneous gastric inflammation, metaplasia, dysplasia, and carcinoma.

Overexpression of IL-1 β also mobilizes myeloid-derived suppressor cells and induces NF- κ B activation as well as the expression of downstream genes such as IL-6 and TNF- α in these cells. In addition, IL-1 β alone is sufficient to induce gastric preneoplasia. However, the mechanisms by which IL-1 β overexpression itself finally results in oncogenic transformation is unclear. Interestingly, other inflammatory mediators can exert opposite effects. One example is IFN- γ , which is produced primarily by activated T cells, and natural killer cells and is a key mediator of innate and adaptive immunity. IFN- γ mediates responses to bacterial infection and autoimmune disease, and acts as a tumor suppressor^[134]. In mice, stomach specific overexpression of IFN- γ alone has minimal effects on the gastric mucosa, but inhibits IL-1 β - and *Helicobacter felis*-induced gastritis and neoplasia. The mechanism has been attributed to IFN- γ induced inhibition of gastric epithelial cell proliferation, acceleration of apoptosis of gastric T lymphocytes and decrease in the production of pro-inflammatory Th1 and Th17 cytokines. These effects may balance epithelial cell proliferation, restrain inflammation, and ultimately inhibit tumor formation^[134]. Therefore, disruption of host cell inflammatory cytokine production is involved in gastric oncogenesis.

Chemokines are involved in the chemoattraction of leukocytes to inflammatory sites and can be produced by many kinds of cells in the TME including leukocytes, endothelial cells, fibroblasts and epithelial cells^[135,136]. Recent reports described that chemokines not only play a role in

the immune system, but also promote tumorigenesis and metastasis of cancer. CXC chemokines and their receptors (CXCR) modulate tumor behavior by three important mechanisms: regulation of angiogenesis, activation of a tumor-specific immune response and stimulation of tumor cell proliferation in an autocrine or paracrine fashion^[137].

CXC chemokines containing the ELR (Glu-Leu-Arg)-motif such as IL-8/CXCL8 have been described to promote tumor growth by stimulation of angiogenesis and chemoattraction of neutrophilic granulocytes^[138-140]. Previous studies have shown that IL-1, TNF- α and infection with *H. pylori* induce or enhance the secretion of IL-8 by several gastric adenocarcinoma cell lines *in vitro*^[141,142]. In addition, CXCR1 and CXCR2 expression increased in gastric carcinoma cells after infection by *H. pylori*^[143,144]. In GC, expression of IL-8 in gastric adenocarcinoma is associated with increased tumor vascularization, aggressiveness, invasion, and metastasis. In addition, IL-8 may act as a diagnostic marker as it was demonstrated to be significantly elevated in serum samples of patients with gastric cancer^[145,146]. IL-8 also enhances the expression of the EGFR, MMP-9, VEGF and IL-8 itself^[122,147,148]. Furthermore, the polymorphism of *IL-8* promoter gene is associated with higher IL-8 protein expression, more severe neutrophil infiltration, enhanced angiogenesis, especially with secretion of MMP-9 and angiopoietin-1, and increased risk of poorly differentiated gastric cancer, lymph node, and liver metastasis^[149-151].

In contrast, CXC chemokines lacking the ELR-motif such as interferon- γ , inducible protein-10 (IP-10)/CXCL10, possess angiostatic activities and chemoattract anti-tumoral lymphocytes through binding to CXCR3^[139,140]. It has been described that Mig, IP-10 and I-TAC were constitutively express in GC cell lines, and the production can be enhanced by IFN- γ in synergy with TNF- α . In contrast, *in vitro* infection with *H. pylori* inhibited the IFN- γ /TNF- α induced Mig and IP-10 production by GC cells. Increased expression of CXCR3 ligands by endothelial cells and mononuclear cells, especially antigen-presenting cells within GC, results in the chemoattraction and activation of cytotoxic T lymphocytes that favor tumor regression.

Stromal cell-derived factor-1 (SDF-1)/CXCL12 is an exception on this rule as this chemokine lacks the ELR-motif, has angiogenic properties and mediates the dissemination of CXCR4-positive tumor cells to distant organs^[140]. SDF-1 modulates the angiogenic process directly by binding to its receptors CXCR4 and/or CXCR7 expressed on endothelial cells or indirectly by the induced secretion of matrix-metalloproteinases or angiogenic factors such as IL-8, VEGF, respectively^[152,153]. Many studies have demonstrated that both CXCL12 and CXCR4 are differentially expressed in GC^[154,155], and overexpression of CXCR4 in gastric cancer cells is associated with aggressive tumor behavior, such as tumor invasion, lymph node metastasis, liver metastasis, and poor differentiation as well as peritoneal carcinomatosis^[156]. In addition, peritoneal mesothelial cells contained high concentrations of SDF-1 indicating that SDF-1 induces the migration

of CXCR4-positive tumor cells to the peritoneum^[157]. *H. pylori* increased CXCR4 expression in gastric cancer through increased secretion of TNF- α . CXCR4 has also been found in leukocytes and microvascular blood vessels, confirming that SDF-1 binds to endothelial cells^[158]. In addition to cancer cells, stromal cells such as endothelial cells, tumor-infiltrating lymphocytes and cancer-associated fibroblasts have been demonstrated to produce elevated levels of SDF-1^[158,159].

Matrix metalloproteinases

MMPs lead to tissue remodeling, inflammation, tumor cell growth, migration, invasion and metastasis in many cancers. They are major modulators of the tumor microenvironment, playing key roles in tumorigenesis^[160]. Different stromal and cancer cells produce various types of MMPs whose main subtypes are collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), matrilysins (MMP-7, MMP-26), membrane type MMPs (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, MMP-25) and stromelysins (MMP-3, MMP-10, MMP-11).

Previous studies reported that MMP-1^[161], MMP-7^[162-164] and MMP-9^[165,166] are important in development of gastritis during infection by *H. pylori* and these molecules are utilized as molecular markers. It has been suggested that overexpression of MMP-1^[167] and MMP-7^[162,168] is dependent upon the pathogenicity island of *H. pylori* and, interestingly, it is known that MMP-7 participates in the epithelial mesenchymal transition^[169] and is also overexpressed in GC^[170]. Moreover, the activity of MMP-9 is increased in macrophages resident in the gastric mucosa of subjects infected with *H. pylori*^[171] and its activity is known to be reduced by the eradication of *H. pylori*^[172].

MMPs are noncovalently inhibited by the tissue inhibitors known as TIMP, a family comprising four members (TIMP-1, TIMP-2, TIMP-3, TIMP-4). TIMP-3 is the only inhibitor associated with the extra cellular matrix (ECM) and the rest of the TIMP are soluble proteins^[173].

The disintegrins and metalloproteinase (ADAM) family are proteases related to the MMP and comprise more than 20 proteins that are anchored to the cell membrane and present various functions which are cell adhesion, cell fusion, activation of signaling pathways and release of substrates such as cytokines and growth factors from the cell membrane or the ECM^[174]. In patients with gastritis and *H. pylori* infection, levels of ADAM-10 and ADAM-17 are elevated^[175], and these play key roles in cell signaling^[174]. E-cadherin is a substrate of ADAM-10 and the Notch signaling pathway, in which ADAM-17 participates, and these pathways are also involved in the development of GC. ADAM-17 has been associated with the generation of transient hypochlorhydria in patients infected with *H. pylori*^[176] and interestingly, high levels of hypochlorhydria are founded in GC patients.

NEW THERAPEUTIC APPROACHES

According to our understanding of the molecular basis

of TME of GC, targeted agents have led to a modest improvement in the outcome of advanced gastric cancer (AGC) patients.

Previous studies showed that EGFR, HER-2, tyrosine kinase inhibitors (TKIs) as well as VEGF were most attractive target for molecular therapy. The ToGA trial targeted HER-2 and AVAGAST trial targeted VEGF have marked the beginning of a new era in AGC treatment. A number of other phase III clinical trials that target different target molecules are ongoing.

Notably, the ToGA trial, which is a large, phase III, randomized controlled multicenter trial^[177], showed that trastuzumab in combination with chemotherapy led to a significantly higher overall response rate (ORR 47% *vs* 35%, $P = 0.0017$), significantly longer progression free survival interval (PFS; 6.7 mo *vs* 5.5 mo, $P = 0.0002$), and significantly longer overall survival duration (OS; 13.8 mo *vs* 11.1 mo, $P = 0.0046$) than that of the controls. Moreover, the trastuzumab-containing regimen was generally well tolerated and did not affect quality of life. To date, trastuzumab is the first and only targeted agent for gastric cancer approved by both the United States^[178] and European^[179] authorities.

Although the phase III Avastin® in Gastric Cancer (AVAGAST) trial did not meet its primary endpoint of OS and was thus a negative trial for this endpoint, the ORR was significantly better in the bevacizumab arm (46% *vs* 37%, $P = 0.0315$) and the PFS interval was significantly longer (6.7 mo *vs* 5.3 mo, HR = 0.8; $P = 0.0037$) than that of the controls^[180].

In first-line phase II trials, cetuximab, a recombinant human-mouse chimeric monoclonal antibody targeting EGFR, showed that the ORR was in the range of 40%-60%, the time to progression (T_{0P}) was 5.5-8.0 mo, and the OS time was 9.5-16.0 mo^[181,182]. Other study reported that cetuximab showed no clinically significant benefit in combination with docetaxel plus oxaliplatin^[183]. Other EGFR targeted therapy including Erbitux®, panitumumab, matuzumab, and nimotuzumab are under evaluation in phase II / III trials in combination with chemotherapy. The EGFR TKIs such as gefitinib and erlotinib were evaluated in phase II trials but produced disappointing results as monotherapy for AGC.

Lapatinib (Tykerb), a dual TKI inhibiting both HER-2 and EGFR are under investigation in two phase III trials. One is the LoGIC trial that is the lapatinib Optimization Study in ErbB2 (HER-2)⁺ GC patient^[184], and the other is TYTAN trial that is investigating the lapatinib with paclitaxel (Taxol) in Asian ErbB2⁺ (HER2⁺) GC patients^[185].

A few signaling pathways have attracted a lot of enthusiasm. The ubiquitin-proteasome pathway that is involved in cell cycle control is one good target.

Bortezomib, a proteasome inhibitor, was shown to induce apoptosis and suppress tumor growth in GC cell lines^[186]. The overexpression/activation of c-Met, a receptor for hepatocyte growth factor, leads to proliferation and antiapoptotic signals^[80]. A phase II study of GSK1363089 (GSK089, formerly XL880), a c-Met TKI,

showed minimal activity in a cohort of metastatic GS patients unselected for c-Met^[187]. The Hedgehog (Hh) pathway further complicates the complex signaling in gastric cancer cells^[83]. Clinical use of Hh inhibitors is currently only in the early phases of development^[188].

Inhibition of other biological pathways in AGC is in preclinical or early clinical evaluation. Insulin like growth factor-1 receptor antibody, figitumumab, in combination with docetaxel was well tolerated in a phase I trial of patients with advanced solid tumors^[188]. FGFR inhibitors, HSP90 inhibitors, histone deacetylase and IL-6 antibody also may play a role in AGC treatment^[189-193].

CONCLUSION

Although recent phase III clinical trials with conventional chemotherapeutic agents have shown encouraging results in advanced GC, overall survival rates continue to be suboptimal. This highlights the need for new therapeutic strategy using targeted therapy to improve the result of GC treatment.

The association between chronic gastritis and tumors is well documented in the step-wise histopathologic (Correa) model of GC. A better understanding of the molecular pathogenesis of GC would help for improving the knowledge on this relationship and would be crucial for the design of novel molecular targets.

Previous studies reported that a synergistic interplay among the components of TME of GC, including *H. pylori* infection, immune cells and mediators, and several proteins along with matrix metalloproteinases, is essential for the initiation, progression and metastasis of GC. The understanding of how these mechanisms regulate the relationship among those components of TME of GC would contribute strongly to identifying key signaling pathways that serve as both novel biomarkers for early detection and molecular targets for new therapeutic strategies.

REFERENCES

- 1 Torpy JM, Lynn C, Glass RM. JAMA patient page. Stomach cancer. *JAMA* 2010; **303**: 1771 [PMID: 20442395 DOI: 10.1001/jama.303.17.1771]
- 2 Terry MB, Gaudet MM, Gammon MD. The epidemiology of gastric cancer. *Semin Radiat Oncol* 2002; **12**: 111-127 [PMID: 11979413 DOI: 10.1053/srao.30814]
- 3 Hohenberger P, Gretscher S. Gastric cancer. *Lancet* 2003; **362**: 305-315 [PMID: 12892963 DOI: 10.1016/S0140-6736(03)13975-X]
- 4 Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 2001; **357**: 539-545 [PMID: 11229684]
- 5 Hussain SP, Harris CC. Inflammation and cancer: an ancient link with novel potentials. *Int J Cancer* 2007; **121**: 2373-2380 [PMID: 17893866 DOI: 10.1002/ijc.23173]
- 6 Witz IP, Levy-Nissenbaum O. The tumor microenvironment in the post-PAGET era. *Cancer Lett* 2006; **242**: 1-10 [PMID: 16413116 DOI: 10.1016/j.canlet.2005.12.005]
- 7 Witz IP. Yin-yang activities and vicious cycles in the tumor microenvironment. *Cancer Res* 2008; **68**: 9-13 [PMID: 18172289 DOI: 10.1158/0008-5472.CAN-07-2917]
- 8 Witz IP. Tumor-microenvironment interactions: dangerous liaisons. *Adv Cancer Res* 2008; **100**: 203-229 [PMID: 18620097]

- DOI: 10.1016/S0065-230X(08)00007-9]
- 9 **Balkwill F**, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* 2005; **7**: 211-217 [PMID: 15766659 DOI: 10.1016/j.ccr.2005.02.013]
- 10 **Koehne CH**, Dubois RN. COX-2 inhibition and colorectal cancer. *Semin Oncol* 2004; **31**: 12-21 [PMID: 15252926 DOI: 10.1053/j.seminoncol.2004.03.041]
- 11 **Flossmann E**, Rothwell PM. Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. *Lancet* 2007; **369**: 1603-1613 [PMID: 17499602 DOI: 10.1016/S0140-6736(07)60747-8]
- 12 **De Wever O**, Demetter P, Mareel M, Bracke M. Stromal myofibroblasts are drivers of invasive cancer growth. *Int J Cancer* 2008; **123**: 2229-2238 [PMID: 18777559 DOI: 10.1002/ijc.23925]
- 13 **Xing F**, Saidou J, Watabe K. Cancer associated fibroblasts (CAFs) in tumor microenvironment. *Front Biosci* (Landmark Ed) 2010; **15**: 166-179 [PMID: 20036813 DOI: 10.2741/3613]
- 14 **Peek RM**, Crabtree JE. Helicobacter infection and gastric neoplasia. *J Pathol* 2006; **208**: 233-248 [PMID: 16362989 DOI: 10.1002/path.1868]
- 15 **Howlader N**, Ries LA, Mariotto AB, Reichman ME, Ruhl J, Cronin KA. Improved estimates of cancer-specific survival rates from population-based data. *J Natl Cancer Inst* 2010; **102**: 1584-1598 [PMID: 20937991 DOI: 10.1093/jnci/djq366]
- 16 **Ding SZ**, Zheng PY. Helicobacter pylori infection induced gastric cancer; advance in gastric stem cell research and the remaining challenges. *Gut Pathog* 2012; **4**: 18 [PMID: 23217022 DOI: 10.1186/1757-4749-4-18]
- 17 **Segal ED**, Cha J, Lo J, Falkow S, Tompkins LS. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by Helicobacter pylori. *Proc Natl Acad Sci USA* 1999; **96**: 14559-14564 [PMID: 10588744 DOI: 10.1073/pnas.96.25.14559]
- 18 **Suganuma M**, Yamaguchi K, Ono Y, Matsumoto H, Hayashi T, Ogawa T, Imai K, Kuzuhara T, Nishizono A, Fujiki H. TNF-alpha-inducing protein, a carcinogenic factor secreted from H. pylori, enters gastric cancer cells. *Int J Cancer* 2008; **123**: 117-122 [PMID: 18412243 DOI: 10.1002/ijc.23484]
- 19 **Hatakeyama M**. Linking epithelial polarity and carcinogenesis by multitasking Helicobacter pylori virulence factor CagA. *Oncogene* 2008; **27**: 7047-7054 [PMID: 19029944 DOI: 10.1038/onc.2008.353]
- 20 **Sharma SA**, Tummuru MK, Blaser MJ, Kerr LD. Activation of IL-8 gene expression by Helicobacter pylori is regulated by transcription factor nuclear factor-kappa B in gastric epithelial cells. *J Immunol* 1998; **160**: 2401-2407 [PMID: 9498783]
- 21 **Isomoto H**, Mizuta Y, Miyazaki M, Takeshima F, Omagari K, Murase K, Nishiyama T, Inoue K, Murata I, Kohno S. Implication of NF-kappaB in Helicobacter pylori-associated gastritis. *Am J Gastroenterol* 2000; **95**: 2768-2776 [PMID: 11051346]
- 22 **Hatz RA**, Rieder G, Stolte M, Bayerdörffer E, Meimarakis G, Schildberg FW, Enders G. Pattern of adhesion molecule expression on vascular endothelium in Helicobacter pylori-associated antral gastritis. *Gastroenterology* 1997; **112**: 1908-1919 [PMID: 9178683 DOI: 10.1053/gast.1997.v112.pm9178683]
- 23 **Nakanishi C**, Toi M. Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. *Nat Rev Cancer* 2005; **5**: 297-309 [PMID: 15803156 DOI: 10.1038/nrc1588]
- 24 **Liu XJ**, Chen ZF, Li HL, Hu ZN, Liu M, Tian AP, Zhao D, Wu J, Zhou YN, Qiao L. Interaction between cyclooxygenase-2, Snail, and E-cadherin in gastric cancer cells. *World J Gastroenterol* 2013; **19**: 6265-6271 [PMID: 24115825]
- 25 **Williams CS**, Smalley W, DuBois RN. Aspirin use and potential mechanisms for colorectal cancer prevention. *J Clin Invest* 1997; **100**: 1325-1329 [PMID: 9294096 DOI: 10.1172/JCI119651]
- 26 **Tsuji M**, DuBois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell* 1995; **83**: 493-501 [PMID: 8521479 DOI: 10.1016/0092-8674(95)90127-2]
- 27 **Jaiswal M**, LaRusso NF, Gores GJ. Nitric oxide in gastrointestinal epithelial cell carcinogenesis: linking inflammation to oncogenesis. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G626-G634 [PMID: 11518674]
- 28 **Goto T**, Haruma K, Kitadai Y, Ito M, Yoshihara M, Sumii K, Hayakawa N, Kajiyama G. Enhanced expression of inducible nitric oxide synthase and nitrotyrosine in gastric mucosa of gastric cancer patients. *Clin Cancer Res* 1999; **5**: 1411-1415 [PMID: 10389926]
- 29 **Murata-Kamiya N**, Kurashima Y, Teishikata Y, Yamahashi Y, Saito Y, Higashi H, Aburatani H, Akiyama T, Peek RM, Azuma T, Hatakeyama M. Helicobacter pylori CagA interacts with E-cadherin and deregulates the beta-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. *Oncogene* 2007; **26**: 4617-4626 [PMID: 17237808]
- 30 **Lauren P**. The two histological main types of gastric carcinoma: Diffuse and so-called intestinal-type carcinoma. an attempt at a histo-clinical classification. *Acta Pathol Microbiol Scand* 1965; **64**: 31-49 [PMID: 14320675]
- 31 **Correa P**. Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Res* 1992; **52**: 6735-6740 [PMID: 1458460]
- 32 **Mantovani A**, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* 2008; **454**: 436-444 [PMID: 18650914 DOI: 10.1038/nature07205]
- 33 **Karin M**. Nuclear factor-kappaB in cancer development and progression. *Nature* 2006; **441**: 431-436 [PMID: 16724054 DOI: 10.1038/nature04870]
- 34 **Greten FR**, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, Kagnoff MF, Karin M. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* 2004; **118**: 285-296 [PMID: 15294155 DOI: 10.1016/j.cell.2004.07.013]
- 35 **Ditsworth D**, Zong WX. NF-kappaB: key mediator of inflammation-associated cancer. *Cancer Biol Ther* 2004; **3**: 1214-1216 [PMID: 15611628 DOI: 10.4161/cbt.3.12.1391]
- 36 **Aihara M**, Tsuchimoto D, Takizawa H, Azuma A, Wakebe H, Ohmoto Y, Imagawa K, Kikuchi M, Mukaida N, Matsushima K. Mechanisms involved in Helicobacter pylori-induced interleukin-8 production by a gastric cancer cell line, MKN45. *Infect Immun* 1997; **65**: 3218-3224 [PMID: 9234778]
- 37 **Yasumoto K**, Okamoto S, Mukaida N, Murakami S, Mai M, Matsushima K. Tumor necrosis factor alpha and interferon gamma synergistically induce interleukin 8 production in a human gastric cancer cell line through acting concurrently on AP-1 and NF-kB-like binding sites of the interleukin 8 gene. *J Biol Chem* 1992; **267**: 22506-22511 [PMID: 1331059]
- 38 **Kwon HC**, Kim SH, Oh SY, Lee S, Lee JH, Jang JS, Kim MC, Kim KH, Kim SJ, Kim SG, Kim HJ. Clinicopathologic significance of expression of nuclear factor-kB RelA and its target gene products in gastric cancer patients. *World J Gastroenterol* 2012; **18**: 4744-4750 [PMID: 23002344 DOI: 10.3748/wjg.v18.i34.4744]
- 39 **Crone SG**, Jacobsen A, Federspiel B, Bardram L, Krogh A, Lund AH, Friis-Hansen L. microRNA-146a inhibits G protein-coupled receptor-mediated activation of NF-kB by targeting CARD10 and COPS8 in gastric cancer. *Mol Cancer* 2012; **11**: 71 [PMID: 22992343 DOI: 10.1186/1476-4598-11-71]
- 40 **Talks KL**, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL. The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. *Am J Pathol* 2000; **157**: 411-421 [PMID: 10934146 DOI: 10.1016/S0002-9440(10)64554-3]
- 41 **Rius J**, Guma M, Schachtrup C, Akassoglou K, Zinkernagel AS, Nizet V, Johnson RS, Haddad GG, Karin M. NF-kappaB links innate immunity to the hypoxic response through

- transcriptional regulation of HIF-1 α . *Nature* 2008; **453**: 807-811 [PMID: 18432192 DOI: 10.1038/nature06905]
- 42 **Zhong H**, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, Simons JW. Overexpression of hypoxia-inducible factor 1 α in common human cancers and their metastases. *Cancer Res* 1999; **59**: 5830-5835 [PMID: 10582706]
- 43 **Zhou J**, Schmid T, Schnitzer S, Brüne B. Tumor hypoxia and cancer progression. *Cancer Lett* 2006; **237**: 10-21 [PMID: 16002209 DOI: 10.1016/j.canlet.2005.05.028]
- 44 **Liu L**, Ning X, Sun L, Zhang H, Shi Y, Guo C, Han S, Liu J, Sun S, Han Z, Wu K, Fan D. Hypoxia-inducible factor-1 α contributes to hypoxia-induced chemoresistance in gastric cancer. *Cancer Sci* 2008; **99**: 121-128 [PMID: 17953712]
- 45 **Giaccia A**, Siim BG, Johnson RS. HIF-1 as a target for drug development. *Nat Rev Drug Discov* 2003; **2**: 803-811 [PMID: 14526383 DOI: 10.1038/nrd1199]
- 46 **Rohwer N**, Lobitz S, Daskalow K, Jöns T, Vieth M, Schlag PM, Kemmner W, Wiedenmann B, Cramer T, Höcker M. HIF-1 α determines the metastatic potential of gastric cancer cells. *Br J Cancer* 2009; **100**: 772-781 [PMID: 19223895 DOI: 10.1038/sj.bjc.6604919]
- 47 **Yu H**, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nat Rev Immunol* 2007; **7**: 41-51 [PMID: 17186030 DOI: 10.1038/nri1995]
- 48 **Tye H**, Kennedy CL, Najdovska M, McLeod L, McCormack W, Hughes N, Dev A, Sievert W, Ooi CH, Ishikawa TO, Oshima H, Bhathal PS, Parker AE, Oshima M, Tan P, Jenkins BJ. STAT3-driven upregulation of TLR2 promotes gastric tumorigenesis independent of tumor inflammation. *Cancer Cell* 2012; **22**: 466-478 [PMID: 23079657 DOI: 10.1016/j.ccr.2012.08.010]
- 49 **Yu H**, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer* 2009; **9**: 798-809 [PMID: 19851315 DOI: 10.1038/nrc2734]
- 50 **Kim DY**, Cha ST, Ahn DH, Kang HY, Kwon CI, Ko KH, Hwang SG, Park PW, Rim KS, Hong SP. STAT3 expression in gastric cancer indicates a poor prognosis. *J Gastroenterol Hepatol* 2009; **24**: 646-651 [PMID: 19175826 DOI: 10.1111/j.1440-1746.2008.05671.x]
- 51 **Ernst M**, Najdovska M, Grail D, Lundgren-May T, Buchert M, Tye H, Matthews VB, Armes J, Bhathal PS, Hughes NR, Marcusson EG, Karras JG, Na S, Sedgwick JD, Hertzog PJ, Jenkins BJ. STAT3 and STAT1 mediate IL-11-dependent and inflammation-associated gastric tumorigenesis in gp130 receptor mutant mice. *J Clin Invest* 2008; **118**: 1727-1738 [PMID: 18431520]
- 52 **Ellmark P**, Ingvarsson J, Carlsson A, Lundin BS, Wingren C, Borrebaeck CA. Identification of protein expression signatures associated with *Helicobacter pylori* infection and gastric adenocarcinoma using recombinant antibody microarrays. *Mol Cell Proteomics* 2006; **5**: 1638-1646 [PMID: 16844680 DOI: 10.1074/mcp.M600170-MCP200]
- 53 **Bollrath J**, Phesse TJ, von Burstin VA, Putoczki T, Bennecke M, Bateman T, Nebelsiek T, Lundgren-May T, Canli O, Schwitalla S, Matthews V, Schmid RM, Kirchner T, Arkan MC, Ernst M, Greten FR. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. *Cancer Cell* 2009; **15**: 91-102 [PMID: 19185844 DOI: 10.1016/j.ccr.2009.01.002]
- 54 **Wang T**, Niu G, Kortylewski M, Burdelya L, Shain K, Zhang S, Bhattacharya R, Gabrilovich D, Heller R, Coppola D, Dalton W, Jove R, Pardoll D, Yu H. Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. *Nat Med* 2004; **10**: 48-54 [PMID: 14702634 DOI: 10.1038/nm976]
- 55 **Kortylewski M**, Kujawski M, Wang T, Wei S, Zhang S, Pilon-Thomas S, Niu G, Kay H, Mulé J, Kerr WG, Jove R, Pardoll D, Yu H. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nat Med* 2005; **11**: 1314-1321 [PMID: 16288283 DOI: 10.1038/nm1325]
- 56 **Kortylewski M**, Xin H, Kujawski M, Lee H, Liu Y, Harris T, Drake C, Pardoll D, Yu H. Regulation of the IL-23 and IL-12 balance by Stat3 signaling in the tumor microenvironment. *Cancer Cell* 2009; **15**: 114-123 [PMID: 19185846 DOI: 10.1016/j.ccr.2008.12.018]
- 57 **Mantovani G**, Macciò A, Madeddu C, Serpe R, Antoni G, Massa E, Dessì M, Panzone F. Phase II nonrandomized study of the efficacy and safety of COX-2 inhibitor celecoxib on patients with cancer cachexia. *J Mol Med (Berl)* 2010; **88**: 85-92 [PMID: 19802504 DOI: 10.1007/s00109-009-0547-z]
- 58 **Chan AT**, Ogino S, Fuchs CS. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. *N Engl J Med* 2007; **356**: 2131-2142 [PMID: 17522398 DOI: 10.1056/NEJMoa067208]
- 59 **Honjo S**, Kase S, Osaki M, Ardyanto TD, Kaibara N, Ito H. Cyclooxygenase-2 expression in human gastric tubular adenomas and carcinomas; correlation with intratumoral microvessel density and apoptotic index. *Anticancer Res* 2004; **24**: 1439-1444 [PMID: 15274307]
- 60 **Yoshida K**, Yokozaki H, Niimoto M, Ito H, Ito M, Tahara E. Expression of TGF- β and procollagen type I and type III in human gastric carcinomas. *Int J Cancer* 1989; **44**: 394-398 [PMID: 2777404 DOI: 10.1002/ijc.2910440303]
- 61 **Mahara K**, Kato J, Terui T, Takimoto R, Horimoto M, Murakami T, Mogi Y, Watanabe N, Kohgo Y, Niitsu Y. Transforming growth factor β 1 secreted from scirrhous gastric cancer cells is associated with excess collagen deposition in the tissue. *Br J Cancer* 1994; **69**: 777-783 [PMID: 8142266 DOI: 10.1038/bjc.1994.147]
- 62 **Horimoto M**, Kato J, Takimoto R, Terui T, Mogi Y, Niitsu Y. Identification of a transforming growth factor β -1 activator derived from a human gastric cancer cell line. *Br J Cancer* 1995; **72**: 676-682 [PMID: 7669580 DOI: 10.1038/bjc.1995.393]
- 63 **Maehara Y**, Kakeji Y, Kabashima A, Emi Y, Watanabe A, Akazawa K, Baba H, Kohno S, Sugimachi K. Role of transforming growth factor- β 1 in invasion and metastasis in gastric carcinoma. *J Clin Oncol* 1999; **17**: 607-614 [PMID: 10080606]
- 64 **Dhanasekaran DN**, Johnson GL. MAPKs: function, regulation, role in cancer and therapeutic targeting. *Oncogene* 2007; **26**: 3097-3099 [PMID: 17496908 DOI: 10.1038/sj.onc.1210395]
- 65 **Schlessinger J**. Common and distinct elements in cellular signaling via EGF and FGF receptors. *Science* 2004; **306**: 1506-1507 [PMID: 15567848 DOI: 10.1126/science.1105396]
- 66 **Matsubara J**, Nishina T, Yamada Y, Moriwaki T, Shimoda T, Kajiwarra T, Nakajima TE, Kato K, Hamaguchi T, Shimada Y, Okayama Y, Oka T, Shirao K. Impacts of excision repair cross-complementing gene 1 (ERCC1), dihydropyrimidine dehydrogenase, and epidermal growth factor receptor on the outcomes of patients with advanced gastric cancer. *Br J Cancer* 2008; **98**: 832-839 [PMID: 18231104 DOI: 10.1038/sj.bjc.6604211]
- 67 **Yano T**, Doi T, Ohtsu A, Boku N, Hashizume K, Nakanishi M, Ochiai A. Comparison of HER2 gene amplification assessed by fluorescence in situ hybridization and HER2 protein expression assessed by immunohistochemistry in gastric cancer. *Oncol Rep* 2006; **15**: 65-71 [PMID: 16328035]
- 68 **Grávalos C**, Gómez-Martín C, Rivera F, Alés I, Queralt B, Márquez A, Jiménez U, Alonso V, García-Carbonero R, Sastre J, Colomer R, Cortés-Funes H, Jimeno A. Phase II study of trastuzumab and cisplatin as first-line therapy in patients with HER2-positive advanced gastric or gastroesophageal junction cancer. *Clin Transl Oncol* 2011; **13**: 179-184 [PMID: 21421462 DOI: 10.1007/s12094-011-0637-6]
- 69 **Zheng Y**, Wang L, Zhang JP, Yang JY, Zhao ZM, Zhang XY. Expression of p53, c-erbB-2 and Ki67 in intestinal metaplasia and gastric carcinoma. *World J Gastroenterol* 2010; **16**: 339-344 [PMID: 20082479 DOI: 10.3748/wjg.v16.i3.339]

- 70 **Im SA**, Lee KE, Nam E, Kim DY, Lee JH, Han HS, Seoh JY, Park HY, Cho MS, Han WS, Lee SN. Potential prognostic significance of p185(HER2) overexpression with loss of PTEN expression in gastric carcinomas. *Tumori* 2005; **91**: 513-521 [PMID: 16457151]
- 71 **Uchino S**, Tsuda H, Maruyama K, Kinoshita T, Sasako M, Saito T, Kobayashi M, Hirohashi S. Overexpression of c-erbB-2 protein in gastric cancer. Its correlation with long-term survival of patients. *Cancer* 1993; **72**: 3179-3184 [PMID: 7902202]
- 72 **Grabsch H**, Sivakumar S, Gray S, Gabbert HE, Müller W. HER2 expression in gastric cancer: Rare, heterogeneous and of no prognostic value - conclusions from 924 cases of two independent series. *Cell Oncol* 2010; **32**: 57-65 [PMID: 20208134]
- 73 **Chua TC**, Merrett ND. Clinicopathologic factors associated with HER2-positive gastric cancer and its impact on survival outcomes—a systematic review. *Int J Cancer* 2012; **130**: 2845-2856 [PMID: 21780108 DOI: 10.1002/ijc.26292]
- 74 **Byun DS**, Cho K, Ryu BK, Lee MG, Park JI, Chae KS, Kim HJ, Chi SG. Frequent monoallelic deletion of PTEN and its reciprocal association with PIK3CA amplification in gastric carcinoma. *Int J Cancer* 2003; **104**: 318-327 [PMID: 12569555 DOI: 10.1002/ijc.10962]
- 75 **Li VS**, Wong CW, Chan TL, Chan AS, Zhao W, Chu KM, So S, Chen X, Yuen ST, Leung SY. Mutations of PIK3CA in gastric adenocarcinoma. *BMC Cancer* 2005; **5**: 29 [PMID: 15784156 DOI: 10.1186/1471-2407-5-29]
- 76 **Kang YH**, Lee HS, Kim WH. Promoter methylation and silencing of PTEN in gastric carcinoma. *Lab Invest* 2002; **82**: 285-291 [PMID: 11896207 DOI: 10.1038/labinvest.3780422]
- 77 **Lee SH**, Lee JW, Soung YH, Kim HS, Park WS, Kim SY, Lee JH, Park JY, Cho YG, Kim CJ, Nam SW, Kim SH, Lee JY, Yoo NJ. BRAF and KRAS mutations in stomach cancer. *Oncogene* 2003; **22**: 6942-6945 [PMID: 14534542 DOI: 10.1038/sj.onc.1206749]
- 78 **Hiyama T**, Haruma K, Kitadai Y, Masuda H, Miyamoto M, Tanaka S, Yoshihara M, Shimamoto F, Chayama K. K-ras mutation in helicobacter pylori-associated chronic gastritis in patients with and without gastric cancer. *Int J Cancer* 2002; **97**: 562-566 [PMID: 11807778 DOI: 10.1002/ijc.1644]
- 79 **Kim IJ**, Park JH, Kang HC, Shin Y, Park HW, Park HR, Ku JL, Lim SB, Park JG. Mutational analysis of BRAF and K-ras in gastric cancers: absence of BRAF mutations in gastric cancers. *Hum Genet* 2003; **114**: 118-120 [PMID: 14513361 DOI: 10.1007/s00439-003-1027-0]
- 80 **Migliore C**, Giordano S. Molecular cancer therapy: can our expectation be MET? *Eur J Cancer* 2008; **44**: 641-651 [PMID: 18295476 DOI: 10.1016/j.ejca.2008.01.022]
- 81 **Inoue T**, Kataoka H, Goto K, Nagaike K, Igami K, Naka D, Kitamura N, Miyazawa K. Activation of c-Met (hepatocyte growth factor receptor) in human gastric cancer tissue. *Cancer Sci* 2004; **95**: 803-808 [PMID: 15504247 DOI: 10.1111/j.1349-7006.2004.tb02185.x]
- 82 **Oliveira MJ**, Costa AC, Costa AM, Henriques L, Suriano G, Atherton JC, Machado JC, Carneiro F, Seruca R, Mareel M, Leroy A, Figueiredo C. Helicobacter pylori induces gastric epithelial cell invasion in a c-Met and type IV secretion system-dependent manner. *J Biol Chem* 2006; **281**: 34888-34896 [PMID: 16990273 DOI: 10.1074/jbc.M607067200]
- 83 **Han ME**, Lee YS, Baek SY, Kim BS, Kim JB, Oh SO. Hedgehog signaling regulates the survival of gastric cancer cells by regulating the expression of Bcl-2. *Int J Mol Sci* 2009; **10**: 3033-3043 [PMID: 19742123 DOI: 10.3390/ijms10073033]
- 84 **Allavena P**, Mantovani A. Immunology in the clinic review series; focus on cancer: tumour-associated macrophages: undisputed stars of the inflammatory tumour microenvironment. *Clin Exp Immunol* 2012; **167**: 195-205 [PMID: 22235995 DOI: 10.1111/j.1365-2249.2011.04515.x]
- 85 **Lewis CE**, Pollard JW. Distinct role of macrophages in different tumor microenvironments. *Cancer Res* 2006; **66**: 605-612 [PMID: 16423985 DOI: 10.1158/0008-5472.CAN-05-4005]
- 86 **Condeelis J**, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 2006; **124**: 263-266 [PMID: 16439202 DOI: 10.1016/j.cell.2006.01.007]
- 87 **Ohta M**, Kitadai Y, Tanaka S, Yoshihara M, Yasui W, Mukaida N, Haruma K, Chayama K. Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human esophageal squamous cell carcinomas. *Int J Cancer* 2002; **102**: 220-224 [PMID: 12397639 DOI: 10.1002/ijc.10705]
- 88 **Ohta M**, Kitadai Y, Tanaka S, Yoshihara M, Yasui W, Mukaida N, Haruma K, Chayama K. Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human gastric carcinomas. *Int J Oncol* 2003; **22**: 773-778 [PMID: 12632067]
- 89 **Jonuleit H**, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 2001; **193**: 1285-1294 [PMID: 11390435 DOI: 10.1084/jem.193.11.1285]
- 90 **Ng WF**, Duggan PJ, Ponchel F, Matarese G, Lombardi G, Edwards AD, Isaacs JD, Lechler RI. Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. *Blood* 2001; **98**: 2736-2744 [PMID: 11675346 DOI: 10.1182/blood.V98.9.2736]
- 91 **Sakaguchi S**, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995; **155**: 1151-1164 [PMID: 7636184]
- 92 **Beyer M**, Schultze JL. Regulatory T cells in cancer. *Blood* 2006; **108**: 804-811 [PMID: 16861339 DOI: 10.1182/blood-2006-02-002774]
- 93 **Ichihara F**, Kono K, Takahashi A, Kawaida H, Sugai H, Fujii H. Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. *Clin Cancer Res* 2003; **9**: 4404-4408 [PMID: 14555512]
- 94 **Mizukami Y**, Kono K, Kawaguchi Y, Akaike H, Kamimura K, Sugai H, Fujii H. CCL17 and CCL22 chemokines within tumor microenvironment are related to accumulation of Foxp3+ regulatory T cells in gastric cancer. *Int J Cancer* 2008; **122**: 2286-2293 [PMID: 18224687 DOI: 10.1002/ijc.23392]
- 95 **Kono K**, Kawaida H, Takahashi A, Sugai H, Mimura K, Miyagawa N, Omata H, Fujii H. CD4(+)CD25high regulatory T cells increase with tumor stage in patients with gastric and esophageal cancers. *Cancer Immunol Immunother* 2006; **55**: 1064-1071 [PMID: 16328385 DOI: 10.1007/s00262-005-0092-8]
- 96 **Lu X**, Liu J, Li H, Li W, Wang X, Ma J, Tong Q, Wu K, Wang G. Conversion of intratumoral regulatory T cells by human gastric cancer cells is dependent on transforming growth factor-β1. *J Surg Oncol* 2011; **104**: 571-577 [PMID: 21695703 DOI: 10.1002/jso.22005]
- 97 **Lin Y**, Kikuchi S, Obata Y, Yagyu K. Serum levels of transforming growth factor beta1 are significantly correlated with venous invasion in patients with gastric cancer. *J Gastroenterol Hepatol* 2006; **21**: 432-437 [PMID: 16509870 DOI: 10.1111/j.1440-1746.2005.03939.x]
- 98 **Vagenas K**, Spyropoulos C, Gavala V, Tsamandas AC. TGF-beta1, TGFbeta2, and TGFbeta3 protein expression in gastric carcinomas: correlation with prognostic factors and patient survival. *J Surg Res* 2007; **139**: 182-188 [PMID: 17270215 DOI: 10.1016/j.jss.2006.10.005]
- 99 **Allan SE**, Crome SQ, Crellin NK, Passerini L, Steiner TS, Bacchetta R, Roncarolo MG, Levings MK. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol* 2007; **19**:

- 345-354 [PMID: 17329235 DOI: 10.1093/intimm/dxm014]
- 100 **Zheng SG**, Wang JH, Gray JD, Soucier H, Horwitz DA. Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. *J Immunol* 2004; **172**: 5213-5221 [PMID: 15100259]
 - 101 **Shen LS**, Wang J, Shen DF, Yuan XL, Dong P, Li MX, Xue J, Zhang FM, Ge HL, Xu D. CD4(+)CD25(+)CD127(low/-) regulatory T cells express Foxp3 and suppress effector T cell proliferation and contribute to gastric cancers progression. *Clin Immunol* 2009; **131**: 109-118 [PMID: 19153062 DOI: 10.1016/j.clim.2008.11.010]
 - 102 **Maruyama T**, Kono K, Mizukami Y, Kawaguchi Y, Mimura K, Watanabe M, Izawa S, Fujii H. Distribution of Th17 cells and FoxP3(+) regulatory T cells in tumor-infiltrating lymphocytes, tumor-draining lymph nodes and peripheral blood lymphocytes in patients with gastric cancer. *Cancer Sci* 2010; **101**: 1947-1954 [PMID: 20550524 DOI: 10.1111/j.1349-7006.2010.01624.x]
 - 103 **Orimo A**, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 2005; **121**: 335-348 [PMID: 15882617 DOI: 10.1016/j.cell.2005.02.034]
 - 104 **Pietras K**, Ostman A. Hallmarks of cancer: interactions with the tumor stroma. *Exp Cell Res* 2010; **316**: 1324-1331 [PMID: 20211171 DOI: 10.1016/j.yexcr.2010.02.045]
 - 105 **Bhowmick NA**, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature* 2004; **432**: 332-337 [PMID: 15549095 DOI: 10.1038/nature03096]
 - 106 **Allinen M**, Beroukhi R, Cai L, Brennan C, Lahti-Domenici J, Huang H, Porter D, Hu M, Chin L, Richardson A, Schnitt S, Sellers WR, Polyak K. Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell* 2004; **6**: 17-32 [PMID: 15261139 DOI: 10.1016/j.ccr.2004.06.010]
 - 107 **Kalluri R**, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer* 2006; **6**: 392-401 [PMID: 16572188 DOI: 10.1038/nrc1877]
 - 108 **Zeisberg EM**, Potenta S, Xie L, Zeisberg M, Kalluri R. Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. *Cancer Res* 2007; **67**: 10123-10128 [PMID: 17974953 DOI: 10.1158/0008-5472.CAN-07-3127]
 - 109 **Direkze NC**, Hodivala-Dilke K, Jeffery R, Hunt T, Poulson R, Oukrif D, Alison MR, Wright NA. Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts. *Cancer Res* 2004; **64**: 8492-8495 [PMID: 15574751 DOI: 10.1158/0008-5472.CAN-04-1708]
 - 110 **Worthley DL**, Ruzsiewicz A, Davies R, Moore S, Nivison-Smith I, Bik To L, Browett P, Western R, Durrant S, So J, Young GP, Mullighan CG, Bardy PG, Michael MZ. Human gastrointestinal neoplasia-associated myofibroblasts can develop from bone marrow-derived cells following allogeneic stem cell transplantation. *Stem Cells* 2009; **27**: 1463-1468 [PMID: 19492298 DOI: 10.1002/stem.63]
 - 111 **Semba S**, Kodama Y, Ohnuma K, Mizuuchi E, Masuda R, Yashiro M, Hirakawa K, Yokozaki H. Direct cancer-stromal interaction increases fibroblast proliferation and enhances invasive properties of scirrhous-type gastric carcinoma cells. *Br J Cancer* 2009; **101**: 1365-1373 [PMID: 19773759 DOI: 10.1038/sj.bjc.6605309]
 - 112 **Hewitt RE**, Powe DG, Carter GI, Turner DR. Desmoplasia and its relevance to colorectal tumour invasion. *Int J Cancer* 1993; **53**: 62-69 [PMID: 7677932 DOI: 10.1002/ijc.2910530113]
 - 113 **Dvorak HF**. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 1986; **315**: 1650-1659 [PMID: 3537791 DOI: 10.1056/NEJM198612253152606]
 - 114 **Matsumoto T**, Kuroda R, Mifune Y, Kawamoto A, Shoji T, Miwa M, Asahara T, Kurosaka M. Circulating endothelial/skeletal progenitor cells for bone regeneration and healing. *Bone* 2008; **43**: 434-439 [PMID: 18547890 DOI: 10.1016/j.bone.2008.05.001]
 - 115 **Takahashi Y**, Kitadai Y, Bucana CD, Cleary KR, Ellis LM. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 1995; **55**: 3964-3968 [PMID: 7664263]
 - 116 **Tanigawa N**, Amaya H, Matsumura M, Shimomatsuya T, Horiuchi T, Muraoka R, Iki M. Extent of tumor vascularization correlates with prognosis and hematogenous metastasis in gastric carcinomas. *Cancer Res* 1996; **56**: 2671-2676 [PMID: 8653715]
 - 117 **Takahashi Y**, Cleary KR, Mai M, Kitadai Y, Bucana CD, Ellis LM. Significance of vessel count and vascular endothelial growth factor and its receptor (KDR) in intestinal-type gastric cancer. *Clin Cancer Res* 1996; **2**: 1679-1684 [PMID: 9816116]
 - 118 **Folkman J**. How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial Award lecture. *Cancer Res* 1986; **46**: 467-473 [PMID: 2416426]
 - 119 **Folkman J**. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 1990; **82**: 4-6 [PMID: 1688381 DOI: 10.1093/jnci/82.1.4]
 - 120 **Maeda K**, Chung YS, Ogawa Y, Takatsuka S, Kang SM, Ogawa M, Sawada T, Sowa M. Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. *Cancer* 1996; **77**: 858-863 [PMID: 8608475 DOI: 10.1002/(SICI)1097-0142]
 - 121 **Kitadai Y**, Haruma K, Sumii K, Yamamoto S, Ue T, Yokozaki H, Yasui W, Ohmoto Y, Kajiyama G, Fidler IJ, Tahara E. Expression of interleukin-8 correlates with vascularity in human gastric carcinomas. *Am J Pathol* 1998; **152**: 93-100 [PMID: 9422527]
 - 122 **Tanimoto H**, Yoshida K, Yokozaki H, Yasui W, Nakayama H, Ito H, Ohama K, Tahara E. Expression of basic fibroblast growth factor in human gastric carcinomas. *Virchows Arch B Cell Pathol Incl Mol Pathol* 1991; **61**: 263-267 [PMID: 1685819 DOI: 10.1007/BF02890427]
 - 123 **Takahashi Y**, Bucana CD, Akagi Y, Liu W, Cleary KR, Mai M, Ellis LM. Significance of platelet-derived endothelial cell growth factor in the angiogenesis of human gastric cancer. *Clin Cancer Res* 1998; **4**: 429-434 [PMID: 9516932]
 - 124 **Amioka T**, Kitadai Y, Tanaka S, Haruma K, Yoshihara M, Yasui W, Chayama K. Vascular endothelial growth factor-C expression predicts lymph node metastasis of human gastric carcinomas invading the submucosa. *Eur J Cancer* 2002; **38**: 1413-1419 [DOI: 10.1016/S0959-8049(02)00106-5]
 - 125 **Yonemura Y**, Endo Y, Fujita H, Fushida S, Ninomiya I, Bandou E, Taniguchi K, Miwa K, Ohoyama S, Sugiyama K, Sasaki T. Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. *Clin Cancer Res* 1999; **5**: 1823-1829 [PMID: 10430087]
 - 126 **Onogawa S**, Kitadai Y, Amioka T, Kodama M, Cho S, Kuroda T, Ochiuni T, Kimura S, Kuwai T, Tanaka S, Chayama K. Expression of vascular endothelial growth factor (VEGF)-C and VEGF-D in early gastric carcinoma: correlation with clinicopathological parameters. *Cancer Lett* 2005; **226**: 85-90 [PMID: 16004935 DOI: 10.1016/j.canlet.2004.12.030]
 - 127 **Whiteside TL**. The tumor microenvironment and its role in promoting tumor growth. *Oncogene* 2008; **27**: 5904-5912 [PMID: 18836471 DOI: 10.1038/onc.2008.271]
 - 128 **Cabarcas SM**, Mathews LA, Farrar WL. The cancer stem cell niche--there goes the neighborhood? *Int J Cancer* 2011; **129**: 2315-2327 [PMID: 21792897 DOI: 10.1002/ijc.26312]
 - 129 **Korkaya H**, Paulson A, Charafe-Jauffret E, Ginestier C, Brown M, Dutcher J, Clouthier SG, Wicha MS. Regulation of mammary stem/progenitor cells by PTEN/Akt/beta-catenin signaling. *PLoS Biol* 2009; **7**: e1000121 [PMID: 19492080 DOI: 10.1371/journal.pbio.1000121]

- 130 **Liu S**, Dontu G, Mantle ID, Patel S, Ahn NS, Jackson KW, Suri P, Wicha MS. Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res* 2006; **66**: 6063-6071 [PMID: 16778178 DOI: 10.1158/0008-5472.CAN-06-0054]
- 131 **Iliopoulos D**, Hirsch HA, Struhl K. An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. *Cell* 2009; **139**: 693-706 [PMID: 19878981 DOI: 10.1016/j.cell.2009.10.014]
- 132 **El-Omar EM**, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF, Rabkin CS. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 2000; **404**: 398-402 [PMID: 10746728 DOI: 10.1038/35006081]
- 133 **El-Omar EM**, Rabkin CS, Gammon MD, Vaughan TL, Risch HA, Schoenberg JB, Stanford JL, Mayne ST, Goedert J, Blot WJ, Fraumeni JF, Chow WH. Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. *Gastroenterology* 2003; **124**: 1193-1201 [PMID: 12730860 DOI: 10.1016/S0016-5085(03)00157-4]
- 134 **Tu SP**, Quante M, Bhagat G, Takaishi S, Cui G, Yang XD, Muthuplani S, Shibata W, Fox JG, Pritchard DM, Wang TC. IFN- γ inhibits gastric carcinogenesis by inducing epithelial cell autophagy and T-cell apoptosis. *Cancer Res* 2011; **71**: 4247-4259 [PMID: 21512143 DOI: 10.1158/0008-5472.CAN-10-4009]
- 135 **Le Y**, Zhou Y, Iribarren P, Wang J. Chemokines and chemokine receptors: their manifold roles in homeostasis and disease. *Cell Mol Immunol* 2004; **1**: 95-104 [PMID: 16212895]
- 136 **Luster AD**. Chemokines--chemotactic cytokines that mediate inflammation. *N Engl J Med* 1998; **338**: 436-445 [PMID: 9459648]
- 137 **Raman D**, Baugher PJ, Thu YM, Richmond A. Role of chemokines in tumor growth. *Cancer Lett* 2007; **256**: 137-165 [PMID: 17629396 DOI: 10.1016/j.canlet.2007.05.013]
- 138 **Strieter RM**, Polverini PJ, Kunkel SL, Arenberg DA, Burdick MD, Kasper J, Dzuiba J, Van Damme J, Walz A, Marriott D. The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *J Biol Chem* 1995; **270**: 27348-27357 [PMID: 7592998 DOI: 10.1074/jbc.270.45.27348]
- 139 **Strieter RM**, Burdick MD, Gomperts BN, Belperio JA, Keane MP. CXC chemokines in angiogenesis. *Cytokine Growth Factor Rev* 2005; **16**: 593-609 [PMID: 16046180 DOI: 10.1016/j.cytogfr.2005.04.007]
- 140 **Vandercappellen J**, Van Damme J, Struyf S. The role of CXC chemokines and their receptors in cancer. *Cancer Lett* 2008; **267**: 226-244 [PMID: 18579287 DOI: 10.1016/j.canlet.2008.04.050]
- 141 **Crabtree JE**, Farmery SM, Lindley IJ, Figura N, Peichl P, Tompkins DS. CagA/cytotoxic strains of *Helicobacter pylori* and interleukin-8 in gastric epithelial cell lines. *J Clin Pathol* 1994; **47**: 945-950 [PMID: 7962609 DOI: 10.1136/jcp.47.10.945]
- 142 **Beales IL**, Calam J. Stimulation of IL-8 production in human gastric epithelial cells by *Helicobacter pylori*, IL-1 β and TNF- α requires tyrosine kinase activity, but not protein kinase C. *Cytokine* 1997; **9**: 514-520 [PMID: 9237814 DOI: 10.1006/cyto.1996.0195]
- 143 **Bäckhed F**, Torstensson E, Seguin D, Richter-Dahlfors A, Rokbi B. *Helicobacter pylori* infection induces interleukin-8 receptor expression in the human gastric epithelium. *Infect Immun* 2003; **71**: 3357-3360 [PMID: 12761119 DOI: 10.1128/IAI.71.6.3357-3360.2003]
- 144 **Beswick EJ**, Das S, Pinchuk IV, Adegboyega P, Suarez G, Yamaoka Y, Reyes VE. *Helicobacter pylori*-induced IL-8 production by gastric epithelial cells up-regulates CD74 expression. *J Immunol* 2005; **175**: 171-176 [PMID: 15972644]
- 145 **Konturek PC**, Nikiforuk A, Kania J, Raithel M, Hahn EG, Mühlendorfer S. Activation of NF-kappaB represents the central event in the neoplastic progression associated with Barrett's esophagus: a possible link to the inflammation and over-expression of COX-2, PPARgamma and growth factors. *Dig Dis Sci* 2004; **49**: 1075-1083 [PMID: 15387324]
- 146 **Macri A**, Versaci A, Loddo S, Scuderi G, Travagliante M, Trimarchi G, Teti D, Famulari C. Serum levels of interleukin 1 β , interleukin 8 and tumour necrosis factor alpha as markers of gastric cancer. *Biomarkers* 2006; **11**: 184-193 [PMID: 16766394 DOI: 10.1080/13547500600565677]
- 147 **Kitadai Y**, Haruma K, Mukaida N, Ohmoto Y, Matsutani N, Yasui W, Yamamoto S, Sumii K, Kajiyama G, Fidler IJ, Tahara E. Regulation of disease-progression genes in human gastric carcinoma cells by interleukin 8. *Clin Cancer Res* 2000; **6**: 2735-2740 [PMID: 10914718]
- 148 **Opdenakker G**, Van den Steen PE, Dubois B, Nelissen I, Van Coillie E, Masure S, Proost P, Van Damme J. Gelatinase B functions as regulator and effector in leukocyte biology. *J Leukoc Biol* 2001; **69**: 851-859 [PMID: 11404367]
- 149 **Taguchi A**, Ohmiya N, Shirai K, Mabuchi N, Itoh A, Hirooka Y, Niwa Y, Goto H. Interleukin-8 promoter polymorphism increases the risk of atrophic gastritis and gastric cancer in Japan. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 2487-2493 [PMID: 16284368 DOI: 10.1158/1055-9965.EPI-05-0326]
- 150 **Ye BD**, Kim SG, Park JH, Kim JS, Jung HC, Song IS. The interleukin-8-251 A allele is associated with increased risk of noncardia gastric adenocarcinoma in *Helicobacter pylori*-infected Koreans. *J Clin Gastroenterol* 2009; **43**: 233-239 [PMID: 18542040 DOI: 10.1097/MCG.0b013e3181646701]
- 151 **Song JH**, Kim SG, Jung SA, Lee MK, Jung HC, Song IS. The interleukin-8-251 AA genotype is associated with angiogenesis in gastric carcinogenesis in *Helicobacter pylori*-infected Koreans. *Cytokine* 2010; **51**: 158-165 [PMID: 20621718 DOI: 10.1016/j.cyt.2010.05.001]
- 152 **Kucia M**, Reza R, Miekus K, Wanzeck J, Wojakowski W, Janowska-Wieczorek A, Ratajczak J, Ratajczak MZ. Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: pivotal role of the SDF-1-CXCR4 axis. *Stem Cells* 2005; **23**: 879-894 [PMID: 15888687 DOI: 10.1634/stemcells.2004-0342]
- 153 **Zheng K**, Li HY, Su XL, Wang XY, Tian T, Li F, Ren GS. Chemokine receptor CXCR7 regulates the invasion, angiogenesis and tumor growth of human hepatocellular carcinoma cells. *J Exp Clin Cancer Res* 2010; **29**: 31 [PMID: 20380740 DOI: 10.1186/1756-9966-29-31]
- 154 **Lee HJ**, Kim SW, Kim HY, Li S, Yun HJ, Song KS, Kim S, Jo DY. Chemokine receptor CXCR4 expression, function, and clinical implications in gastric cancer. *Int J Oncol* 2009; **34**: 473-480 [PMID: 19148483]
- 155 **Zhao BC**, Zhao B, Han JG, Ma HC, Wang ZJ. Adipose-derived stem cells promote gastric cancer cell growth, migration and invasion through SDF-1/CXCR4 axis. *Hepatogastroenterology* 2010; **57**: 1382-1389 [PMID: 21443090]
- 156 **Zhao BC**, Wang ZJ, Mao WZ, Ma HC, Han JG, Zhao B, Xu HM. CXCR4/SDF-1 axis is involved in lymph node metastasis of gastric carcinoma. *World J Gastroenterol* 2011; **17**: 2389-2396 [PMID: 21633638 DOI: 10.3748/wjg.v17.i19.2389]
- 157 **Yasumoto K**, Koizumi K, Kawashima A, Saitoh Y, Arita Y, Shinohara K, Minami T, Nakayama T, Sakurai H, Takahashi Y, Yoshie O, Saiki I. Role of the CXCL12/CXCR4 axis in peritoneal carcinomatosis of gastric cancer. *Cancer Res* 2006; **66**: 2181-2187 [PMID: 16489019 DOI: 10.1158/0008-5472.CAN-05-3393]
- 158 **Ingold B**, Simon E, Ungethüm U, Kuban RJ, Müller BM, Lupp A, Neumann U, Ebert MP, Denkert C, Weichert W, Schulz S, Röcken C. Vascular CXCR4 expression - a novel antiangiogenic target in gastric cancer? *PLoS One* 2010; **5**: e10087 [PMID: 20386750 DOI: 10.1371/journal.pone.0010087]
- 159 **Ishigami S**, Natsugoe S, Okumura H, Matsumoto M, Nakajo A, Uenosono Y, Arigami T, Uchikado Y, Setoyama T, Arima H, Hokita S, Aikou T. Clinical implication of CXCL12 expression in gastric cancer. *Ann Surg Oncol* 2007; **14**: 3154-3158

- [PMID: 17653799 DOI: 10.1245/s10434-007-9521-6]
- 160 **Nathoo N**, Chaharvi A, Barnett GH, Toms SA. Pathobiology of brain metastases. *J Clin Pathol* 2005; **58**: 237-242 [PMID: 15735152 DOI: 10.1136/jcp.2003.013623]
 - 161 **Krueger S**, Hundertmark T, Kalinski T, Peitz U, Wex T, Malfertheiner P, Naumann M, Roessner A. Helicobacter pylori encoding the pathogenicity island activates matrix metalloproteinase 1 in gastric epithelial cells via JNK and ERK. *J Biol Chem* 2006; **281**: 2868-2875 [PMID: 16321971 DOI: 10.1074/jbc.M511053200]
 - 162 **Crawford HC**, Krishna US, Israel DA, Matrisian LM, Washington MK, Peek RM. Helicobacter pylori strain-selective induction of matrix metalloproteinase-7 in vitro and within gastric mucosa. *Gastroenterology* 2003; **125**: 1125-1136 [PMID: 14517796 DOI: 10.1016/S0016-5085(03)01206-X]
 - 163 **Wroblewski LE**, Noble PJ, Pagliocca A, Pritchard DM, Hart CA, Campbell F, Dodson AR, Dockray GJ, Varro A. Stimulation of MMP-7 (matrilysin) by Helicobacter pylori in human gastric epithelial cells: role in epithelial cell migration. *J Cell Sci* 2003; **116**: 3017-3026 [PMID: 12808021 DOI: 10.1242/jcs.00518]
 - 164 **McCaig C**, Duval C, Hemers E, Steele I, Pritchard DM, Przemek S, Dimaline R, Ahmed S, Bodger K, Kerrigan DD, Wang TC, Dockray GJ, Varro A. The role of matrix metalloproteinase-7 in redefining the gastric microenvironment in response to Helicobacter pylori. *Gastroenterology* 2006; **130**: 1754-1763 [PMID: 16697739 DOI: 10.1053/j.gastro.2006.02.031]
 - 165 **Rautelin HI**, Oksanen AM, Veijola LI, Sipponen PI, Tervahartia TI, Sorsa TA, Lauhio A. Enhanced systemic matrix metalloproteinase response in Helicobacter pylori gastritis. *Ann Med* 2009; **41**: 208-215 [PMID: 18979291 DOI: 10.1080/07853890802482452]
 - 166 **Bergin PJ**, Raghavan S, Svensson H, Starckx S, Van Aelst I, Gjertsson I, Opendakker G, Quiding-Järbrink M. Gastric gelatinase B/matrix metalloproteinase-9 is rapidly increased in Helicobacter felis-induced gastritis. *FEMS Immunol Med Microbiol* 2008; **52**: 88-98 [PMID: 17995959 DOI: 10.1111/j.1574-695X.2007.00349.x]
 - 167 **Wu JY**, Lu H, Sun Y, Graham DY, Cheung HS, Yamaoka Y. Balance between polyoma enhancing activator 3 and activator protein 1 regulates Helicobacter pylori-stimulated matrix metalloproteinase 1 expression. *Cancer Res* 2006; **66**: 5111-5120 [PMID: 16707434 DOI: 10.1158/0008-5472.CAN-06-0383]
 - 168 **Ogden SR**, Wroblewski LE, Weydig C, Romero-Gallo J, O'Brien DP, Israel DA, Krishna US, Fingleton B, Reynolds AB, Wessler S, Peek RM. p120 and Kaiso regulate Helicobacter pylori-induced expression of matrix metalloproteinase-7. *Mol Biol Cell* 2008; **19**: 4110-4121 [PMID: 18653469 DOI: 10.1091/mbc.E08-03-0283]
 - 169 **Yonemura Y**, Endou Y, Fujita H, Fushida S, Bandou E, Taniguchi K, Miwa K, Sugiyama K, Sasaki T. Role of MMP-7 in the formation of peritoneal dissemination in gastric cancer. *Gastric Cancer* 2000; **3**: 63-70 [PMID: 11984713 DOI: 10.1007/PL00011698]
 - 170 **Yin Y**, Grabowska AM, Clarke PA, Whelband E, Robinson K, Argent RH, Tobias A, Kumari R, Atherton JC, Watson SA. Helicobacter pylori potentiates epithelial: mesenchymal transition in gastric cancer: links to soluble HB-EGF, gastrin and matrix metalloproteinase-7. *Gut* 2010; **59**: 1037-1045 [PMID: 20584780 DOI: 10.1136/gut.2009.199794]
 - 171 **Bergin PJ**, Anders E, Sicheng W, Erik J, Jennie A, Hans L, Pierre M, Qiang PH, Marianne QJ. Increased production of matrix metalloproteinases in Helicobacter pylori-associated human gastritis. *Helicobacter* 2004; **9**: 201-210 [PMID: 15165255 DOI: 10.1111/j.1083-4389.2004.00232.x]
 - 172 **Kubben FJ**, Sier CF, Schram MT, Witte AM, Veenendaal RA, van Duijn W, Verheijen JH, Hanemaaijer R, Lamers CB, Verspaget HW. Eradication of Helicobacter pylori infection favourably affects altered gastric mucosal MMP-9 levels. *Helicobacter* 2007; **12**: 498-504 [PMID: 17760717 DOI: 10.1111/j.1523-5378.2007.00527.x]
 - 173 **Baker AH**, Edwards DR, Murphy G. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J Cell Sci* 2002; **115**: 3719-3727 [PMID: 12235282 DOI: 10.1242/jcs.00063]
 - 174 **Edwards DR**, Handsley MM, Pennington CJ. The ADAM metalloproteinases. *Mol Aspects Med* 2008; **29**: 258-289 [PMID: 18762209 DOI: 10.1016/j.mam.2008.08.001]
 - 175 **Yoshimura T**, Tomita T, Dixon MF, Axon AT, Robinson PA, Crabtree JE. ADAMs (a disintegrin and metalloproteinase) messenger RNA expression in Helicobacter pylori-infected, normal, and neoplastic gastric mucosa. *J Infect Dis* 2002; **185**: 332-340 [PMID: 11807715 DOI: 10.1086/338191]
 - 176 **Saha A**, Backert S, Hammond CE, Gooz M, Smolka AJ. Helicobacter pylori CagL activates ADAM17 to induce repression of the gastric H, K-ATPase alpha subunit. *Gastroenterology* 2010; **139**: 239-248 [PMID: 20303353 DOI: 10.1053/j.gastro.2010.03.036]
 - 177 **Bang YJ**, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Rüschoff J, Kang YK; ToGA Trial Investigators. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): A phase 3, open-label, randomized controlled trial. *Lancet* 2010; **376**: 687-697 [DOI: 10.1016/S0140-6736(10)61121-X]
 - 178 **United States Food and Drug Administration**. Herceptin (trastuzumab) [prescribing information]. Available from: URL: http://www.accessdata.fda.gov/drugsatfda_docs/label/2010/103792s5250lbl.pdf Accessed October 26, 2011.
 - 179 **European Medicines Agency**. Herceptin [summary of product characteristics]. Available from: URL: http://www.ema.europa.eu/docs/enMGB/document_library/EPAR_-_Product_Information/human/000278/WC500074922.pdf Accessed October 26, 2011.
 - 180 **Ohtsu A**, Shah MA, Van Cutsem E, Rha SY, Sawaki A, Park SR, Lim HY, Yamada Y, Wu J, Langer B, Starnawski M, Kang YK. Bevacizumab in combination with chemotherapy as first-line therapy in advanced gastric cancer: a randomized, double-blind, placebo-controlled phase III study. *J Clin Oncol* 2011; **29**: 3968-3976 [PMID: 21844504 DOI: 10.1200/JCO.2011.36.2236]
 - 181 **Pinto C**, Di Fabio F, Siena S, Cascinu S, Rojas Llimpe FL, Ceccarelli C, Mutri V, Giannetta L, Giaquinta S, Funaioli C, Berardi R, Longobardi C, Piana E, Martoni AA. Phase II study of cetuximab in combination with FOLFIRI in patients with untreated advanced gastric or gastroesophageal junction adenocarcinoma (FOLCETUX study). *Ann Oncol* 2007; **18**: 510-517 [PMID: 17164226 DOI: 10.1093/annonc/mdl459]
 - 182 **Moehler M**, Mueller A, Trarbach T, Lordick F, Seufferlein T, Kubicka S, Geissler M, Schwarz S, Galle PR, Kanzler S. Cetuximab with irinotecan, folinic acid and 5-fluorouracil as first-line treatment in advanced gastroesophageal cancer: a prospective multi-center biomarker-oriented phase II study. *Ann Oncol* 2011; **22**: 1358-1366 [PMID: 21119032 DOI: 10.1093/annonc/mdq591]
 - 183 **Richards D**, Kocs DM, Spira AI, David McCollum A, Diab S, Hecker LI, Cohn A, Zhan F, Asmar L. Results of docetaxel plus oxaliplatin (DOCOX) ± cetuximab in patients with metastatic gastric and/or gastroesophageal junction adenocarcinoma: results of a randomised Phase 2 study. *Eur J Cancer* 2013; **49**: 2823-2831 [PMID: 23747051 DOI: 10.1016/j.ejca.2013.04.022]
 - 184 **Optimization Study in ErbB2 (HER2) Positive Gastric Cancer: A Phase III Global, Blinded Study Designed to Evaluate Clinical Endpoints and Safety of Chemotherapy Plus Lapatinib**. accessed January 21, 2012. Available from: URL:

- <http://www.clinicaltrials.gov/ct/show/NCT00680901>
- 185 **Satoh T**, Bang Y, Wang J. Interim safety analysis from TY-TAN: A phase III Asian study of lapatinib in combination with paclitaxel as second line therapy in gastric cancer. *J Clin Oncol* 2010; **28** suppl15: 4057
 - 186 **Bae SH**, Ryoo HM, Kim MK, Lee KH, Sin JI, Hyun MS. Effects of the proteasome inhibitor bortezomib alone and in combination with chemotherapeutic agents in gastric cancer cell lines. *Oncol Rep* 2008; **19**: 1027-1032 [PMID: 18357392]
 - 187 **Shah MA**, Wainberg ZA, Catenacci DV, Hochster HS, Ford J, Kunz P, Lee FC, Kallender H, Cecchi F, Rabe DC, Keer H, Martin AM, Liu Y, Gagnon R, Bonate P, Liu L, Gilmer T, Bottaro DP. Phase II study evaluating 2 dosing schedules of oral foretinib (GSK1363089), cMET/VEGFR2 inhibitor, in patients with metastatic gastric cancer. *PLoS One* 2013; **8**: e54014 [PMID: 23516391 DOI: 10.1371/journal.pone.0054014]
 - 188 **Molife LR**, Fong PC, Paccagnella L, Reid AH, Shaw HM, Vidal L, Arkenau HT, Karavasilis V, Yap TA, Olmos D, Spicer J, Postel-Vinay S, Yin D, Lipton A, Demers L, Leitzel K, Gualberto A, de Bono JS. The insulin-like growth factor-I receptor inhibitor figitumumab (CP-751,871) in combination with docetaxel in patients with advanced solid tumours: results of a phase Ib dose-escalation, open-label study. *Br J Cancer* 2010; **103**: 332-339 [PMID: 20628389 DOI: 10.1038/sj.bjc.6605767]
 - 189 **Squires M**, Ward G, Saxty G, Berdini V, Cleasby A, King P, Angibaud P, Perera T, Fazal L, Ross D, Jones CG, Madin A, Benning RK, Vickerstaffe E, O'Brien A, Frederickson M, Reader M, Hamlett C, Batey MA, Rich S, Carr M, Miller D, Feltell R, Thiru A, Bethell S, Devine LA, Graham BL, Pike A, Cosme J, Lewis EJ, Freyne E, Lyons J, Irving J, Murray C, Newell DR, Thompson NT. Potent, selective inhibitors of fibroblast growth factor receptor define fibroblast growth factor dependence in preclinical cancer models. *Mol Cancer Ther* 2011; **10**: 1542-1552 [PMID: 21764904 DOI: 10.1158/1535-7163.MCT-11-0426]
 - 190 **Lang SA**, Klein D, Moser C, Gaumann A, Glockzin G, Dahlke MH, Dietmaier W, Bolder U, Schlitt HJ, Geissler EK, Stoeltzing O. Inhibition of heat shock protein 90 impairs epidermal growth factor-mediated signaling in gastric cancer cells and reduces tumor growth and vascularization in vivo. *Mol Cancer Ther* 2007; **6**: 1123-1132 [PMID: 17363505 DOI: 10.1158/1535-7163.MCT-06-0628]
 - 191 **Weichert W**, Röske A, Gekeler V, Beckers T, Ebert MP, Pross M, Dietel M, Denkert C, Röcken C. Association of patterns of class I histone deacetylase expression with patient prognosis in gastric cancer: a retrospective analysis. *Lancet Oncol* 2008; **9**: 139-148 [PMID: 18207460 DOI: 10.1016/S1470-2045(08)70004-4]
 - 192 **Fetterly GJ**, Brady WE, LeVea CM. A phase I pharmacokinetic (PK) study of vorinostat (V) in combination with irinotecan (I), 5-fluorouracil (5FU), and leucovorin (FOLFIRI) in advanced upper gastrointestinal cancers (AGC). *J Clin Oncol* 2009; **27** suppl15: e15540
 - 193 **Chiba T**, Marusawa H, Seno H, Watanabe N. Mechanism for gastric cancer development by *Helicobacter pylori* infection. *J Gastroenterol Hepatol* 2008; **23**: 1175-1181 [PMID: 18637055 DOI: 10.1111/j.1440-1746.2008.05472.x]

P- Reviewers: De Lusong MAA, Hasanein P, Lambrecht NW

S- Editor: Qi Y **L- Editor:** A **E- Editor:** Wu HL



WJG 20th Anniversary Special Issues (8): Gastric cancer

CXC chemokines and chemokine receptors in gastric cancer: From basic findings towards therapeutic targeting

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Supported by Basic Science Research Program through the National Research of Korea (NRF) funded by the Ministry of Education, Science and Technology, NRF-2009-0076540, NRF-2009-0067256

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Received: August 23, 2013 Revised: October 1, 2013

Accepted: November 12, 2013

Published online: February 21, 2014

Abstract

Gastric cancer is the fourth most common cancer, and the second-highest cause of cancer-related deaths worldwide. Despite extensive research to identify novel diagnostic and therapeutic agents, patients with advanced gastric cancer suffer from a poor quality of life and poor prognosis, and treatment is dependent mainly on conventional cytotoxic chemotherapy. To improve the quality of life and survival of gastric cancer patients, a better understanding of the underlying molecular pathologies, and their application towards the development of novel targeted therapies, is urgently needed.

Chemokines are a group of small proteins associated with cytoskeletal rearrangements, the directional migration of several cell types during development and physiology, and the host immune response *via* interactions with G-protein coupled receptors. There is also growing evidence to suggest that chemokines not only play a role in the immune system, but are also involved in the development and progression of tumors. In gastric cancer, CXC chemokines and chemokine receptors regulate the trafficking of cells in and out of the tumor microenvironment. CXC chemokines and their receptors can also directly influence tumorigenesis by modulating tumor transformation, survival, growth, invasion and metastasis, as well as indirectly by regulating angiogenesis, and tumor-leukocyte interactions. In this review, we will focus on the roles of CXC chemokines and their receptors in the development, progression, and metastasis of gastric tumors, and discuss their therapeutic potential for gastric cancer.

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Key words: Chemokine; Chemokine receptor; Gastric neoplasm; Therapeutic target

Core tip: Chemokines were traditionally believed to regulate the directional migration of leukocytes to inflammatory sites. However, it is now clear that chemokines and chemokine receptors also regulate the processes underlying the development and progression of malignant disease. In gastric cancer, CXC chemokines and their receptors directly influence tumorigenesis by modulating tumor transformation, survival, growth, invasion, and metastasis, as well as indirectly by regulating angiogenesis and interactions between tumor and microenvironment. Aim of this review is to discuss the involvement of CXC chemokines and their receptors in the development, progression, and metastasis of gastric cancer and their therapeutic potential.

Lee HJ, Song IC, Yun HJ, Jo DY, Kim S. CXC chemokines and chemokine receptors in gastric cancer: From basic findings towards therapeutic targeting. *World J Gastroenterol* 2014; 20(7): 1681-1693 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1681.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1681>

INTRODUCTION

Gastric cancer is the fourth most common cancer and the second-highest cause of cancer-related deaths worldwide although the incidence is decreasing in many developed countries. Approximately 8% of newly diagnosed malignant tumors are gastric cancer, and over 700000 people die from gastric cancer annually^[1-3]. Despite intensive research into novel diagnostic and therapeutic interventions, the prognosis of patients with advanced gastric cancer remains poor, and little improvement in survival has been achieved^[4]. In recent years, many new advances have enhanced our understanding of the molecular mechanisms and alterations that lead to initiation and progression of gastric cancer, including multiple genetic and molecular alterations and mutations^[4-8]. Molecular alterations in gastric carcinogenesis have been identified in *Her-2/neu* (*c-erbB2*)^[9-11], *c-Myc*^[12,13], semaphorin-5A^[14], *BCL2*-like-12 (*BCL2L12*)^[15], *c-MET*^[16], and *K-sam*^[17], while mutations have been reported in *TP53*^[18], adenomatous polyposis coli (*APC*)^[19], *K-ras*^[20], and E-cadherin^[21]. Importantly, the ToGA (Trastuzumab for Gastric Cancer) trial recently demonstrated that the addition of trastuzumab, a monoclonal antibody against *Her-2/neu*, to conventional chemotherapy significantly improved the survival of patients with advanced gastric or gastro-esophageal junction cancer compared with chemotherapy alone^[22]. In spite of these advances, the successful treatment of advanced or metastatic gastric cancer depends predominantly on the response of the tumor to conventional cytotoxic chemotherapy. Understanding the distinct molecular pathways behind the progression and treatment resistance of gastric cancer may therefore lead to novel therapeutic opportunities, and improve the quality of life and overall survival of patients.

Chemokines are a group of small (8-14 kDa) proteins that interact with their cell-surface receptors during development, the host immune response, and other physiological processes, to direct cells to specific sites throughout the body^[23,24]. The term chemokines was originally introduced in 1992 as an abbreviated form of chemotactic cytokines, shortly after the characterization of the first chemokine, interleukin-8 (IL-8; also known as CXCL8)^[25,26]. Subsequently, chemokines were characterized as a large family of heparin-binding proteins that modulate cell trafficking and the targeting of immune cells^[25,27,28]. The chemokine system evolved with vertebrates, and approximately 50 human genes encode chemokine ligands, together with more than 20 chemokine receptor genes, which encode seven-transmembrane G protein-coupled receptors^[23,29]. Chemokines are catego-

rized into four major groups (CXC, CC, CX3C or C), depending on the position of their cysteine residues near the N-terminus, in which X represents any amino acid. Most chemokines are in the CXC and CC groups^[30-32]. With the exception of the "C" subgroup, all chemokines include a common four-cysteine residue motif linked by disulfide bonds at conserved sites: one between the first and the third Cys, and one between the second and the fourth Cys, leading to the formation of a triple-stranded β -sheet structure. CXC chemokines can be further subclassified based on the presence or absence of a glutamic acid-leucine-arginine (ELR) motif situated before the first conserved cysteine residue (ELR⁺ or ELR⁻)^[32-35].

Chemokines are produced by many cell types, including leukocytes, endothelial cells, fibroblasts, epithelial cells, and tumor cells^[32,36]. Recent evidence has revealed that, in addition to their role in the immune system, chemokines and their receptors are also involved in tumor initiation and progression^[37,38]. Chemokines bind to the extracellular domain of chemokine receptors, which comprises the N-terminus and extracellular loops. Following activation, the intracellular domains (consisting of three loops and the C-terminus) dissociate from G-proteins, which are composed of three distinct subunits (α , β and γ heterotrimers). This results in the formation of the second messengers inositol triphosphate (IP3) and diacylglycerol (DAG), leading to cytoplasmic calcium mobilization, and the activation of multiple downstream signaling cascades, including the phosphatidylinositol 3-kinase (PI3K)/Akt, Ras/mitogen-activated protein kinase (MAPK), and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways^[23,25].

In cancer, chemokines and their receptors play a crucial role in the trafficking of cells in and out of the tumor microenvironment, modulating the behavior of the tumor. Chemokines induce directional cell migration, particularly of leukocytes, during inflammation. Prolonged inflammation can facilitate carcinogenesis by providing a favorable microenvironment around the tumor for its growth and development^[30,37]. Chemokines can influence tumorigenesis indirectly by regulating angiogenesis and tumor-leukocyte interactions, and directly by modulating tumor transformation, survival, growth, invasion, and metastasis. However, the roles of chemokines and their receptors in tumorigenesis are complex, since some family members promote conditions favorable for tumor growth and progression, while others demonstrate anti-tumor activity^[30]. For example, ELR⁺ CXC chemokines such as CXCL8 can enhance tumor growth by inducing angiogenesis and the chemoattraction of neutrophilic granulocytes. Neutrophils then further facilitate angiogenesis, tumor growth, and metastasis by releasing matrix-degrading enzymes [such as matrix-metalloprotease (MMP)-9] and angiogenic tumor-promoting factors such as vascular endothelial growth factor (VEGF)^[30,39-41]. In contrast, ELR⁻ CXC chemokines such as interferon- γ inducible protein-10 (IP-10; also known as CXCL10) are angiostatic factors, and attract

anti-tumoral lymphocytes by binding to CXCR3. Stromal cell-derived factor-1 (SDF-1; also known as CXCL12) is an exception to this characterization, since it is an ELR⁺ chemokine that can mediate angiogenesis *via* its cognate receptor CXCR4^[30,37,40,42]. Furthermore, in contrast to the anti-tumoral activities of the CXCR3-binding ELR⁺ CXC chemokines, these chemokines also promote the metastasis of CXCR3-positive tumor cells to lymph nodes and distant sites^[37,43,44]. The balance of chemokines and chemokine receptors within the tumor environment is highly complex, and organ-dependent. In this review we will focus on the involvement of CXC chemokines and their receptors in the development, progression, and metastasis of gastric cancer, and their therapeutic potential.

CXC CHEMOKINES AND THEIR RECEPTORS IN GASTRIC CANCER

CXCL12-CXCR4/CXCR7

CXCR4 is differentially expressed in gastric adenocarcinoma at the transcriptional and protein levels, and in the cell membrane^[5,45-59]. The differential expression of CXCR4 in gastric cancer is also identified by gene expression profiling^[57,60,61]. In addition, pre-operative circulating CXCR4 mRNA levels in the plasma of patients with gastric cancer are elevated compared with normal controls, but then decrease after surgery^[62]. Increased CXCR4 expression in gastric cancer cells is associated with peritoneal carcinomatosis, which occurs frequently and is a major cause of mortality in patients with gastric cancer^[47,63-65]. In addition, elevated expression of CXCL12 was detected in peritoneal mesothelial cells, suggesting that CXCR4-positive gastric cancer cells are preferentially attracted to the peritoneum, where high levels of its ligand CXCL12 are produced^[47,53]. CXCR4 expression is also associated with aggressive tumor behavior, such as poor differentiation, tumor invasion and metastasis^[45,50,54,55,58,66-70], and it could therefore be an independent prognostic marker for the overall survival of patients with gastric cancer^[71]. Several studies have revealed that gastric cancer cells also show altered expression of CXCL12. However, the data are controversial, since increased expression of CXCL12 was associated with tumor size, invasion, lymph node metastasis, and poor prognosis^[51,68,72-75], but the opposite data have also been reported^[76]. Up-regulation of the *CXCL12* gene was demonstrated by cDNA microarrays, while the secretion of CXCL12 was also reported in gastric cancer cells^[77-79]. In addition, Schimanski *et al.*^[80] reported that a CXCL12 (SNP rs1801157) polymorphism of GA/AA was correlated with distant metastasis. The circulating levels of CXCL12 in gastric cancer patients are elevated pre-treatment, and higher in metastatic than non-metastatic patients, suggesting that secretion correlates with the presence of distant metastases^[81]. However, the precise mechanism by which tumor-derived CXCL12 contributes to tumor progression is unclear.

CXCL12 may regulate tumorigenesis in an autocrine and/or paracrine manner. The concomitant expression

of CXCL12 and its receptor CXCR4 in tumor cells can lead to the autocrine/paracrine stimulation of cancer cells, resulting in aggressive tumor behavior^[5,73,82,83]. Subsequently, autocrine/paracrine mitogenic effects of CXCL12 were reported in glioblastoma multiforme, gall bladder cancer, and pituitary tumors^[83-86]. Furthermore, immunohistochemical analysis demonstrated that the staining of CXCR4 and CXCL12 in gastric cancer was more prominent and intense in tumor cells at the invasion front and in lymphatic vessels, respectively. Patients with elevated expression of CXCR4 and CXCL12 therefore exhibit significantly poorer surgical outcomes^[5,37,45,66,72].

Another possible mechanism by which CXCL12 contributes to tumor progression is by inducing a favorable tumor microenvironment by attracting endothelial cells or recruiting immune suppressive cells to the tumor site, resulting in angiogenesis and immune evasion, respectively^[75,86]. Zhuang *et al.*^[87] recently demonstrated that CD8⁺ T cells secrete IL-17, which induces gastric cancer cells to produce CXCL12. CXCL12 then recruits myeloid-derived suppressor cells (MDSCs) into the tumor microenvironment in a CXCR4-dependent manner, where MDSCs promote the progression of gastric cancer. In addition, Ingold *et al.*^[51] reported that the expression of CXCL12 in tumor cells and CXCR4 in the microvessels surrounding the tumor is associated with increased local tumor growth and a more advanced tumor stage, suggesting an important role of the CXCL12-CXCR4 axis in tumor neo-angiogenesis in gastric cancer.

Lastly Shibata *et al.*^[88] used CXCL12 transgenic mouse models to demonstrate that overexpression of CXCL12 contributed to the early stages of gastric carcinogenesis by recruiting CXCR4-positive mesenchymal stem cells and stimulating the expansion of myofibroblasts in the gastric stem cell niche, leading to increased numbers of epithelial progenitors.

CXCR4 mediates several biological processes in cancer cells such as directional migration, invasion and adhesion, all of which are associated with the aggressive behavior of tumors. The ligation of CXCL12 to CXCR4 activates actin polymerization to induce cell motility^[5,89]. In gastric cancer cells, CXCL12 stimulation induced the formation of lamellipodia and filopodia. Within lamellipodia, the condensation of F-actin at the leading edge suggested that stimulation of the cells with CXCL12 resulted in the reorganization of F-actin. In addition, compounds targeting the PI3K/mammalian target of rapamycin (mTOR) pathway inhibited CXCL12/CXCR4-mediated cell migration by preventing F-actin reorganization and lamellipodia formation, and by reducing the expression of GTPases, particularly RhoA^[74]. CXCR4 also activates members of the Src family of protein tyrosine kinases, thereby inducing the activation of focal adhesion complexes such as related adhesion focal tyrosine kinase/Pyk2, focal adhesion kinase, Crk and paxillin. CXCR4 also facilitates the adhesion of tumor cells to components of the extracellular matrix *via* integrins^[5,90-92]. CXCL12-activated CXCR4 progressively upregulated the expression

of MMP-2 and MMP-7 in gastric cancer cells^[5,64], while CXCR4 expression was also correlated with the expression of MMP-7 and MMP-9 in gastric cancer tissue^[58]. MMP-7 activates MMP-2 and MMP-9, and plays a central role in the degradation of the extracellular matrix, including type IV collagen. In addition, MMP-7 expression is related to the transformation of cancer cells, suggesting a possible mechanism by which CXCL12 stimulates the invasion, metastasis, and aggressive behavior of gastric cancer^[5,58,93,94].

The binding of CXCL12 to CXCR4 activates a number of intracellular signaling cascades and effector molecules that regulate the proliferation, migration, invasion, and metastasis of cancer cells. The large number of downstream effectors modulated by CXCR4 likely account for the varying effects of the CXCL12-CXCR4 axis in the biology of gastric cancer. However, the roles of the various effectors induced by CXCR4 on the individual gastric cancer processes—such as cell proliferation and adhesion—remains unclear. Nevertheless, identifying downstream effectors of CXCR4 *in vivo* is important to clarify the molecular mechanisms by which CXCR4 promotes gastric cancer^[5,92]. CXCL12 interacts with and activates CXCR4, which in turn activates the p110 β isoform PI3K, leading to the generation of phosphatidylinositol (3,4,5)-triphosphate, and the phosphorylation of the protein kinase B/Akt and mTOR pathways. Activated mTOR subsequently induces the activation of p70S6K (S6K), and eukaryotic initiation factor 4E binding protein 1 (4E-BP1)^[5,47,64,74,95,96]. The treatment of gastric cancer cells with CXCL12 stimulates Akt kinase activity, which leads to the activation of its downstream targets S6K and 4E-BP1. In addition, CXCL12-induced activation of S6K and 4E-BP1 can be inhibited selectively using the mTOR inhibitor rapamycin^[5,47,64,74]. Activated Akt/mTOR signaling leads to the phosphorylation of a variety of intracellular targets (including S6K1 and 4E-BP1) that are involved in increased survival and decreased apoptosis in a variety of cancer cells. Akt and mTOR have also been implicated in the effects of CXCR4 on cell proliferation and chemotactic migration, which play a role in cell growth and metastasis^[5,47,64]. Accordingly, inhibiting the Akt/mTOR pathway blocked migration and reduced the proliferation of gastric cancer cells. Interestingly, gastric cancer cells expressing high levels of CXCL12 were more sensitive to rapamycin-mediated inhibition of migration and proliferation compared with cells expressing low levels of CXCL12. The correlation between CXCL12 gene expression profiles and the anti-proliferative activities of rapamycin were confirmed in NCI-60 cells^[74].

The MAPK pathway is also modulated by CXCR4. In response to CXCL12, CXCR4 activates mitogen-activated protein kinase kinase, the upstream activator of the p42/44 MAPK [also known as extracellular receptor kinase (ERK)-1/2]^[5,92]. In gastric cancer cells, treatment with CXCL12 rapidly induced the phosphorylation of MAPK, which could be blocked by AMD3100, a small molecule that specifically inhibits the CXCR4 recep-

tor^[5,45,47,95]. Collectively, these data suggest that the activation of MAPK is another mechanism by which CXCR4 may promote the progression of gastric cancer.

The JAK/STAT pathway is involved in the migration and invasion of cancer cells^[5,97], and is a third potential pathway by which CXCR4 regulates the growth and progression of gastric cancer. After treatment with CXCL12, JAK kinases associate with CXCR4, leading to the activation of members of the STAT family of transcription factors. In a study using a single gastric cancer cell line, it was reported that CXCR4 signaling is independent of the JAK/STAT pathway. However, the role of this pathway in the biological actions of CXCR4 in gastric cancer remains unclear due to insufficient data^[5,45].

Recently, CXCR7 was identified as a novel receptor for CXCL12, which has complicated our understanding of the role of the CXCL12-CXCR4 axis in regulating cancer development and progression^[86]. CXCR7 is highly expressed on the surface of malignant cells compared with cells in normal adult tissues. It binds CXCL12 with a high affinity, and exerts various biological effects depending on cell type, either by activating intracellular signaling pathways, or *via* its role as a scavenger-type receptor. Importantly, CXCR7 was implicated in cancer cell growth, survival, and metastasis in various cancers, including breast and lung cancer^[98-100]. In gastric cancer, Lee *et al.*^[101] demonstrated that CXCR7 was differentially expressed in gastric cancer tissues, and that elevated expression of both CXCR7 and CXCL12 in tumor cells correlated with aggressive tumor behavior and poor prognosis. This suggests that additional studies should be carried out to elucidate the role of the CXCL12-CXCR7 axis in gastric carcinogenesis.

CXCL8-CXCR1/CXCR2

Several reports have suggested that gastric cancer cells produce CXCL8 both *in vitro* and *in vivo*^[102-107]. The gastric cancer cell lines AGS and KATO III secreted CXCL8 following infection with *H. pylori*, which is associated with gastric carcinogenesis^[103,108]. The up regulation of CXCL8 was demonstrated in gastric cancer cell lines by gene expression profiling, and in gastric cancer tissue by immunostaining^[78,104,109,110]. In addition, the serum levels of circulating CXCL8 were higher in gastric cancer patients than healthy controls^[111]. The expression of CXCL8 was associated with increased venous and lymphatic invasion, and increased depth of invasion in gastric cancer^[106]. Elevated mRNA and protein expression of CXCL8 is also significantly correlated with tumor vascularization, suggesting that it may play a role in gastric cancer^[104,109]. Consistent with these observations, CXCL8 was identified as a strong angiogenic factor in lung, ovarian, and prostate cancer^[112-114]. Direct evidence for the role of CXCL8 in the angiogenesis and tumorigenesis of gastric cancer was first provided by Kitadai *et al.*^[115] who used CXCL8-stably transfected gastric cancer cells to demonstrate angiogenic activity *in vitro*. In addition, the orthotopic implantation of CXCL8-transfected gastric cancer cells into nude mice

in vivo led to the development of rapidly growing and highly vascularized tumors.

CXCL8 also plays a role in the migration, invasion, and adhesion of gastric cancer^[116,117]. Ju *et al*^[116] treated the human gastric cancer cell line SCG-7901 with recombinant CXCL8, and observed enhanced adhesion to endothelial cells and extracellular matrix components, and increased migration and invasion, possibly by regulating the expression of MMP-9, intracellular adhesion molecule (ICAM)-1, and E-cadherin. Similarly, Kuai *et al*^[117] demonstrated that constitutive expression of CXCL8 in MKN45 cells facilitated cell adhesion, migration, and invasion, all of which were inhibited by silencing CXCL8 expression in KATO III cells. In addition, they demonstrated that overexpression of CXCL8 increased the expression of the adhesion molecules ICAM-1, vascular cell adhesion molecule-1 and CD44, as well as the activities of activated nuclear factor (NF)- κ B and Akt. Finally, CXCL8 decreased the sensitivity of gastric cancer cells to the cytotoxic effects of the chemotherapy agent oxaliplatin.

Polymorphisms of the CXCL8 251 allele have also been linked to gastric cancer risk^[118-124]. Wang *et al*^[122] demonstrated that the AA genotype at the CXCL8 251 allele was a risk factor for gastric cancer, particularly in Asian populations. Consistent with this, Song *et al*^[124] reported an increased incidence of this allele in *H. pylori*-infected Korean populations. The gastric mucosal concentration of MMP-9 and angiopoietin (Ang)-1 were correlated with disease progression in patients with the CXCL8 251 AA allele, suggesting that this genotype may be associated with angiogenesis in gastric carcinogenesis. Finally, Vinagre *et al*^[123] revealed an interaction between CXCL8 251 polymorphism, particularly with carriers of the A allele, and *s1m1 cagA* positive *H. pylori* infection. Taken together, these data suggest that polymorphisms of CXCL8 251 allele play an important role in the development of gastric cancer.

CXCR1 and CXCR2 are the receptors for CXCL8, and both are expressed on the surface of gastric cancer cells^[107,109,125-127], suggesting that tumor-derived CXCL8 exerts biological effects on the tumor cells in an autocrine or paracrine manner. Wang *et al*^[125] reported that increased CXCR1 expression was associated with advanced stage, and was an independent risk factor for a higher nodal stage. They also demonstrated that CXCR1/2 expression was higher in gastric cancer compared with corresponding non-neoplastic tissue, and was correlated with increased invasion, metastasis, and microvessel density, as well as increased levels of phospho-Akt, phospho-ERK, cyclin D1, epidermal growth factor receptor (EGFR), Bcl-2, MMP-9 and MMP-2. This suggests that CXCR1/2 signaling plays a crucial role in the progression of gastric cancer, possibly *via* the phosphorylation of ERK and Akt^[126]. Elevated expression of CXCR1 was also detected in most, and CXCR2 in only a few, tumor-infiltrating neutrophils, suggesting that tumor-secreted CXCL8 recruits neutrophils to the tumor microenvironment *via*

CXCR1^[109]. This is consistent with previous observations that the over-expression of CXCL8 by tumor cells attracts a large number of CXCL8 receptor-expressing leukocytes to the tumor site by chemotaxis, and that these leukocytes then secrete growth factors to further facilitate the growth and progression of tumors^[128,129].

CXCL1-CXCR2

CXCL1 and its receptor CXCR2 are differentially expressed in gastric cancer^[109,130-132]. Gastric cancer cells produce CXCL1, and its gene expression was detected both *in vitro* and in gastric cancer mouse models *in vivo*^[103,105,133-135]. In addition, the expression of CXCL1 mRNA and protein was higher in gastric cancer tissue compared with non-cancerous gastric tissues^[130,131,136]. Further studies analyzed gene expression profiles, and revealed the upregulation of circulating CXCL1 levels in patients with gastric cancer compared with healthy controls^[78,130,131,137]. In addition, the expression of CXCR2, the CXCL1 receptor, was increased significantly in tumor tissue compared with non-cancerous adjacent tissue. The immunostaining of consecutive sections revealed that CXCL1 and CXCR2 were predominantly co-expressed in tumor epithelial cells, with a significant correlation between the staining scores of CXCL1 and CXCR2 in gastric cancer tissue^[131]. Increased expression of both proteins was also associated with tumor progression, and more advanced stages of gastric cancer^[131,132], while elevated CXCL1 expression was an independent prognostic factor for patient survival^[131]. However, the role of CXCL1 in gastric cancer remains controversial. Some studies revealed that increased circulating levels of CXCL1 correlated with aggressive tumor behavior, such as lymph node metastasis and higher tumor stage^[131,137]. In contrast, Junnila *et al*^[130] reported that increased levels of CXCL1 mRNA transcripts in gastric cancer tissue were associated with increased survival, although protein levels of CXCL1 were not measured.

CXCL1 and CXCR2 play important regulatory roles in the migration, invasion and metastasis of tumor cells, in a variety of cancers, such as melanoma, colon and breast cancer^[138-140]. In gastric cancer, CXCL1-overexpressing cells showed increased migratory and invasive potential, whereas depletion of CXCL1 or CXCR2 significantly decreased the migration and invasion of the same cells^[131]. CXCL1-overexpressing cells also expressed significantly higher levels of MMP-2 and MMP-9 activity than control cells, and upregulation of Ras and STAT3^[131], suggesting a potential mechanism by which CXCL1-CXCR2 contributes to the progression of gastric cancer.

Additional CXC chemokines and chemokine receptors

CXCL5 is expressed in gastric cancer, and is correlated with nodal and overall stage^[141,142]. Elevated serum CXCL5 expression is also observed in late-stage gastric cancer patients compared with those with benign tumors, suggesting that CXCL5 may play a role in the progression of gastric cancer^[142]. In addition, CXCL5 gene ex-

pression was up regulated in the gastric mucosa of the *A49nt^{-/-}* gastric cancer mouse model compared with wild-type mice, which was confirmed by quantitative reverse transcription-polymerase chain reaction^[134]. However, the role of the chemokine CXCL5 in gastric carcinogenesis is still unclear, and additional *in vitro* and *in vivo* studies are needed to characterize its effects.

Recently Yanagi *et al.*^[78] used a cDNA microarray to carry out a comparative analysis on the differential expression of chemokines and chemokine receptors in gastric cancer cell lines. They showed that CXCL7 and CXCL14 were upregulated (along with CXCL1, CXCL8 and CXCL12), suggesting that these chemokines play a role in the progression of gastric cancer.

CXCL9, CXCL10 and CXCL11 have also been linked to gastric cancer, and are constitutively expressed in gastric cancer cell lines such as AGS, KATO III and NCI. In addition, their secretion was strongly induced by treatment with a combination of interferon- γ and tumor necrosis factor- α ^[143]. Jung *et al.*^[137] used genome-wide gene expression databases to show that the *CXCL9* and *CXCL10* genes were overexpressed more than twofold in gastric cancer compared to normal tissues. Consistent with this, Rajkumar *et al.*^[144] demonstrated that the plasma levels of CXCL9 and CXCL10 were decreased significantly in gastric cancer patients after surgery. Additional studies revealed that a small portion of gastric cancer cells expressed both CXCL9 and CXCL10, and that their expression co-localized with cytokeratin^[145]. In the same study, cells in lymphocyte-rich gastric cancers were more frequently positive for CXCL9 and CXCL10 than were conventional gastric cancer cells.

The chemokine CXCL16 and its receptor CXCR6 are up regulated in multiple cancer tissues and cell lines compared with normal samples and cells. In addition, both CXCL16 and CXCR6 levels increase as tumor malignancy increases. The CXCL16 exists both in a soluble form and a transmembrane form. Soluble CXCL16 promotes cell proliferation and migration while transmembranous CXCL16 suppresses proliferation^[146]. For example, Darash-Yahana *et al.*^[147] showed that CXCL16-CXCR6 expression level was closely related to high malignant degree in prostate cancer. Soluble CXCL16 facilitated the migration of CXCR6-expressing cancer cells and promoted the proliferation *in vitro*. Hojo *et al.*^[148] demonstrated that CXCL16 expression was up regulated in colorectal tumor tissue compared with normal mucosa, and suggested that the transmembranous expression of CXCL16 by tumor cells enhanced the recruitment of tumor-infiltrating lymphocytes, thereby improving prognosis. In gastric cancer, Xing *et al.*^[149] recently reported that eight gastric cancer cell lines and the gastric epithelial cell line GES-1 differentially expressed CXCL16 and CXCR6 mRNA. They demonstrated that CXCL16 mRNA expression was elevated in cancer tissue compared with adjacent mucosa, while CXCR6 was expressed in the opposite manner. Nuclear CXCL16 expression inversely correlated with the invasion depth of the tumor, lymphatic invasion, and

stage, suggesting that CXCL16 and its receptor CXCR6 may play a role in gastric tumorigenesis.

THERAPEUTIC TARGETING OF CHEMOKINES AND THEIR RECEPTORS IN GASTRIC CANCER

CXCL12-CXCR4/CXCR7 axis

The CXCL12-CXCR4/CXCR7 axis is a potential novel therapeutic target for the treatment of cancer, and so multiple agents that modulate this pathway are being developed for use in malignant tumors. Examples include the anti-CXCR4 drug AMD3100 (also known as plerixafor, or Mozobil), the CXCL12 analog CTCE-9908 (Chemokine Therapeutics), the anti-CXCL12 aptamer Nox-A12 (Noxxon), and the CXCR7-specific inhibitor CCX2066 (ChemoCentryx). Additional strategies to inhibit CXCL12 signaling, including chalcone 4 (C7870) or RNA interference, could also be assessed for the treatment of solid tumors^[150].

In gastric cancer, several preclinical studies have demonstrated that blocking the CXCL12-CXCR4 axis showed anti-tumor activity *in vitro* and *in vivo*. CXCL12-induced migration, cell proliferation, and cell survival were significantly blocked by treatment with a neutralizing anti-CXCR4 antibody or AMD3100, a specific CXCR4 antagonist^[45,47,50,63]. AMD3100 also significantly reduced tumor growth, inhibited the formation of malignant ascitic fluid, and increased survival in nude mice inoculated with NUGC-4 cells compared with control. Importantly, none of the mice showed signs of drug-associated toxicity^[47,151,152]. In addition, Xie *et al.*^[153] demonstrated that elevated CXCR4 mRNA levels in gastric cancer tissues were significantly correlated with docetaxel sensitivity, and that AMD3100 enhanced docetaxel cytotoxicity *in vitro*. Additional studies reported that plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), an analogue of vitamin K3, inhibited CXCL12-induced migration and invasion of gastric cancer cells by down-regulating the expression of functional CXCR4 at the transcriptional level^[154]. Plumbagin suppressed the binding of NF- κ B to the CXCR4 promoter, suggesting that it inhibits CXCR4 expression by suppressing NF- κ B-mediated CXCR4 transcription. Taken together, these data suggest CXCR4 antagonists to be attractive therapeutic candidates, necessitating a better understanding of the CXCL12-CXCR4 axis in gastric tumorigenesis.

Recent studies suggested that fluorescent magnetic nanoparticle-labeled mesenchymal stem cells (MSCs) could target *in vivo* mouse gastric cancer cells *via* the CXCL12-CXCR4 axis to inhibit tumor growth during hyperthermic therapy^[79]. In this study, gastric cancer cells produced CXCL12, which attracted CXCR4-expressing MSCs to gastric tumor sites. These data suggest that gastric tumor-expressed CXCL12 could be targeted during treatments, such as hyperthermia combined with fluorescent magnetic nanoparticle-labeled MSCs, to attract CXCR4-expressing MSCs.

CXCL8-CXCR1/CXCR2 axis

The chemokine CXCL8 and its receptors CXCR1/2 are potential therapeutic targets in a variety of solid tumors such as malignant melanoma, colon, breast, and bladder cancer. As such, several antagonists of CXCL8-CXCR1/CXCR2-mediated signaling are in development, including neutralizing antibodies and small-molecule antagonists^[140,155-158]. In melanoma, neutralizing antibodies to CXCR1 and CXCR2 inhibited cell proliferation and invasive potential, while knock down of CXCR1 or CXCR2 using small interfering RNA inhibited melanoma tumor growth and invasion *in vitro* and *in vivo*^[156,159]. Varney *et al*^[160] reported that orally active small molecular antagonists against CXCR2 and CXCR1 (such as SCH-527123 and SCH-479833) inhibited liver metastasis by decreasing neovascularization and enhancing malignant cell apoptosis in colon cancer. Consistent with this, Ning *et al*^[155] demonstrated that treatment with SCH-527123 alone or in combination with oxaliplatin synergistically inhibited proliferation and angiogenesis, and enhanced chemosensitivity in colorectal cancer cells and xenograft models. However, little data in gastric cancer have explored the potential of the CXCL8-CXCR1/CXCR2 axis as therapeutic targets until recently. Ju *et al*^[107] reported that Xiaotan Sanjie Decoction, a traditional Chinese herbal medicine, inhibited tumor growth by decreasing the expression of CXCL8, CXCR1, and CXCR2 in gastric cancer xenograft models, suggesting that inhibiting this axis may be one of mechanisms by which the herb inhibits tumor growth and prevents recurrence. However, with the recent advances in understanding the role of CXCL8 and its receptors in the development and progression of gastric cancer, additional studies are needed to maximize the therapeutic potential of this axis for the treatment of gastric cancer.

CONCLUSION

Advanced gastric cancer patients, particularly those with peritoneal seeding, have a very poor quality of life, and poor prognosis. To improve quality of life and survival in these patients, a better understanding of the underlying molecular pathogenesis of gastric carcinogenesis, and its application for the development of novel targeted therapies, are urgently needed. Chemokines, also known as chemotactic cytokines, were traditionally believed to regulate the directional migration of leukocytes to inflammatory sites. However, it is now clear that chemokines and their receptors also regulate the processes underlying the development and progression of malignant diseases, including tumor growth, survival, angiogenesis, invasion, and metastasis. CXC chemokines and their receptors are widely expressed in gastrointestinal tumors, including gastric cancer, and are associated with prognosis. CXCL12 and its receptor CXCR4 play a crucial role in aspects of gastric carcinogenesis including cell proliferation, migration, invasion, peritoneal seeding, and resistance to treatment. In addition, there is accumulating

evidence to suggest that modulating CXCL12-CXCR4 signaling could be an important therapeutic strategy, either alone or in combination with conventional treatment modalities. CXCL8-CXCR1/2 and CXCL1-CXCR2 are differentially expressed in gastric cancer, and are involved in its progression. This suggests that they may also be future therapeutic candidates. About CXCL16-CXCR6, both CXCL16 and its receptor CXCR6 are aberrantly expressed in gastric cancer suggesting their involvement in gastric carcinogenesis. However, the role and significance of CXCL16-CXCR6 in gastric cancer remain uncertain due to insufficient data. Overall, the role of the various chemokines and chemokine receptors in the development and progression of gastric cancer is complex. In addition, little is known of the roles of other CXC chemokines and chemokine receptors in gastric cancer. More extensive studies are therefore needed to elucidate the roles of the complex chemokine and chemokine receptor network in gastric tumorigenesis, which may result in therapeutic applications for patients with gastric cancer.

REFERENCES

- 1 Thiel A, Ristimäki A. Gastric cancer: basic aspects. *Helicobacter* 2012; **17** Suppl 1: 26-29 [PMID: 22958152 DOI: 10.1111/j.1523-5378.2012.00979.x]
- 2 Pisani P, Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers in 1990. *Int J Cancer* 1999; **83**: 18-29 [PMID: 10449602]
- 3 Lee HJ, Cho do Y, Park JC, Bae SB, Lee KT, Cho IS, Han CS, Park SY, Yun HJ, Kim S. Phase II trial of biweekly paclitaxel plus infusional 5-fluorouracil and leucovorin in patients with advanced or recurrent inoperable gastric cancer. *Cancer Chemother Pharmacol* 2009; **63**: 427-432 [PMID: 18415100 DOI: 10.1007/s00280-008-0752-4]
- 4 Zheng L, Wang L, Ajani J, Xie K. Molecular basis of gastric cancer development and progression. *Gastric Cancer* 2004; **7**: 61-77 [PMID: 15224192 DOI: 10.1007/s10120-004-0277-4]
- 5 Lee HJ, Jo DY. The role of the CXCR4/CXCL12 axis and its clinical implications in gastric cancer. *Histol Histopathol* 2012; **27**: 1155-1161 [PMID: 22806902]
- 6 Ebert MP, Fei G, Kahmann S, Müller O, Yu J, Sung JJ, Malfertheiner P. Increased beta-catenin mRNA levels and mutational alterations of the APC and beta-catenin gene are present in intestinal-type gastric cancer. *Carcinogenesis* 2002; **23**: 87-91 [PMID: 11756228]
- 7 Panani AD. Cytogenetic and molecular aspects of gastric cancer: clinical implications. *Cancer Lett* 2008; **266**: 99-115 [PMID: 18381231 DOI: 10.1016/j.canlet.2008.02.053]
- 8 Resende C, Ristimäki A, Machado JC. Genetic and epigenetic alteration in gastric carcinogenesis. *Helicobacter* 2010; **15** Suppl 1: 34-39 [PMID: 21054651 DOI: 10.1111/j.1523-5378.2010.00782.x]
- 9 Oda N, Tsujino T, Tsuda T, Yoshida K, Nakayama H, Yasui W, Tahara E. DNA ploidy pattern and amplification of ERBB and ERBB2 genes in human gastric carcinomas. *Virchows Arch B Cell Pathol Incl Mol Pathol* 1990; **58**: 273-277 [PMID: 1970690]
- 10 Ross JS, McKenna BJ. The HER-2/neu oncogene in tumors of the gastrointestinal tract. *Cancer Invest* 2001; **19**: 554-568 [PMID: 11458821]
- 11 Shun CT, Wu MS, Lin JT, Chen SY, Wang HP, Lee WJ, Wang TH, Chuang SM. Relationship of p53 and c-erbB-2 expression to histopathological features, *Helicobacter pylori* infection and prognosis in gastric cancer. *Hepatogastroenterology* 1997; **44**: 604-609 [PMID: 9164544]

- 12 **Han S**, Kim HY, Park K, Cho HJ, Lee MS, Kim HJ, Kim YD. c-Myc expression is related with cell proliferation and associated with poor clinical outcome in human gastric cancer. *J Korean Med Sci* 1999; **14**: 526-530 [PMID: 10576148]
- 13 **Kozma L**, Kiss I, Hajdú J, Szentkereszty Z, Szakáll S, Ember I. C-myc amplification and cluster analysis in human gastric carcinoma. *Anticancer Res* 2001; **21**: 707-710 [PMID: 11299830]
- 14 **Pan G**, Lv H, Ren H, Wang Y, Liu Y, Jiang H, Wen J. Elevated expression of semaphorin 5A in human gastric cancer and its implication in carcinogenesis. *Life Sci* 2010; **86**: 139-144 [PMID: 20026339 DOI: 10.1016/j.lfs.2009.12.004]
- 15 **Florou D**, Papadopoulos IN, Scorilas A. Molecular analysis and prognostic impact of the novel apoptotic gene BCL2L12 in gastric cancer. *Biochem Biophys Res Commun* 2010; **391**: 214-218 [PMID: 19903463 DOI: 10.1016/j.bbrc.2009.11.034]
- 16 **Kuniyasu H**, Yasui W, Yokozaki H, Kitadai Y, Tahara E. Aberrant expression of c-met mRNA in human gastric carcinomas. *Int J Cancer* 1993; **55**: 72-75 [PMID: 8344755]
- 17 **Hattori Y**, Odagiri H, Nakatani H, Miyagawa K, Naito K, Sakamoto H, Katoh O, Yoshida T, Sugimura T, Terada M. K-sam, an amplified gene in stomach cancer, is a member of the heparin-binding growth factor receptor genes. *Proc Natl Acad Sci USA* 1990; **87**: 5983-5987 [PMID: 2377625]
- 18 **Liu XP**, Tsushima K, Tsushima M, Oga A, Kawauchi S, Furuya T, Sasaki K. Expression of p53 protein as a prognostic indicator of reduced survival time in diffuse-type gastric carcinoma. *Pathol Int* 2001; **51**: 440-444 [PMID: 11422805]
- 19 **Nakatsuru S**, Yanagisawa A, Ichii S, Tahara E, Kato Y, Nakamura Y, Horii A. Somatic mutation of the APC gene in gastric cancer: frequent mutations in very well differentiated adenocarcinoma and signet-ring cell carcinoma. *Hum Mol Genet* 1992; **1**: 559-563 [PMID: 1338691]
- 20 **Lee KH**, Lee JS, Suh C, Kim SW, Kim SB, Lee JH, Lee MS, Park MY, Sun HS, Kim SH. Clinicopathologic significance of the K-ras gene codon 12 point mutation in stomach cancer. An analysis of 140 cases. *Cancer* 1995; **75**: 2794-2801 [PMID: 7773929]
- 21 **More H**, Humar B, Weber W, Ward R, Christian A, Lintott C, Graziano F, Ruzzo AM, Acosta E, Boman B, Harlan M, Ferreira P, Seruca R, Suriano G, Guilford P. Identification of seven novel germline mutations in the human E-cadherin (CDH1) gene. *Hum Mutat* 2007; **28**: 203 [PMID: 17221870 DOI: 10.1002/humu.9473]
- 22 **Bang YJ**, Van Cutsem E, Feyerislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Rüschhoff J, Kang YK. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet* 2010; **376**: 687-697 [PMID: 20728210 DOI: 10.1016/s0140-6736(10)61121-x]
- 23 **Balkwill FR**. The chemokine system and cancer. *J Pathol* 2012; **226**: 148-157 [PMID: 21989643 DOI: 10.1002/path.3029]
- 24 **Koizumi K**, Hojo S, Akashi T, Yasumoto K, Saiki I. Chemokine receptors in cancer metastasis and cancer cell-derived chemokines in host immune response. *Cancer Sci* 2007; **98**: 1652-1658 [PMID: 17894551 DOI: 10.1111/j.1349-7006.2007.00606.x]
- 25 **Burger JA**. Chemokines and chemokine receptors in chronic lymphocytic leukemia (CLL): from understanding the basics towards therapeutic targeting. *Semin Cancer Biol* 2010; **20**: 424-430 [PMID: 20883788 DOI: 10.1016/j.semcancer.2010.09.005]
- 26 **Kunkel SL**, Strieter RM, Lindley IJ, Westwick J. Chemokines: new ligands, receptors and activities. *Immunol Today* 1995; **16**: 559-561 [PMID: 8579746 DOI: 10.1016/0167-5699(95)80076-x]
- 27 **Keeley EC**, Mehrad B, Strieter RM. CXC chemokines in cancer angiogenesis and metastases. *Adv Cancer Res* 2010; **106**: 91-111 [PMID: 20399957 DOI: 10.1016/s0065-230x(10)06003-3]
- 28 **Charo IF**, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med* 2006; **354**: 610-621 [PMID: 16467548 DOI: 10.1056/NEJMra052723]
- 29 **Vicari AP**, Caux C. Chemokines in cancer. *Cytokine Growth Factor Rev* 2002; **13**: 143-154 [PMID: 11900990]
- 30 **Vandercappellen J**, Van Damme J, Struyf S. The role of CXC chemokines and their receptors in cancer. *Cancer Lett* 2008; **267**: 226-244 [PMID: 18579287 DOI: 10.1016/j.canlet.2008.04.050]
- 31 **Zhu Q**, Han X, Peng J, Qin H, Wang Y. The role of CXC chemokines and their receptors in the progression and treatment of tumors. *J Mol Histol* 2012; **43**: 699-713 [PMID: 22752457 DOI: 10.1007/s10735-012-9435-x]
- 32 **Verbeke H**, Struyf S, Laureys G, Van Damme J. The expression and role of CXC chemokines in colorectal cancer. *Cytokine Growth Factor Rev* 2011; **22**: 345-358 [PMID: 22000992 DOI: 10.1016/j.cytogfr.2011.09.002]
- 33 **Strieter RM**, Burdick MD, Mestas J, Gomperts B, Keane MP, Belperio JA. Cancer CXC chemokine networks and tumour angiogenesis. *Eur J Cancer* 2006; **42**: 768-778 [PMID: 16510280 DOI: 10.1016/j.ejca.2006.01.006]
- 34 **Rainczuk A**, Rao J, Gathercole J, Stephens AN. The emerging role of CXC chemokines in epithelial ovarian cancer. *Reproduction* 2012; **144**: 303-317 [PMID: 22771929 DOI: 10.1530/rep-12-0153]
- 35 **Fernandez EJ**, Lolis E. Structure, function, and inhibition of chemokines. *Annu Rev Pharmacol Toxicol* 2002; **42**: 469-499 [PMID: 11807180 DOI: 10.1146/annurev.pharmtox.42.091901.115838]
- 36 **Arya M**, Patel HR, Williamson M. Chemokines: key players in cancer. *Curr Med Res Opin* 2003; **19**: 557-564 [PMID: 14594528 DOI: 10.1185/030079903125002216]
- 37 **Verbeke H**, Geboes K, Van Damme J, Struyf S. The role of CXC chemokines in the transition of chronic inflammation to esophageal and gastric cancer. *Biochim Biophys Acta* 2012; **1825**: 117-129 [PMID: 22079531 DOI: 10.1016/j.bbcan.2011.10.008]
- 38 **Luster AD**. Chemokines--chemotactic cytokines that mediate inflammation. *N Engl J Med* 1998; **338**: 436-445 [PMID: 9459648 DOI: 10.1056/nejm199802123380706]
- 39 **Tazzyman S**, Lewis CE, Murdoch C. Neutrophils: key mediators of tumour angiogenesis. *Int J Exp Pathol* 2009; **90**: 222-231 [PMID: 19563607 DOI: 10.1111/j.1365-2613.2009.00641.x]
- 40 **Strieter RM**, Polverini PJ, Kunkel SL, Arenberg DA, Burdick MD, Kasper J, Dzuiba J, Van Damme J, Walz A, Marriott D. The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *J Biol Chem* 1995; **270**: 27348-27357 [PMID: 7592998]
- 41 **Strieter RM**, Burdick MD, Gomperts BN, Belperio JA, Keane MP. CXC chemokines in angiogenesis. *Cytokine Growth Factor Rev* 2005; **16**: 593-609 [PMID: 16046180 DOI: 10.1016/j.cytogfr.2005.04.007]
- 42 **Strieter RM**, Belperio JA, Phillips RJ, Keane MP. CXC chemokines in angiogenesis of cancer. *Semin Cancer Biol* 2004; **14**: 195-200 [PMID: 15246055 DOI: 10.1016/j.semcancer.2003.10.006]
- 43 **Zipin-Roitman A**, Meshel T, Sagi-Assif O, Shalmon B, Avivi C, Pfeffer RM, Witz IP, Ben-Baruch A. CXCL10 promotes invasion-related properties in human colorectal carcinoma cells. *Cancer Res* 2007; **67**: 3396-3405 [PMID: 17409450 DOI: 10.1158/0008-5472.can-06-3087]
- 44 **Kawada K**, Sonoshita M, Sakashita H, Takabayashi A, Yamaoka Y, Manabe T, Inaba K, Minato N, Oshima M, Taketo MM. Pivotal role of CXCR3 in melanoma cell metastasis to lymph nodes. *Cancer Res* 2004; **64**: 4010-4017 [PMID: 15173015 DOI: 10.1158/0008-5472.can-03-1757]
- 45 **Lee HJ**, Kim SW, Kim HY, Li S, Yun HJ, Song KS, Kim S, Jo DY. Chemokine receptor CXCR4 expression, function, and clinical implications in gastric cancer. *Int J Oncol* 2009; **34**:

- 473-480 [PMID: 19148483]
- 46 **Li S**, Huang S, Peng SB. Overexpression of G protein-coupled receptors in cancer cells: involvement in tumor progression. *Int J Oncol* 2005; **27**: 1329-1339 [PMID: 16211229]
 - 47 **Yasumoto K**, Koizumi K, Kawashima A, Saitoh Y, Arita Y, Shinohara K, Minami T, Nakayama T, Sakurai H, Takahashi Y, Yoshie O, Saiki I. Role of the CXCL12/CXCR4 axis in peritoneal carcinomatosis of gastric cancer. *Cancer Res* 2006; **66**: 2181-2187 [PMID: 16489019 DOI: 10.1158/0008-5472.can-05-3393]
 - 48 **Zhao BC**, Zhao B, Han JG, Ma HC, Wang ZJ. Adipose-derived stem cells promote gastric cancer cell growth, migration and invasion through SDF-1/CXCR4 axis. *Hepatogastroenterology* 2010; **57**: 1382-1389 [PMID: 21443090]
 - 49 **Mitra P**, Shibuta K, Mathai J, Shimoda K, Banner BF, Mori M, Barnard GF. CXCR4 mRNA expression in colon, esophageal and gastric cancers and hepatitis C infected liver. *Int J Oncol* 1999; **14**: 917-925 [PMID: 10200342]
 - 50 **Zhao BC**, Wang ZJ, Mao WZ, Ma HC, Han JG, Zhao B, Xu HM. CXCR4/SDF-1 axis is involved in lymph node metastasis of gastric carcinoma. *World J Gastroenterol* 2011; **17**: 2389-2396 [PMID: 21633638 DOI: 10.3748/wjg.v17.i19.2389]
 - 51 **Ingold B**, Simon E, Ungethüm U, Kuban RJ, Müller BM, Lupp A, Neumann U, Ebert MP, Denkert C, Weichert W, Schulz S, Röcken C. Vascular CXCR4 expression - a novel antiangiogenic target in gastric cancer? *PLoS One* 2010; **5**: e10087 [PMID: 20386750 DOI: 10.1371/journal.pone.0010087]
 - 52 **Pituch-Noworolska A**, Drabik G, Szatanek R, Białas M, Kołodziejczyk P, Szczepanik A, Stachura J, Zembala M. Immunophenotype of isolated tumour cells in the blood, bone marrow and lymph nodes of patients with gastric cancer. *Pol J Pathol* 2007; **58**: 93-97 [PMID: 17715675]
 - 53 **Yasumoto K**, Yamada T, Kawashima A, Wang W, Li Q, Donev IS, Tacheuchi S, Mourii H, Yamashita K, Ohtsubo K, Yano S. The EGFR ligands amphiregulin and heparin-binding egf-like growth factor promote peritoneal carcinomatosis in CXCR4-expressing gastric cancer. *Clin Cancer Res* 2011; **17**: 3619-3630 [PMID: 21482691 DOI: 10.1158/1078-0432.ccr-10-2475]
 - 54 **Ying J**, Xu Q, Zhang G, Liu B, Zhu L. The expression of CXCL12 and CXCR4 in gastric cancer and their correlation to lymph node metastasis. *Med Oncol* 2012; **29**: 1716-1722 [PMID: 21630055 DOI: 10.1007/s12032-011-9990-0]
 - 55 **Zhao C**, Ma H, Bu X, Wang W, Zhang N. SFRP5 inhibits gastric epithelial cell migration induced by macrophage-derived Wnt5a. *Carcinogenesis* 2013; **34**: 146-152 [PMID: 23054609 DOI: 10.1093/carcin/bgs309]
 - 56 **Xue Z**, Yan H, Li J, Liang S, Cai X, Chen X, Wu Q, Gao L, Wu K, Nie Y, Fan D. Identification of cancer stem cells in vincristine preconditioned SGC7901 gastric cancer cell line. *J Cell Biochem* 2012; **113**: 302-312 [PMID: 21913215 DOI: 10.1002/jcb.23356]
 - 57 **Graziosi L**, Mencarelli A, Santorelli C, Renga B, Cipriani S, Cavazzoni E, Palladino G, Laufer S, Burnet M, Donini A, Fiorucci S. Mechanistic role of p38 MAPK in gastric cancer dissemination in a rodent model peritoneal metastasis. *Eur J Pharmacol* 2012; **674**: 143-152 [PMID: 22119383 DOI: 10.1016/j.ejphar.2011.11.015]
 - 58 **Fanelli MF**, Chinen LT, Begnami MD, Costa WL, Fregnami JH, Soares FA, Montagnini AL. The influence of transforming growth factor- α , cyclooxygenase-2, matrix metalloproteinase (MMP)-7, MMP-9 and CXCR4 proteins involved in epithelial-mesenchymal transition on overall survival of patients with gastric cancer. *Histopathology* 2012; **61**: 153-161 [PMID: 22582975 DOI: 10.1111/j.1365-2559.2011.04139.x]
 - 59 **Kwak MK**, Hur K, Park DJ, Lee HJ, Lee HS, Kim WH, Lee KU, Choe KJ, Yang HK. Expression of chemokine receptors in human gastric cancer. *Tumour Biol* 2005; **26**: 65-70 [PMID: 15867478 DOI: 10.1159/000085587]
 - 60 **Sun XJ**, Sun KL, Zheng ZH, Fu WN, Hao DM, Xu HM, Li XM. Gene expression patterns in gastric cancer. *Zhonghua Yixue Yichuanxue Zazhi* 2006; **23**: 142-146 [PMID: 16604482]
 - 61 **Graziosi L**, Mencarelli A, Renga B, Santorelli C, Cantarella F, Bugiantella W, Cavazzoni E, Donini A, Fiorucci S. Gene expression changes induced by HIPEC in a murine model of gastric cancer. *In Vivo* 2012; **26**: 39-45 [PMID: 22210714]
 - 62 **Xu W**, Zhou H, Qian H, Bu X, Chen D, Gu H, Zhu W, Yan Y, Mao F. Combination of circulating CXCR4 and Bmi-1 mRNA in plasma: A potential novel tumor marker for gastric cancer. *Mol Med Rep* 2012; **2**: 765-771 [PMID: 21475899 DOI: 10.3892/mmr.00000170]
 - 63 **Ding YL**, Zhang JL, Tang SF, Fu QY, Li ZT. [Effect of chemokine stromal cell derived factor-1 and its receptor CXCR4 on the peritoneal carcinometastasis of gastric cancer]. *Zhonghua Yixue Zazhi* 2008; **88**: 202-205 [PMID: 18361822]
 - 64 **Hashimoto I**, Koizumi K, Tatematsu M, Minami T, Cho S, Takeno N, Nakashima A, Sakurai H, Saito S, Tsukada K, Saiki I. Blocking on the CXCR4/mTOR signalling pathway induces the anti-metastatic properties and autophagic cell death in peritoneal disseminated gastric cancer cells. *Eur J Cancer* 2008; **44**: 1022-1029 [PMID: 18375114 DOI: 10.1016/j.ejca.2008.02.043]
 - 65 **Zieker D**, Königsrainer I, Traub F, Nieselt K, Knapp B, Schilling C, Stirnkorb C, Fend F, Northoff H, Kupka S, Brücher BL, Königsrainer A. PGK1 a potential marker for peritoneal dissemination in gastric cancer. *Cell Physiol Biochem* 2008; **21**: 429-436 [PMID: 18453750 DOI: 10.1159/000129635]
 - 66 **Tsuboi K**, Kodaera Y, Nakanishi H, Ito S, Mochizuki Y, Nakayama G, Koike M, Fujiwara M, Yamamura Y, Nakao A. Expression of CXCL12 and CXCR4 in pT3-stage gastric cancer does not correlate with peritoneal metastasis. *Oncol Rep* 2008; **20**: 1117-1123 [PMID: 18949410]
 - 67 **Arigami T**, Natsugoe S, Uenosono Y, Yanagita S, Arima H, Hirata M, Ishigami S, Aikou T. CCR7 and CXCR4 expression predicts lymph node status including micrometastasis in gastric cancer. *Int J Oncol* 2009; **35**: 19-24 [PMID: 19513547]
 - 68 **Iwasa S**, Yanagawa T, Fan J, Katoh R. Expression of CXCR4 and its ligand SDF-1 in intestinal-type gastric cancer is associated with lymph node and liver metastasis. *Anticancer Res* 2009; **29**: 4751-4758 [PMID: 20032431]
 - 69 **Zhu S**, Hong J, Tripathi MK, Sehdev V, Belkhir A, El-Rifai W. Regulation of CXCR4-mediated invasion by DARPP-32 in gastric cancer cells. *Mol Cancer Res* 2013; **11**: 86-94 [PMID: 23160836 DOI: 10.1158/1541-7786.mcr-12-0243-t]
 - 70 **Bao W**, Fu HJ, Xie QS, Wang L, Zhang R, Guo ZY, Zhao J, Meng YL, Ren XL, Wang T, Li Q, Jin BQ, Yao LB, Wang RA, Fan DM, Chen SY, Jia LT, Yang AG. HER2 interacts with CD44 to up-regulate CXCR4 via epigenetic silencing of microRNA-139 in gastric cancer cells. *Gastroenterology* 2011; **141**: 2076-2087.e6 [PMID: 21925125 DOI: 10.1053/j.gastro.2011.08.050]
 - 71 **He H**, Wang C, Shen Z, Fang Y, Wang X, Chen W, Liu F, Qin X, Sun Y. Upregulated expression of C-X-C chemokine receptor 4 is an independent prognostic predictor for patients with gastric cancer. *PLoS One* 2013; **8**: e71864 [PMID: 23936528 DOI: 10.1371/journal.pone.0071864]
 - 72 **Ishigami S**, Natsugoe S, Okumura H, Matsumoto M, Nakajo A, Uenosono Y, Arigami T, Uchikado Y, Setoyama T, Arima H, Hokita S, Aikou T. Clinical implication of CXCL12 expression in gastric cancer. *Ann Surg Oncol* 2007; **14**: 3154-3158 [PMID: 17653799 DOI: 10.1245/s10434-007-9521-6]
 - 73 **Lee HJ**, Huang SM, Kim HY, Oh YS, Hwang JY, Liang ZL, Ki Min J, Yun HJ, Sul JY, Kim S, Jo DY, Kim JM. Evaluation of the combined expression of chemokine SDF-1 α and its receptor CXCR4 as a prognostic marker for gastric cancer. *Exp Ther Med* 2011; **2**: 499-504 [PMID: 22977531 DOI: 10.3892/etm.2011.228]
 - 74 **Chen G**, Chen SM, Wang X, Ding XF, Ding J, Meng LH. Inhibition of chemokine (CXC motif) ligand 12/chemokine

- (CXC motif) receptor 4 axis (CXCL12/CXCR4)-mediated cell migration by targeting mammalian target of rapamycin (mTOR) pathway in human gastric carcinoma cells. *J Biol Chem* 2012; **287**: 12132-12141 [PMID: 22337890 DOI: 10.1074/jbc.M111.302299]
- 75 **Song IC**, Liang ZL, Lee JC, Huang SM, Kim HY, Oh YS, Yun HJ, Sul JY, Jo DY, Kim S, Kim JM, Lee HJ. Expression of stromal cell-derived factor-1 α is an independent risk factor for lymph node metastasis in early gastric cancer. *Oncol Lett* 2011; **2**: 1197-1202 [PMID: 22848288 DOI: 10.3892/ol.2011.389]
 - 76 **Zhi Y**, Chen J, Zhang S, Chang X, Ma J, Dai D. Down-regulation of CXCL12 by DNA hypermethylation and its involvement in gastric cancer metastatic progression. *Dig Dis Sci* 2012; **57**: 650-659 [PMID: 21960286 DOI: 10.1007/s10620-011-1922-5]
 - 77 **Ruan J**, Song H, Li C, Bao C, Fu H, Wang K, Ni J, Cui D. DiR-labeled Embryonic Stem Cells for Targeted Imaging of in vivo Gastric Cancer Cells. *Theranostics* 2012; **2**: 618-628 [PMID: 22768029 DOI: 10.7150/thno.4561]
 - 78 **Yanagie H**, Hisa T, Ono M, Eriguchi M. [Chemokine and chemokine receptor related to cancer metastasis]. *Gan To Kagaku Ryoho* 2010; **37**: 2052-2057 [PMID: 21084802]
 - 79 **Ruan J**, Ji J, Song H, Qian Q, Wang K, Wang C, Cui D. Fluorescent magnetic nanoparticle-labeled mesenchymal stem cells for targeted imaging and hyperthermia therapy of in vivo gastric cancer. *Nanoscale Res Lett* 2012; **7**: 309 [PMID: 22709686 DOI: 10.1186/1556-276x-7-309]
 - 80 **Schimanski CC**, Jordan M, Schlaegel F, Schmidtmann I, Lang H, Galle PR, Moehler M, Gockel I. SNP rs1801157 significantly correlates with distant metastasis in CXCL12 expressing esophagogastric cancer. *Int J Oncol* 2011; **39**: 515-520 [PMID: 21584490 DOI: 10.3892/ijo.2011.1044]
 - 81 **Woo IS**, Hong SH, Byun JH, Kang JH, Jeon HM, Choi MG. Circulating stromal cell derived factor-1 α (SDF-1 α) is predictive of distant metastasis in gastric carcinoma. *Cancer Invest* 2008; **26**: 256-261 [PMID: 18317966 DOI: 10.1080/07357900701684057]
 - 82 **Rempel SA**, Dudas S, Ge S, Gutiérrez JA. Identification and localization of the cytokine SDF1 and its receptor, CXC chemokine receptor 4, to regions of necrosis and angiogenesis in human glioblastoma. *Clin Cancer Res* 2000; **6**: 102-111 [PMID: 10656438]
 - 83 **Barbieri F**, Bajetto A, Stumm R, Pattarozzi A, Porcile C, Zona G, Dorcaratto A, Ravetti JL, Minuto F, Spaziante R, Schettini G, Ferone D, Florio T. Overexpression of stromal cell-derived factor 1 and its receptor CXCR4 induces autocrine/paracrine cell proliferation in human pituitary adenomas. *Clin Cancer Res* 2008; **14**: 5022-5032 [PMID: 18698020 DOI: 10.1158/1078-0432.ccr-07-4717]
 - 84 **Barbero S**, Bonavia R, Bajetto A, Porcile C, Pirani P, Ravetti JL, Zona GL, Spaziante R, Florio T, Schettini G. Stromal cell-derived factor 1 α stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinases 1/2 and Akt. *Cancer Res* 2003; **63**: 1969-1974 [PMID: 12702590]
 - 85 **Bajetto A**, Barbieri F, Dorcaratto A, Barbero S, Daga A, Porcile C, Ravetti JL, Zona G, Spaziante R, Corte G, Schettini G, Florio T. Expression of CXC chemokine receptors 1-5 and their ligands in human glioma tissues: role of CXCR4 and SDF1 in glioma cell proliferation and migration. *Neurochem Int* 2006; **49**: 423-432 [PMID: 16621164 DOI: 10.1016/j.neuint.2006.03.003]
 - 86 **Lee HJ**, Lee K, Lee DG, Bae KH, Kim JS, Liang ZL, Huang SM, Suk Oh Y, Kim HY, Jo DY, Min JK, Kim JM, Lee HJ. Chemokine (C-X-C motif) ligand 12 is associated with gallbladder carcinoma progression and is a novel independent poor prognostic factor. *Clin Cancer Res* 2012; **18**: 3270-3280 [PMID: 22553346 DOI: 10.1158/1078-0432.ccr-11-2417]
 - 87 **Zhuang Y**, Peng LS, Zhao YL, Shi Y, Mao XH, Chen W, Pang KC, Liu XF, Liu T, Zhang JY, Zeng H, Liu KY, Guo G, Tong WD, Shi Y, Tang B, Li N, Yu S, Luo P, Zhang WJ, Lu DS, Yu PW, Zou QM. CD8(+) T cells that produce interleukin-17 regulate myeloid-derived suppressor cells and are associated with survival time of patients with gastric cancer. *Gastroenterology* 2012; **143**: 951-962.e8 [PMID: 22710190 DOI: 10.1053/j.gastro.2012.06.010]
 - 88 **Shibata W**, Ariyama H, Westphalen CB, Worthley DL, Muthupalani S, Asfaha S, Dubeykovskaya Z, Quante M, Fox JG, Wang TC. Stromal cell-derived factor-1 overexpression induces gastric dysplasia through expansion of stromal myofibroblasts and epithelial progenitors. *Gut* 2013; **62**: 192-200 [PMID: 22362916 DOI: 10.1136/gutjnl-2011-301824]
 - 89 **Oh YS**, Kim HY, Song IC, Yun HJ, Jo DY, Kim S, Lee HJ. Hypoxia induces CXCR4 expression and biological activity in gastric cancer cells through activation of hypoxia-inducible factor-1 α . *Oncol Rep* 2012; **28**: 2239-2246 [PMID: 23023480 DOI: 10.3892/or.2012.2063]
 - 90 **Fernandis AZ**, Prasad A, Band H, Klösel R, Ganju RK. Regulation of CXCR4-mediated chemotaxis and chemoinvasion of breast cancer cells. *Oncogene* 2004; **23**: 157-167 [PMID: 14712221 DOI: 10.1038/sj.onc.1206910]
 - 91 **Hartmann TN**, Burger JA, Glodek A, Fujii N, Burger M. CXCR4 chemokine receptor and integrin signaling co-operate in mediating adhesion and chemoresistance in small cell lung cancer (SCLC) cells. *Oncogene* 2005; **24**: 4462-4471 [PMID: 15806155 DOI: 10.1038/sj.onc.1208621]
 - 92 **Luker KE**, Luker GD. Functions of CXCL12 and CXCR4 in breast cancer. *Cancer Lett* 2006; **238**: 30-41 [PMID: 16046252 DOI: 10.1016/j.canlet.2005.06.021]
 - 93 **Yamashita K**, Azumano I, Mai M, Okada Y. Expression and tissue localization of matrix metalloproteinase 7 (matrilysin) in human gastric carcinomas. Implications for vessel invasion and metastasis. *Int J Cancer* 1998; **79**: 187-194 [PMID: 9583735]
 - 94 **Shim KN**, Jung SA, Joo YH, Yoo K. Clinical significance of tissue levels of matrix metalloproteinases and tissue inhibitors of metalloproteinases in gastric cancer. *J Gastroenterol* 2007; **42**: 120-128 [PMID: 17351800 DOI: 10.1007/s00535-006-1975-y]
 - 95 **Dubeykovskaya Z**, Dubeykovskiy A, Solal-Cohen J, Wang TC. Secreted trefoil factor 2 activates the CXCR4 receptor in epithelial and lymphocytic cancer cell lines. *J Biol Chem* 2009; **284**: 3650-3662 [PMID: 19064997 DOI: 10.1074/jbc.M804935200]
 - 96 **Koizumi K**, Kato S, Sakurai H, Hashimoto I, Yasumoto K, Saiki I. Therapeutics target of CXCR4 and its downstream in peritoneal carcinomatosis of gastric cancer. *Front Biosci (Schol Ed)* 2012; **4**: 269-276 [PMID: 22202059]
 - 97 **Soriano SF**, Serrano A, Hernanz-Falcón P, Martín de Ana A, Monterrubio M, Martínez C, Rodríguez-Frade JM, Mellado M. Chemokines integrate JAK/STAT and G-protein pathways during chemotaxis and calcium flux responses. *Eur J Immunol* 2003; **33**: 1328-1333 [PMID: 12731058 DOI: 10.1002/eji.200323897]
 - 98 **Maksym RB**, Tarnowski M, Grymula K, Tarnowska J, Wysoczynski M, Liu R, Czerny B, Ratajczak J, Kucia M, Ratajczak MZ. The role of stromal-derived factor-1--CXCR7 axis in development and cancer. *Eur J Pharmacol* 2009; **625**: 31-40 [PMID: 19835865 DOI: 10.1016/j.ejphar.2009.04.071]
 - 99 **Burns JM**, Summers BC, Wang Y, Melikian A, Berahovich R, Miao Z, Penfold ME, Sunshine MJ, Littman DR, Kuo CJ, Wei K, McMaster BE, Wright K, Howard MC, Schall TJ. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *J Exp Med* 2006; **203**: 2201-2213 [PMID: 16940167 DOI: 10.1084/jem.20052144]
 - 100 **Miao Z**, Luker KE, Summers BC, Berahovich R, Bhojani MS, Rehemtulla A, Kleer CG, Essner JJ, Nasevicius A, Luker GD, Howard MC, Schall TJ. CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed

- on tumor-associated vasculature. *Proc Natl Acad Sci USA* 2007; **104**: 15735-15740 [PMID: 17898181 DOI: 10.1073/pnas.0610444104]
- 101 **Lee HJ**, Lee KS, Ryu H, Song IC, Huang SM, Yun HJ, Kim J, Jo DY, Kim S. The combined expression of CXCR7 and its ligand CXCL12 is a marker for unfavorable prognosis in gastric cancer. *Ann Oncol* 2012; **23** suppl 9: 541-541 Available from: URL: http://annonc.oxfordjournals.org/content/23/suppl_9/ix541.full?sid=956a7a70-63a0-4e96-80d1-7d8982754c6c
 - 102 **Miyazaki H**, Takabe K, Yeudall WA. Chemokines, chemokine receptors and the gastrointestinal system. *World J Gastroenterol* 2013; **19**: 2847-2863 [PMID: 23704819 DOI: 10.3748/wjg.v19.i19.2847]
 - 103 **Sieveking D**, Mitchell HM, Day AS. Gastric epithelial cell CXC chemokine secretion following *Helicobacter pylori* infection in vitro. *J Gastroenterol Hepatol* 2004; **19**: 982-987 [PMID: 15304113 DOI: 10.1111/j.1440-1746.2004.03413.x]
 - 104 **Kitadai Y**, Haruma K, Sumii K, Yamamoto S, Ue T, Yokozaki H, Yasui W, Ohmoto Y, Kajiyama G, Fidler IJ, Tahara E. Expression of interleukin-8 correlates with vascularity in human gastric carcinomas. *Am J Pathol* 1998; **152**: 93-100 [PMID: 9422527]
 - 105 **Torti D**, Sassi F, Galimi F, Gastaldi S, Perera T, Comoglio PM, Trusolino L, Bertotti A. A preclinical algorithm of soluble surrogate biomarkers that correlate with therapeutic inhibition of the MET oncogene in gastric tumors. *Int J Cancer* 2012; **130**: 1357-1366 [PMID: 21500189 DOI: 10.1002/ijc.26137]
 - 106 **Kido S**, Kitadai Y, Hattori N, Haruma K, Kido T, Ohta M, Tanaka S, Yoshihara M, Sumii K, Ohmoto Y, Chayama K. Interleukin 8 and vascular endothelial growth factor -- prognostic factors in human gastric carcinomas? *Eur J Cancer* 2001; **37**: 1482-1487 [PMID: 11506954]
 - 107 **Ju DW**, Wei PK, Lin HM, Sun DZ, Yu S, Xiu LJ. Effects of Xiaotan Sanjie Decoction on expressions of interleukin-8 and its receptors in gastric tumor xenografts and gastric tissue adjacent to the tumor in mice. *Zhongxi Yijiehe Xuebao* 2010; **8**: 74-79 [PMID: 20082763]
 - 108 **Sakitani K**, Hirata Y, Hayakawa Y, Serizawa T, Nakata W, Takahashi R, Kinoshita H, Sakamoto K, Nakagawa H, Akanuma M, Yoshida H, Maeda S, Koike K. Role of interleukin-32 in *Helicobacter pylori*-induced gastric inflammation. *Infect Immun* 2012; **80**: 3795-3803 [PMID: 22890997 DOI: 10.1128/iai.00637-12]
 - 109 **Eck M**, Schmausser B, Scheller K, Brändlein S, Müller-Hermelink HK. Pleiotropic effects of CXC chemokines in gastric carcinoma: differences in CXCL8 and CXCL1 expression between diffuse and intestinal types of gastric carcinoma. *Clin Exp Immunol* 2003; **134**: 508-515 [PMID: 14632759]
 - 110 **Allison CC**, Ferrand J, McLeod L, Hassan M, Kaparakis-Liaskos M, Grubman A, Bhathal PS, Dev A, Sievert W, Jenkins BJ, Ferrero RL. Nucleotide oligomerization domain 1 enhances IFN- γ signaling in gastric epithelial cells during *Helicobacter pylori* infection and exacerbates disease severity. *J Immunol* 2013; **190**: 3706-3715 [PMID: 23460743 DOI: 10.4049/jimmunol.1200591]
 - 111 **Macri A**, Versaci A, Loddo S, Scuderi G, Travagliante M, Trimarchi G, Teti D, Famulari C. Serum levels of interleukin 1beta, interleukin 8 and tumour necrosis factor alpha as markers of gastric cancer. *Biomarkers* 2006; **11**: 184-193 [PMID: 16766394 DOI: 10.1080/13547500600565677]
 - 112 **Smith DR**, Polverini PJ, Kunkel SL, Orringer MB, Whyte RI, Burdick MD, Wilke CA, Strieter RM. Inhibition of interleukin 8 attenuates angiogenesis in bronchogenic carcinoma. *J Exp Med* 1994; **179**: 1409-1415 [PMID: 7513008]
 - 113 **Yoneda J**, Kuniyasu H, Crispens MA, Price JE, Bucana CD, Fidler IJ. Expression of angiogenesis-related genes and progression of human ovarian carcinomas in nude mice. *J Natl Cancer Inst* 1998; **90**: 447-454 [PMID: 9521169]
 - 114 **Inoue K**, Slaton JW, Eve BY, Kim SJ, Perrotte P, Balbay MD, Yano S, Bar-Eli M, Radinsky R, Pettaway CA, Dinney CP. Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer. *Clin Cancer Res* 2000; **6**: 2104-2119 [PMID: 10815938]
 - 115 **Kitadai Y**, Takahashi Y, Haruma K, Naka K, Sumii K, Yokozaki H, Yasui W, Mukaida N, Ohmoto Y, Kajiyama G, Fidler IJ, Tahara E. Transfection of interleukin-8 increases angiogenesis and tumorigenesis of human gastric carcinoma cells in nude mice. *Br J Cancer* 1999; **81**: 647-653 [PMID: 10574250 DOI: 10.1038/sj.bjc.6690742]
 - 116 **Ju D**, Sun D, Xiu L, Meng X, Zhang C, Wei P. Interleukin-8 is associated with adhesion, migration and invasion in human gastric cancer SCG-7901 cells. *Med Oncol* 2012; **29**: 91-99 [PMID: 21191670 DOI: 10.1007/s12032-010-9780-0]
 - 117 **Kuai WX**, Wang Q, Yang XZ, Zhao Y, Yu R, Tang XJ. Interleukin-8 associates with adhesion, migration, invasion and chemosensitivity of human gastric cancer cells. *World J Gastroenterol* 2012; **18**: 979-985 [PMID: 22408359 DOI: 10.3748/wjg.v18.i9.979]
 - 118 **Taguchi A**, Ohmiya N, Shirai K, Mabuchi N, Itoh A, Hirooka Y, Niwa Y, Goto H. Interleukin-8 promoter polymorphism increases the risk of atrophic gastritis and gastric cancer in Japan. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 2487-2493 [PMID: 16284368 DOI: 10.1158/1055-9965.epi-05-0326]
 - 119 **Ye BD**, Kim SG, Park JH, Kim JS, Jung HC, Song IS. The interleukin-8-251 A allele is associated with increased risk of noncardia gastric adenocarcinoma in *Helicobacter pylori*-infected Koreans. *J Clin Gastroenterol* 2009; **43**: 233-239 [PMID: 18542040 DOI: 10.1097/MCG.0b013e3181646701]
 - 120 **Ohyauchi M**, Imatani A, Yonechi M, Asano N, Miura A, Iijima K, Koike T, Sekine H, Ohara S, Shimosegawa T. The polymorphism interleukin 8 -251 A/T influences the susceptibility of *Helicobacter pylori* related gastric diseases in the Japanese population. *Gut* 2005; **54**: 330-335 [PMID: 15710978 DOI: 10.1136/gut.2003.033050]
 - 121 **Lu W**, Pan K, Zhang L, Lin D, Miao X, You W. Genetic polymorphisms of interleukin (IL)-1B, IL-1RN, IL-8, IL-10 and tumor necrosis factor {alpha} and risk of gastric cancer in a Chinese population. *Carcinogenesis* 2005; **26**: 631-636 [PMID: 15579481 DOI: 10.1093/carcin/bgh349]
 - 122 **Wang J**, Pan HF, Hu YT, Zhu Y, He Q. Polymorphism of IL-8 in 251 allele and gastric cancer susceptibility: a meta-analysis. *Dig Dis Sci* 2010; **55**: 1818-1823 [PMID: 19777350 DOI: 10.1007/s10620-009-0978-y]
 - 123 **Vinagre RM**, Corvelo TC, Arnaud VC, Leite AC, Barile KA, Martins LC. Determination of strains of *Helicobacter pylori* and of polymorphism in the interleukin-8 gene in patients with stomach cancer. *Arq Gastroenterol* 2011; **48**: 46-51 [PMID: 21537542]
 - 124 **Song JH**, Kim SG, Jung SA, Lee MK, Jung HC, Song IS. The interleukin-8-251 AA genotype is associated with angiogenesis in gastric carcinogenesis in *Helicobacter pylori*-infected Koreans. *Cytokine* 2010; **51**: 158-165 [PMID: 20621718 DOI: 10.1016/j.cyto.2010.05.001]
 - 125 **Wang JP**, Hu WM, Wang KS, Luo BH, Wu C, Chen ZH, Luo GQ, Liu YW, Liu QL, Yu J, Li JH, Wen JF. Upregulation of C-X-C chemokine receptor type 1 expression is associated with late-stage gastric adenocarcinoma. *Exp Ther Med* 2012; **4**: 55-60 [PMID: 23060922 DOI: 10.3892/etm.2012.568]
 - 126 **Wang JP**, Hu WM, Wang KS, Yu J, Luo BH, Wu C, Chen ZH, Luo GQ, Liu YW, Liu QL, Xiao Y, Zhou HY, Yang XJ, Jiang HY, Li JH, Wen JF. Expression of C-X-C chemokine receptor types 1/2 in patients with gastric carcinoma: Clinicopathological correlations and significance. *Oncol Lett* 2013; **5**: 574-582 [PMID: 23420470 DOI: 10.3892/ol.2012.1043]
 - 127 **Kitadai Y**, Haruma K, Mukaida N, Ohmoto Y, Matsutani N, Yasui W, Yamamoto S, Sumii K, Kajiyama G, Fidler IJ, Tahara E. Regulation of disease-progression genes in human gastric carcinoma cells by interleukin 8. *Clin Cancer Res* 2000;

- 6: 2735-2740 [PMID: 10914718]
- 128 **Ewington L**, Taylor A, Sriraksa R, Horimoto Y, Lam EW, El-Bahrawy MA. The expression of interleukin-8 and interleukin-8 receptors in endometrial carcinoma. *Cytokine* 2012; **59**: 417-422 [PMID: 22626766 DOI: 10.1016/j.cyto.2012.04.036]
- 129 **Waugh DJ**, Wilson C. The interleukin-8 pathway in cancer. *Clin Cancer Res* 2008; **14**: 6735-6741 [PMID: 18980965 DOI: 10.1158/1078-0432.ccr-07-4843]
- 130 **Junnila S**, Kokkola A, Mizuguchi T, Hirata K, Karjalainen-Lindsberg ML, Puolakkainen P, Monni O. Gene expression analysis identifies over-expression of CXCL1, SPARC, SPP1, and SULF1 in gastric cancer. *Genes Chromosomes Cancer* 2010; **49**: 28-39 [PMID: 19780053 DOI: 10.1002/gcc.20715]
- 131 **Cheng WL**, Wang CS, Huang YH, Tsai MM, Liang Y, Lin KH. Overexpression of CXCL1 and its receptor CXCR2 promote tumor invasion in gastric cancer. *Ann Oncol* 2011; **22**: 2267-2276 [PMID: 21343381 DOI: 10.1093/annonc/mdq739]
- 132 **Xu J**, Zhang C, He Y, Wu H, Wang Z, Song W, Li W, He W, Cai S, Zhan W. Lymphatic endothelial cell-secreted CXCL1 stimulates lymphangiogenesis and metastasis of gastric cancer. *Int J Cancer* 2012; **130**: 787-797 [PMID: 21387301 DOI: 10.1002/ijc.26035]
- 133 **Kuzuhara T**, Suganuma M, Kurusu M, Fujiki H. Helicobacter pylori-secreting protein Tipalpha is a potent inducer of chemokine gene expressions in stomach cancer cells. *J Cancer Res Clin Oncol* 2007; **133**: 287-296 [PMID: 17393199 DOI: 10.1007/s00432-006-0169-6]
- 134 **Karasawa F**, Shiota A, Goso Y, Kobayashi M, Sato Y, Masumoto J, Fujiwara M, Yokosawa S, Muraki T, Miyagawa S, Ueda M, Fukuda MN, Fukuda M, Ishihara K, Nakayama J. Essential role of gastric gland mucin in preventing gastric cancer in mice. *J Clin Invest* 2012; **122**: 923-934 [PMID: 22307328 DOI: 10.1172/jci59087]
- 135 **Sheh A**, Ge Z, Parry NM, Muthupalani S, Rager JE, Raczynski AR, Mobley MW, McCabe AF, Fry RC, Wang TC, Fox JG. 17 β -estradiol and tamoxifen prevent gastric cancer by modulating leukocyte recruitment and oncogenic pathways in Helicobacter pylori-infected INS-GAS male mice. *Cancer Prev Res (Phila)* 2011; **4**: 1426-1435 [PMID: 21680705 DOI: 10.1158/1940-6207.capr-11-0219]
- 136 **Tang W**, Morgan DR, Meyers MO, Dominguez RL, Martinez E, Kakudo K, Kuan PF, Banet N, Muallem H, Woodward K, Speck O, Gulley ML. Epstein-barr virus infected gastric adenocarcinoma expresses latent and lytic viral transcripts and has a distinct human gene expression profile. *Infect Agent Cancer* 2012; **7**: 21 [PMID: 22929309 DOI: 10.1186/1750-9378-7-21]
- 137 **Jung JJ**, Noh S, Jeung HC, Jung M, Kim TS, Noh SH, Roh JK, Chung HC, Rha SY. Chemokine growth-regulated oncogene 1 as a putative biomarker for gastric cancer progression. *Cancer Sci* 2010; **101**: 2200-2206 [PMID: 20731665 DOI: 10.1111/j.1349-7006.2010.01666.x]
- 138 **Sharma B**, Nawandar DM, Nannuru KC, Varney ML, Singh RK. Targeting CXCR2 enhances chemotherapeutic response, inhibits mammary tumor growth, angiogenesis, and lung metastasis. *Mol Cancer Ther* 2013; **12**: 799-808 [PMID: 23468530 DOI: 10.1158/1535-7163.mct-12-0529]
- 139 **Yamamoto M**, Kikuchi H, Ohta M, Kawabata T, Hiramatsu Y, Kondo K, Baba M, Kamiya K, Tanaka T, Kitagawa M, Konno H. TSU68 prevents liver metastasis of colon cancer xenografts by modulating the premetastatic niche. *Cancer Res* 2008; **68**: 9754-9762 [PMID: 19047154 DOI: 10.1158/0008-5472.can-08-1748]
- 140 **Singh S**, Sadanandam A, Nannuru KC, Varney ML, Mayer-Ezell R, Bond R, Singh RK. Small-molecule antagonists for CXCR2 and CXCR1 inhibit human melanoma growth by decreasing tumor cell proliferation, survival, and angiogenesis. *Clin Cancer Res* 2009; **15**: 2380-2386 [PMID: 19293256 DOI: 10.1158/1078-0432.ccr-08-2387]
- 141 **Okayama H**, Kumamoto K, Saitou K, Hayase S, Kofunato Y, Sato Y, Miyamoto K, Nakamura I, Ohki S, Sekikawa K, Takenoshita S. CD44v6, MMP-7 and nuclear Cdx2 are significant biomarkers for prediction of lymph node metastasis in primary gastric cancer. *Oncol Rep* 2009; **22**: 745-755 [PMID: 19724852]
- 142 **Park JY**, Park KH, Bang S, Kim MH, Lee JE, Gang J, Koh SS, Song SY. CXCL5 overexpression is associated with late stage gastric cancer. *J Cancer Res Clin Oncol* 2007; **133**: 835-840 [PMID: 17479287 DOI: 10.1007/s00432-007-0225-x]
- 143 **Kraft M**, Riedel S, Maaser C, Kucharzik T, Steinbuechel A, Domschke W, Luegering N. IFN-gamma synergizes with TNF-alpha but not with viable H. pylori in up-regulating CXC chemokine secretion in gastric epithelial cells. *Clin Exp Immunol* 2001; **126**: 474-481 [PMID: 11737065]
- 144 **Rajkumar T**, Vijayalakshmi N, Gopal G, Sabitha K, Shirley S, Raja UM, Ramakrishnan SA. Identification and validation of genes involved in gastric tumorigenesis. *Cancer Cell Int* 2010; **10**: 45 [PMID: 21092330 DOI: 10.1186/1475-2867-10-45]
- 145 **Ohtani H**, Jin Z, Takegawa S, Nakayama T, Yoshie O. Abundant expression of CXCL9 (MIG) by stromal cells that include dendritic cells and accumulation of CXCR3+ T cells in lymphocyte-rich gastric carcinoma. *J Pathol* 2009; **217**: 21-31 [PMID: 18980207 DOI: 10.1002/path.2448]
- 146 **Deng L**, Chen N, Li Y, Zheng H, Lei Q. CXCR6/CXCL16 functions as a regulator in metastasis and progression of cancer. *Biochim Biophys Acta* 2010; **1806**: 42-49 [PMID: 20122997 DOI: 10.1016/j.bbcan.2010.01.004]
- 147 **Darash-Yahana M**, Gillespie JW, Hewitt SM, Chen YY, Maeda S, Stein I, Singh SP, Bedolla RB, Peled A, Troyer DA, Pikarsky E, Karin M, Farber JM. The chemokine CXCL16 and its receptor, CXCR6, as markers and promoters of inflammation-associated cancers. *PLoS One* 2009; **4**: e6695 [PMID: 19690611 DOI: 10.1371/journal.pone.0006695]
- 148 **Hojo S**, Koizumi K, Tsuneyama K, Arita Y, Cui Z, Shinohara K, Minami T, Hashimoto I, Nakayama T, Sakurai H, Takano Y, Yoshie O, Tsukada K, Saiki I. High-level expression of chemokine CXCL16 by tumor cells correlates with a good prognosis and increased tumor-infiltrating lymphocytes in colorectal cancer. *Cancer Res* 2007; **67**: 4725-4731 [PMID: 17510400 DOI: 10.1158/0008-5472.can-06-3424]
- 149 **Xing YN**, Xu XY, Nie XC, Yang X, Yu M, Xu HM, Liu YP, Takano Y, Zheng HC. Role and clinicopathologic significance of CXC chemokine ligand 16 and chemokine (C-X-C motif) receptor 6 expression in gastric carcinomas. *Hum Pathol* 2012; **43**: 2299-2307 [PMID: 22863086 DOI: 10.1016/j.humpath.2011.08.027]
- 150 **Duda DG**, Kozin SV, Kirkpatrick ND, Xu L, Fukumura D, Jain RK. CXCL12 (SDF1alpha)-CXCR4/CXCR7 pathway inhibition: an emerging sensitizer for anticancer therapies? *Clin Cancer Res* 2011; **17**: 2074-2080 [PMID: 21349998 DOI: 10.1158/1078-0432.ccr-10-2636]
- 151 **Iwanaga T**, Iwasaki Y, Ohashi M, Nunobe S, Iwagami S. [Establishment of a CXCR4-expressing gastric cancer cell line in nude mice and the effect of AMD 3100 on tumor regression]. *Gan To Kagaku Ryoho* 2007; **34**: 1917-1919 [PMID: 18219852]
- 152 **Iwanaga T**, Iwasaki Y, Ohashi M, Ohinata R, Takahashi K, Yamaguchi T, Matsumoto H, Nakano D. [Inhibitory effect of CXCR4 blockers on a CXCR4-expressing gastric cancer cell line in nude mice]. *Gan To Kagaku Ryoho* 2012; **39**: 1788-1790 [PMID: 23267887]
- 153 **Xie L**, Wei J, Qian X, Chen G, Yu L, Ding Y, Liu B. CXCR4, a potential predictive marker for docetaxel sensitivity in gastric cancer. *Anticancer Res* 2010; **30**: 2209-2216 [PMID: 20651371]
- 154 **Manu KA**, Shanmugam MK, Rajendran P, Li F, Ramachandran L, Hay HS, Kannaiyan R, Swamy SN, Vali S, Kapoor S, Ramesh B, Bist P, Koay ES, Lim LH, Ahn KS, Kumar AP, Sethi G. Plumbagin inhibits invasion and migration of breast and gastric cancer cells by downregulating the expression of chemokine receptor CXCR4. *Mol Cancer* 2011; **10**: 107 [PMID: 21349998 DOI: 10.1158/1078-0432.ccr-10-2636]

- 21880153 DOI: 10.1186/1476-4598-10-107]
- 155 **Ning Y**, Labonte MJ, Zhang W, Bohanes PO, Gerger A, Yang D, Benhaim L, Paez D, Rosenberg DO, Nagulapalli Venkata KC, Louie SG, Petasis NA, Ladner RD, Lenz HJ. The CXCR2 antagonist, SCH-527123, shows antitumor activity and sensitizes cells to oxaliplatin in preclinical colon cancer models. *Mol Cancer Ther* 2012; **11**: 1353-1364 [PMID: 22391039 DOI: 10.1158/1535-7163.mct-11-0915]
 - 156 **Varney ML**, Li A, Dave BJ, Bucana CD, Johansson SL, Singh RK. Expression of CXCR1 and CXCR2 receptors in malignant melanoma with different metastatic potential and their role in interleukin-8 (CXCL-8)-mediated modulation of metastatic phenotype. *Clin Exp Metastasis* 2003; **20**: 723-731 [PMID: 14713106]
 - 157 **Singh JK**, Farnie G, Bundred NJ, Simões BM, Shergill A, Landberg G, Howell SJ, Clarke RB. Targeting CXCR1/2 significantly reduces breast cancer stem cell activity and increases the efficacy of inhibiting HER2 via HER2-dependent and -independent mechanisms. *Clin Cancer Res* 2013; **19**: 643-656 [PMID: 23149820 DOI: 10.1158/1078-0432.ccr-12-1063]
 - 158 **Mian BM**, Dinney CP, Bermejo CE, Sweeney P, Tellez C, Yang XD, Gudas JM, McConkey DJ, Bar-Eli M. Fully human anti-interleukin 8 antibody inhibits tumor growth in orthotopic bladder cancer xenografts via down-regulation of matrix metalloproteases and nuclear factor-kappaB. *Clin Cancer Res* 2003; **9**: 3167-3175 [PMID: 12912969]
 - 159 **Singh S**, Sadanandam A, Varney ML, Nannuru KC, Singh RK. Small interfering RNA-mediated CXCR1 or CXCR2 knock-down inhibits melanoma tumor growth and invasion. *Int J Cancer* 2010; **126**: 328-336 [PMID: 19585580 DOI: 10.1002/ijc.24714]
 - 160 **Varney ML**, Singh S, Li A, Mayer-Ezell R, Bond R, Singh RK. Small molecule antagonists for CXCR2 and CXCR1 inhibit human colon cancer liver metastases. *Cancer Lett* 2011; **300**: 180-188 [PMID: 21035946 DOI: 10.1016/j.canlet.2010.10.004]

P- Reviewer: Yun S **S- Editor:** Wen LL **L- Editor:** A
E- Editor: Wu HL



WJG 20th Anniversary Special Issues (8): Gastric cancer

Cyr61/CTGF/Nov family proteins in gastric carcinogenesis

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Received: October 29, 2013 Revised: December 7, 2013

Accepted: January 3, 2014

Published online: February 21, 2014

Core tip: Cyr61/CTGF/Nov (CCN) proteins are matricellular proteins responsible for many physiological and pathological processes, including carcinogenesis. The prototypical CCN family protein is composed of an N-terminal secretory signal peptide and four structural modules. Several truncated variants participate in the carcinogenesis of gastrointestinal tract cancers. The role of CCNs in carcinogenesis is tumor-type and context-dependent. The evidence suggests that CCN family proteins play important roles in gastric cancer (GC) carcinogenic processes. Recent CCN targeting agents, including monoclonal antibodies, antisense oligonucleotides and RNA interference compounds, may be helpful in future GC therapeutics.

Abstract

Gastric cancer (GC) is the second leading cause of cancer-related death. The poor survival rate may reflect the relatively aggressive tumor biology of GC. Recently, the importance of the tumor microenvironment in carcinogenesis has emerged. In the tumor microenvironment, tumor cells and the surrounding stromal cells aberrantly secrete matricellular proteins capable of modulating carcinogenesis and regulating metastasis. The Cyr61/CTGF/Nov (CCN) proteins are a family of matricellular proteins with variable roles in many physiological and pathological processes. The evidence suggests that CCN family proteins contribute to GC carcinogenic processes. Here, we briefly review recent research on the effects of CCN family proteins in GC carcinogenesis and the development of new targeted agents in this field.

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Key words: Cyr61/CTGF/Nov proteins; Cysteine-rich angiogenic inducer 61; Connective tissue growth factor; Nephroblastoma over-expressed; Gastric cancer; Gastric carcinogenesis

Cheng TY, Wu MS, Hua KT, Kuo ML, Lin MT. Cyr61/CTGF/Nov family proteins in gastric carcinogenesis. *World J Gastroenterol* 2014; 20(7): 1694-1700 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1694.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1694>

INTRODUCTION

Gastric cancer (GC) is the second leading cause of cancer-related death, accounting for approximately 10% of total cancer deaths worldwide^[1]. Despite significant advances in cancer treatment modalities, the prognosis for GC has only modestly improved. The five-year relative survival rate for all stages combined was 28% between 2002 and 2008, compared to 20% between 1987 and 1989^[2]. The poor survival rate may reflect the relatively aggressive tumor biology of GC.

The interplay between the tumor and its microenvironment is crucial for both tumor development and progression. In the tumor microenvironment, tumor cells and the surrounding stromal cells aberrantly secrete matricellular proteins, a group of proteins that function as regulators of cell-cell and cell-matrix interactions that modulate carcinogenesis and the regulatory networks of

Table 1 Nomenclature and characterization of the Cyr61/CTGF/Nov family proteins

CCN proteins (synonyms)	Chromosomal location	Molecular mass, kDa (number of amino acids)
CCN1 (Cyr61; CTGF-2; IGFBP-10/IBP-10; IGFBP-rP4; Cef-10)	1p22.3	42.0 (381)
CCN2 (CTGF; IGFBP-8/IBP-8; IGFBP-rP2; hypertrophic chondrocyte-specific protein 24)	6q23.1	38.1 (349)
CCN3 (Nov; IGFBP-9/IBP-9; IGFBP-rP3)	8q24.1	39.2 (357)
CCN4 (WISP-1; Elm-1)	8q24.22	40.3 (367)
CCN5 (WISP-2; CTGF-3; CTGF-L; Cop-1)	20q13.12	26.8 (250)
CCN6 (WISP-3; LIBC)	6q21	39.3 (354)

CCN: Cyr61/CTGF/Nov; Cyr61: Cysteine-rich angiogenic inducer 61; CTGF: Connective tissue growth factor; IGFBP: Insulin-like growth factor-binding protein; IGFBP-rP: IGFBP-related protein; Cef: Chicken embryo fibroblasts; Nov: Nephroblastoma over-expressed; WISP: Wnt1-inducible signaling pathway protein; Elm: Expressed in low-metastatic cells; Cop-1: Card-only protein 1; LIBC: Lost in inflammatory breast cancer.

metastasis^[3]. The Cyr61/CTGF/Nov (CCN) proteins are a family of matricellular proteins that play pivotal roles in many physiological and pathological processes, including carcinogenesis^[4]. The CCN family proteins include six members, as summarized in Table 1. The expression of CCN family proteins is dependent on cell type and context. CCN family proteins can act both positively and negatively in carcinogenesis for different tumor types. The positive or negative effect depends on whether angiogenic factors are limiting and whether conditions that favor apoptosis or senescence prevail^[5,6]. The prototypical CCN family protein is composed of an N-terminal secretory signal peptide and four structural modules: an insulin-like growth factor binding protein-like module, a von Willebrand factor type C repeat (VWC) module, a thrombospondin-homology type 1 repeat (TSP1) module, and a C-terminal cysteine-knot-containing (CT) module^[7]. Except for CCN5, which lacks the CT module, all CCN proteins contain the four complete structural modules. However, there are biologically active CCN variants with less than four modules after translational processing or alternative splicing. Some of these truncated variants may participate in the carcinogenesis of gastrointestinal tract cancers^[7-10], including GC. More recent evidence suggests that CCN family proteins contribute to GC carcinogenesis (Figure 1). Of the CCN family proteins, only CCN2 has been reported to be involved in *Helicobacter pylori*-associated chronic gastritis. There is a positive correlation between the density of CCN2-producing mononuclear cells and the severity of chronic gastritis. The actual role of CCN family proteins in the initiation stage of GC carcinogenesis will be clarified with future studies^[11]. In this brief review, we focus on the roles that the CCN family proteins play in the promotion and progression of GC, the cell signaling pathways involved in the GC regulatory processes, and the development of new agents in targeted therapy.

INDIVIDUAL CCN FAMILY PROTEINS WITH GC DEVELOPMENT AND PROGRESSION

CCN1

CCN1 was the first cloned member of the CCN family proteins^[12] and has been reported to regulate diverse cellular functions through binding to distinct integrins^[13]. CCN1 supports cell adhesion, stimulates cell migration, augments growth factor-induced DNA synthesis, promotes cell survival, inhibits apoptosis, and enhances angiogenesis^[14]. Although much more data have been reported from cancer cell lines, CCN1 expression is up-regulated in patients with breast cancer, gliomas, hepatocellular carcinoma, prostate cancer, and oral squamous cell carcinoma^[15-20] but is down-regulated in leiomyoma and non-small cell lung cancer^[21,22]. The role of CCN1 in carcinogenesis may be cell type- and context-dependent. CCN1 mediates its activities primarily through interaction with cell adhesion receptor integrins and co-receptor heparan sulfate proteoglycans (HSPGs). CCN1 as a ligand of integrins was first demonstrated by the direct binding of CCN1 to integrin $\alpha v \beta 3$ to mediate endothelial cell adhesion^[23]. Several other integrins, such as $\alpha 2 \beta 1$, $\alpha 6 \beta 1$, $\alpha v \beta 5$, $\alpha II b \beta 3$, $\alpha M \beta 2$, and $\alpha D \beta 2$, have also been identified as signaling receptors mediating CCN1 functions^[5].

In patients with GC, high expression levels of CCN1 correlate with more lymph node metastases, more advanced tumor stage, a histologic diffuse type, and early recurrence^[24]. Forced expression of CCN1 can induce angiogenesis, a process essential for nourishing the growing tumor. CCN1 promotes angiogenesis either directly by effects on endothelial cells or indirectly by regulating the angiogenic factors vascular endothelial growth factor (VEGF)-A and VEGF-C^[25,26]. However, there are no data illustrating the relationship among CCN1, VEGF-A and VEGF-C in GC. CCN1 promotes tumor growth and increases tumor vascularization upon over-expression in GC cells in the severe combined immunodeficiency mouse model^[27]. In addition, *in vitro* studies have shown that more invasive GC cell lines contain higher levels of CCN1. The forced expression of CCN1 or treatment with recombinant CCN1 in GC cells significantly increases invasive ability. CCN1 regulates GC cell motility/invasion through integrin $\alpha v \beta 3$ and induces nuclear factor- κB (NF- κB) activation as well as the subsequent cyclooxygenase-2 (COX-2) up-regulation to promote cell invasion^[24]. The importance of COX-2 expression in GC is well established, with its correlation with depth of invasion, lymph node metastasis and advanced stage^[28-30].

In addition to the NF- κB -dependent pathway, CCN1 regulates GC cell invasiveness by the hypoxia-inducing factor-1 α (HIF-1 α)-dependent up-regulation of plasminogen activator inhibitor-1 (PAI-1). Both phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin and extracellular signal-regulated kinase 1/2 signaling pathways are essential for HIF-1 α accumulation^[31]. CCN1 may also contribute to the peritoneal

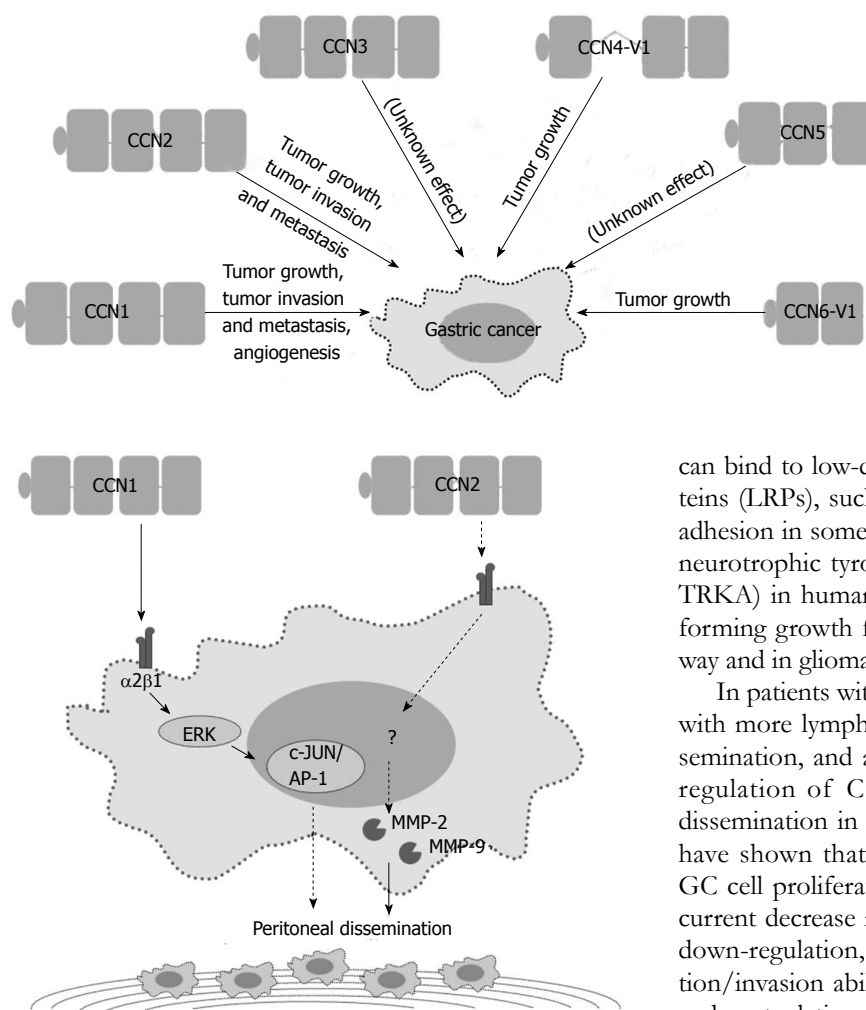


Figure 1 An overview of the roles of individual cysteine-rich angiogenic inducer Cyr61/CTGF/Nov proteins in gastric cancer carcinogenesis based on current evidence. CCN: Cyr61/CTGF/Nov; Cyr61: Cysteine-rich angiogenic inducer 61; CTGF: Connective tissue growth factor; Nov: Nephroblastoma over-expressed.

Figure 2 Summary of the impacts of Cyr61/CTGF/Nov 1 and Cyr61/CTGF/Nov 2 on the peritoneal dissemination of gastric cancer. CCN: Cyr61/CTGF/Nov; Cyr61: Cysteine-rich angiogenic inducer 61; CTGF: Connective tissue growth factor; Nov: Nephroblastoma over-expressed; MMP: Matrix metalloproteinase.

dissemination of GC by promoting tumor-cell adhesion ability. High CCN1 expression levels correlate with peritoneal dissemination in advanced stage GC patients. GC cells over-expressing CCN1 up-regulate integrin $\alpha 2 \beta 1$ via an activator protein-1 (AP-1)-dependent pathway (Figure 2)^[32].

CCN2

CCN2 was first recognized as the major platelet-derived growth factor (PDGF)-related mitogen secreted by human vascular endothelial cells^[33]. CCN2 is involved in a wide variety of regulatory processes, such as angiogenesis, chondrogenesis, osteogenesis, fibrosis formation, diabetic nephropathy, and tumor development^[5]. CCN2 expression is up-regulated in patients with breast cancer, gliomas, esophageal adenocarcinoma, pancreatic cancer, and melanoma^[15,17,34-36] but is down-regulated in lung adenocarcinoma and colon cancer^[22,37,38]. Similar to CCN1, CCN2 achieves functional versatility through its interaction with different integrins, including $\alpha \nu \beta 3$, $\alpha 5 \beta 1$, $\alpha 6 \beta 1$, $\alpha \text{II} \beta 3$, and $\alpha \text{M} \beta 2$. In addition to HSPGs, CCN2

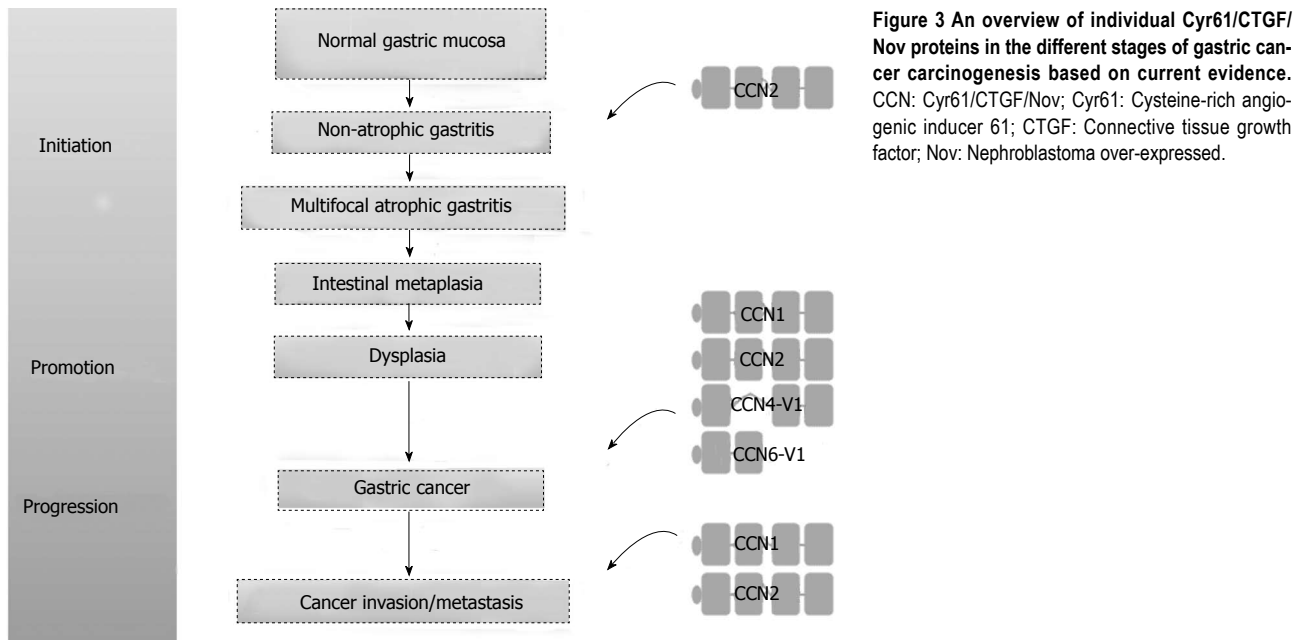
can bind to low-density lipoprotein receptor-related proteins (LRPs), such as LRP-1 and LRP-6, to mediate cell adhesion in some cell types. CCN2 can also interact with neurotrophic tyrosine kinase receptor type 1 (NTRK1/TRKA) in human mesangial cells to enhance the transforming growth factor- β (TGF- β)/Smad signaling pathway and in glioma cells to facilitate NF- κ B activation^[5,6].

In patients with GC, high CCN2 expression correlates with more lymph node metastases, more peritoneal dissemination, and a shorter five-year survival^[39-41]. Down-regulation of CCN2 in GC cells reduces peritoneal dissemination in the nude mouse model. *In vitro* studies have shown that down-regulation of CCN2 decreases GC cell proliferation and colony formation with a concurrent decrease in cyclin D1 expression^[42]. After CCN2 down-regulation, GC cells also show attenuated migration/invasion abilities with decreased protein expression and proteolytic activity of both matrix metalloproteinase (MMP)-2 and MMP-9 (Figure 2)^[41].

In GC specimens, CCN2 expression is in agreement with the expression of vascular endothelial growth factor VEGF-C and VEGF-D, as shown by immunohistochemical staining^[39]. CCN2 can induce angiogenesis, and it can also regulate VEGF-induced angiogenesis through the TSP1 and CT modules^[43]. In addition, CCN2 is transcriptionally induced under hypoxia^[44], a condition favoring blood vessel growth by the induction of angiogenic factors such as VEGF. Further studies are necessary to elucidate the complex interaction between CCN2 and the VEGF family proteins in GC.

CCN3

CCN3 was first discovered as an over-expressed gene in a myeloblastosis-associated virus type-1-induced nephroblastoma in chickens^[45]. CCN3 is implicated in many diverse biological processes, such as proliferation, differentiation, and angiogenesis, as well as some pathological conditions, including fibrosis and cancer^[46]. CCN3 is up-regulated in patients with Wilms' tumor with predominantly stromal elements and metastatic Ewing's sarcoma^[47,48] but is down-regulated in malignant adrenocortical tumors and poorly differentiated prostate cancer^[49,50]. CCN3 can mediate its various activities through interacting with integrins, such as $\alpha \nu \beta 3$, $\alpha \nu \beta 5$, $\alpha 5 \beta 1$ and $\alpha 6 \beta 1$ ^[5,6]. CCN3 expression has not been reported in GC samples.



CCN4

CCN4 was first identified in low-metastatic cells by comparing mRNA differential display data from high- and low-metastatic murine melanoma cells^[51]. CCN4 is involved in regulating morphological transformation, cell growth, and tumor growth^[52,53]. Although CCN4 over-expression suppresses the growth of melanoma tumors in a mouse model, CCN4 is up-regulated in patients with breast cancer, non-small cell lung cancer, colorectal cancer, esophageal squamous cell carcinoma, endometrial endometrioid adenocarcinoma, and prostate cancer^[15,22,54-59].

In patients with GC, a truncated variant of CCN4-V1 completely lacking the VWC module is up-regulated in scirrhous GC. *In vitro* experiments have shown that forced expression of CCN4-V1 in fibroblast cells induces cellular transformation and a rapid growth characterized by cell piling. CCN4-V1 transfectants can enhance the invasive abilities of co-cultured GC cells^[8].

CCN5

The rat homologue of CCN5 was first reported to be down-regulated in rat embryo fibroblasts transformed by the cooperation of the activated H-ras oncogene and the inactivated p53 tumor suppressor gene^[60]. CCN5 is involved in regulating cell growth, morphological transformation, and attenuating cell migration^[61,62]. CCN5 is down-regulated in patients with colon cancer, pancreatic cancer, and invasive breast cancer^[53,61,62]. CCN5 expression has not been reported in cases of GC.

CCN6

CCN6 was first identified as an expressed sequence tag after database screening for differentially expressed cDNAs after Wnt1-induction in mouse mammary epithelial cells^[54]. CCN6 is involved in regulating morphological transformation, inhibiting cell growth, attenuating cell migration, and inhibiting tumor-induced angiogenesis^[63,64]. CCN6 is down-regulated in patients with inflammatory

breast cancer^[65].

In patients with GC, the truncated variant CCN6-V1 lacking TSP1 and CT modules is noted in 11%-20% of microsatellite unstable GCs. A frameshift mutation in the (A)₉ repeat in exon 4 of CCN6 leads to a premature stop codon in exon 4 and consequently to truncated CCN6-V1. Forced expression of CCN6 in GC cells can inhibit cell invasive abilities, but CCN6-V1 transfectants lose the inhibitory effect^[9,66].

CCN-TARGETED THERAPY

With the greater understanding of the molecular biology of carcinogenesis, more targeted agents have been developed and are associated with improved outcomes in some advanced cancers. Trastuzumab, the first and only targeted agent approved for the treatment of GC, has shown clinical benefits in response rates and survival in combination treatment with chemotherapy for HER-2 positive advanced GC^[67]. Because CCN family proteins are implicated in many processes of carcinogenesis, it is reasonable to develop treatment strategies for these potential targets. For this family of secreted proteins, monoclonal antibodies are good therapeutic candidates. Among the six family members, CCN2 has received the most attention because of previous detailed studies and its strong clinical association with fibrosis. Blocking CCN2 with FG-3019, a CCN2 monoclonal antibody, inhibits pancreatic tumor growth and metastases in both xenograft and orthotopic mouse models^[68,69]. A phase I study that assessed the safety and tolerance of FG-3019 has been performed in patients with idiopathic pulmonary fibrosis, and FG-3019 was shown to be safe and well-tolerated^[70]. Further phase II clinical trials for evaluating its efficacy are underway. For cancer therapy, there is only one ongoing phase I study evaluating FG-3019 therapy in combination with gemcitabine and erlotinib for patients with locally advanced or metastatic pancreatic can-

cer (ClinicalTrials.gov identifier: NCT01181245). There are currently no clinical trials of CCN-targeted therapy in GC.

In addition to monoclonal antibodies, antisense oligonucleotides (EXC 001) and RNA interference compounds (RXI-109) have been recently developed to reduce scar formation by inhibiting CCN2 expression^[6]. Further application of these targeting agents as cancer therapeutics may be helpful for patients with GC.

CONCLUSION

In summary, CCN family proteins play important roles in mediating GC carcinogenesis (Figure 3), including their involvement in cell signaling pathways, angiogenesis, tumor formation, tumor invasion and metastasis. The recognition of the matricellular protein nature of CCNs with a corresponding biological niche in GC carcinogenesis, as illustrated in this review, may allow oncologic issues to be considered in a new way that will have a positive impact on the future management of GC.

REFERENCES

- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855 DOI: 10.3322/caac.20107]
- Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin* 2013; **63**: 11-30 [PMID: 23335087 DOI: 10.3322/caac.21166]
- Chong HC, Tan CK, Huang RL, Tan NS. Matricellular proteins: a sticky affair with cancers. *J Oncol* 2012; **2012**: 351089 [PMID: 22481923 DOI: 10.1155/2012/351089]
- Perbal B. CCN proteins: A centralized communication network. *J Cell Commun Signal* 2013; **7**: 169-177 [PMID: 23420091 DOI: 10.1007/s12079-013-0193-7]
- Chen CC, Lau LF. Functions and mechanisms of action of CCN matricellular proteins. *Int J Biochem Cell Biol* 2009; **41**: 771-783 [PMID: 18775791 DOI: 10.1016/j.biocel.2008.07.025]
- Jun JI, Lau LF. Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. *Nat Rev Drug Discov* 2011; **10**: 945-963 [PMID: 22129992 DOI: 10.1038/nrd3599]
- Bork P. The modular architecture of a new family of growth regulators related to connective tissue growth factor. *FEBS Lett* 1993; **327**: 125-130 [PMID: 7687569]
- Tanaka S, Sugimachi K, Saeki H, Kinoshita J, Ohga T, Shimada M, Maehara Y, Sugimachi K. A novel variant of WISP1 lacking a Von Willebrand type C module overexpressed in scirrhous gastric carcinoma. *Oncogene* 2001; **20**: 5525-5532 [PMID: 11571650 DOI: 10.1038/sj.onc.1204723]
- Thorstensen L, Diep CB, Meling GI, Aagesen TH, Ahrens CH, Rognum TO, Lothe RA. WNT1 inducible signaling pathway protein 3, WISP-3, a novel target gene in colorectal carcinomas with microsatellite instability. *Gastroenterology* 2001; **121**: 1275-1280 [PMID: 11729105 DOI: 10.1053/gast.2001.29570]
- Tanaka S, Sugimachi K, Shimada M, Maehara Y, Sugimachi K. Variant WISPs as targets for gastrointestinal carcinomas. *Gastroenterology* 2002; **123**: 392-393 [PMID: 12105881 DOI: 10.1053/gast.2002.34589]
- Li Z, Li J. Local expressions of TGF-beta1, TGF-beta1RI, CTGF, and Smad-7 in Helicobacter pylori-associated gastritis. *Scand J Gastroenterol* 2006; **41**: 1007-1012 [PMID: 16938712 DOI: 10.1080/00365520600554477]
- Lau LF, Nathans D. Identification of a set of genes expressed during the G0/G1 transition of cultured mouse cells. *EMBO J* 1985; **4**: 3145-3151 [PMID: 3841511]
- Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell* 2002; **110**: 673-687 [PMID: 12297042 DOI: 10.1016/S0092-8674(02)00971-6]
- Lau LF. CCN1/CYR61: the very model of a modern matricellular protein. *Cell Mol Life Sci* 2011; **68**: 3149-3163 [PMID: 21805345 DOI: 10.1007/s00018-011-0778-3]
- Xie D, Nakachi K, Wang H, Elashoff R, Koeffler HP. Elevated levels of connective tissue growth factor, WISP-1, and CYR61 in primary breast cancers associated with more advanced features. *Cancer Res* 2001; **61**: 8917-8923 [PMID: 11751417]
- Tsai MS, Hornby AE, Lakins J, Lupu R. Expression and function of CYR61, an angiogenic factor, in breast cancer cell lines and tumor biopsies. *Cancer Res* 2000; **60**: 5603-5607 [PMID: 11059746]
- Xie D, Yin D, Wang HJ, Liu GT, Elashoff R, Black K, Koeffler HP. Levels of expression of CYR61 and CTGF are prognostic for tumor progression and survival of individuals with gliomas. *Clin Cancer Res* 2004; **10**: 2072-2081 [PMID: 15041728]
- Zeng ZJ, Yang LY, Ding X, Wang W. Expressions of cysteine-rich61, connective tissue growth factor and Nov genes in hepatocellular carcinoma and their clinical significance. *World J Gastroenterol* 2004; **10**: 3414-3418 [PMID: 15526358]
- D'Antonio KB, Toubaji A, Albadini R, Mondul AM, Platz EA, Netto GJ, Getzenberg RH. Extracellular matrix associated protein CYR61 is linked to prostate cancer development. *J Urol* 2010; **183**: 1604-1610 [PMID: 20172544 DOI: 10.1016/j.juro.2009.12.006]
- Kok SH, Chang HH, Tsai JY, Hung HC, Lin CY, Chiang CP, Liu CM, Kuo MY. Expression of Cyr61 (CCN1) in human oral squamous cell carcinoma: An independent marker for poor prognosis. *Head Neck* 2010; **32**: 1665-1673 [PMID: 20848406 DOI: 10.1002/hed.21381]
- Sampath D, Zhu Y, Winneker RC, Zhang Z. Aberrant expression of Cyr61, a member of the CCN (CTGF/Cyr61/Cef10/NOVH) family, and dysregulation by 17 beta-estradiol and basic fibroblast growth factor in human uterine leiomyomas. *J Clin Endocrinol Metab* 2001; **86**: 1707-1715 [PMID: 11297607 DOI: 10.1210/jc.86.4.1707]
- Chen PP, Li WJ, Wang Y, Zhao S, Li DY, Feng LY, Shi XL, Koeffler HP, Tong XJ, Xie D. Expression of Cyr61, CTGF, and WISP-1 correlates with clinical features of lung cancer. *PLoS One* 2007; **2**: e534 [PMID: 17579708 DOI: 10.1371/journal.pone.0000534]
- Kireeva ML, Lam SC, Lau LF. Adhesion of human umbilical vein endothelial cells to the immediate-early gene product Cyr61 is mediated through integrin alphavbeta3. *J Biol Chem* 1998; **273**: 3090-3096 [PMID: 9446626 DOI: 10.1074/jbc.273.5.3090]
- Lin MT, Zuon CY, Chang CC, Chen ST, Chen CP, Lin BR, Wang MY, Jeng YM, Chang KJ, Lee PH, Chen WJ, Kuo ML. Cyr61 induces gastric cancer cell motility/invasion via activation of the integrin/nuclear factor-kappaB/cyclooxygenase-2 signaling pathway. *Clin Cancer Res* 2005; **11**: 5809-5820 [PMID: 16115920]
- Chen CC, Mo FE, Lau LF. The angiogenic factor Cyr61 activates a genetic program for wound healing in human skin fibroblasts. *J Biol Chem* 2001; **276**: 47329-47337 [PMID: 11584015 DOI: 10.1074/jbc.M107666200]
- Mo FE, Muntean AG, Chen CC, Stolz DB, Watkins SC, Lau LF. CYR61 (CCN1) is essential for placental development and vascular integrity. *Mol Cell Biol* 2002; **22**: 8709-8720 [PMID: 12446788 DOI: 10.1128/MCB.22.24.8709-8720.2002]
- Babic AM, Kireeva ML, Kolesnikova TV, Lau LF. CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth. *Proc Natl Acad Sci USA* 1998; **95**: 6355-6360 [PMID: 9600969]

- 28 **Ohno R**, Yoshinaga K, Fujita T, Hasegawa K, Iseki H, Tsunozaki H, Ichikawa W, Nihei Z, Sugihara K. Depth of invasion parallels increased cyclooxygenase-2 levels in patients with gastric carcinoma. *Cancer* 2001; **91**: 1876-1881 [PMID: 11346869 DOI: 10.1002/1097-0142(20010515)91]
- 29 **Murata H**, Kawano S, Tsuji S, Tsuji M, Sawaoka H, Kimura Y, Shiozaki H, Hori M. Cyclooxygenase-2 overexpression enhances lymphatic invasion and metastasis in human gastric carcinoma. *Am J Gastroenterol* 1999; **94**: 451-455 [PMID: 10022645 DOI: 10.1111/j.1572-0241.1999.876_e.x]
- 30 **Yu HG**, Li JY, Yang YN, Luo HS, Yu JP, Meier JJ, Schrader H, Bastian A, Schmidt WE, Schmitz F. Increased abundance of cyclooxygenase-2 correlates with vascular endothelial growth factor-A abundance and tumor angiogenesis in gastric cancer. *Cancer Lett* 2003; **195**: 43-51 [PMID: 12767510]
- 31 **Lin MT**, Kuo IH, Chang CC, Chu CY, Chen HY, Lin BR, Sureshbabu M, Shih HJ, Kuo ML. Involvement of hypoxia-inducing factor-1 α -dependent plasminogen activator inhibitor-1 up-regulation in Cyr61/CCN1-induced gastric cancer cell invasion. *J Biol Chem* 2008; **283**: 15807-15815 [PMID: 18381294 DOI: 10.1074/jbc.M708933200]
- 32 **Lin MT**, Chang CC, Lin BR, Yang HY, Chu CY, Wu MH, Kuo ML. Elevated expression of Cyr61 enhances peritoneal dissemination of gastric cancer cells through integrin α -2 β 1. *J Biol Chem* 2007; **282**: 34594-34604 [PMID: 17905740 DOI: 10.1074/jbc.M706600200]
- 33 **Bradham DM**, Igarashi A, Potter RL, Grotendorst GR. Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J Cell Biol* 1991; **114**: 1285-1294 [PMID: 1654338]
- 34 **Koliopanos A**, Friess H, di Mola FF, Tang WH, Kubulus D, Brigstock D, Zimmermann A, Büchler MW. Connective tissue growth factor gene expression alters tumor progression in esophageal cancer. *World J Surg* 2002; **26**: 420-427 [PMID: 11910473 DOI: 10.1007/s00268-001-0242-x]
- 35 **Hartel M**, Di Mola FF, Gardini A, Zimmermann A, Di Sebastiano P, Guweidhi A, Innocenti P, Giese T, Giese N, Büchler MW, Friess H. Desmoplastic reaction influences pancreatic cancer growth behavior. *World J Surg* 2004; **28**: 818-825 [PMID: 15457365]
- 36 **Braig S**, Wallner S, Junglas B, Fuchshofer R, Bosserhoff AK. CTGF is overexpressed in malignant melanoma and promotes cell invasion and migration. *Br J Cancer* 2011; **105**: 231-238 [PMID: 21673687 DOI: 10.1038/bjc.2011.226]
- 37 **Chang CC**, Shih JY, Jeng YM, Su JL, Lin BZ, Chen ST, Chau YP, Yang PC, Kuo ML. Connective tissue growth factor and its role in lung adenocarcinoma invasion and metastasis. *J Natl Cancer Inst* 2004; **96**: 364-375 [PMID: 14996858 DOI: 10.1093/jnci/djh059]
- 38 **Lin BR**, Chang CC, Che TF, Chen ST, Chen RJ, Yang CY, Jeng YM, Liang JT, Lee PH, Chang KJ, Chau YP, Kuo ML. Connective tissue growth factor inhibits metastasis and acts as an independent prognostic marker in colorectal cancer. *Gastroenterology* 2005; **128**: 9-23 [PMID: 15633118 DOI: 10.1053/j.gastro.2004.10.007]
- 39 **Liu L**, Li Z, Feng G, You W, Li J. Expression of connective tissue growth factor is in agreement with the expression of VEGF, VEGF-C, -D and associated with shorter survival in gastric cancer. *Pathol Int* 2007; **57**: 712-718 [PMID: 17922682 DOI: 10.1111/j.1440-1827.2007.02162.x]
- 40 **Liu LY**, Han YC, Wu SH, Lv ZH. Expression of connective tissue growth factor in tumor tissues is an independent predictor of poor prognosis in patients with gastric cancer. *World J Gastroenterol* 2008; **14**: 2110-2114 [PMID: 18395916]
- 41 **Jiang CG**, Lv L, Liu FR, Wang ZN, Liu FN, Li YS, Wang CY, Zhang HY, Sun Z, Xu HM. Downregulation of connective tissue growth factor inhibits the growth and invasion of gastric cancer cells and attenuates peritoneal dissemination. *Mol Cancer* 2011; **10**: 122 [PMID: 21955589 DOI: 10.1186/1476-4598-10-122]
- 42 **Walker JL**, Assoian RK. Integrin-dependent signal transduction regulating cyclin D1 expression and G1 phase cell cycle progression. *Cancer Metastasis Rev* 2005; **24**: 383-393 [PMID: 16258726 DOI: 10.1007/s10555-005-5130-7]
- 43 **Inoki I**, Shiomi T, Hashimoto G, Enomoto H, Nakamura H, Makino K, Ikeda E, Takata S, Kobayashi K, Okada Y. Connective tissue growth factor binds vascular endothelial growth factor (VEGF) and inhibits VEGF-induced angiogenesis. *FASEB J* 2002; **16**: 219-221 [PMID: 11744618 DOI: 10.1096/fj.01-0332fje]
- 44 **Higgins DF**, Biju MP, Akai Y, Wutz A, Johnson RS, Haase VH. Hypoxic induction of Ctgf is directly mediated by Hif-1. *Am J Physiol Renal Physiol* 2004; **287**: F1223-F1232 [PMID: 15315937 DOI: 10.1152/ajprenal.00245.2004]
- 45 **Joliet V**, Martinerie C, Dambrine G, Plassiart G, Brisac M, Crochet J, Perbal B. Proviral rearrangements and overexpression of a new cellular gene (nov) in myeloblastosis-associated virus type 1-induced nephroblastomas. *Mol Cell Biol* 1992; **12**: 10-21 [PMID: 1309586]
- 46 **Ouellet V**, Siegel PM. CCN3 modulates bone turnover and is a novel regulator of skeletal metastasis. *J Cell Commun Signal* 2012; **6**: 73-85 [PMID: 22427255 DOI: 10.1007/s12079-012-0161-7]
- 47 **Martinerie C**, Huff V, Joubert I, Badzioch M, Saunders G, Strong L, Perbal B. Structural analysis of the human nov proto-oncogene and expression in Wilms tumor. *Oncogene* 1994; **9**: 2729-2732 [PMID: 7520150]
- 48 **Manara MC**, Perbal B, Benini S, Strammiello R, Cerisano V, Perdichizzi S, Serra M, Astolfi A, Bertoni F, Alami J, Yeager H, Picci P, Scotlandi K. The expression of ccn3(nov) gene in musculoskeletal tumors. *Am J Pathol* 2002; **160**: 849-859 [PMID: 11891184 DOI: 10.1016/S0002-9440(10)64908-5]
- 49 **Martinerie C**, Gicquel C, Louvel A, Laurent M, Schofield PN, Le Bouc Y. Altered expression of novH is associated with human adrenocortical tumorigenesis. *J Clin Endocrinol Metab* 2001; **86**: 3929-3940 [PMID: 11502835 DOI: 10.1210/jc.86.8.3929]
- 50 **Wu L**, Runkle C, Jin HJ, Yu J, Li J, Yang X, Kuzel T, Lee C, Yu J. CCN3/NOV gene expression in human prostate cancer is directly suppressed by the androgen receptor. *Oncogene* 2013 Jan 14; Epub ahead of print [PMID: 23318417 DOI: 10.1038/onc.2012.602]
- 51 **Hashimoto Y**, Shindo-Okada N, Tani M, Takeuchi K, Toma H, Yokota J. Identification of genes differentially expressed in association with metastatic potential of K-1735 murine melanoma by messenger RNA differential display. *Cancer Res* 1996; **56**: 5266-5271 [PMID: 8912867]
- 52 **Xu L**, Corcoran RB, Welsh JW, Pennica D, Levine AJ. WISP-1 is a Wnt-1- and beta-catenin-responsive oncogene. *Genes Dev* 2000; **14**: 585-595 [PMID: 10716946 DOI: 10.1101/gad.14.5.585]
- 53 **Hashimoto Y**, Shindo-Okada N, Tani M, Nagamachi Y, Takeuchi K, Shiroishi T, Toma H, Yokota J. Expression of the Elm1 gene, a novel gene of the CCN (connective tissue growth factor, Cyr61/Cef10, and neuroblastoma overexpressed gene) family, suppresses In vivo tumor growth and metastasis of K-1735 murine melanoma cells. *J Exp Med* 1998; **187**: 289-296 [PMID: 9449709]
- 54 **Pennica D**, Swanson TA, Welsh JW, Roy MA, Lawrence DA, Lee J, Brush J, Taneyhill LA, Deuel B, Lew M, Watanabe C, Cohen RL, Melhem MF, Finley GG, Quirke P, Goddard AD, Hillan KJ, Gurney AL, Botstein D, Levine AJ. WISP genes are members of the connective tissue growth factor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors. *Proc Natl Acad Sci USA* 1998; **95**: 14717-14722 [PMID: 9843955]
- 55 **Tian C**, Zhou ZG, Meng WJ, Sun XF, Yu YY, Li L, Luo HZ, Yang L, Zhou B, Gu J. Overexpression of connective tissue growth factor WISP-1 in Chinese primary rectal cancer

- patients. *World J Gastroenterol* 2007; **13**: 3878-3882 [PMID: 17657846]
- 56 **Davies SR**, Davies ML, Sanders A, Parr C, Torkington J, Jiang WG. Differential expression of the CCN family member WISP-1, WISP-2 and WISP-3 in human colorectal cancer and the prognostic implications. *Int J Oncol* 2010; **36**: 1129-1136 [PMID: 20372786 DOI: 10.3892/ijo_00000595]
 - 57 **Nagai Y**, Watanabe M, Ishikawa S, Karashima R, Kurashige J, Iwagami S, Iwatsuki M, Baba Y, Imamura Y, Hayashi N, Baba H. Clinical significance of Wnt-induced secreted protein-1 (WISP-1/CCN4) in esophageal squamous cell carcinoma. *Anticancer Res* 2011; **31**: 991-997 [PMID: 21498727]
 - 58 **Tang Q**, Jiang X, Li H, Lin Z, Zhou X, Luo X, Liu L, Chen G. Expression and prognostic value of WISP-1 in patients with endometrial endometrioid adenocarcinoma. *J Obstet Gynaecol Res* 2011; **37**: 606-612 [PMID: 21564416 DOI: 10.1111/j.1447-0756.2011.01631.x]
 - 59 **Ono M**, Inkson CA, Sonn R, Kilts TM, de Castro LF, Maeda A, Fisher LW, Robey PG, Berendsen AD, Li L, McCartney-Francis N, Brown AC, Crawford NP, Molinolo A, Jain A, Fedarko NS, Young MF. WISP1/CCN4: a potential target for inhibiting prostate cancer growth and spread to bone. *PLoS One* 2013; **8**: e71709 [PMID: 23977121 DOI: 10.1371/journal.pone.0071709]
 - 60 **Zhang R**, Averboukh L, Zhu W, Zhang H, Jo H, Dempsey PJ, Coffey RJ, Pardee AB, Liang P. Identification of rCop-1, a new member of the CCN protein family, as a negative regulator for cell transformation. *Mol Cell Biol* 1998; **18**: 6131-6141 [PMID: 9742130]
 - 61 **Dhar G**, Mehta S, Banerjee S, Gardner A, McCarty BM, Mathur SC, Campbell DR, Kambhampati S, Banerjee SK. Loss of WISP-2/CCN5 signaling in human pancreatic cancer: a potential mechanism for epithelial-mesenchymal-transition. *Cancer Lett* 2007; **254**: 63-70 [PMID: 17383817]
 - 62 **Banerjee S**, Dhar G, Haque I, Kambhampati S, Mehta S, Sengupta K, Tawfik O, Phillips TA, Banerjee SK. CCN5/WISP-2 expression in breast adenocarcinoma is associated with less frequent progression of the disease and suppresses the invasive phenotypes of tumor cells. *Cancer Res* 2008; **68**: 7606-7612 [PMID: 18794149 DOI: 10.1158/0008-5472.CAN-08-1461]
 - 63 **Kleer CG**, Zhang Y, Pan Q, van Golen KL, Wu ZF, Livant D, Merajver SD. WISP3 is a novel tumor suppressor gene of inflammatory breast cancer. *Oncogene* 2002; **21**: 3172-3180 [PMID: 12082632 DOI: 10.1038/sj.onc.1205462]
 - 64 **Huang W**, Zhang Y, Varambally S, Chinnaiyan AM, Banerjee M, Merajver SD, Kleer CG. Inhibition of CCN6 (Wnt-1-induced signaling protein 3) down-regulates E-cadherin in the breast epithelium through induction of snail and ZEB1. *Am J Pathol* 2008; **172**: 893-904 [PMID: 18321996 DOI: 10.2353/ajpath.2008.070899]
 - 65 **van Golen KL**, Davies S, Wu ZF, Wang Y, Bucana CD, Root H, Chandrasekharappa S, Strawderman M, Ethier SP, Merajver SD. A novel putative low-affinity insulin-like growth factor-binding protein, LIBC (lost in inflammatory breast cancer), and RhoC GTPase correlate with the inflammatory breast cancer phenotype. *Clin Cancer Res* 1999; **5**: 2511-2519 [PMID: 10499627]
 - 66 **Tanaka S**, Sugimachi K, Maehara S, Shimada M, Maehara Y. A loss of function mutation in WISP3 derived from microsatellite unstable gastric carcinoma. *Gastroenterology* 2003; **125**: 1563-1564 [PMID: 14628823 DOI: 10.1053/S0016-5085(03)01467-7]
 - 67 **Bang YJ**, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Rüschoff J, Kang YK. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet* 2010; **376**: 687-697 [PMID: 20728210 DOI: 10.1016/S0140-6736(10)61121-X]
 - 68 **Mageto Y**, Flaherty K, Brown K, Fong A, Raghu G. Safety and tolerability of human monoclonal antibody FG-3019, anti-connective tissue growth factor, in patients with idiopathic pulmonary fibrosis. *Chest* 2004; **126**: 773S [DOI: 10.1378/chest.126.4_MeetingAbstracts.773S-a]
 - 69 **Dornhöfer N**, Spong S, Bennewith K, Salim A, Klaus S, Kambham N, Wong C, Kaper F, Sutphin P, Nacamuli R, Höckel M, Le Q, Longaker M, Yang G, Koong A, Giaccia A. Connective tissue growth factor-specific monoclonal antibody therapy inhibits pancreatic tumor growth and metastasis. *Cancer Res* 2006; **66**: 5816-5827 [PMID: 16740721 DOI: 10.1158/0008-5472.CAN-06-0081]
 - 70 **Aikawa T**, Gunn J, Spong SM, Klaus SJ, Korc M. Connective tissue growth factor-specific antibody attenuates tumor growth, metastasis, and angiogenesis in an orthotopic mouse model of pancreatic cancer. *Mol Cancer Ther* 2006; **5**: 1108-1116 [PMID: 16731742 DOI: 10.1158/1535-7163.MCT-05-0516]

P- Reviewers: Aoyagi K, Ding SZ, Higgins PJ, Lee HC, Stanojevic GZ

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WJG 20th Anniversary Special Issues (8): Gastric cancer

Diabetes and gastric cancer: The potential links

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Received: October 21, 2013 **Revised:** November 18, 2013

Accepted: December 5, 2013

Published online: February 21, 2014

rate and higher reinfection rate of *H. pylori*. High salt intake can act synergistically with *H. pylori* infection in the induction of gastric cancer. Whether a higher risk of gastric cancer in patients with diabetes may be ascribed to a higher intake of salt due to the loss of taste sensation awaits further investigation. The use of medications such as insulin, metformin, sulfonylureas, aspirin, statins and antibiotics may also influence the risk of gastric cancer, but most of them have not been extensively studied. Comorbidities may affect the development of gastric cancer through the use of medications and changes in lifestyle, dietary intake, and the metabolism of drugs. Finally, a potential detection bias related to gastrointestinal symptoms more commonly seen in patients with diabetes and with multiple comorbidities should be pointed out. Taking into account the inconsistent findings and the potential confounders and detection bias in previous epidemiological studies, it is expected that there are still more to be explored for the clarification of the association between diabetes and gastric cancer.

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Key words: Diabetes mellitus; Gastric cancer; Epidemiology; Meta-analysis; Literature review

Abstract

This article reviews the epidemiological evidence linking diabetes and gastric cancer and discusses some of the potential mechanisms, confounders and biases in the evaluation of such an association. Findings from four meta-analyses published from 2011 to 2013 suggest a positive link, which may be more remarkable in females and in the Asian populations. Putative mechanisms may involve shared risk factors, hyperglycemia, *Helicobacter pylori* (*H. pylori*) infection, high salt intake, medications and comorbidities. Diabetes may increase the risk of gastric cancer through shared risk factors including obesity, insulin resistance, hyperinsulinemia and smoking. Hyperglycemia, even before the clinical diagnosis of diabetes, may predict gastric cancer in some epidemiological studies, which is supported by *in vitro*, and *in vivo* studies. Patients with diabetes may also have a higher risk of gastric cancer through the higher infection rate, lower eradication

Core tip: Epidemiological studies suggested a possible higher risk of gastric cancer in patients with diabetes. This article summarizes the findings in four meta-analyses and proposes some mechanisms explaining the association. Findings in the meta-analyses suggested that the association between diabetes and gastric cancer is more remarkable in females and in the Asian populations. Although the mechanisms for such a link remain to be explored, these may involve shared risk factors between diabetes and gastric cancer (such as obesity, insulin resistance, hyperinsulinemia and smoking), hyperglycemia, *Helicobacter pylori* infection, high salt intake, medications and comorbidities.

Tseng CH, Tseng FH. Diabetes and gastric cancer: The potential links. *World J Gastroenterol* 2014; 20(7): 1701-1711 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1701.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1701>

INTRODUCTION

Diabetes mellitus may increase the risk of several cancers involving the breast^[1,2], liver^[3,4], pancreas^[5-7], colorectum^[8-10], endometrium^[11,12], kidney^[13], non-Hodgkin lymphoma^[14,15] and urinary bladder^[16-20]. The underlying mechanisms for a higher risk of cancer in patients with diabetes may be due to insulin resistance, poor glycemic control, oxidative stress and pro-inflammatory status^[21,22]. In addition, the use of anti-diabetic drugs, diabetes duration and the severity of diabetes status accompanied by various comorbidities may play some roles^[22-24].

Gastric cancer is more common in men and in people aged 50 years or older^[25-27]. Obesity, smoking, salt intake and *Helicobacter pylori* (*H. pylori*) infection are important risk factors^[28,29]. Gastric cancer is very common in developing countries in East Asia, East Europe and South America; while the incidence is low in North America and most parts of Africa^[26]. The prognosis of gastric cancer is very poor, with a 5-year survival < 20% for advanced disease^[27]. The incidence of gastric cancer has decreased in most parts of the world in recent years, probably due to the increasing use of refrigerators and less dependence on salt for food preservation, increasing availability of fresh fruits and vegetables, and the control of chronic infection with *H. pylori*. However, it remains as a major cancer affecting human health, and in 2008 it may account for 8% of the total cancer incidence and 10% of the total cancer death worldwide^[26].

Recent observational studies suggested that diabetes or hyperglycemia may increase the risk of gastric cancer incidence or mortality^[30-36]. In this article, we review the current evidence, and discuss the potential mechanisms, confounders and biases in the evaluation of such an association.

EPIDEMIOLOGICAL EVIDENCE FOR A LINK BETWEEN DIABETES AND GASTRIC CANCER

Whether diabetes may increase the risk of gastric cancer has become a focus of attention in recent years. On October 1, 2013, we used the keywords of “diabetes, gastric cancer, meta-analysis” to search the Pubmed, eight papers were available. After further scrutiny, four of them were excluded because they are not related to the topic under review. Finally, there are four meta-analyses^[33-36] published within a 3-year period from 2011 to 2013. The main findings of these four meta-analyses are summarized in Table 1 and briefly described below.

In the first meta-analysis by Ge *et al*^[33], which in-

cluded 21 (4 case-control and 17 cohort) studies evaluating either incidence or mortality of gastric cancer, patients with diabetes did not show an overall higher risk of gastric cancer when sex was not analyzed separately. The summary relative risk (SRR) was 1.09, 95%CI: 0.98-1.22. However, when men and women were analyzed separately, diabetes was associated with a significantly increased risk of gastric cancer in women (SRR = 1.18, 95%CI: 1.01-1.39) but not in men (SRR = 1.04, 95%CI: 0.94-1.15)^[33]. In other subgroup analyses including both sexes, studies with a follow-up duration < 10 years showed a null association, but those with a follow-up duration ≥ 10 years showed a significant SRR (1.14, 95%CI: 1.01-1.29)^[33].

The second meta-analysis by Marimuthu *et al*^[34] included 20 population-based cohort studies evaluating gastric cancer incidence and mortality separately. The overall SRR for gastric cancer incidence was 1.01 (95%CI: 0.90-1.11). The null association was similarly observed in studies conducted in Europe, Asia and United States. It is interesting that the link with gastric cancer incidence was more remarkable, though not significant, in patients with type 1 diabetes (< 30 years of age at diagnosis), with SRR of 1.60 (95%CI: 0.56-2.64) derived from two studies^[34]. When gastric cancer mortality was evaluated, patients with diabetes had a significantly higher risk in overall analysis (SRR = 1.62, 95%CI: 1.36-1.89) and in studies from Asian populations (SRR = 1.98, 95%CI: 1.57-2.39), but not in studies from Europe or the United States^[34].

The third meta-analysis by Tian *et al*^[35] included 25 (7 case-control and 18 cohort) studies involving incidence and mortality of gastric cancer. The overall analysis showed a significant link between diabetes and gastric cancer incidence and mortality with respective SRR of 1.11 (95%CI: 1.00-1.24) ($P = 0.045$) and 1.29 (95%CI: 1.04-1.59)^[35]. Subgroup analyses from various numbers of studies with a mixture of incidence and mortality of gastric cancer showed a positive association in studies conducted in Asian countries, in cohort study design, in patients with type 2 diabetes and in studies adjusted for more confounders, with respective SRR of 1.19 (95%CI: 1.07-1.32), 1.14 (95%CI: 1.01-1.30), 1.16 (95%CI: 1.01-1.33) and 1.16 (95%CI: 1.03-1.30)^[35].

The latest meta-analysis by Yoon *et al*^[36] included 17 (6 case-control and 11 cohort) studies comparing gastric cancer incidence between patients with diabetes and control subjects. This meta-analysis excluded studies investigating only mortality but not incidence or studies reporting only standardized incidence ratios without control groups. The overall SRR was 1.19 (95%CI: 1.08-1.31), and was consistently significant in subgroup analyses conducted in cohort studies, in studies done in populations of either Western or Eastern countries, in females, and in studies with high quality^[36]. The significantly higher risk was also demonstrated in analyses confined to studies controlling well-known risk factors such as smoking or *H. pylori* infection, with respective SRR of 1.17 (95%CI:

Table 1 Main findings in four meta-analyses on the association between diabetes and incidence or mortality of gastric cancer

Ref.	Studies included	Summary RR (95%CI)		Notes and comments for specific studies	Limitations common to the meta-analysis studies
		Overall	Subgroup analysis		
Ge <i>et al</i> ^[33] , 2011	4 case-control and 17 cohort	1.09 (0.98-1.22)	Women: 1.18 (1.01-1.39) Men: 1.04 (0.94-1.15) Duration of follow-up < 10 yr: 0.95 (0.72-1.26) Duration of follow-up ≥ 10 yr: 1.14 (1.01-1.29)	Evaluating incidence and mortality together A mixture of incidence and mortality studies may not be appropriate Ethnicity differences not considered	Heterogeneity in terms of study design, population demographics, diabetes ascertainment, duration of follow-up, and confounders Type 1 and type 2 diabetes not distinguished in most studies
Marimuthu <i>et al</i> ^[34] , 2011	20 population-based cohort	Incidence: 1.01 (0.90-1.11) Mortality: 1.62 (1.36-1.89)	Type 1 diabetes (incidence): 1.60 (0.56-2.64) Asians (mortality): 1.98 (1.57-2.39)	Evaluating incidence and mortality separately in overall analysis Considering type 1 diabetes and ethnicity differences in subgroup analyses	Cardia and non-cardia gastric cancer not discerned in most studies Confounding effects of <i>H. pylori</i> , smoking and diet are not considered in most studies
Tian <i>et al</i> ^[35] , 2012	7 case-control and 18 cohort	Incidence: 1.11 (1.00-1.24) Mortality: 1.29 (1.04-1.59)	Asians: 1.19 (1.07-1.32) Cohort design: 1.14 (1.01-1.30) Type 2 diabetes: 1.16 (1.01-1.33) Studies adjusted for more confounders: 1.16 (1.03-1.30)	Evaluating incidence and mortality separately in overall analysis Subgroup analysis was conducted with a mixture of incidence and mortality	Numbers of studies in subgroup analyses varied and may be too small for some analyses Most studies included in meta-analyses were conducted in developed western countries and not primarily designed for evaluating the association between diabetes and gastric cancer Publication bias is possible
Yoon <i>et al</i> ^[36] , 2013	6 case-control and 11 cohort	1.19 (1.08-1.31)	Cohort design: 1.20 (1.08-1.34) Case-control design: 1.12 (0.87-1.45) East Asian countries: 1.19 (1.02-1.38) Western countries: 1.18 (1.03-1.36) Men: 1.10 (0.97-1.24) Women: 1.24 (1.01-1.52) Studies adjusted for smoking: 1.17 (1.01-1.34) Studies adjusted for infection of <i>H. pylori</i> : 2.35 (1.24-4.46) Cardia cancer: 1.39 (0.72-2.69) Noncardia cancer: 1.19 (0.80-1.77)	Evaluating only incidence Strengths include considering subgroup analyses in studies with adjustment for smoking and <i>H. pylori</i> infection Subgroup analyses on cardia and noncardia cancer are available, but only 2 studies are included	

H. pylori: *Helicobacter pylori*.

1.01-1.34) and 2.35 (95%CI: 1.24-4.46). Another strength is the subgroup analysis for cardia and noncardia gastric cancer, with respective SRR of 1.39 (95%CI: 0.72-2.69) and 1.19 (95%CI: 0.80-1.77). But only two studies are available for these site-specific analyses.

SOME COMMENTS ON THE OBSERVATIONAL STUDIES

Because the findings are inconsistent from observational studies^[30-36], a consensus report does not support diabetes as a risk factor for gastric cancer^[22]. However, some common limitations in the above meta-analysis studies should be pointed out (Table 1).

First, heterogeneity exists in study design, diabetes diagnosis, cancer ascertainment, use of incidence, prevalence or mortality, consideration of confound-

ers (*e.g.*, age, sex, obesity, smoking, salt intake and *H. pylori* infection), follow-up duration, and population demography.

Second, most studies did not differentiate type 1 and type 2 diabetes, and did not discern between different histopathology (adenocarcinoma, lymphoma or other types) or anatomical sites (cardia or noncardia) of gastric cancer. It is worth to point out that patients with diabetes may increase the risk of adenocarcinoma located specifically at the gastric cardia by 89% in one United States population-based study^[37]. On the other hand, *H. pylori* infection-related gastric cancer may be primarily located at the noncardiac portion^[38].

Third, because most studies were conducted in the developed western countries where gastric cancer is less common, and these studies were mainly designed to evaluate the risk of all or multiple cancer sites and not specifically of gastric cancer, they might not have suf-

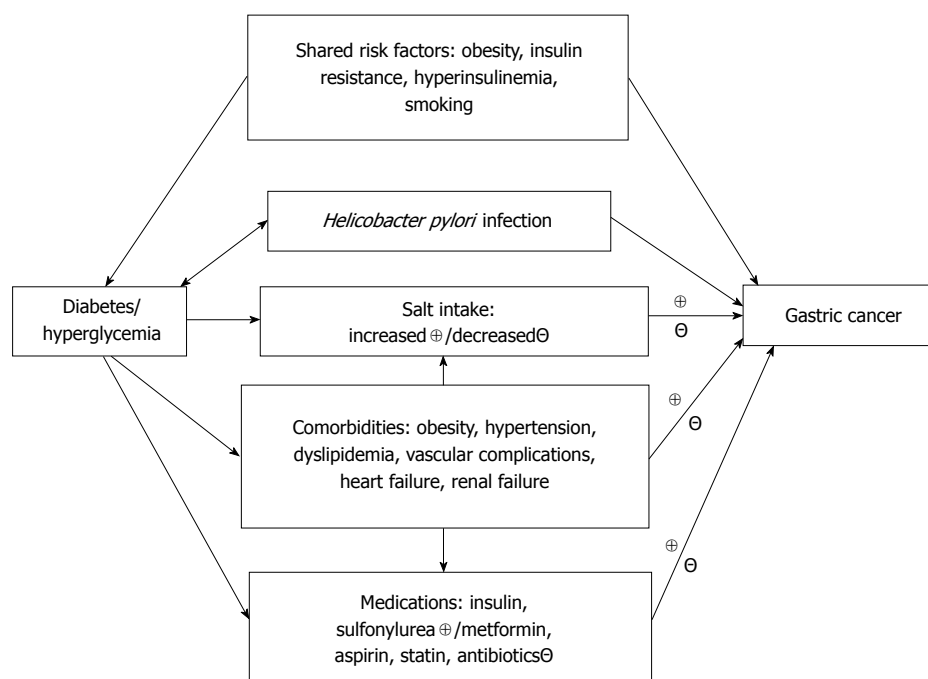


Figure 1 Putative mechanisms linking diabetes and gastric cancer (“ \oplus ” denotes a positive effect and “ \ominus ” denotes a negative effect). A direct effect of hyperglycemia and a synergistic effect between salt intake and *Helicobacter pylori* infection are both possible but not shown in the dendrogram. Comorbidities may affect the development of gastric cancer, either positively or negatively, through the use of medications and changes in lifestyle, salt intake, dietary components, and the metabolism of drugs. A summary of the explanations on the links can be seen in Table 2.

ficient power for investigating the association between diabetes and gastric cancer. If the effect of diabetes on gastric cancer is smaller than its effect on the other types of cancer, then a much larger sample size will be required.

Fourth, dietary factors may also modify the development of gastric cancer induced by carcinogens^[27], but most of these factors have not been considered in previous studies.

As described in the above meta-analyses, there are signals indicating a positive link between diabetes and gastric cancer, especially in females^[33,36] and in Asian populations^[34,35]. Estrogen has been shown to interact with insulin/insulin-like growth factor 1 (IGF-1) in the development of breast cancer^[39], and gastrointestinal tissues may express estrogen receptor^[40,41]. Therefore, estrogen may play a role in the differential effect between men and women on the link between diabetes and gastric cancer. The stronger link found in studies conducted in the Asian populations may either indicate a higher *H. pylori* infection rate in the patients with diabetes in these populations, or it may suggest an effect of different ethnic/genetic backgrounds, dietary habits, lifestyle, or disease prevalence.

PUTATIVE MECHANISMS EXPLAINING THE LINK BETWEEN DIABETES AND GASTRIC CANCER

The putative mechanisms linking diabetes and gastric

cancer are shown in Figure 1. Table 2 summarizes the explanations for such links and discusses some limitations for each possible link. These potential links will be discussed below under the subtitles of: (1) shared risk factors; (2) hyperglycemia; (3) *H. pylori* infection; (4) salt intake; (5) medications; and (6) comorbidities.

SHARED RISK FACTORS

Diabetes and gastric cancer may share common risk factors such as obesity, insulin resistance, hyperinsulinemia and smoking.

Obesity is associated with inflammation, oxidative stress, insulin resistance and hyperinsulinemia. All of these may contribute to a higher risk of gastric cancer^[42]. Because some patients with diabetes may be obese^[43-46], it is possible that this shared risk factor may partly explain the higher risk of gastric cancer in patients with diabetes.

Insulin has both metabolic and mitogenic properties^[47,48]. Hyperinsulinemia, especially in the presence of insulin resistance, may promote cancer cell growth either through the mitogenic pathways triggered by insulin receptor or IGF-1 receptor, or *via* increased bioavailability of free IGF-1 by inhibiting the expression of IGF binding proteins^[47-49]. These effects have also been well demonstrated in gastric cancer cell lines and in *in vivo* studies^[50-54].

Smoking is another common risk factor for diabetes^[55] and gastric cancer^[27]. Therefore, smoking may also confound the association between diabetes and gastric cancer. However, because the higher risk of gastric can-

Table 2 Explanations on the putative mechanisms linking diabetes and gastric cancer and the limitations of current studies

Factors	Explanations/limitations
Shared risk factors	<p>Explanations: Diabetes and gastric cancer may share common risk factors such as obesity, insulin resistance, hyperinsulinemia and smoking</p> <p>Limitations: These shared risk factors are known to cause cancer. Therefore, if these shared risk factors are in play, they may also increase the risk of other types of cancer, like colorectal cancer and lung cancer. As demonstrated in some studies, the link between diabetes and gastric cancer may be independent of smoking. Evidence for such an effect in humans needs to be fortified by further studies</p>
Hyperglycemia	<p>Explanations: Hyperglycemia is associated with pro-inflammatory status, oxidative stress, impaired immune function and increased insulin secretion. All of these may contribute to the development of gastric cancer. Epidemiological studies conducted in Japan support hyperglycemia as a risk factor for gastric cancer, and an interaction between hyperglycemia and <i>H. pylori</i> infection. Such a link may also be supported by findings from <i>in vitro</i> studies</p> <p>Limitations: Confirmation of such a link in other ethnicities is necessary</p>
<i>H. pylori</i> infection	<p>Explanations: Diabetes and <i>H. pylori</i> infection may be mutually causative. Patients with diabetes may have a higher infection rate, a lower eradication rate, and/or a higher reinfection rate of <i>H. pylori</i>. On the other hand, the inflammatory process induced by <i>H. pylori</i> infection may also increase the risk of diabetes</p> <p>Limitations: Findings in epidemiological studies are controversial with regards to the higher infection rate of <i>H. pylori</i> in patients with diabetes. Detection bias can not be excluded because patients with diabetes may suffer from more gastrointestinal symptoms leading to the diagnosis of <i>H. pylori</i> infection and gastric cancer</p>
Salt intake	<p>Explanations: A synergistic effect between <i>H. pylori</i> infection and salt intake on gastric cancer is supported by recent human studies and by <i>in vivo</i> and <i>in vitro</i> studies. Patients with diabetes may consume more salt because of loss of sensitivity to taste</p> <p>Limitations: Patients with diabetes may also be advised to take less salt especially in those with hypertension, kidney disease or congestive heart failure. Epidemiological studies evaluating the link between salt intake and gastric cancer in patients with diabetes are lacking</p>
Medications	<p>Explanations: Insulin and sulfonylureas may increase the risk of cancer. On the other hand, metformin, aspirin and statin may potentially reduce the risk of gastric cancer. Patients who repeatedly use antibiotics may have a lower risk of infection with <i>H. pylori</i></p> <p>Limitations: Research of well quality on the use of medications and gastric cancer risk is lacking</p>
Comorbidities	<p>Explanations: Patients with diabetes may have multiple comorbidities including obesity, hypertension, dyslipidemia, vascular complications and heart failure. All of these may affect the development of gastric cancer, either positively or negatively, through the use of medications and changes in lifestyle, salt intake, dietary components, and the metabolism of drugs</p> <p>Limitations: A detection bias on <i>H. pylori</i> infection or gastric cancer is possible in patients with multiple comorbidities. Studies clarifying such links are still lacking</p>

H. pylori: *Helicobacter pylori*.

cer associated with diabetes remains significant after adjustment for multiple risk factors including smoking^[35,36], the link between diabetes and gastric cancer can also be independent of smoking.

It should also be pointed out that if shared risk factors are in play, their effects may not be site-specific and the risk of other types of cancer like colorectal cancer and lung cancer may also be increased. Furthermore, evidence for such a link through shared risk factors is not sufficient in humans and needs to be fortified by further studies.

HYPERGLYCEMIA

Patients with diabetes are characterized by an increased serum level of glucose. Similar observation of an increased risk of gastric cancer in patients with type 1 diabetes^[34,56,57] and type 2 diabetes^[35,36] may imply a mechanism involving hyperglycemia, which is independent of insulin effect because type 1 diabetes is characterized by insulin deficiency. This is supported by human studies conducted in Japan showing an association between hyperglycemia even before the diagnosis of diabetes with a higher risk of gastric cancer^[31,58]. Furthermore an interaction between hyperglycemia and *H. pylori* infection was reported to markedly increase the risk^[31,58]. However,

such a link with hyperglycemia needs to be confirmed in other ethnicities.

It is worth mentioning that a higher risk of gastric cancer in patients with type 1 diabetes may not completely exclude a mechanism involving insulin resistance or hyperinsulinemia because of the following facts: (1) recent studies strongly support the presence of insulin resistance in patients with type 1 diabetes^[59]; (2) insulin injected subcutaneously bypasses the first-pass clearance by the liver; and (3) therapeutic insulin dose can not always be adjusted exactly to the physiological demands and hyperinsulinemia should be the usual phenomenon if glycemic control is aimed close to the normal range.

Some *in vitro* and *in vivo* studies may support a link between hyperglycemia and gastric cancer. An *in vitro* study indicated that glucose *per se* may affect the development of cancer *via* β -catenin acetylation with increased Wnt signaling^[60], which is also a characteristic of gastric cancer^[61]. Patients with diabetes may have an increased expression of pro-inflammatory cytokines such as interleukin-1, interleukin-6 and tumor necrosis factor- α ^[62]. It is also shown that these factors may upregulate and activate the Wnt/ β -catenin pathway^[63]. An animal study supported that gastric cancer induced by *N*-methyl-*N*-nitrosourea is enhanced in diabetic (*db/db*) mice through the effects of hyperglycemia and/or hyperinsulinemia^[64].

Hyperglycemia may also promote carcinogenesis *via* increasing reactive oxygen species resulting in DNA damage^[65] or increasing the expression of vascular endothelial growth factor, which is correlated with tumor vascularity and metastasis^[66]. Furthermore, hyperglycemia may impair immune function rendering susceptibility to *H. pylori* infection and delaying wound healing in gastric ulcer following *H. pylori* infection. Hyperglycemia may also trigger insulin secretion, leading to hyperinsulinemia, especially in the presence of insulin resistance, which may increase the risk of cancer through insulin signaling. Because cancer cells are less efficient in using glucose for energy expenditure and they may consume more glucose than normal cells (the Warburg effect)^[67], hyperglycemia provides a more suitable condition for tumor cells to grow.

H. PYLORI INFECTION

H. pylori infection is well known as a risk factor for gastric ulcer and cancer^[27,68,69], possibly through DNA damage induced by reactive oxygen species in the infected gastric epithelial cells^[70]. A research conducted in Taiwan suggested that early *H. pylori* eradication decreases the risk of gastric cancer in patients with peptic ulcer disease^[71]. However, the role of diabetes on the relation between *H. pylori* infection and gastric cancer is still under investigation.

The relation between *H. pylori* infection and diabetes can be mutually causative. The increased risk of gastric cancer in patients with diabetes may be explained by either one of the following conditions related to *H. pylori* infection^[72-76]: (1) higher infection rate; (2) lower eradication rate; or (3) higher reinfection rate. Patients with diabetes may be more susceptible to *H. pylori* infection because of impaired immune function associated with hyperglycemia^[77]. However, whether patients with diabetes may really have a higher rate of *H. pylori* infection is controversial in epidemiological studies. Although studies from Qatar^[78] and Egypt^[79] suggested an increased infection rate in the patients with diabetes, this could not be similarly observed in studies conducted in Turkey^[80] and Japan^[81]. Because diabetes or poor glycemic control may be associated with an increased prevalence of gastrointestinal symptoms^[82,83], it is not known whether the higher rate of *H. pylori* infection in some of the studies may be due to detection bias related to the symptoms^[84]. Furthermore, it should be pointed out that an evaluation of the prevalence rate of *H. pylori* infection may not necessarily indicate an increased risk in terms of incidence. Patients with diabetes may have a lower eradication rate^[72,76,85-88] and a higher reinfection rate after *H. pylori* infection^[73,75,76]. Therefore, even in the condition that the incidence of *H. pylori* infection may not be increased in patients with diabetes, the prevalence rate may be significantly higher.

On the other hand, *H. pylori* infection can lead to diabetes because the active chronic inflammation may af-

fect the normal secretion and function of insulin leading to glucose dysregulation^[89-91]. For example, in a human study measuring the HOMA-IR (homeostasis model assessment of insulin resistance) levels in patients with and without *H. pylori* infection, insulin resistance is well demonstrated in those having *H. pylori* infection^[89]. *H. pylori* infection may also affect the secretion of gastrointestinal hormones, such that basal and stimulated levels of serum gastrin are elevated but somatostatin level is decreased^[92,93]. Gastrin increases food- or glucose-stimulated insulin secretion; but somatostatin inhibits the release of insulin. As a result, hyperinsulinemia may be seen following *H. pylori* infection. Whether *H. pylori* infection may directly affect insulin secretion from pancreas is not known. If the inflammatory process and oxidative stress induced by *H. pylori* infection^[91] could also be demonstrated in the pancreas, it is expected that insulin secretion may be impaired. Insulin resistance, as induced by *H. pylori* infection, may also accelerate β -cell loss and leads finally to the clinical onset of diabetes^[94]. Therefore, insulin deficiency as well as insulin resistance might be seen in chronic *H. pylori* infection.

SALT INTAKE

High salt intake has long been recognized as an important risk factor for gastric cancer^[95-101], which can be independent of *H. pylori* infection^[101]. However, some recent human studies showed a synergistic effect between salt intake and *H. pylori* infection^[96,100]. Evidence from an *in vivo* study using Mongolian gerbils confirmed that high salt intake may exacerbate the risk of gastric cancer induced by *H. pylori* infection^[102], which could probably be due to the upregulation of CagA synthesis in the bacteria in response to increased concentration of salt. The CagA protein is a bacterial oncoprotein related to the *H. pylori*-induced gastric cancer^[102].

Whether high salt intake could be responsible for the increased risk of gastric cancer in patients with diabetes remains to be answered. It has been speculated that people with easy access to sugary, salty and fatty foods, which are calorie-rich but micronutrient-poor, may cause diseases such as obesity and diabetes^[103]. On the other hand, patients with diabetes may consume more salt than people without diabetes because of the loss of sensitivity to taste, especially in those with a late stage of the disease complicated with neuropathy^[104,105]. However, it is also possible that patients with diabetes may be advised to consume less salt than people without diabetes by their physicians, especially when the patients also suffer from hypertension, renal disease or congestive heart failure.

MEDICATIONS

Exogenous insulin use has also been shown to increase the risk of several cancer types^[106,107]. Whether this could also be applied to gastric cancer has not been extensively

studied. In studies conducted in Taiwan, patients with diabetes who used insulin had a significantly higher risk of *H. pylori* eradication, but none of the other anti-diabetic drugs including sulfonylurea, metformin, acarbose, pioglitazone or rosiglitazone was associated with *H. pylori* eradication^[84]. However, insulin use was not associated with an increased risk of gastric cancer^[108]. It has been explained that the use of insulin might indicate poor glycemic control with more severe disease conditions in the *H. pylori* eradication study^[84], suggesting a deteriorating metabolic control following *H. pylori* infection.

Insulin glargine, a long-acting insulin analog, may increase the risk of certain cancers involving colon, pancreas and breast^[107,109,110]. This has always been ascribed to the very high affinity of insulin glargine to the IGF-1 receptor in *in vitro* studies^[111]. However, this may not be the case when insulin glargine is injected subcutaneously because it is converted at the injection site to less mitogenic metabolites^[112]. It remains unknown whether clinical use of exogenous human insulin or insulin analogs may affect the risk of gastric cancer.

Metformin may protect against a number of cancers^[10,107,113], but sulfonylureas may be associated with an increased risk^[106,114]. Whether these medications may affect the risk of gastric cancer in humans has rarely been studied. An inhibitory effect of metformin on gastric cancer cell proliferation can be demonstrated in *in vitro* and *in vivo* studies^[115]. Similarly, an early *in vitro* study suggested that glibenclamide (a sulfonylurea) may exert antitumor activity in a human gastric cancer cell line^[116]. However, a preliminary human study conducted in Taiwan showed a slightly higher but not significant risk ratio while comparing users of sulfonylureas only to users of metformin only in patients with type 2 diabetes (age-sex-adjusted OR = 1.855, 95%CI: 0.779-4.419)^[106]. Thiazolidinediones may also demonstrate some antitumor effects on gastric cancer cells in *in vitro* and *in vivo* studies^[117,118]. However, whether this can be translated into a preventive effect on gastric cancer growth in humans remains unknown.

From meta-analyses, use of statins is associated with a significantly 32% lower risk of gastric cancer^[119], and aspirin may significantly reduce the risk with a SRR of 0.71 (95%CI: 0.60-0.82)^[120]. Although without evidence, patients who repeatedly use antibiotics may happen to have a reduced risk of *H. pylori* infection. The confounding effects of these commonly used medications have rarely been controlled in previous studies investigating the association between diabetes and gastric cancer. Some studies suggested a sex difference in the use of insulin (more common in women)^[121] and statins (more common in men)^[122] in patients with type 2 diabetes. Whether this may contribute to a sex difference in the association between diabetes and gastric cancer awaits further investigation.

COMORBIDITIES

Obesity, hypertension and dyslipidemia are common

comorbidities observed in patients with diabetes^[43-46,123]. All of these may be associated with insulin resistance. Patients with ischemic heart disease, other vascular complications, congestive heart failure or chronic kidney disease/end-stage renal disease may have changed their lifestyle, daily activity, salt intake and dietary components or may have taken some other medications, supplements or alternative treatment. Hepatic or renal insufficiency may also affect the metabolism of medications. The confounding effects of comorbidities in the association between diabetes and gastric cancer have rarely been addressed in previous studies.

A detection bias related to multiple comorbidities is also possible. Patients with more comorbidities may have a higher probability of receiving laboratory examinations leading to the diagnosis of gastric cancer. This detection bias should be seriously taken into account in future studies.

CONCLUSION

Epidemiological evidence signals a higher risk of gastric cancer in patients with diabetes, which is more remarkable in females and in the Asian populations. Potential mechanisms may include shared risk factors, hyperglycemia, *H. pylori* infection, high salt intake, medications and comorbidities. It should be recognized that epidemiological findings are inconsistent, the estimated relative risk is moderate, and most studies have inherent limitations related to study design, sample size, confounders and biases. Therefore, more well-designed epidemiological studies are required to confirm the association between diabetes and gastric cancer in humans, and in-depth mechanistic studies are necessary to explain the possible links.

ACKNOWLEDGMENTS

The authors thank the following institutes in Taiwan for their continuous support on epidemiological studies of diabetes and arsenic-related health hazards: the Department of Health (DOH97-TD-D-113-97009); and the National Science Council (NSC 101-2314-B-002-117, NSC-102-2314-B-002-067).

REFERENCES

- 1 Larsson SC, Mantzoros CS, Wolk A. Diabetes mellitus and risk of breast cancer: a meta-analysis. *Int J Cancer* 2007; **121**: 856-862 [PMID: 17397032 DOI: 10.1002/ijc.22717]
- 2 Tseng CH, Chong CK, Tai TY. Secular trend for mortality from breast cancer and the association between diabetes and breast cancer in Taiwan between 1995 and 2006. *Diabetologia* 2009; **52**: 240-246 [PMID: 19018510 DOI: 10.1007/s00125-008-1204-8]
- 3 El-Serag HB, Hampel H, Javadi F. The association between diabetes and hepatocellular carcinoma: a systematic review of epidemiologic evidence. *Clin Gastroenterol Hepatol* 2006; **4**: 369-380 [PMID: 16527702 DOI: 10.1016/j.cgh.2005.12.007]
- 4 Tseng CH. Type 2 diabetes, smoking, insulin use and mortality from hepatocellular carcinoma: a 12-year follow-up of

- a national cohort in Taiwan. *Hepatol Int* 2013; **7**: 693-702 [DOI: 10.1007/s12072-012-9405-0]
- 5 Ben Q, Cai Q, Li Z, Yuan Y, Ning X, Deng S, Wang K. The relationship between new-onset diabetes mellitus and pancreatic cancer risk: a case-control study. *Eur J Cancer* 2011; **47**: 248-254 [PMID: 20709528 DOI: 10.1016/j.ejca.2010.07.010]
 - 6 Tseng CH. New-onset diabetes with a history of dyslipidemia predicts pancreatic cancer. *Pancreas* 2013; **42**: 42-48 [PMID: 22750971 DOI: 10.1097/MPA.0b013e3182571ba9]
 - 7 Tseng CH. Diabetes, insulin use, smoking, and pancreatic cancer mortality in Taiwan. *Acta Diabetol* 2013; **50**: 879-886 [PMID: 23508375 DOI: 10.1007/s00592-013-0471-0]
 - 8 Larsson SC, Orsini N, Wolk A. Diabetes mellitus and risk of colorectal cancer: a meta-analysis. *J Natl Cancer Inst* 2005; **97**: 1679-1687 [PMID: 16288121 DOI: 10.1093/jnci/dji375]
 - 9 Tseng CH. Diabetes but not insulin is associated with higher colon cancer mortality. *World J Gastroenterol* 2012; **18**: 4182-4190 [PMID: 22919252 DOI: 10.3748/wjg.v18.i31.4182]
 - 10 Tseng CH. Diabetes, metformin use, and colon cancer: a population-based cohort study in Taiwan. *Eur J Endocrinol* 2012; **167**: 409-416 [PMID: 22778198 DOI: 10.1530/EJE-12-0369]
 - 11 Friberg E, Orsini N, Mantzoros CS, Wolk A. Diabetes mellitus and risk of endometrial cancer: a meta-analysis. *Diabetologia* 2007; **50**: 1365-1374 [PMID: 17476474 DOI: 10.1007/s00125-007-0681-5]
 - 12 Saltzman BS, Doherty JA, Hill DA, Beresford SA, Voigt LF, Chen C, Weiss NS. Diabetes and endometrial cancer: an evaluation of the modifying effects of other known risk factors. *Am J Epidemiol* 2008; **167**: 607-614 [PMID: 18071194 DOI: 10.1093/aje/kwm333]
 - 13 Larsson SC, Wolk A. Diabetes mellitus and incidence of kidney cancer: a meta-analysis of cohort studies. *Diabetologia* 2011; **54**: 1013-1018 [PMID: 21274512 DOI: 10.1007/s00125-011-2051-6]
 - 14 Tseng CH. Diabetes and non-Hodgkin's lymphoma: analyses of prevalence and annual incidence in 2005 using the National Health Insurance database in Taiwan. *Ann Oncol* 2012; **23**: 153-158 [PMID: 21765043 DOI: 10.1093/annonc/mdr334]
 - 15 Tseng CH. Diabetes, insulin use, and non-Hodgkin lymphoma mortality in Taiwan. *Metabolism* 2012; **61**: 1003-1009 [PMID: 22237115 DOI: 10.1016/j.metabol.2011.11.015]
 - 16 Tseng CH. Insulin use and smoking jointly increase the risk of bladder cancer mortality in patients with type 2 diabetes. *Clin Genitourin Cancer* 2013; **11**: 508-514 [PMID: 23791436 DOI: 10.1016/j.clgc.2013.04.019]
 - 17 Tseng CH, Chong CK, Tseng CP, Chan TT. Age-related risk of mortality from bladder cancer in diabetic patients: a 12-year follow-up of a national cohort in Taiwan. *Ann Med* 2009; **41**: 371-379 [PMID: 19191082 DOI: 10.1080/07853890902729778]
 - 18 Tseng CH. Diabetes and risk of bladder cancer: a study using the National Health Insurance database in Taiwan. *Diabetologia* 2011; **54**: 2009-2015 [PMID: 21544514 DOI: 10.1007/s00125-011-2171-z]
 - 19 Tseng CH. Pioglitazone and bladder cancer: a population-based study of Taiwanese. *Diabetes Care* 2012; **35**: 278-280 [PMID: 22210574 DOI: 10.2337/dc11-1449]
 - 20 Tseng CH. Benign prostatic hyperplasia is a significant risk factor for bladder cancer in diabetic patients: a population-based cohort study using the National Health Insurance in Taiwan. *BMC Cancer* 2013; **13**: 7 [PMID: 23286275 DOI: 10.1186/1471-2407-13-7]
 - 21 Arcidiacono B, Iiritano S, Nocera A, Possidente K, Nevolo MT, Ventura V, Foti D, Chiefari E, Brunetti A. Insulin resistance and cancer risk: an overview of the pathogenetic mechanisms. *Exp Diabetes Res* 2012; **2012**: 789174 [PMID: 22701472 DOI: 10.1155/2012/789174]
 - 22 Giovannucci E, Harlan DM, Archer MC, Bergenstal RM, Gapstur SM, Habel LA, Pollak M, Regensteiner JG, Yee D. Diabetes and cancer: a consensus report. *Diabetes Care* 2010; **33**: 1674-1685 [PMID: 20587728 DOI: 10.2337/dc10-0666]
 - 23 Tseng CH, Tseng FH. Peroxisome proliferator-activated receptor agonists and bladder cancer: lessons from animal studies. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 2012; **30**: 368-402 [PMID: 23167631 DOI: 10.1080/10590501.2012.735519]
 - 24 Tseng CH. Pioglitazone and bladder cancer in human studies: is it diabetes itself, diabetes drugs, flawed analyses or different ethnicities? *J Formos Med Assoc* 2012; **111**: 123-131 [PMID: 22423665 DOI: 10.1016/j.jfma.2011.10.003]
 - 25 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]
 - 26 Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855 DOI: 10.3322/caac.20107]
 - 27 Nagini S. Carcinoma of the stomach: A review of epidemiology, pathogenesis, molecular genetics and chemoprevention. *World J Gastrointest Oncol* 2012; **4**: 156-169 [PMID: 22844547 DOI: 10.4251/wjgo.v4.i7.156]
 - 28 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]
 - 29 Correa P, Piazuelo MB. Helicobacter pylori Infection and Gastric Adenocarcinoma. *US Gastroenterol Hepatol Rev* 2011; **7**: 59-64 [PMID: 21857882]
 - 30 Tseng CH. Diabetes conveys a higher risk of gastric cancer mortality despite an age-standardised decreasing trend in the general population in Taiwan. *Gut* 2011; **60**: 774-779 [PMID: 21193459 DOI: 10.1136/gut.2010.226522]
 - 31 Ikeda F, Doi Y, Yonemoto K, Ninomiya T, Kubo M, Shikata K, Hata J, Tanizaki Y, Matsumoto T, Iida M, Kiyohara Y. Hyperglycemia increases risk of gastric cancer posed by Helicobacter pylori infection: a population-based cohort study. *Gastroenterology* 2009; **136**: 1234-1241 [PMID: 19236964 DOI: 10.1053/j.gastro.2008.12.045]
 - 32 Gong Y, Yang YS, Zhang XM, Su M, Wang J, Han JD, Guo MZ. ABO blood type, diabetes and risk of gastrointestinal cancer in northern China. *World J Gastroenterol* 2012; **18**: 563-569 [PMID: 22363124 DOI: 10.3748/wjg.v18.i6.563]
 - 33 Ge Z, Ben Q, Qian J, Wang Y, Li Y. Diabetes mellitus and risk of gastric cancer: a systematic review and meta-analysis of observational studies. *Eur J Gastroenterol Hepatol* 2011; **23**: 1127-1135 [PMID: 21934509 DOI: 10.1097/MEG.0b013e32834b8d73]
 - 34 Marimuthu SP, Vijayaragavan P, Moysich KB, Jayaprakash V. Diabetes mellitus and gastric carcinoma: Is there an association? *J Carcinog* 2011; **10**: 30 [PMID: 22190872 DOI: 10.4103/1477-3163.90481]
 - 35 Tian T, Zhang LQ, Ma XH, Zhou JN, Shen J. Diabetes mellitus and incidence and mortality of gastric cancer: a meta-analysis. *Exp Clin Endocrinol Diabetes* 2012; **120**: 217-223 [PMID: 22187293 DOI: 10.1055/s-0031-1297969]
 - 36 Yoon JM, Son KY, Eom CS, Durrance D, Park SM. Pre-existing diabetes mellitus increases the risk of gastric cancer: a meta-analysis. *World J Gastroenterol* 2013; **19**: 936-945 [PMID: 23429469]
 - 37 Lin SW, Freedman ND, Hollenbeck AR, Schatzkin A, Abnet CC. Prospective study of self-reported diabetes and risk of upper gastrointestinal cancers. *Cancer Epidemiol Biomarkers Prev* 2011; **20**: 954-961 [PMID: 21415356 DOI: 10.1158/1055-9965.EPI-10-1244]
 - 38 Cavaleiro-Pinto M, Peleteiro B, Lunet N, Barros H. Helicobacter pylori infection and gastric cardia cancer: systematic review and meta-analysis. *Cancer Causes Control* 2011; **22**: 375-387 [PMID: 21184266 DOI: 10.1007/s10552-010-9707-2]
 - 39 Lanzino M, Morelli C, Garofalo C, Panno ML, Mauro L, Andò S, Sisci D. Interaction between estrogen receptor alpha and insulin/IGF signaling in breast cancer. *Curr Cancer Drug Targets* 2008; **8**: 597-610 [PMID: 18991569 DOI: 10.2174/15680

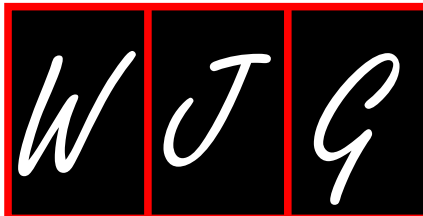
- 0908786241104]
- 40 **Matsuyama S**, Ohkura Y, Eguchi H, Kobayashi Y, Akagi K, Uchida K, Nakachi K, Gustafsson JA, Hayashi S. Estrogen receptor beta is expressed in human stomach adenocarcinoma. *J Cancer Res Clin Oncol* 2002; **128**: 319-324 [PMID: 12073050 DOI: 10.1007/s00432-002-0336-3]
 - 41 **Enmark E**, Peltto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjöld M, Gustafsson JA. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J Clin Endocrinol Metab* 1997; **82**: 4258-4265 [PMID: 9398750 DOI: 10.1210/jc.82.12.4258]
 - 42 **Yang P**, Zhou Y, Chen B, Wan HW, Jia GQ, Bai HL, Wu XT. Overweight, obesity and gastric cancer risk: results from a meta-analysis of cohort studies. *Eur J Cancer* 2009; **45**: 2867-2873 [PMID: 19427197 DOI: 10.1016/j.ejca.2009.04.019]
 - 43 **Tseng CH**. Body composition as a risk factor for coronary artery disease in Chinese type 2 diabetic patients in Taiwan. *Circ J* 2003; **67**: 479-484 [PMID: 12808262 DOI: 10.1253/circj.67.479]
 - 44 **Tseng CH**. Body mass index and blood pressure in adult type 2 diabetic patients in Taiwan. *Circ J* 2007; **71**: 1749-1754 [PMID: 17965496 DOI: 10.1253/circj.71.1749]
 - 45 **Tseng CH**, Chong CK, Tseng CP, Shau WY, Tai TY. Hypertension is the most important component of metabolic syndrome in the association with ischemic heart disease in Taiwanese type 2 diabetic patients. *Circ J* 2008; **72**: 1419-1424 [PMID: 18724015 DOI: 10.1253/circj.CJ-08-0009]
 - 46 **Tseng CH**. Obesity paradox: differential effects on cancer and noncancer mortality in patients with type 2 diabetes mellitus. *Atherosclerosis* 2013; **226**: 186-192 [PMID: 23040832 DOI: 10.1016/j.atherosclerosis.2012.09.004]
 - 47 **Pollak M**. Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer* 2008; **8**: 915-928 [PMID: 19029956 DOI: 10.1038/nrc2536]
 - 48 **Pollak M**. The insulin and insulin-like growth factor receptor family in neoplasia: an update. *Nat Rev Cancer* 2012; **12**: 159-169 [PMID: 22337149 DOI: 10.1038/nrc3215]
 - 49 **Giovannucci E**. Insulin, insulin-like growth factors and colon cancer: a review of the evidence. *J Nutr* 2001; **131**: 3109S-3120S [PMID: 11694656]
 - 50 **Yi HK**, Hwang PH, Yang DH, Kang CW, Lee DY. Expression of the insulin-like growth factors (IGFs) and the IGF-binding proteins (IGFBPs) in human gastric cancer cells. *Eur J Cancer* 2001; **37**: 2257-2263 [PMID: 11677116 DOI: 10.1016/S0959-8049(01)00269-6]
 - 51 **Lee DY**, Yi HK, Hwang PH, Oh Y. Enhanced expression of insulin-like growth factor binding protein-3 sensitizes the growth inhibitory effect of anticancer drugs in gastric cancer cells. *Biochem Biophys Res Commun* 2002; **294**: 480-486 [PMID: 12051736 DOI: 10.1016/S0006-291X(02)00491-6]
 - 52 **Adachi Y**, Li R, Yamamoto H, Min Y, Piao W, Wang Y, Imsumran A, Li H, Arimura Y, Lee CT, Imai K, Carbone DP, Shinomura Y. Insulin-like growth factor-I receptor blockade reduces the invasiveness of gastrointestinal cancers via blocking production of matrilysin. *Carcinogenesis* 2009; **30**: 1305-1313 [PMID: 19493905 DOI: 10.1093/carcin/bgp134]
 - 53 **Pavelić K**, Kolak T, Kapitanović S, Radošević S, Spaventi S, Kruslin B, Pavelić J. Gastric cancer: the role of insulin-like growth factor 2 (IGF 2) and its receptors (IGF 1R and M6-P/IGF 2R). *J Pathol* 2003; **201**: 430-438 [PMID: 14595755 DOI: 10.1002/path.1465]
 - 54 **Thompson MA**, Cox AJ, Whitehead RH, Jonas HA. Autocrine regulation of human tumor cell proliferation by insulin-like growth factor II: an in-vitro model. *Endocrinology* 1990; **126**: 3033-3042 [PMID: 1693565 DOI: 10.1210/endo-126-6-3033]
 - 55 **Bi Y**, Wang T, Xu M, Xu Y, Li M, Lu J, Zhu X, Ning G. Advanced research on risk factors of type 2 diabetes. *Diabetes Metab Res Rev* 2012; **28** Suppl 2: 32-39 [PMID: 23280864 DOI: 10.1002/dmrr.2352]
 - 56 **Zendehdel K**, Nyrén O, Ostenson CG, Adami HO, Ekblom A, Ye W. Cancer incidence in patients with type 1 diabetes mellitus: a population-based cohort study in Sweden. *J Natl Cancer Inst* 2003; **95**: 1797-1800 [PMID: 14652242 DOI: 10.1093/jnci/djg105]
 - 57 **Swerdlow AJ**, Laing SP, Qiao Z, Slater SD, Burden AC, Botha JL, Waugh NR, Morris AD, Gatling W, Gale EA, Patterson CC, Keen H. Cancer incidence and mortality in patients with insulin-treated diabetes: a UK cohort study. *Br J Cancer* 2005; **92**: 2070-2075 [PMID: 15886700 DOI: 10.1038/sj.bjc.6602611]
 - 58 **Yamagata H**, Kiyohara Y, Nakamura S, Kubo M, Tanizaki Y, Matsumoto T, Tanaka K, Kato I, Shirota T, Iida M. Impact of fasting plasma glucose levels on gastric cancer incidence in a general Japanese population: the Hisayama study. *Diabetes Care* 2005; **28**: 789-794 [PMID: 15793174 DOI: 10.2337/diacare.28.4.789]
 - 59 **Fourlanos S**, Narendran P, Byrnes GB, Colman PG, Harrison LC. Insulin resistance is a risk factor for progression to type 1 diabetes. *Diabetologia* 2004; **47**: 1661-1667 [PMID: 15480539 DOI: 10.1007/s00125-004-1507-3]
 - 60 **Chocarro-Calvo A**, García-Martínez JM, Ardila-González S, De la Vieja A, García-Jiménez C. Glucose-induced β -catenin acetylation enhances Wnt signaling in cancer. *Mol Cell* 2013; **49**: 474-486 [PMID: 23273980 DOI: 10.1016/j.molcel.2012.11.022]
 - 61 **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]
 - 62 **Wieser V**, Moschen AR, Tilg H. Inflammation, cytokines and insulin resistance: a clinical perspective. *Arch Immunol Ther Exp (Warsz)* 2013; **61**: 119-125 [PMID: 23307037 DOI: 10.1007/s00005-012-0210-1]
 - 63 **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]
 - 64 **Yoshizawa N**, Yamaguchi H, Yamamoto M, Shimizu N, Furihata C, Tatematsu M, Seto Y, Kaminishi M. Gastric carcinogenesis by N-Methyl-N-nitrosourea is enhanced in db/db diabetic mice. *Cancer Sci* 2009; **100**: 1180-1185 [PMID: 19432903 DOI: 10.1111/j.1349-7006.2009.01157.x]
 - 65 **Lorenzi M**, Montisano DF, Toledo S, Barrioux A. High glucose induces DNA damage in cultured human endothelial cells. *J Clin Invest* 1986; **77**: 322-325 [PMID: 3944257 DOI: 10.1172/JCI112295]
 - 66 **Mahdy RA**, Nada WM. Evaluation of the role of vascular endothelial growth factor in diabetic retinopathy. *Ophthalmic Res* 2011; **45**: 87-91 [PMID: 20720438 DOI: 10.1159/000317062]
 - 67 **Warburg O**. On the origin of cancer cells. *Science* 1956; **123**: 309-314 [PMID: 13298683 DOI: 10.1126/science.123.3191.309]
 - 68 **Shimizu H**, Monden T, Matsumura M, Domeki N, Kasai K. Effects of twice-daily injections of premixed insulin analog on glycemic control in type 2 diabetic patients. *Yonsei Med J* 2010; **51**: 845-849 [PMID: 20879049 DOI: 10.3349/ymj.2010.51.6.845]
 - 69 **Yamagata H**, Kiyohara Y, Aoyagi K, Kato I, Iwamoto H, Nakayama K, Shimizu H, Tanizaki Y, Arima H, Shinohara N, Kondo H, Matsumoto T, Fujishima M. Impact of Helicobacter pylori infection on gastric cancer incidence in a general Japanese population: the Hisayama study. *Arch Intern Med* 2000; **160**: 1962-1968 [PMID: 10888970 DOI: 10.1001/archinte.160.13.1962]
 - 70 **Obst B**, Wagner S, Sewing KF, Beil W. Helicobacter pylori causes DNA damage in gastric epithelial cells. *Carcinogenesis* 2000; **21**: 1111-1115 [PMID: 10836997 DOI: 10.1093/carcin/21.6.1111]
 - 71 **Wu CY**, Kuo KN, Wu MS, Chen YJ, Wang CB, Lin JT. Early

- Helicobacter pylori eradication decreases risk of gastric cancer in patients with peptic ulcer disease. *Gastroenterology* 2009; **137**: 1641-8.e1-2 [PMID: 19664631 DOI: 10.1053/j.gastro.2009.07.060]
- 72 **Gasbarrini A**, Ojetti V, Pitocco D, Franceschi F, Candelli M, Torre ES, Gabrielli M, Cammarota G, Armuzzi A, Pola R, Pola P, Ghirlanda G, Gasbarrini G. Insulin-dependent diabetes mellitus affects eradication rate of Helicobacter pylori infection. *Eur J Gastroenterol Hepatol* 1999; **11**: 713-716 [PMID: 10445788 DOI: 10.1097/00042737-199907000-00005]
 - 73 **Ojetti V**, Pitocco D, Bartolozzi F, Danese S, Migneco A, Lupascu A, Pola P, Ghirlanda G, Gasbarrini G, Gasbarrini A. High rate of helicobacter pylori re-infection in patients affected by type 1 diabetes. *Diabetes Care* 2002; **25**: 1485 [PMID: 12145262 DOI: 10.2337/diacare.25.8.1485]
 - 74 **Ojetti V**, Migneco A, Silveri NG, Ghirlanda G, Gasbarrini G, Gasbarrini A. The role of H. pylori infection in diabetes. *Curr Diabetes Rev* 2005; **1**: 343-347 [PMID: 18220610 DOI: 10.2174/157339905774574275]
 - 75 **Ojetti V**, Migneco A, Nista EC, Gasbarrini G, Gasbarrini A, Pitocco D, Ghirlanda G. H pylori re-infection in type 1 diabetes: a 5 years follow-up. *Dig Liver Dis* 2007; **39**: 286-287 [PMID: 17275424 DOI: 10.1016/j.dld.2006.11.008]
 - 76 **Ojetti V**, Pellicano R, Fagoonee S, Migneco A, Berrutti M, Gasbarrini A. Helicobacter pylori infection and diabetes. *Minerva Med* 2010; **101**: 115-119 [PMID: 20467410]
 - 77 **Daoud AK**, Tayyar MA, Fouda IM, Harfeil NA. Effects of diabetes mellitus vs. in vitro hyperglycemia on select immune cell functions. *J Immunotoxicol* 2009; **6**: 36-41 [PMID: 19519161 DOI: 10.1080/15476910802604564]
 - 78 **Bener A**, Micallef R, Afifi M, Derbala M, Al-Mulla HM, Usmani MA. Association between type 2 diabetes mellitus and Helicobacter pylori infection. *Turk J Gastroenterol* 2007; **18**: 225-229 [PMID: 18080918]
 - 79 **Hamed SA**, Amine NF, Galal GM, Helal SR, Tag El-Din LM, Shawky OA, Ahmed EA, Abdel Rahman MS. Vascular risks and complications in diabetes mellitus: the role of helicobacter pylori infection. *J Stroke Cerebrovasc Dis* 2008; **17**: 86-94 [PMID: 18346651 DOI: 10.1016/j.jstrokecerebrovasdis]
 - 80 **Demir M**, Gokturk HS, Ozturk NA, Kulaksizoglu M, Serin E, Yilmaz U. Helicobacter pylori prevalence in diabetes mellitus patients with dyspeptic symptoms and its relationship to glycemic control and late complications. *Dig Dis Sci* 2008; **53**: 2646-2649 [PMID: 18320319 DOI: 10.1007/s10620-007-0185-7]
 - 81 **Ariizumi K**, Koike T, Ohara S, Inomata Y, Abe Y, Iijima K, Imatani A, Oka T, Shimosegawa T. Incidence of reflux esophagitis and H pylori infection in diabetic patients. *World J Gastroenterol* 2008; **14**: 3212-3217 [PMID: 18506928 DOI: 10.3748/wjg.14.3212]
 - 82 **Bytzer P**, Talley NJ, Hammer J, Young LJ, Jones MP, Horowitz M. GI symptoms in diabetes mellitus are associated with both poor glycemic control and diabetic complications. *Am J Gastroenterol* 2002; **97**: 604-611 [PMID: 11922554 DOI: 10.1111/j.1572-0241.2002.05537.x]
 - 83 **Kim JH**, Park HS, Ko SY, Hong SN, Sung IK, Shim CS, Song KH, Kim DL, Kim SK, Oh J. Diabetic factors associated with gastrointestinal symptoms in patients with type 2 diabetes. *World J Gastroenterol* 2010; **16**: 1782-1787 [PMID: 20380013 DOI: 10.3748/wjg.v16.i14.1782]
 - 84 **Tseng CH**. Diabetes, insulin use and Helicobacter pylori eradication: a retrospective cohort study. *BMC Gastroenterol* 2012; **12**: 46 [PMID: 22571603 DOI: 10.1186/1471-230X-12-46]
 - 85 **Selinger C**, Robinson A. Helicobacter pylori eradication in diabetic patients: still far off the treatment targets. *South Med J* 2010; **103**: 975-976 [PMID: 20818306 DOI: 10.1097/SMJ.0b013e3181ee7dce]
 - 86 **Demir M**, Gokturk HS, Ozturk NA, Serin E, Yilmaz U. Efficacy of two different Helicobacter pylori eradication regimens in patients with type 2 diabetes and the effect of Helicobacter pylori eradication on dyspeptic symptoms in patients with diabetes: a randomized controlled study. *Am J Med Sci* 2009; **338**: 459-464 [PMID: 19884816 DOI: 10.1097/MAJ.0b013e3181b5d3cf]
 - 87 **Demir M**, Gokturk HS, Ozturk NA, Arslan H, Serin E, Yilmaz U. Clarithromycin resistance and efficacy of clarithromycin-containing triple eradication therapy for Helicobacter pylori infection in type 2 diabetes mellitus patients. *South Med J* 2009; **102**: 1116-1120 [PMID: 19864973 DOI: 10.1097/SMJ.0b013e3181bca538]
 - 88 **Sargyn M**, Uygur-Bayramicli O, Sargyn H, Orbay E, Yavuzer D, Yayla A. Type 2 diabetes mellitus affects eradication rate of Helicobacter pylori. *World J Gastroenterol* 2003; **9**: 1126-1128 [PMID: 12717872]
 - 89 **Aydemir S**, Bayraktaroglu T, Sert M, Sokmen C, Atmaca H, Mungan G, Gun BD, Borazan A, Ustundag Y. The effect of Helicobacter pylori on insulin resistance. *Dig Dis Sci* 2005; **50**: 2090-2093 [PMID: 16240220 DOI: 10.1007/s10620-005-3012-z]
 - 90 **Gunji T**, Matsushashi N, Sato H, Fujibayashi K, Okumura M, Sasabe N, Urabe A. Helicobacter pylori infection significantly increases insulin resistance in the asymptomatic Japanese population. *Helicobacter* 2009; **14**: 144-150 [PMID: 19751440 DOI: 10.1111/j.1523-5378.2009.00705.x]
 - 91 **Aslan M**, Horoz M, Nazligul Y, Bolukbas C, Bolukbas FF, Selek S, Celik H, Erel O. Insulin resistance in H pylori infection and its association with oxidative stress. *World J Gastroenterol* 2006; **12**: 6865-6868 [PMID: 17106938]
 - 92 **Kaneko H**, Konagaya T, Kusugami K. Helicobacter pylori and gut hormones. *J Gastroenterol* 2002; **37**: 77-86 [PMID: 11871770 DOI: 10.1007/s005350200000]
 - 93 **Calam J**. Helicobacter pylori modulation of gastric acid. *Yale J Biol Med* 1999; **72**: 195-202 [PMID: 10780581]
 - 94 **Wilkin TJ**. Is autoimmunity or insulin resistance the primary driver of type 1 diabetes? *Curr Diab Rep* 2013; **13**: 651-656 [PMID: 24005814 DOI: 10.1007/s11892-013-0407-7]
 - 95 **Ge S**, Feng X, Shen L, Wei Z, Zhu Q, Sun J. Association between Habitual Dietary Salt Intake and Risk of Gastric Cancer: A Systematic Review of Observational Studies. *Gastroenterol Res Pract* 2012; **2012**: 808120 [PMID: 23125851 DOI: 10.1155/2012/808120]
 - 96 **Shikata K**, Kiyohara Y, Kubo M, Yonemoto K, Ninomiya T, Shirota T, Tanizaki Y, Doi Y, Tanaka K, Oishi Y, Matsumoto T, Iida M. A prospective study of dietary salt intake and gastric cancer incidence in a defined Japanese population: the Hisayama study. *Int J Cancer* 2006; **119**: 196-201 [PMID: 16450397 DOI: 10.1002/ijc.21822]
 - 97 **Wang XQ**, Terry PD, Yan H. Review of salt consumption and stomach cancer risk: epidemiological and biological evidence. *World J Gastroenterol* 2009; **15**: 2204-2213 [PMID: 19437559 DOI: 10.3748/wjg.15.2204]
 - 98 **Tsugane S**, Sasazuki S. Diet and the risk of gastric cancer: review of epidemiological evidence. *Gastric Cancer* 2007; **10**: 75-83 [PMID: 17577615 DOI: 10.1007/s10120-007-0420-0]
 - 99 **Tsugane S**. Salt, salted food intake, and risk of gastric cancer: epidemiologic evidence. *Cancer Sci* 2005; **96**: 1-6 [PMID: 15649247 DOI: 10.1111/j.1349-7006.2005.00006.x]
 - 100 **Zhong C**, Li KN, Bi JW, Wang BC. Sodium intake, salt taste and gastric cancer risk according to Helicobacter pylori infection, smoking, histological type and tumor site in China. *Asian Pac J Cancer Prev* 2012; **13**: 2481-2484 [PMID: 22938408 DOI: 10.7314/APJCP.2012.13.6.2481]
 - 101 **Peleteiro B**, Lopes C, Figueiredo C, Lunet N. Salt intake and gastric cancer risk according to Helicobacter pylori infection, smoking, tumour site and histological type. *Br J Cancer* 2011; **104**: 198-207 [PMID: 21081930 DOI: 10.1038/sj.bjc.6605993]
 - 102 **Gaddy JA**, Radin JN, Loh JT, Zhang F, Washington MK, Peek RM, Algood HM, Cover TL. High dietary salt intake exacerbates Helicobacter pylori-induced gastric carcinogenesis. *Infect Immun* 2013; **81**: 2258-2267 [PMID: 23569116 DOI: 10.1128/IAI.01271-12]
 - 103 **Breslin PA**. An evolutionary perspective on food and hu-

- man taste. *Curr Biol* 2013; **23**: R409-R418 [PMID: 23660364 DOI: 10.1016/j.cub.2013.04.010]
- 104 **Bajaj S**, Prasad S, Gupta A, Singh VB. Oral manifestations in type-2 diabetes and related complications. *Indian J Endocrinol Metab* 2012; **16**: 777-779 [PMID: 23087863 DOI: 10.4103/2230-8210.100673]
 - 105 **Gondivkar SM**, Indurkar A, Degwekar S, Bhowate R. Evaluation of gustatory function in patients with diabetes mellitus type 2. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2009; **108**: 876-880 [PMID: 19913725 DOI: 10.1016/j.tripleo.2009.08.015]
 - 106 **Hsieh MC**, Lee TC, Cheng SM, Tu ST, Yen MH, Tseng CH. The influence of type 2 diabetes and glucose-lowering therapies on cancer risk in the Taiwanese. *Exp Diabetes Res* 2012; **2012**: 413782 [PMID: 22719752 DOI: 10.1155/2012/413782]
 - 107 **Currie CJ**, Poole CD, Gale EA. The influence of glucose-lowering therapies on cancer risk in type 2 diabetes. *Diabetologia* 2009; **52**: 1766-1777 [PMID: 19572116 DOI: 10.1007/s00125-009-1440-6]
 - 108 **Tseng CH**. Diabetes, insulin use, and gastric cancer: a population-based analysis of the Taiwanese. *J Clin Gastroenterol* 2013; **47**: e60-e64 [PMID: 23269314 DOI: 10.1097/MCG.0b013e31827245eb]
 - 109 **Jonasson JM**, Ljung R, Talbäck M, Haglund B, Gudbjörnsdóttir S, Steineck G. Insulin glargine use and short-term incidence of malignancies-a population-based follow-up study in Sweden. *Diabetologia* 2009; **52**: 1745-1754 [PMID: 19588120 DOI: 10.1007/s00125-009-1444-2]
 - 110 **Hemkens LG**, Grouven U, Bender R, Günster C, Gutschmidt S, Selke GW, Sawicki PT. Risk of malignancies in patients with diabetes treated with human insulin or insulin analogues: a cohort study. *Diabetologia* 2009; **52**: 1732-1744 [PMID: 19565214 DOI: 10.1007/s00125-009-1418-4]
 - 111 **Smith U**, Gale EA. Does diabetes therapy influence the risk of cancer? *Diabetologia* 2009; **52**: 1699-1708 [PMID: 19597799 DOI: 10.1007/s00125-009-1441-5]
 - 112 **Sommerfeld MR**, Müller G, Tschank G, Seipke G, Habermann P, Kurrle R, Tennagels N. In vitro metabolic and mitogenic signaling of insulin glargine and its metabolites. *PLoS One* 2010; **5**: e9540 [PMID: 20209060 DOI: 10.1371/journal.pone.0009540]
 - 113 **Lee MS**, Hsu CC, Wahlqvist ML, Tsai HN, Chang YH, Huang YC. Type 2 diabetes increases and metformin reduces total, colorectal, liver and pancreatic cancer incidences in Taiwanese: a representative population prospective cohort study of 800,000 individuals. *BMC Cancer* 2011; **11**: 20 [PMID: 21241523 DOI: 10.1186/1471-2407-11-20]
 - 114 **Tseng CH**. Thyroid cancer risk is not increased in diabetic patients. *PLoS One* 2012; **7**: e53096 [PMID: 23300866 DOI: 10.1371/journal.pone.0053096]
 - 115 **Kato K**, Gong J, Iwama H, Kitanaka A, Tani J, Miyoshi H, Nomura K, Mimura S, Kobayashi M, Aritomo Y, Kobara H, Mori H, Himoto T, Okano K, Suzuki Y, Murao K, Masaki T. The antidiabetic drug metformin inhibits gastric cancer cell proliferation in vitro and in vivo. *Mol Cancer Ther* 2012; **11**: 549-560 [PMID: 22222629 DOI: 10.1158/1535-7163.MCT-11-0594]
 - 116 **Qian X**, Li J, Ding J, Wang Z, Duan L, Hu G. Glibenclamide exerts an antitumor activity through reactive oxygen species-c-jun NH2-terminal kinase pathway in human gastric cancer cell line MGC-803. *Biochem Pharmacol* 2008; **76**: 1705-1715 [PMID: 18840412 DOI: 10.1016/j.bcp.2008.09.009]
 - 117 **Zhang L**, Hu JF, Li GQ, Xiao X, Su Q. Rosiglitazone reverses mitomycin C resistance in human gastric cancer cells. *Am J Med Sci* 2012; **343**: 382-387 [PMID: 22052411 DOI: 10.1097/MAJ.0b013e31822f3c63]
 - 118 **Leung WK**, Bai AH, Chan VY, Yu J, Chan MW, To KF, Wu JR, Chan KK, Fu YG, Chan FK, Sung JJ. Effect of peroxisome proliferator activated receptor gamma ligands on growth and gene expression profiles of gastric cancer cells. *Gut* 2004; **53**: 331-338 [PMID: 14960510 DOI: 10.1136/gut.2003.021105]
 - 119 **Singh PP**, Singh S. Statins are associated with reduced risk of gastric cancer: a systematic review and meta-analysis. *Ann Oncol* 2013; **24**: 1721-1730 [PMID: 23599253 DOI: 10.1093/annonc/mdt150]
 - 120 **Ye X**, Fu J, Yang Y, Gao Y, Liu L, Chen S. Frequency-risk and duration-risk relationships between aspirin use and gastric cancer: a systematic review and meta-analysis. *PLoS One* 2013; **8**: e71522 [PMID: 23936269 DOI: 10.1371/journal.pone.0071522]
 - 121 **Franzini L**, Ardigo D, Cavalot F, Miccoli R, Rivellese AA, Trovati M, Zavaroni I, Vaccaro O. Women show worse control of type 2 diabetes and cardiovascular disease risk factors than men: results from the MIND.IT Study Group of the Italian Society of Diabetology. *Nutr Metab Cardiovasc Dis* 2013; **23**: 235-241 [PMID: 22397873 DOI: 10.1016/j.numecd.2011.12.003]
 - 122 **Fu AZ**, Zhang Q, Davies MJ, Pentakota SR, Radican L, Seck T. Underutilization of statins in patients with type 2 diabetes in US clinical practice: a retrospective cohort study. *Curr Med Res Opin* 2011; **27**: 1035-1040 [PMID: 21410303 DOI: 10.1185/03007995.2011.567257]
 - 123 **Tseng CH**, Chong CK, Chan TT, Bai CH, You SL, Chiou HY, Su TC, Chen CJ. Optimal anthropometric factor cutoffs for hyperglycemia, hypertension and dyslipidemia for the Taiwanese population. *Atherosclerosis* 2010; **210**: 585-589 [PMID: 20053403 DOI: 10.1016/j.atherosclerosis.2009.12.015]

P- Reviewers: Buchler C, Marchesini G, Naoaki S
S- Editor: Ma YJ **L- Editor:** A **E- Editor:** Liu XM





WJG 20th Anniversary Special Issues (12): Fatty liver

Clinical approaches to non-alcoholic fatty liver disease

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Received: October 29, 2013 Revised: December 5, 2013

Accepted: January 3, 2014

Published online: February 21, 2014

Abstract

Non-alcoholic fatty liver disease (NAFLD) ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), leading to fibrosis and potentially cirrhosis, and it is one of the most common causes of liver disease worldwide. NAFLD is associated with other medical conditions such as metabolic syndrome, obesity, cardiovascular disease and diabetes. NASH can only be diagnosed through liver biopsy, but noninvasive techniques have been developed to identify patients who are most likely to have NASH or fibrosis, reducing the need for liver biopsy and risk to patients. Disease progression varies between individuals and is linked to a number of risk factors. Mechanisms involved in the pathogenesis are associated with diet and lifestyle, influx of free fatty acids to the liver from adipose tissue due to insulin resistance, hepatic oxidative stress, cytokines production, reduced very low-density lipoprotein secretion and intestinal microbiome. Weight loss through improved diet and increased physical activity has been the cornerstone therapy of NAFLD. Recent therapies such as pioglitazone and vitamin E have been shown to be beneficial. Omega 3 polyunsaturated fatty acids and statins may offer additional benefits. Bariatric surgery should be considered in morbidly obese patients. More research is needed to assess the impact of these treatments on a

long-term basis. The objective of this article is to briefly review the diagnosis, management and treatment of this disease in order to aid clinicians in managing these patients.

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Key words: Non-alcoholic fatty liver disease; Steatohepatitis; Cirrhosis; Steatosis; Pathogenesis; Diagnosis; Management; Treatment

Core tip: Non-alcoholic fatty liver disease (NAFLD) is associated with the metabolic syndrome and patients who present with nonalcoholic steatohepatitis can progress to cirrhosis and liver failure requiring transplantation. NAFLD is becoming a public health issue due to its increased prevalence. It is important to recognize the disease early to prevent its progression. Proper management is required in order to reduce associated with it. This review discusses what current practices are and provides suggestions for future research.

Schwenger KJP, Allard JP. Clinical approaches to non-alcoholic fatty liver disease. *World J Gastroenterol* 2014; 20(7): 1712-1723
Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1712.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1712>

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of liver disease worldwide, with a prevalence of 15%-30% in Western populations^[1-4]. The prevalence increases to 58% in overweight individuals and can be as high as 98% in non-diabetic obese individuals^[5]. NAFLD ranges from simple steatosis to nonalcoholic steatohepatitis (NASH) and potentially cirrhosis^[6]. NASH is the picture of hepatocellular injury and inflammation of the liver^[7]. Cirrhosis, which occurs in 25% of patients

with NASH, can result in liver failure, portal hypertension, and hepatocellular carcinoma and patients with cirrhosis are at a high risk for developing cardiovascular disease^[8]. Not only the presence of excess weight and obesity, but also the location of fat storage plays a role in NAFLD pathogenesis. Visceral fat stores increase the risk for NAFLD in both obese and non-obese individuals^[9]. NAFLD is associated with metabolic syndrome and obesity^[10]. The diagnosis of NAFLD is made when there is evidence of liver steatosis on imaging modalities and this is associated with features of the metabolic syndrome in the context of a patient who does not have other causes of liver disease^[11] and where alcohol consumption is less than 21 drinks and 14 drinks per week for men and women, respectively^[12]. Diagnosis for NASH is confirmed when a liver biopsy shows the presence of perilobular inflammation, or the presence of hepatocyte ballooning, Mallory hyaline and acidophil bodies with or without fibrosis. Non-invasive tests such as liver enzymes, medical imaging, Fatty Liver Index, NAFLD fibrosis score, FibroMeter and Fibroscan^[13] may suggest the presence of NASH by detecting fibrosis and research is on-going to assess surrogate markers for NASH such as CK18, but this remains experimental^[14]. Therefore, for a definite diagnosis of NASH, patients still need a liver biopsy.

PATHOGENESIS

In NAFLD, the accumulation of fat in the liver^[15] is a result of increased delivery of free fatty acid (FFA) to the liver, increased synthesis, decreased triglyceride export through very-low density lipoprotein (VLDL) and reduced beta-oxidation^[16]. Universally, patients with NAFLD have insulin resistance (IR) which increases lipolysis from the adipose tissue^[17]. The resulting FFA will be taken up by the liver and can cause lipid peroxidation^[17]. Lipid peroxidation can increase the production of pro-inflammatory cytokines^[17]. The increase in FFA can also exceed mitochondrial beta-oxidation further increasing the oxidative stress^[18] and inflammation^[18].

Liver *de novo* lipogenesis (DNL)^[19] also contributes to the steatosis. *De novo* lipogenesis is due to the hyperinsulinemia associated with IR, which stimulates the enzymes in the DNL pathway, increasing the production and storage of triglycerides. In NAFLD, DNL contributes to 26% of hepatic triglyceride accumulation where it accounts for < 5% in healthy individuals^[19]. Hyperinsulinemia can also cause a reduction in VLDL secretion^[20], leading to triglyceride accumulation in the liver.

The presence of inflammation or steatohepatitis depends on a number of factors such as the presence of free fatty acids (FFA), inflammatory cytokines and adipokines, oxidative stress and mitochondrial dysfunction^[16].

Proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6) are elevated and generally produced by the liver and adipose tissue, from NF- κ B activation^[21] (e.g., from lipid peroxidation or activation of Toll-like receptors). Increased TNF- α

and IL-6 are also associated with IR by interfering with insulin signaling^[7,22]. On the other hand, adiponectin^[7,22] is produced by the adipose tissue and is an anti-inflammatory adipokine that can increase insulin sensitivity^[23]. In NAFLD adiponectin is reduced which decreases fatty acid oxidation and hepatic gluconeogenesis^[24]. The role of other adipokines, like visfatin, leptin and resistin are still controversial^[25-27]. Potential pathways are promotion of IR, oxidative stress and inflammation as well as fibrogenesis^[28].

Diet is an important contributor to NAFLD, mainly because excessive energy intake leads to obesity, which in turn increases the risk for NAFLD. However, not only the amount of energy but also the quality of the diet could play an important role for the development and progression of NAFLD. Diets rich in saturated fat, cholesterol, and low in polyunsaturated fat, fiber and antioxidant vitamins C and E^[29] have been associated with NASH. High saturated fat diets are associated with IR and hepatic inflammation^[29]. Other research has also demonstrated a relationship between increased dietary fat consumption and NAFLD^[30,31]. Conversely, a study investigating pre-surgical bariatric patients in the United States reported that increased carbohydrate intake was associated with hepatic inflammation^[32]. Among carbohydrates, specifically fructose might contribute to NAFLD progression. Fructose intake has been linked to increasing hepatic fat, inflammation and possibly fibrosis^[33]. Fructose has also been associated with both an increase in visceral adipose tissue^[34] and plasma triglycerides^[35].

Recently, new evidence has linked intestinal microbiota to NAFLD pathogenesis. Intestinal microbiota (IM) may play a role in the development of NAFLD, however very few human studies have been conducted and most were cross-sectional^[36-39]. One study suggested an association between low percentage of fecal bacteroidetes and the presence of NASH, independent of diet and body mass index (BMI)^[36]. Other studies showed an increased abundance of *E. coli* associated with higher blood alcohol levels^[39] or differences in IM associated with differences in volatile organic compounds^[38]. Development of fatty liver on a choline deficient diet was also associated with IM at baseline and single nucleotide polymorphism in the phosphatidylethanolamine methyl transferase gene region^[37]. IM can be altered by the type of diet consumed and may contribute to NAFLD through several mechanisms. These include salvaging energy from food, contributing to inflammation *via* cytokines by increasing intestinal permeability leading to endotoxemia, modulating the innate immune system such as activation of Toll-like receptors and inflammasomes, regulating bile acid, metabolizing dietary choline and increasing endogenous ethanol by bacteria^[37,40].

DIAGNOSIS NAFLD

NAFLD should be suspected in individuals who are either obese, diabetic or have metabolic syndrome^[40].

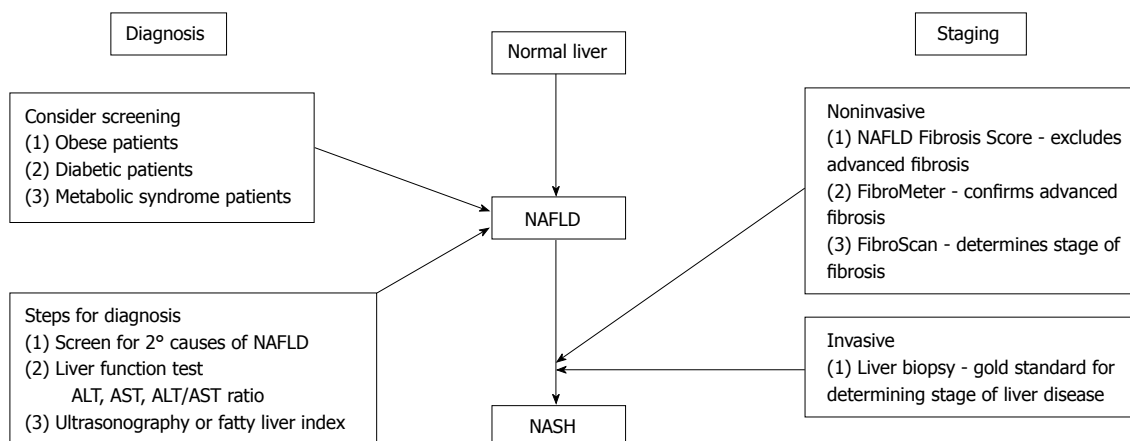


Figure 1 Diagnosis and staging of non-alcoholic fatty liver disease. NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

However, the majority of patients with NAFLD are asymptomatic and the disease may be detected *via* routine blood tests showing elevated liver enzymes or when an ultrasound is performed for various reasons and detects liver steatosis (Figure 1). However, secondary causes of hepatic steatosis or elevated liver enzymes, such as excess alcohol consumption, medications, toxins, lipodystrophy, autoimmune and inflammatory diseases, nutrition (malnutrition, total parenteral nutrition, severe weight loss, and refeeding syndrome), viral hepatitis and metabolic liver disease should be excluded by reviewing patient's history and proper investigation^[40,41].

Although it is still not possible to diagnose NAFLD based solely on blood work, elevated transaminases can be used as a first step^[42]. Elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in the absence of other liver diseases may support NAFLD^[41,43], and have been found in up to approximately 50% of simple steatosis patients and 80% of NASH patients^[42]. An AST:ALT ratio less than 1 is also seen in NAFLD^[44] and supports NASH. However, it is important to note that patients with normal transaminases and liver steatosis on imaging may also have NASH^[45].

Ultrasonography is a non-invasive tool that is used in the detection of liver steatosis^[40,46]. Other imaging techniques such as computed tomography and nuclear magnetic resonance imaging can also detect liver steatosis, but neither of these more expensive techniques provide more information than ultrasonography^[46,47] except for fat quantification^[48]. A review conducted by Festi *et al.*^[46] concluded that ultrasonography should be used as a first-line diagnostic tool because of its evaluation of liver steatosis and other abdominal organs.

The Fatty Liver Index is an algorithm based on four markers; BMI, waist circumference, triglyceride and γ -glutamyltransferase (GGT)^[26], which is confirmed to accurately identify NAFLD^[49,50]. This index has been used in population studies^[40] and has achieved an accuracy of 0.84 in detecting fatty liver^[51]. The Fatty Liver Index provides a score out of 100, indicating that a score < 30 can rule out and a score \geq 60 to rule in hepatic ste-

atosis^[51]. The formula for the Fatty Liver Index is $[e^{0.953 \times \log_e(\text{triglycerides}) + 0.139 \times \text{BMI} + 0.718 \times \log_e(\text{GGT}) + 0.053 \times \text{waist circumference} - 15.745}] / [1 + e^{0.953 \times \log_e(\text{triglycerides}) + 0.139 \times \text{BMI} + 0.718 \times \log_e(\text{GGT}) + 0.053 \times \text{waist circumference} - 15.745}] \times 100$ ^[51].

Liver biopsy is currently the gold standard for diagnosing NASH^[41], as it also establishes the stage of NASH^[52]. This invasive procedure is used to analyze the degree of hepatocyte injury and level of fibrosis and inflammation^[46]. However, it is used after imaging techniques, laboratory abnormalities and/or non-invasive methods suggest the presence and severity of NASH^[46,52].

STAGING OF NAFLD

Recent advances have allowed for non-invasive techniques to be used to diagnose the level of inflammation/fibrosis (Figure 1)^[40].

The NAFLD fibrosis score (NFS) evaluates six variables; age, hyperglycemia, BMI, platelet count, albumin and AST/ALT ratio^[40,53]. The NAFLD fibrosis score formula is $-1.675 + 0.037 \times \text{age (year)} + 0.094 \times \text{BMI (kg/m}^2\text{)} + 1.13 \times \text{IFG/diabetes (yes = 1; no = 0)} + 0.99 \times \text{AST/ALT ratio} - 0.013 \times \text{platelets (} \times 10^9/\text{L)} - 0.66 \times \text{albumin (g/dL)}$ ^[53]. This equation is used to classify the probability of fibrosis as < -1.5 for low probability and > -1.5 to < 0.67 for intermediate probability and > 0.67 for high probability^[54]. Angulo *et al.*^[53] validated NFS to a liver biopsy and found that this accurately classifies NAFLD patients with and without advanced fibrosis. Furthermore, a study investigating NAFLD in a morbidly obese population undergoing bariatric surgery found that the NFS is accurate at excluding advanced fibrosis within this population^[55]. Overall, this tool is widely used in practice to exclude advanced fibrosis.

Another tool used in clinical practice is the FibroMeter. This tool uses age, weight, fasting glucose, AST, ALT, ferritin and platelet count to diagnose significant fibrosis^[46,56,57]. The formula for the FibroMeter is $-0.007 \times \text{platelets (} \times 10^9/\text{L)} - 0.049 \times \text{prothrombin time (\%)} + 0.012 \times \text{AST (U/L)} + 0.005 \times \alpha 2 \text{ macroglobulin (g/L)}$

Table 1 Summary of lifestyle intervention studies: diet and/or physical activity

Ref.	Population, Study Design	Intervention	Results
[71]	<i>n</i> = 96, 12-mo intervention on adults with hepatic steatosis and type 2 diabetes	Combination of moderate caloric restriction (1200-1800 kcal/d) and increased moderate physical activity (175 min per week)	Significant decreases in BMI, weight, waist circumference, percent body fat and A1C
[72]	<i>n</i> = 50, longitudinal study with lifestyle intervention in NAFLD adults	10 concealing sessions with a dietitian, and moderate intensity activity 3 h/wk	Significantly decreased body fat and liver fat and increased fitness. NAFLD at baseline resolved in 20 participants
[68]	<i>n</i> = 28, randomized control trial adults with elevated ALT or AST, BMI of 25-40	Combination of diet (1000-1500/d), exercise (10000 steps per day and 200 min/wk of moderate physical activity) and behavior modification	Weight in intervention group decreased by 9.3%, significant improvement of NASH. > 7% weight loss significantly improved steatosis
[73]	<i>n</i> = 152, randomized intervention of adults with elevated liver enzymes, central obesity and metabolic risk factors	Randomized to moderate (6 sessions/10 wk) or low-intensity (3 sessions/4 wk) or control. Physical activity 150 min/wk and low saturated fat and process food diet (1700-2400 kcal/d)	Moderate intensity – improvement in all risk factors, greater reduction in liver enzymes and weight loss than low-intensity
[74]	<i>n</i> = 19, 8 wk exercise intervention in NAFLD adults	8 wk (3 × wk) of resistance exercise (<i>n</i> = 11) <i>vs</i> control (<i>n</i> = 8)	13% reduction in liver lipid. Lipid oxidation, glucose and IR improved. No effect on weight or body fat

BMI: Body mass index; NAFLD: Non-alcoholic fatty liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NASH: Nonalcoholic steatohepatitis; IR: Insulin resistance.

+ 0.021 × hyaluronate (ng/mL) -0.270 × urea (mmol/L) + 0.270 × age (year) + 3.718^[58]. The FibroMeter provides the probability of significant fibrosis and the percentage of hepatic fibrosis^[58]. Calès *et al*^[56] compared the FibroMeter to NFS and found that the FibroMeter provides a more reliable diagnosis for significant fibrosis than the NFS. This tool can be used to confirm or disconfirm advanced fibrosis in NAFLD patients^[57].

FibroScan, also known as transient elastography, is another noninvasive method to assess liver fibrosis^[59]. This method measures liver stiffness, which was originally designed for the hepatitis C population^[60], but is now being used in the NAFLD population^[61]. The FibroScan sends a pulse through the skin, which is circulated through the liver. The velocity of the wave, which is correlated with liver stiffness, is measured by ultrasound. The stiffer the liver the greater the degree of fibrosis^[62]. The liver stiffness measurement (LSM) is used to assess the current stage of liver fibrosis. The cutoffs are 4.85, 7.38, 9.28, 13.33 and 25.34 kPa which represent stages, 0 (no steatosis), 1 (perivenular and/or perisinusoidal fibrosis), 2 (combined pericellular portal fibrosis), 3 (septal fibrosis) and 4 (cirrhosis)^[59]. Yoneda *et al*^[59] investigated the usefulness of the transient elastography in NAFLD patients. They found that there is a significant correlation between liver stiffness and fibrosis stage, which was confirmed by liver biopsy^[59]. Therefore, this measurement can be used in the NAFLD population to determine the stage of fibrosis. However, special consideration is needed for overweight and obese patients. Studies have shown that obesity, (BMI > 30 kg/m²) provides inaccurate LSMs. The use of a FibroScan XL probe has been shown to provide reliable LSM^[63,64].

Overall, these noninvasive measurements to assess NAFLD/NASH should be used prior to a liver biopsy as they pose minimal risk to the patient. However, liver biopsy should be considered in patients when these noninvasive tests suggesting fibrosis are inconclusive^[40], or the

patients have risk factors associated with advanced fibrosis, such as age > 50 years, presence of diabetes, morbid obesity or metabolic syndrome^[65].

MANAGEMENT OF NAFLD

The goal of managing NAFLD is to improve steatosis and prevent fibrosis. No standard treatment currently exists, however, treating risk factors such as obesity and IR, remains the focus of managing NAFLD. Currently, lifestyle interventions, medical treatments, alternative therapies and surgery are being used to treat risk factors associated with NAFLD.

Lifestyle interventions

As previously mentioned IR and obesity increase the risk of developing NAFLD and are instrumental in NAFLD progression. In today's society obesity and IR have been linked to poor diet choices, as well as sedentary lifestyles. Weight management through improvements in diet and increased physical activity can help to improve liver histology as well as delay disease progression (Table 1)^[66].

NAFLD patients have been found to have an increased energy intake when compared to healthy individuals^[67]. Several studies have shown that weight loss is successful in improving liver enzymes, insulin sensitivity, reducing inflammation and liver histology^[68-72]. Recent studies use diet, physical activity and behavior modification to help promote weight loss in NAFLD patients^[69]. A randomized controlled trial conducted by Promrat *et al*^[68] used a combination of diet, physical activity and behavior modification to trigger 7%-10% weight loss in obese NASH patients. Those who achieved a minimum of 7% weight loss had improvements in their liver histology^[68]. A similar study used NAFLD patients with elevated liver enzymes and central obesity to assess the effectiveness of lifestyle interventions. Patients were randomly assigned to either low (3 sessions/4 wk) or moder-

Table 2 Summary of medication intervention studies

Ref.	Population, Study Design	Intervention	Results
[79]	<i>n</i> = 15, open label study with histologically confirmed NAFLD adults	All patients received 20 mg/kg per day of metformin for 48 wk	In the initial 3 mo there was improvement in ALT and AST levels and insulin sensitivity, after 3 mo no further improvement noted
[80]	<i>n</i> = 57 24-mo observational study with NAFLD or NASH overweight and obese children	Metformin was progressively titrated from 250-500 mg <i>tid</i> at weekly intervals and patients were given a hypocaloric or isocaloric diet and recommended to engage in 45 min/d of physical activity (<i>n</i> = 57) compared to control group (<i>n</i> = 30) with the same diet and physical activity recommendations	ALT significantly improved with decreasing body weight. NAS score decreased in both groups, no significant changes in fibrosis
[85]	<i>n</i> = 63, randomized, double-blind placebo - controlled in NASH adults	32 patients were given rosiglitazone (4 mg/d for 1 mo then 8 mg/d for 11 mo) <i>vs</i> placebo (<i>n</i> = 31)	Improved steatosis and normalized transaminase, only ½ responded. Improvement of insulin sensitivity
[86]	<i>n</i> = 47, randomized control study in adults with impaired glucose tolerance or type 2 diabetes with NASH	6 mo of hypocaloric diet and 45 mg (<i>n</i> = 26) of pioglitazone <i>vs</i> 6 mo of hypocaloric diet (<i>n</i> = 21)	Diet and pioglitazone improved glucose tolerance and normalized ALT. Histologic features of NASH improved, no significant reduction in fibrosis
[87]	<i>n</i> = 13 patient cohort with NASH adults	All were treated with 30 mg/d of pioglitazone for 48 wk, then followed up 48 wk after stopping pioglitazone.	Stopping pioglitazone increased ALT, decreased adiponectin, worsened insulin sensitivity and increased hepatic fat, no change in fibrosis
[89]	<i>n</i> = 247, randomization of adults with NASH without diabetes	96 wk of either 30 mg pioglitazone (<i>n</i> = 80), vitamin E (800 IU/d) (<i>n</i> = 84) or placebo (<i>n</i> = 83)	Vitamin E significantly improved NASH. AST and ALT significantly improved in vitamin E and pioglitazone groups, and reduction in hepatic steatosis with no improvement in fibrosis score.
[90]	<i>n</i> = 45 prospective, double-blind randomized, placebo controlled trial in NASH adults	Received vitamin E and C (1000 IU and 1000 mg) (<i>n</i> = 23) or placebo for 6 mo (<i>n</i> = 22) additionally patients received weight loss counselling and encouraged to follow a low fat diet	Vitamin treatment significantly improved fibrosis score

NAFLD: Non-alcoholic fatty liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; *tid*: Three times a day; NASH: Nonalcoholic steatohepatitis.

ate (6 sessions/10 wk) physical activity intensity groups and were compared to a control group. The lifestyle interventions included physical activity and dietary guidance as well as behavior modification^[73]. St George *et al*^[73] found that there was a reduction in liver enzymes, which was greater in the moderate-intensity lifestyle intervention group in comparison to the control.

Physical activity alone has been found to reduce hepatic steatosis, independent of weight loss. A study on sedentary NAFLD patients examined the effects of resistant exercises on liver lipid levels^[74]. Hallsworth *et al*^[74] found that after 8 wk (3 times per week lasting 45-60 min) of resistance based exercise resulted in a reduction of liver lipids, and improvements of lipid oxidation, glucose control and insulin resistance. A review conducted by Thoma *et al*^[69] analyzed 23 studies using diet modification, physical activity, or a combination of both. Thoma *et al*^[69] found that lifestyle modifications that led to weight reduction and/or increased physical activity greatly reduced liver fat and improved insulin sensitivity.

Overall, lifestyle modification (diet and exercise) resulting in weight loss or increased physical activity can reduce liver enzymes and inflammation and improve liver histology, glucose control, and insulin sensitivity and lipid oxidation. Therefore, when developing a treatment plan for NAFLD patients, lifestyle modification should be used as a first step in clinical settings.

Medical treatment

Lifestyle interventions may not be effective in certain cases and thus other approaches must be considered in the management of NAFLD (Table 2). Pharmacological treatment has been studied in this population, specifically insulin-sensitizing agents. Two insulin-sensitizing agents, metformin and thiazolidinediones (TZD), have been investigated in this population, however there are conflicting results. In addition, vitamin E therapy has been used in the treatment of NAFLD, as it inhibits oxidative stress and reduces the promotion of hepatic fibrosis^[75].

Metformin is used in the treatment for type 2 diabetes, as it lowers blood glucose by decreasing gluconeogenesis in the liver as well as decreasing intestinal glucose absorption which stimulates glucose uptake in muscle, and increases fatty acid oxidation^[76,77], resulting in improved insulin sensitivity^[78]. Clinical studies have investigated the use of metformin in the treatment of NAFLD, specifically looking at liver histology and aminotransferases. Nair *et al*^[79] conducted a pilot study to investigate the efficiency and safety of metformin in NAFLD patients. Patients were prescribed 20 mg/kg per day of metformin for one year, comparing liver histology pre and post treatment regimen^[79]. Three months into the treatment, aminotransferase decreased, which was related to an improvement in insulin sensitivity^[79]. However, this improvement was not sustained for the duration of the treatment; therefore

Nair *et al*^[79] concluded that metformin should not be used for the treatment of NAFLD. More recently, a study conducted on children with NAFLD used lifestyle interventions and metformin (1.5 g/d for 24 mo) to determine the effect on liver enzymes^[80]. Nobili *et al*^[80] found that metformin was no more effective than lifestyle interventions in improving liver enzymes or histology. Additionally, other studies have also failed to prove benefits of using metformin to improve liver histology^[4,81]. In conclusion, metformin should not be used in the treatment of NAFLD, as research has shown that it is ineffective in the management of NAFLD.

TZD are peroxisomal proliferator activated receptor- γ (PPAR- γ) agonists that are used primarily in the type 2 diabetes population to help improve insulin sensitivity within the liver, muscle and adipose tissue, promote hepatic fatty acid oxidation and decrease hepatic lipogenesis^[82,83]. TZD use in NAFLD patients, specifically the effects of pioglitazone and rosiglitazone, have shown to decrease hepatic fat and decrease cellular injury, however these medications have also shown to cause weight gain^[84]. Ratziu *et al*^[85] studied the treatment and safety of rosiglitazone in NASH patients. The treatment group received 4 mg/d for the first month, and then 8 mg/d for 11 mo^[85]. They found that rosiglitazone only improved steatosis and transaminase levels, and resulted in weight gain (mean gain of 1.5 kg)^[85]. Belfort *et al*^[86] studied the effects of a hypocaloric diet (500 kcal reduction) and 45 mg of pioglitazone per day on 55 NASH patients with impaired glucose tolerance or type 2 diabetes. The results indicated that the diet and pioglitazone improved glycemic control, glucose tolerance, improved liver enzymes and increased hepatic sensitivity^[86]. Conversely, Lutchman *et al*^[87] found that discontinuing TZD therapy resulted in NASH recurrence, indicating that long-term use is necessary for successful treatment. In addition, long-term use of TZDs can result in medical complications such as edema, congestive heart failure, osteoporosis and weight gain^[87,88]. Overall, pioglitazone is used in the medical community as a treatment for NASH, however, careful consideration is needed when prescribing this pharmacological treatment to patients.

Vitamin E is an antioxidant used to treat NAFLD, due to its ability to inhibit oxidative stress. Several studies have been conducted to further analyze the benefits of administering high doses of vitamin E to NASH patients. One notable study is the PIVENS clinical trial, which administered high doses of vitamin E (800 IU/d for 96 d) in non-diabetic patients^[89]. Sanyal *et al*^[89] found a reduction in hepatocellular inflammation, hepatic steatosis and improvements in liver function tests were noted. They concluded that vitamin E is an effective treatment for NASH patients without diabetes^[89]. Harrison *et al*^[90] also investigated the effects of a combination of vitamin E (1000 IU/d) and vitamin C (1000 mg/d) on liver histology in 45 diagnosed NASH patients over a 6-mo period. Their findings were that vitamins E and C were effective in improving fibrosis scores, though no improvements in

inflammation or liver function tests were noted^[90]. Caution needs to be taken when prescribing vitamin E as studies have shown that there is a potential harm for patients. A meta-analysis of 135967 people taking 400 IU/d of vitamin E found that there is an increase of all-cause mortality and therefore its use should be avoided^[91]. In addition, a study conducted by Klein *et al*^[92] studied the long-term effects of vitamin E (400 IU/d)^[92]. The study found that vitamin E supplementation significantly increases the risk of developing prostate cancer in healthy men^[92]. Overall, caution needs to be taken when prescribing vitamin E, especially to diabetic NASH patients, as there is no research to support vitamin E at this time for this population.

TZDs and vitamin E medical treatment need to be carefully considered when developing a treatment plan for NAFLD/NASH patients.

Other therapies

Due to the rise in NAFLD cases, as well as other compounding diseases, additional therapies have been investigated and used in clinical practice, such as ursodeoxycholic acid (UDCA), omega-3 polyunsaturated fatty acids (N-3 PUFA), statins and pre and probiotics. These therapies target risk factors of NAFLD, such as obesity, dyslipidemia, cardiovascular disease, insulin resistance and IM.

UDCA has been studied in clinical trials to determine its effectiveness on the NAFLD population. UDCA is a naturally occurring secondary bile acid that has been used in clinical trials to determine its effectiveness for treatment of patients with NAFLD/NASH^[93]. A randomized double blind study investigated using UDCA (10 mg/kg per day) in obese NAFLD patients over a 3 mo period^[94]. The results showed that UDCA was able to reduce liver enzymes, though there was no effect on liver fat content^[94]. Lindor *et al*^[93] conducted a large randomized trial using UDCA (receiving between 13-15 mg/kg per day) on NASH diagnosed patients and found that there was no significant differences between the placebo and UDCA groups. Therefore, UDCA is not recommend for the treatment of NAFLD.

N-3 PUFAs have been used in the treatment of hyperlipidemia and cardiovascular disease, and more recently in the treatment of NAFLD^[95]. Studies have highlighted the correlation between insulin resistance and changes in fatty acids, specifically a deficiency in n-3 PUFA^[96]. As a result Capanni *et al*^[96] investigated the effects of N-3 PUFA supplementation (1 g/d for 12 mo) in 56 NAFLD patients. Their results indicated that n-3 PUFA improves biochemical aspects of NAFLD as well as liver steatosis^[96]. Similarly, a literature review conducted by Masterton *et al*^[95], found that in animal studies N-3 PUFA reduced hepatic steatosis, improved insulin sensitivity and biochemical markers of inflammation; human studies yielded similar results. Masterton *et al*^[95] and Capanni *et al*^[96] concluded that N-3 PUFA is a promising therapeutic approach to the treatment of NAFLD.

Statins are used in the medical field to manage dyslip-

idemia; and are typically used in patients with cardiovascular disease. NAFLD patients often have dyslipidemia along with other features of metabolic syndrome^[97]. Several studies have shown that statin use in NAFLD patients with dyslipidemia can improve liver function tests^[97-99] as well as steatosis^[100]. In addition, these studies have determined that the use of statins producing liver injury is rare^[97], and that statins are safe to use in NAFLD/NASH patients with dyslipidemia^[98,101]. However, there is a lack of evidence to use statins to treat NASH patients without dyslipidemia^[12]. Therefore statin use should be considered for NAFLD/NASH patients with dyslipidemia, but at this time, should not be used for the specific treatment of NAFLD/NASH.

IM has been shown to be beneficial to human health. Research has found that IM regulates energy homeostasis and ectopic fat deposition^[102], which has been related to metabolic diseases. NAFLD is associated with metabolic syndrome, and therefore has been the focus of recent pre-probiotic research.

Prebiotics are non-digestible carbohydrates that stimulate growth and activity on bacteria in the colon^[103]. The majority of research has been conducted using mice-models; however there have been a limited number of human clinical trials. The majority of studies have investigated risk factors associated with NAFLD. Parnell *et al*^[103] conducted a randomized double-blind, placebo-controlled trial to examine the effects of oligofructose (21 g/d for 12 wk) in 48 overweight and obese adults. The results found that oligofructose promoted weight loss and improved glucose regulation^[103]. Daubioul *et al*^[104] also used oligofructose (16 g/d for 8 wk) in a randomized double-blind crossover study and investigated the effects of oligofructose on glucose and lipid metabolism in 7 NASH patients. Compared to the placebo, AST and ALT decreased after 8 wk and insulin levels after 4 wk, supporting the use of prebiotics in management of liver disease^[104]. There is a need for studies to specifically evaluate the use of prebiotics in NAFLD patients with histological end points.

Probiotics (live microorganisms) have been found to improve liver enzymes and liver histology in NAFLD patients^[105]. An open pilot study conducted by Loguerio *et al*^[106] used probiotic VSL#3 (containing 450 billion bacteria in different strains) for 3 mo. This study had 78 participants, 22 that were biopsy proven NAFLD. In the NAFLD group, plasma levels and lipid peroxidation markers (malondialdehyde and 4-hydroxynonenal) improved^[106]. Another study using the same probiotic found that VSL#3 had no beneficial effect on liver disease^[107]. Solga *et al*^[107] studied the effect of VSL#3 on 4 NAFLD adult subjects in an open pilot study over a 4-mo period. All 4 subjects had a significant increase in liver fat, and no significant differences in biochemical or clinical parameters^[107]. As the researchers highlighted, the small sample size is an important limitation to consider^[107]. Another study using a randomized double blind clinical trial evaluated the effects of a different probiotic^[108]. This study evaluated the effects of *Lactobacillus bulgaricus* and

Streptococcus thermophilus (1 tablet/d) in 28 NAFLD patients over a 3-mo period^[108]. The results were that ALT, AST and gamma-glutamyl transferase levels decreased^[108].

Pre and probiotics have been proven to be useful in the NAFLD population. However, there is a need for larger longitudinal clinical trials to be able to determine the optimal dose and pre and probiotic composition.

Bariatric surgery

Obesity is on the rise in today's society, which is taking a toll on today's healthcare system. Obesity is associated with metabolic syndrome, cardiovascular disease, insulin resistance and type 2 diabetes resulting in an increased risk of individuals developing NAFLD. NAFLD is very common in the morbidly obese population; in fact the prevalence of NAFLD in this population is between 75%-100%^[109]. Bariatric surgery induces weight loss by reducing the size of a patient's stomach by either removing a portion of the stomach, using a gastric band, or by gastric bypass^[110], and is considered in patients who have a BMI greater than 40 kg/m² or with a BMI of 35 kg/m² who have obesity related comorbidities^[111]. Prospective and retrospective studies have found that bariatric surgery improved insulin resistance, steatosis and inflammation^[112]. Moschen *et al*^[113] prospectively found that weight loss after bariatric surgery improved insulin resistance, liver function tests and histology in 18 NAFLD patients. Similarly, the prospective study by Furuya *et al*^[114] found that significant weight loss two years post-bariatric surgery significantly improved steatosis and fibrosis in 18 patients with NAFLD. However, a recent Cochrane review concluded that there is insufficient data due to a lack of well-designed randomized control study trials to determine if bariatric surgery is an effective treatment for NAFLD^[115]. Overall, the usefulness of bariatric surgery as a treatment for NAFLD, particularly for inflammation and fibrosis, is not clear and future well designed studies need to be conducted.

CONCLUSION

The increase in NAFLD has and will continue to burden the health care system, especially because of its ties to obesity, IR and metabolic syndrome. Currently, the understanding of its epidemiology and pathogenesis are well understood, guidelines for proper care are constantly changing as new information emerges, but still NAFLD remains a complex multifaceted issue (Figure 2). The development of non-invasive measures to assess inflammation and fibrosis are commonly used in practice, with liver biopsy being used only in specific cases or within research protocol. Addressing the risk factors associated with NAFLD, such as IR, weight loss and lipid levels remain the primary way to improve NAFLD. However, bariatric surgery, insulin sensitizing agents, antioxidants and fish oil, may also be considered although further research is necessary to clearly document the effect.

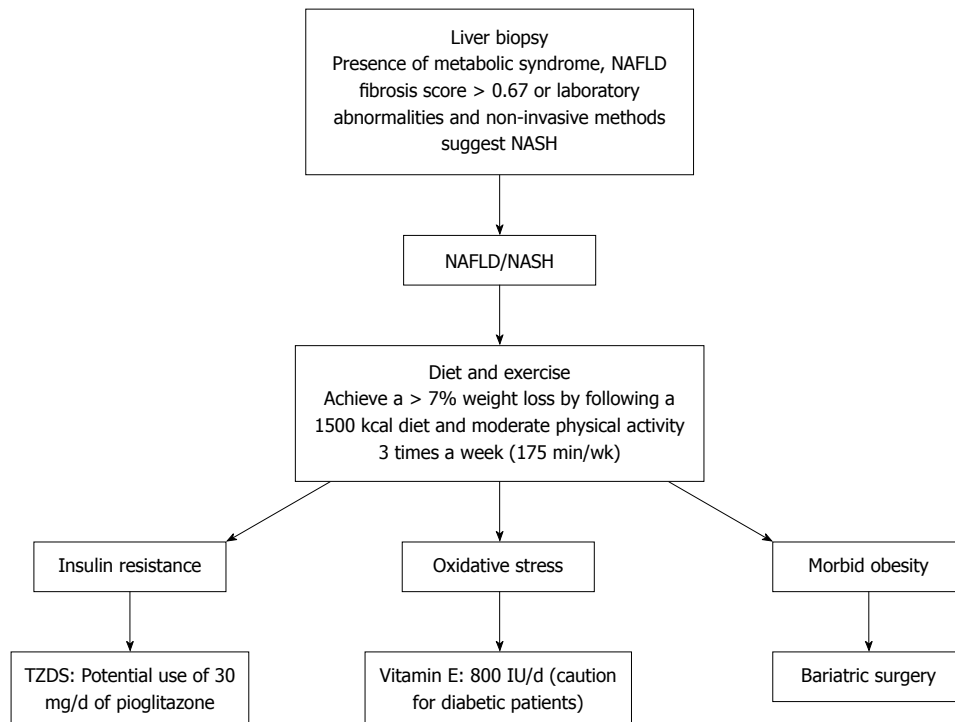


Figure 2 Suggested diagnosing and treatment of non-alcoholic fatty liver disease. NAFLD: Non-alcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

Currently, weight loss and lifestyle modification should be used as first line therapy. In addition, cardiovascular disease needs to be investigated and treated as this increases in NAFLD. Future studies need to have larger high-quality clinical trials with rigorous methodology in order to establish standards of care.

REFERENCES

- 1 **Clark JM**, Diehl AM. Hepatic steatosis and type 2 diabetes mellitus. *Curr Diab Rep* 2002; **2**: 210-215 [PMID: 12643175]
- 2 **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]
- 3 **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]
- 4 **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. *Aliment Pharmacol Ther* 2011; **34**: 274-285 [PMID: 21623852 DOI: 10.1111/j.1365-2036.2011.04724.x]
- 5 **Machado M**, Marques-Vidal P, Cortez-Pinto H. Hepatic histology in obese patients undergoing bariatric surgery. *J Hepatol* 2006; **45**: 600-606 [PMID: 16899321 DOI: 10.1016/j.jhep.2006.06.013]
- 6 **Targher G**, Bertolini L, Padovani R, Rodella S, Tessari R, Zenari L, Day C, Arcaro G. Prevalence of nonalcoholic fatty liver disease and its association with cardiovascular disease among type 2 diabetic patients. *Diabetes Care* 2007; **30**: 1212-1218 [PMID: 17277038 DOI: 10.2337/dc06-2247]
- 7 **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]
- 8 **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]
- 9 **van der Poorten D**, Milner KL, Hui J, Hodge A, Trenell MI, Kench JG, London R, Peduto T, Chisholm DJ, George J. Visceral fat: a key mediator of steatohepatitis in metabolic liver disease. *Hepatology* 2008; **48**: 449-457 [PMID: 18627003 DOI: 10.1002/hep.22350]
- 10 **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]
- 11 **Angulo P**. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231 [PMID: 11961152 DOI: 10.1056/NEJMra011775]
- 12 **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]
- 13 **Lewis JR**, Mohanty SR. Nonalcoholic fatty liver disease: a review and update. *Dig Dis Sci* 2010; **55**: 560-578 [PMID: 20101463 DOI: 10.1007/s10620-009-1081-0]
- 14 **Yilmaz Y**, Ulukaya E, Dolar E. A "biomarker biopsy" for the diagnosis of NASH: promises from CK-18 fragments. *Obes Surg* 2008; **18**: 1507-1508; author reply 1509-1510 [PMID: 18679759 DOI: 10.1007/s11695-008-9639-z]
- 15 **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/jci200523621]

- 16 **Dowman JK**, Tomlinson JW, Newsome PN. Pathogenesis of non-alcoholic fatty liver disease. *QJM* 2010; **103**: 71-83 [PMID: 19914930 DOI: 10.1093/qjmed/hcp158]
- 17 **Bugianesi E**, Marchesini G, Gentilcore E, Cua IH, Vanni E, Rizzetto M, George J. Fibrosis in genotype 3 chronic hepatitis C and nonalcoholic fatty liver disease: Role of insulin resistance and hepatic steatosis. *Hepatology* 2006; **44**: 1648-1655 [PMID: 17133473 DOI: 10.1002/hep.21429]
- 18 **Schreuder TC**, Verwer BJ, van Nieuwkerk CM, Mulder CJ. Nonalcoholic fatty liver disease: an overview of current insights in pathogenesis, diagnosis and treatment. *World J Gastroenterol* 2008; **14**: 2474-2486 [PMID: 18442193]
- 19 **Petta S**, Muratore C, Craxi A. Non-alcoholic fatty liver disease pathogenesis: the present and the future. *Dig Liver Dis* 2009; **41**: 615-625 [PMID: 19223251 DOI: 10.1016/j.dld.2009.01.004]
- 20 **Jacobs RL**, Lingrell S, Zhao Y, Francis GA, Vance DE. Hepatic CTP: phosphocholine cytidyltransferase- α is a critical predictor of plasma high density lipoprotein and very low density lipoprotein. *J Biol Chem* 2008; **283**: 2147-2155 [PMID: 18042552 DOI: 10.1074/jbc.M706628200]
- 21 **Cortez-Pinto H**, de Moura MC, Day CP. Non-alcoholic steatohepatitis: from cell biology to clinical practice. *J Hepatol* 2006; **44**: 197-208 [PMID: 16274837 DOI: 10.1016/j.jhep.2005.09.002]
- 22 **Crespo J**, Cayón A, Fernández-Gil P, Hernández-Guerra M, Mayorga M, Domínguez-Díez A, Fernández-Escalante JC, Pons-Romero F. Gene expression of tumor necrosis factor α and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients. *Hepatology* 2001; **34**: 1158-1163 [PMID: 11732005 DOI: 10.1053/jhep.2001.29628]
- 23 **Berg AH**, Combs TP, Scherer PE. ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. *Trends Endocrinol Metab* 2002; **13**: 84-89 [PMID: 11854024]
- 24 **Widhalm K**, Ghods E. Nonalcoholic fatty liver disease: a challenge for pediatricians. *Int J Obes (Lond)* 2010; **34**: 1451-1467 [PMID: 20838401 DOI: 10.1038/ijo.2010.185]
- 25 **Jarrar MH**, Baranova A, Collantes R, Ranard B, Stepanova M, Bennett C, Fang Y, Elariny H, Goodman Z, Chandhoke V, Younossi ZM. Adipokines and cytokines in non-alcoholic fatty liver disease. *Aliment Pharmacol Ther* 2008; **27**: 412-421 [PMID: 18081738 DOI: 10.1111/j.1365-2036.2007.03586.x]
- 26 **Lemoine M**, Ratzin V, Kim M, Maachi M, Wendum D, Paye F, Bastard JP, Poupon R, Housset C, Capeau J, Serfaty L. Serum adipokine levels predictive of liver injury in non-alcoholic fatty liver disease. *Liver Int* 2009; **29**: 1431-1438 [PMID: 19422483 DOI: 10.1111/j.1478-3231.2009.02022.x]
- 27 **Mirza MS**. Obesity, Visceral Fat, and NAFLD: Querying the Role of Adipokines in the Progression of Nonalcoholic Fatty Liver Disease. *ISRN Gastroenterol* 2011; **2011**: 592404 [PMID: 21991518 DOI: 10.5402/2011/592404]
- 28 **Marra F**, Bertolani C. Adipokines in liver diseases. *Hepatology* 2009; **50**: 957-969 [PMID: 19585655 DOI: 10.1002/hep.23046]
- 29 **Musso G**, Gambino R, De Michieli F, Cassader M, Rizzetto M, Durazzo M, Fagà E, Silli B, Pagano G. Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. *Hepatology* 2003; **37**: 909-916 [PMID: 12668986 DOI: 10.1053/jhep.2003.50132]
- 30 **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]
- 31 **Sathiaraj E**, Chutke M, Reddy MY, Pratap N, Rao PN, Reddy DN, Raghunath M. A case-control study on nutritional risk factors in non-alcoholic fatty liver disease in Indian population. *Eur J Clin Nutr* 2011; **65**: 533-537 [PMID: 21346716 DOI: 10.1038/ejcn.2011.3]
- 32 **Solga S**, Alkhuraishe AR, Clark JM, Torbenson M, Greenwald A, Diehl AM, Magnuson T. Dietary composition and nonalcoholic fatty liver disease. *Dig Dis Sci* 2004; **49**: 1578-1583 [PMID: 15573908]
- 33 **Vos MB**, Lavine JE. Dietary fructose in nonalcoholic fatty liver disease. *Hepatology* 2013; **57**: 2525-2531 [PMID: 23390127 DOI: 10.1002/hep.26299]
- 34 **Pollock NK**, Bundy V, Kanto W, Davis CL, Bernard PJ, Zhu H, Gutin B, Dong Y. Greater fructose consumption is associated with cardiometabolic risk markers and visceral adiposity in adolescents. *J Nutr* 2012; **142**: 251-257 [PMID: 22190023 DOI: 10.3945/jn.111.150219]
- 35 **Lê KA**, Faeh D, Stettler R, Ith M, Kreis R, Vermathen P, Boesch C, Ravussin E, Tappy L. A 4-wk high-fructose diet alters lipid metabolism without affecting insulin sensitivity or ectopic lipids in healthy humans. *Am J Clin Nutr* 2006; **84**: 1374-1379 [PMID: 17158419]
- 36 **Mouzaki M**, Comelli EM, Arendt BM, Bonengel J, Fung SK, Fischer SE, McGilvray ID, Allard JP. Intestinal microbiota in patients with nonalcoholic fatty liver disease. *Hepatology* 2013; **58**: 120-127 [PMID: 23401313 DOI: 10.1002/hep.26319]
- 37 **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]
- 38 **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**: 868-875.e1-3 [PMID: 23454028 DOI: 10.1016/j.cgh.2013.02.015]
- 39 **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]
- 40 **Dowman JK**, Tomlinson JW, Newsome PN. Systematic review: the diagnosis and staging of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. *Aliment Pharmacol Ther* 2011; **33**: 525-540 [PMID: 21198708 DOI: 10.1111/j.1365-2036.2010.04556.x]
- 41 **Adams LA**, Feldstein AE. Non-invasive diagnosis of nonalcoholic fatty liver and nonalcoholic steatohepatitis. *J Dig Dis* 2011; **12**: 10-16 [PMID: 21091933 DOI: 10.1111/j.1751-2980.2010.00471.x]
- 42 **Yan E**, Durazo F, Tong M, Hong K. Nonalcoholic fatty liver disease: pathogenesis, identification, progression, and management. *Nutr Rev* 2007; **65**: 376-384 [PMID: 17867371]
- 43 **Pratt DS**, Kaplan MM. Evaluation of abnormal liver-enzyme results in asymptomatic patients. *N Engl J Med* 2000; **342**: 1266-1271 [PMID: 10781624 DOI: 10.1056/NEJM200004273421707]
- 44 **Sorbi D**, Boynton J, Lindor KD. The ratio of aspartate aminotransferase to alanine aminotransferase: potential value in differentiating nonalcoholic steatohepatitis from alcoholic liver disease. *Am J Gastroenterol* 1999; **94**: 1018-1022 [PMID: 10201476 DOI: 10.1111/j.1572-0241.1999.01006.x]
- 45 **Adams LA**, Angulo P, Lindor KD. Nonalcoholic fatty liver disease. *CMAJ* 2005; **172**: 899-905 [PMID: 15795412 DOI: 10.1503/cmaj.045232]
- 46 **Festi D**, Schiumerini R, Marzi L, Di Biase AR, Mandolesi D, Montrone L, Scafoli 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]
- 47 **Federico A**, Trappoliere M, Loguercio C. Treatment of patients with non-alcoholic fatty liver disease: current views and perspectives. *Dig Liver Dis* 2006; **38**: 789-801 [PMID: 16750661 DOI: 10.1016/j.dld.2006.04.009]

- 48 **Pacifico L**, Celestre M, Anania C, Paolantonio P, Chiesa C, Laghi A. MRI and ultrasound for hepatic fat quantification: relationships to clinical and metabolic characteristics of pediatric nonalcoholic fatty liver disease. *Acta Paediatr* 2007; **96**: 542-547 [PMID: 17306008 DOI: 10.1111/j.1651-2227.2007.00186.x]
- 49 **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]
- 50 **Jiang ZY**, Xu CY, Chang XX, Li WW, Sun LY, Yang XB, Yu LF. Fatty liver index correlates with non-alcoholic fatty liver disease, but not with newly diagnosed coronary artery atherosclerotic disease in Chinese patients. *BMC Gastroenterol* 2013; **13**: 110 [PMID: 23834773 DOI: 10.1186/1471-230X-13-110]
- 51 **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]
- 52 **Cortez-Pinto H**, Camilo ME. Non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD/NASH): diagnosis and clinical course. *Best Pract Res Clin Gastroenterol* 2004; **18**: 1089-1104 [PMID: 15561640 DOI: 10.1016/j.bpg.2004.06.021]
- 53 **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, Thorneau 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]
- 54 **Treeprasertsuk S**, Björnsson E, Enders F, Suwanwalaikorn S, Lindor KD. NAFLD fibrosis score: a prognostic predictor for mortality and liver complications among NAFLD patients. *World J Gastroenterol* 2013; **19**: 1219-1229 [PMID: 23482703 DOI: 10.3748/wjg.v19.i8.1219]
- 55 **Qureshi K**, Clements RH, Abrams GA. The utility of the "NAFLD fibrosis score" in morbidly obese subjects with NAFLD. *Obes Surg* 2008; **18**: 264-270 [PMID: 18214632 DOI: 10.1007/s11695-007-9295-8]
- 56 **Calès P**, Lainé F, Boursier J, Deugnier Y, Moal V, Oberti F, Hunault G, Rousselet MC, Hubert I, Laafi J, Ducluzeaux PH, Lunel F. Comparison of blood tests for liver fibrosis specific or not to NAFLD. *J Hepatol* 2009; **50**: 165-173 [PMID: 18977552 DOI: 10.1016/j.jhep.2008.07.035]
- 57 **Wong VW**, Vergniol J, Wong GL, Foucher J, Chan HL, Le Bail B, Choi PC, Kow M, Chan AW, Merrouche W, Sung JJ, de Lédinghen V. Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. *Hepatology* 2010; **51**: 454-462 [PMID: 20101745 DOI: 10.1002/hep.23312]
- 58 **Wu SD**, Wang JY, Li L. Staging of liver fibrosis in chronic hepatitis B patients with a composite predictive model: a comparative study. *World J Gastroenterol* 2010; **16**: 501-507 [PMID: 20101779 DOI: 10.3748/wjg.v16.i4.501]
- 59 **Yoneda M**, Yoneda M, Mawatari H, Fujita K, Endo H, Iida H, Nozaki Y, Yonemitsu K, Higurashi T, Takahashi H, Kobayashi N, Kirikoshi H, Abe Y, Inamori M, Kubota K, Saito S, Tamano M, Hiraishi H, Maeyama S, Yamaguchi N, Togo S, Nakajima A. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with nonalcoholic fatty liver disease (NAFLD). *Dig Liver Dis* 2008; **40**: 371-378 [PMID: 18083083 DOI: 10.1016/j.dld.2007.10.019]
- 60 **Ziol M**, Handra-Luca A, Kettaneh A, Christidis C, Mal F, Kazemi F, de Lédinghen V, Marcellin P, Dhumeaux D, Trinchet JC, Beaugrand M. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. *Hepatology* 2005; **41**: 48-54 [PMID: 15690481 DOI: 10.1002/hep.20506]
- 61 **Yoneda M**, Yoneda M, Fujita K, Inamori M, Tamano M, Hiriishi H, Nakajima A. Transient elastography in patients with non-alcoholic fatty liver disease (NAFLD). *Gut* 2007; **56**: 1330-1331 [PMID: 17470477 DOI: 10.1136/gut.2007.126417]
- 62 **Sandrin L**, Tanter M, Gennisson JL, Catheline S, Fink M. Shear elasticity probe for soft tissues with 1-D transient elastography. *IEEE Trans Ultrason Ferroelectr Freq Control* 2002; **49**: 436-446 [PMID: 11989699]
- 63 **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]
- 64 **de Lédinghen V**, Wong VW, Vergniol J, Wong GL, Foucher J, Chu SH, Le Bail B, Choi PC, Chermak F, Yiu KK, Merrouche W, Chan HL. Diagnosis of liver fibrosis and cirrhosis using liver stiffness measurement: comparison between M and XL probe of FibroScan®. *J Hepatol* 2012; **56**: 833-839 [PMID: 22173167 DOI: 10.1016/j.jhep.2011.10.017]
- 65 **Adams LA**, Feldstein AE. Nonalcoholic steatohepatitis: risk factors and diagnosis. *Expert Rev Gastroenterol Hepatol* 2010; **4**: 623-635 [PMID: 20932147 DOI: 10.1586/egh.10.56]
- 66 **McCarthy EM**, Rinella ME. The role of diet and nutrient composition in nonalcoholic Fatty liver disease. *J Acad Nutr Diet* 2012; **112**: 401-409 [PMID: 22717200 DOI: 10.1016/j.jada.2011.10.007]
- 67 **Capristo E**, Miele L, Forgione A, Vero V, Farnetti S, Mingrone G, Greco AV, Gasbarrini G, Grieco A. Nutritional aspects in patients with non-alcoholic steatohepatitis (NASH). *Eur Rev Med Pharmacol Sci* 2005; **9**: 265-268 [PMID: 16231587]
- 68 **Promrat K**, Kleiner DE, Niemeier HM, Jackvony E, Kearns M, Wands JR, Fava JL, Wing RR. Randomized controlled trial testing the effects of weight loss on nonalcoholic steatohepatitis. *Hepatology* 2010; **51**: 121-129 [PMID: 19827166 DOI: 10.1002/hep.23276]
- 69 **Thoma C**, Day CP, Trenell MI. Lifestyle interventions for the treatment of non-alcoholic fatty liver disease in adults: a systematic review. *J Hepatol* 2012; **56**: 255-266 [PMID: 21723839 DOI: 10.1016/j.jhep.2011.06.010]
- 70 **Zelber-Sagi S**, Ratzin 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]
- 71 **Lazo M**, Solga SF, Horska A, Bonekamp S, Diehl AM, Brancati FL, Wagenknecht LE, Pi-Sunyer FX, Kahn SE, Clark JM. Effect of a 12-month intensive lifestyle intervention on hepatic steatosis in adults with type 2 diabetes. *Diabetes Care* 2010; **33**: 2156-2163 [PMID: 20664019 DOI: 10.2337/dc10-0856]
- 72 **Kantartzis K**, Thamer C, Peter A, Machann J, Schick F, Schraml C, Königsrainer A, Königsrainer I, Kröber S, Niess A, Fritsche A, Häring HU, Stefan N. High cardiorespiratory fitness is an independent predictor of the reduction in liver fat during a lifestyle intervention in non-alcoholic fatty liver disease. *Gut* 2009; **58**: 1281-1288 [PMID: 19074179 DOI: 10.1136/gut.2008.151977]
- 73 **St George A**, Bauman A, Johnston A, Farrell G, Chey T, George J. Effect of a lifestyle intervention in patients with abnormal liver enzymes and metabolic risk factors. *J Gastroenterol Hepatol* 2009; **24**: 399-407 [PMID: 19067776 DOI: 10.1111/j.1440-1746.2008.05694.x]
- 74 **Hallsworth K**, Fattakhova G, Hollingsworth KG, Thoma C, Moore S, Taylor R, Day CP, Trenell MI. Resistance exercise reduces liver fat and its mediators in non-alcoholic fatty liver disease independent of weight loss. *Gut* 2011; **60**: 1278-1283 [PMID: 21708823 DOI: 10.1136/gut.2011.242073]
- 75 **Attar BM**, Van Thiel DH. Current concepts and management approaches in nonalcoholic fatty liver disease. *Scien-*

- tificWorldJournal 2013; **2013**: 481893 [PMID: 23576902 DOI: 10.1155/2013/481893]
- 76 **Zhou G**, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 2001; **108**: 1167-1174 [PMID: 11602624 DOI: 10.1172/JCI13505]
 - 77 **Stumvoll M**, Nurjhan N, Perriello G, Dailey G, Gerich JE. Metabolic effects of metformin in non-insulin-dependent diabetes mellitus. *N Engl J Med* 1995; **333**: 550-554 [PMID: 7623903 DOI: 10.1056/NEJM199508313330903]
 - 78 **Mazza A**, Fruci B, Garinis GA, Giuliano S, Malaguarnera R, Belfiore A. The role of metformin in the management of NAFLD. *Exp Diabetes Res* 2012; **2012**: 716404 [PMID: 22194737 DOI: 10.1155/2012/716404]
 - 79 **Nair S**, Diehl AM, Wiseman M, Farr GH, Perrillo RP. Metformin in the treatment of non-alcoholic steatohepatitis: a pilot open label trial. *Aliment Pharmacol Ther* 2004; **20**: 23-28 [PMID: 15225167 DOI: 10.1111/j.1365-2036.2004.02025.x]
 - 80 **Nobili V**, Manco M, Ciampalini P, Alisi A, Devito R, Bugianesi E, Marcellini M, Marchesini G. Metformin use in children with nonalcoholic fatty liver disease: an open-label, 24-month, observational pilot study. *Clin Ther* 2008; **30**: 1168-1176 [PMID: 18640473 DOI: 10.1016/j.clinthera.2008.06.012]
 - 81 **Haukeland JW**, Konopski Z, Eggesbø HB, von Volkmann HL, Raschpichler G, Bjørø K, Haaland T, Løberg EM, Birke-land K. Metformin in patients with non-alcoholic fatty liver disease: a randomized, controlled trial. *Scand J Gastroenterol* 2009; **44**: 853-860 [PMID: 19811343 DOI: 10.1080/00365520902845268]
 - 82 **Oh MK**, Winn J, Poordad F. Review article: diagnosis and treatment of non-alcoholic fatty liver disease. *Aliment Pharmacol Ther* 2008; **28**: 503-522 [PMID: 18532991 DOI: 10.1111/j.1365-2036.2008.03752.x]
 - 83 **Van Wagner LB**, Rinella ME. The role of insulin-sensitizing agents in the treatment of nonalcoholic steatohepatitis. *Therap Adv Gastroenterol* 2011; **4**: 249-263 [PMID: 21765869 DOI: 10.1177/1756283X114403809]
 - 84 **Caldwell SH**, Argo CK, Al-Osaimi AM. Therapy of NAFLD: insulin sensitizing agents. *J Clin Gastroenterol* 2006; **40** Suppl 1: S61-S66 [PMID: 16540770]
 - 85 **Ratzu V**, Giral P, Jacqueminet S, Charlotte F, Hartemann-Heurtier A, Serfaty L, Povedin P, Lacorte JM, Bernhardt C, Bruckert E, Grimaldi A, Poynard T. Rosiglitazone for nonalcoholic steatohepatitis: one-year results of the randomized placebo-controlled Fatty Liver Improvement with Rosiglitazone Therapy (FLIRT) Trial. *Gastroenterology* 2008; **135**: 100-110 [PMID: 18503774 DOI: 10.1053/j.gastro.2008.03.078]
 - 86 **Belfort R**, Harrison SA, Brown K, Darland C, Finch J, Hardies J, Balas B, Gastaldelli A, Tio F, Pulcini J, Berria R, Ma JZ, Dwivedi S, Havranek R, Fincke C, DeFronzo R, Bannayan GA, Schenker S, Cusi K. A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. *N Engl J Med* 2006; **355**: 2297-2307 [PMID: 17135584 DOI: 10.1056/NEJMoa060326]
 - 87 **Lutchman G**, Modi A, Kleiner DE, Promrat K, Heller T, Ghany M, Borg B, Loomba R, Liang TJ, Premkumar A, Hoofnagle JH. The effects of discontinuing pioglitazone in patients with nonalcoholic steatohepatitis. *Hepatology* 2007; **46**: 424-429 [PMID: 17559148 DOI: 10.1002/hep.21661]
 - 88 **Aithal GP**, Thomas JA, Kaye PV, Lawson A, Ryder SD, Spendlove I, Austin AS, Freeman JG, Morgan L, Webber J. Randomized, placebo-controlled trial of pioglitazone in nondiabetic subjects with nonalcoholic steatohepatitis. *Gastroenterology* 2008; **135**: 1176-1184 [PMID: 18718471 DOI: 10.1053/j.gastro.2008.06.047]
 - 89 **Sanyal AJ**, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, Neuschwander-Tetri BA, Lavine JE, Tonascia J, Unalp A, Van Natta M, Clark J, Brunt EM, Kleiner DE, Hoofnagle JH, Robuck PR. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 2010; **362**: 1675-1685 [PMID: 20427778 DOI: 10.1056/NEJMoa0907929]
 - 90 **Harrison SA**, Torgerson S, Hayashi P, Ward J, Schenker S. Vitamin E and vitamin C treatment improves fibrosis in patients with nonalcoholic steatohepatitis. *Am J Gastroenterol* 2003; **98**: 2485-2490 [PMID: 14638353 DOI: 10.1111/j.1572-0241.2003.08699.x]
 - 91 **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]
 - 92 **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]
 - 93 **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 DOI: 10.1002/hep.20092]
 - 94 **Santos VN**, Lanzoni VP, Szejnfeld J, Shigueoka D, Parise ER. A randomized double-blind study of the short-time treatment of obese patients with nonalcoholic fatty liver disease with ursodeoxycholic acid. *Braz J Med Biol Res* 2003; **36**: 723-729 [PMID: 12792701]
 - 95 **Masterton GS**, Plevris JN, Hayes PC. Review article: omega-3 fatty acids - a promising novel therapy for non-alcoholic fatty liver disease. *Aliment Pharmacol Ther* 2010; **31**: 679-692 [PMID: 20415840 DOI: 10.1111/j.1365-2036.2010.04230.x]
 - 96 **Capanni M**, Calella F, Biagini MR, Genise S, Raimondi L, Bedogni G, Svegliati-Baroni G, Sofi F, Milani S, Abbate R, Surrenti C, Casini A. Prolonged n-3 polyunsaturated fatty acid supplementation ameliorates hepatic steatosis in patients with non-alcoholic fatty liver disease: a pilot study. *Aliment Pharmacol Ther* 2006; **23**: 1143-1151 [PMID: 16611275 DOI: 10.1111/j.1365-2036.2006.02885.x]
 - 97 **Chatrath H**, Vuppalanchi R, Chalasani N. Dyslipidemia in patients with nonalcoholic fatty liver disease. *Semin Liver Dis* 2012; **32**: 22-29 [PMID: 22418885 DOI: 10.1055/s-0032-1306423]
 - 98 **Athyros VG**, Mikhailidis DP, Papageorgiou AA, Symeonidis AN, Pehlivanidis AN, Bouloukos VI, Elisaf M. The effect of statins versus untreated dyslipidaemia on renal function in patients with coronary heart disease. A subgroup analysis of the Greek atorvastatin and coronary heart disease evaluation (GREACE) study. *J Clin Pathol* 2004; **57**: 728-734 [PMID: 15220366 DOI: 10.1136/jcp.2003.012989]
 - 99 **Maroni L**, Guasti L, Castiglioni L, Marino F, Contini S, Macchi V, De Leo A, Gaudio G, Tozzi M, Grandi AM, Cosentino M, Venco A. Lipid targets during statin treatment in dyslipidemic patients affected by nonalcoholic fatty liver disease. *Am J Med Sci* 2011; **342**: 383-387 [PMID: 21629037 DOI: 10.1097/MAJ.0b013e318213e526]
 - 100 **Ekstedt M**, Franzén LE, Mathiesen UL, Holmqvist M, Bode-mar G, Kechagias S. Statins in non-alcoholic fatty liver disease and chronically elevated liver enzymes: a histopathological follow-up study. *J Hepatol* 2007; **47**: 135-141 [PMID: 17400325 DOI: 10.1016/j.jhep.2007.02.013]
 - 101 **Tandra S**, Vuppalanchi R. Use of statins in patients with liver disease. *Curr Treat Options Cardiovasc Med* 2009; **11**: 272-278 [PMID: 19627660]
 - 102 **Iacono A**, Raso GM, Canani RB, Calignano A, Meli R. Probiotics as an emerging therapeutic strategy to treat NAFLD: focus on molecular and biochemical mechanisms. *J Nutr Biochem* 2011; **22**: 699-711 [PMID: 21292470 DOI: 10.1016/j.jnutbio.2010.10.002]

- 103 **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]
- 104 **Dauboul CA**, Horsmans Y, Lambert P, Danse E, Delzenne NM. Effects of oligofructose on glucose and lipid metabolism in patients with nonalcoholic steatohepatitis: results of a pilot study. *Eur J Clin Nutr* 2005; **59**: 723-726 [PMID: 15770222 DOI: 10.1038/sj.ejcn.1602127]
- 105 **Li Z**, Yang S, Lin H, Huang J, Watkins PA, Moser AB, Desimone C, Song XY, Diehl AM. Probiotics and antibodies to TNF inhibit inflammatory activity and improve nonalcoholic fatty liver disease. *Hepatology* 2003; **37**: 343-350 [PMID: 12540784 DOI: 10.1053/jhep.2003.50048]
- 106 **Loguercio C**, Federico A, Tuccillo C, Terracciano F, D'Auria MV, De Simone C, Del Vecchio Blanco C. Beneficial effects of a probiotic VSL#3 on parameters of liver dysfunction in chronic liver diseases. *J Clin Gastroenterol* 2005; **39**: 540-543 [PMID: 15942443]
- 107 **Solga SF**, Buckley G, Clark JM, Horska A, Diehl AM. The effect of a probiotic on hepatic steatosis. *J Clin Gastroenterol* 2008; **42**: 1117-1119 [PMID: 18936646 DOI: 10.1097/MCG.0b013e31816d920c]
- 108 **Aller R**, De Luis DA, Izaola O, Conde R, Gonzalez Sagrado M, Primo D, De La Fuente B, Gonzalez J. Effect of a probiotic on liver aminotransferases in nonalcoholic fatty liver disease patients: a double blind randomized clinical trial. *Eur Rev Med Pharmacol Sci* 2011; **15**: 1090-1095 [PMID: 22013734]
- 109 **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]
- 110 **Cotrim HP**, Daltro C. Liver: Does bariatric surgery reduce the severity of NAFLD? *Nat Rev Gastroenterol Hepatol* 2010; **7**: 11-13 [PMID: 20051969 DOI: 10.1038/nrgastro.2009.215]
- 111 NIH conference. Gastrointestinal surgery for severe obesity. Consensus Development Conference Panel. *Ann Intern Med* 1991; **115**: 956-961 [PMID: 1952493]
- 112 **Baker MK**, Byrne TK, Feldmann ME. Surgical treatment of obesity. *Prim Care* 2009; **36**: 417-427 [PMID: 19501252 DOI: 10.1016/j.pop.2009.01.001]
- 113 **Moschen AR**, Molnar C, Wolf AM, Weiss H, Graziadei I, Kaser S, Ebenbichler CF, Stadlmann S, Moser PL, Tilg H. Effects of weight loss induced by bariatric surgery on hepatic adipocytokine expression. *J Hepatol* 2009; **51**: 765-777 [PMID: 19664840 DOI: 10.1016/j.jhep.2009.06.016]
- 114 **Furuya CK**, de Oliveira CP, de Mello ES, Faintuch J, Raszkowski A, Matsuda M, Vezozzo DC, Halpern A, Garrido AB, Alves VA, Carrilho FJ. Effects of bariatric surgery on nonalcoholic fatty liver disease: preliminary findings after 2 years. *J Gastroenterol Hepatol* 2007; **22**: 510-514 [PMID: 17376042 DOI: 10.1111/j.1440-1746.2007.04833.x]
- 115 **Chavez-Tapia NC**, Tellez-Avila FI, Barrientos-Gutierrez T, Mendez-Sanchez N, Lizardi-Cervera J, Uribe M. Bariatric surgery for non-alcoholic steatohepatitis in obese patients. *Cochrane Database Syst Rev* 2010; (1): CD007340 [PMID: 20091629 DOI: 10.1002/14651858.CD007340.pub2]

P- Reviewers: Chiu KW, Das UN, Williams GM **S- Editor:** Qi Y
L- Editor: A **E- Editor:** Ma S



WJG 20th Anniversary Special Issues (12): Fatty liver

Risk of cardiovascular, cardiac and arrhythmic complications in patients with non-alcoholic fatty liver disease

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Supported by (in part) the Southampton National Institute for Health Research Biomedical Research Centre (Byrne CD); grants from the School of Medicine of the Verona University (Targher GT)

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Received: September 30, 2013 Revised: October 30, 2013

Accepted: November 18, 2013

Published online: February 21, 2014

ventricular dysfunction and hypertrophy, and heart failure), valvular heart disease (*e.g.*, aortic valve sclerosis) and arrhythmias (*e.g.*, atrial fibrillation). Experimental evidence suggests that NAFLD itself, especially in its more severe forms, exacerbates systemic/hepatic insulin resistance, causes atherogenic dyslipidemia, and releases a variety of pro-inflammatory, pro-coagulant and pro-fibrogenic mediators that may play important roles in the pathophysiology of cardiac and arrhythmic complications. Collectively, these findings suggest that patients with NAFLD may benefit from more intensive surveillance and early treatment interventions to decrease the risk for CHD and other cardiac/arrhythmic complications. The purpose of this clinical review is to summarize the rapidly expanding body of evidence that supports a strong association between NAFLD and cardiovascular, cardiac and arrhythmic complications, to briefly examine the putative biological mechanisms underlying this association, and to discuss some of the current treatment options that may influence both NAFLD and its related cardiac and arrhythmic complications.

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Key words: Non-alcoholic fatty liver disease; Cardiovascular disease; Cardiac complications; Coronary heart disease; Myocardial dysfunction; Valvular heart disease; Arrhythmias; Arrhythmic complications

Abstract

Non-alcoholic fatty liver disease (NAFLD) has emerged as a public health problem of epidemic proportions worldwide. Accumulating clinical and epidemiological evidence indicates that NAFLD is not only associated with liver-related morbidity and mortality but also with an increased risk of coronary heart disease (CHD), abnormalities of cardiac function and structure (*e.g.*, left

Core tip: The purpose of this clinical review is to summarize the rapidly expanding body of evidence that supports a strong association between Nonalcoholic fatty liver disease (NAFLD) and cardiovascular, cardiac and arrhythmic complications, to briefly examine the putative biological mechanisms underlying this association, and to discuss some of the current treatment options that may influence both NAFLD and its related cardiac and arrhythmogenic complications.

Ballestri S, Lonardo A, Bonapace S, Byrne CD, Loria P, Targher G. Risk of cardiovascular, cardiac and arrhythmic complications in patients with non-alcoholic fatty liver disease. *World J Gastroenterol* 2014; 20(7): 1724-1745 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1724.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1724>

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a complex health condition with implications far beyond the liver. Over the last decade, it has been shown that the clinical burden of NAFLD is not only confined to liver-related morbidity or mortality, and the majority of deaths among these patients are due to malignancy, coronary heart disease (CHD) and other cardiovascular (CVD) complications.

Although the anecdotal concurrence of peripheral atherosclerosis and atrial fibrillation (AF) in a patient with diabetes and hepatic steatosis dates back to the early 50's^[1], the traditional paradigm of liver disease protecting against the development of CVD has only been recently challenged. The experimental observations that an atherogenic diet causes hepatic steatosis and gallstones in mice^[2], and the pioneering clinical studies showing that NAFLD is a possible contributor to accelerated atherogenesis^[3-6] suggested that either the relationship between NAFLD and CVD is bidirectional or both diseases result from a common pathogenic ancestor. More recent work has identified NAFLD as a risk factor not only for premature CHD and CVD events, but also for early abnormalities in myocardial structure and function^[7,8]. The finding that NAFLD is associated with an increased risk of AF in people without evidence of co-existing valvular heart disease^[9,10] supports the assertion that NAFLD may also be an emerging risk factor for cardiac arrhythmias.

In this clinical review, we will discuss the clinical evidence linking NAFLD to an increased risk of structural and arrhythmogenic cardiac complications. We will also briefly review the putative biological mechanisms linking NAFLD to the development and progression of such complications, and discuss some of the current treatment options that may influence both NAFLD and its related structural and arrhythmogenic cardiac complications. The potential adverse impact of NAFLD on these complications deserves particular attention, especially with respect to screening and surveillance strategies for the growing number of patients with NAFLD.

Review criteria and evidence acquisition: this is a clinical, narrative review and not a systematic review and meta-analysis. PubMed was extensively searched for articles using the keywords “non-alcoholic fatty liver disease” or “fatty liver” combined with “cardiovascular disease”, “cardiovascular risk”, “cardiovascular mortality”, “cardiac complications”, “coronary heart disease”, “congestive heart failure”, “myocardial dysfunction”, “valvular heart disease”, “atrial fibrillation” or “cardiac arrhythmias”

between 1990 and 2013. Articles published in languages other than English were excluded from the analysis.

CLINICAL EVIDENCE LINKING NAFLD TO RISK OF STRUCTURAL AND ARRHYTHMOGENIC CARDIAC COMPLICATIONS

NAFLD and risk of CHD

Over the last decade, the prognostic value of NAFLD as a risk factor for the development and progression of CHD has attracted considerable scientific interest. To date, there is a large body of clinical and epidemiological evidence supporting the assertion that NAFLD is strongly associated with an increased prevalence and incidence of CHD^[11-16].

Subclinical and clinical CHD

Subclinical CHD: Abundant epidemiological data link NAFLD with markers of subclinical atherosclerosis (*i.e.*, endothelial dysfunction, increased arterial stiffness, increased carotid intima-media thickness, elevated coronary calcium score) both in adults and in adolescents^[12-14].

Some investigators have reported that NAFLD is associated with circulatory endothelial dysfunction, independently of obesity, hypertension and other established CVD risk factors^[17-19]. A systematic review and meta-analysis of seven cross-sectional studies (involving a total of 3497 subjects) showed that NAFLD diagnosed on ultrasonography is strongly associated with increased carotid-artery intimal medial thickness and an increased prevalence of carotid atherosclerotic plaques^[5]. Interestingly, two studies also found a positive, graded relationship between carotid-artery intimal medial thickness and the severity of NAFLD histology, independently of multiple cardiometabolic risk factors^[20,21].

Accumulating evidence also suggests that NAFLD is associated with increased coronary artery calcium (CAC) score on cardiac computed tomography (CT), which is another marker of early coronary atherosclerosis^[22]. A retrospective study showed that NAFLD, assessed by either CT or ultrasonography, was significantly associated with increased CAC score (*i.e.*, CAC score > 100), independently of traditional CVD risk factors^[23]. Another community-based study found that the presence of ultrasound-diagnosed NAFLD with increased serum ALT levels, but not hepatic steatosis alone, independently predicted a high CAC score^[24]. In 2012, Sung *et al.*^[25] reported that in a South Korean occupational cohort of 10153 people, NAFLD on ultrasonography was associated with increased CAC score (*i.e.*, CAC > 0), independently of conventional CVD risk factors, metabolic syndrome features, insulin resistance and pre-existing CVD. In the same year, another large community-based study of Korean people confirmed that increasing CAC scores were associated with NAFLD, independently of classical CVD risk factors, including visceral adiposity^[26]. Almost identi-

cal results were reported by some investigators in other ethnic groups^[27].

Notably, some studies reported an abnormal coronary flow reserve (CFR), an index of impaired coronary microcirculation, in patients with NAFLD. For example, Lautamäki *et al*^[28] reported a strong association between higher intra-hepatic fat content and decreased CFR, as assessed by positron emission tomography, in patients with type 2 diabetes and known CHD, independently of whole-body insulin sensitivity, visceral adiposity and other common CVD risk factors. Other studies confirmed a significantly reduced CFR, assessed by either trans-thoracic doppler echocardiography or cardiac magnetic resonance imaging, in patients with NAFLD, independently of conventional CVD risk factors and metabolic syndrome features^[29,30]. Collectively, the presence of reduced CFR among NAFLD patients suggests that decreased CFR might represent an additional pathogenic mechanism involved in CHD mortality and morbidity in this group of patients.

Clinical CHD: Table 1 shows the main cross-sectional studies relating NAFLD to clinically manifest CHD in both nondiabetic and diabetic individuals^[31-45].

Recent data from the Valpolicella Heart Diabetes Study of 2839 unselected Italian patients with type 2 diabetes have shown that those with NAFLD had a remarkably greater prevalence of clinical CVD (CHD, cerebrovascular and peripheral vascular disease) than their counterparts without NAFLD, independently of classical CVD risk factors, use of medications, glycaemic control and features of the metabolic syndrome^[32]. Similar findings were also reported in adults with type 1 diabetes mellitus^[39]. In a large community-based cohort of 2088 Taiwanese male workers, NAFLD was significantly associated with an increased prevalence of CHD, independently of obesity and other established CVD risk factors^[31].

Mirbagheri *et al*^[34] reported that NAFLD was the strongest, positive predictor of angiographically detected CHD in patients who underwent elective coronary angiography, ranking even before sex and diabetes at multivariate analysis; interestingly, the adjustment for traditional CVD risk factors did not attenuate the strong association between NAFLD and CHD. Similarly, Assy *et al*^[38] reported that patients with NAFLD had a much greater prevalence of both calcified and non-calcified coronary plaques than control subjects without hepatic steatosis, and that NAFLD predicted coronary atherosclerosis, independently of metabolic syndrome features and plasma C-reactive protein levels. Akabame *et al*^[36] found that NAFLD was significantly associated with lower remodeling lesions or lipid core plaques of coronary arteries, thus suggesting NAFLD is a novel risk factor for vulnerable coronary plaques. Interestingly, in a large hospital-based sample of 612 Chinese patients with suspected CHD, Wong *et al*^[41] confirmed that NAFLD on ultrasonography was associated with a greater angiographic severity of

CHD, defined as the presence of $\geq 50\%$ stenosis in at least one coronary artery, independently of multiple risk factors for CVD.

As also shown in Table 1, a number of other studies have documented a positive and independent association between NAFLD and the angiographic severity of CHD among patients with acute coronary syndromes or suspected CHD^[33,35,37,40,43-45]. Finally, NAFLD was associated with poor coronary collateral development in nondiabetic patients with severe CHD, independently of insulin resistance and other features of the metabolic syndrome^[46].

Fatal and non-fatal CHD events

As summarized in Table 2, several retrospective and prospective studies have investigated the relationship between NAFLD and the incidence of CHD or CVD events^[47-71]. These studies have used either biochemical markers, such as elevated serum liver enzymes and fatty liver index (FLI), or radiological imaging or liver biopsy for diagnosing NAFLD.

With regard to biochemistry-diagnosed NAFLD, a systematic review and meta-analysis of 10 population-based cohort studies has shown a strong association between mildly elevated serum levels of gamma glutamyl-transferase (GGT), a surrogate marker for NAFLD, and increased incidence of fatal and non-fatal CVD events, independently of alcohol consumption and classical CVD risk factors^[47]. Conversely, although Schindhelm *et al*^[48] found a significant and independent association between mildly increased serum alanine aminotransferase (ALT) levels and risk of incident CHD events among the Hoorn study participants, other large population-based cohort studies that have examined the association of serum ALT levels with adverse CVD outcomes have provided more conflicting results^[47-50,53]. A recent large population-based cohort study of 2074 Italian subjects with a follow-up period of 15 years showed a significant, positive association between NAFLD as estimated by FLI (*i.e.*, a proxy of fatty liver based on body mass index, waist circumference, serum triglyceride and GGT levels^[72]) and increased CVD mortality that was mainly attributed to insulin resistance^[54]. Again, Lerchbaum *et al*^[55] confirmed that high FLI was independently associated with an increased risk of all-cause, CVD and non-CVD related mortality in a large cohort of consecutive patients with suspected CHD, who were routinely referred to coronary angiography. In contrast, a recent study, involving 713 consecutive Chinese patients with suspected CHD, did not find any significant association between FLI and angiographically detected CHD^[73].

With regard to imaging-diagnosed NAFLD, several prospective studies reported an increased risk of fatal and non-fatal CVD events, independently of several cardio-metabolic risk factors, among NAFLD patients with and without type 2 diabetes (as shown in Table 2)^[56-58,61,63,65]. In the only study having CHD as a pre-specified study outcome, Treeprasertsuk *et al*^[65] confirmed that patients

Table 1 Main cross-sectional study examining the association of non-alcoholic fatty liver disease with the presence and severity of clinical coronary heart disease, ordered by year

Ref.	Study characteristics	NAFLD diagnosis	CHD diagnosis	Main findings
Lin <i>et al</i> ^[31] , 2005	2088 male workers undergoing an annual health examination screening; NAFLD in 29.5%	US	Patient history, ECG	NAFLD associated with higher prevalence of CHD, independently of obesity and other traditional CVD risk factors. The odds for CHD increased progressively with ultrasonographic severity of NAFLD
Targher <i>et al</i> ^[32] , 2007	2839 type 2 diabetic outpatients; NAFLD in 69.5%	US	Patient history, review of patient records, ECG, doppler ultrasound of carotid and lower limb arteries	NAFLD associated with higher prevalence of coronary, cerebrovascular and peripheral vascular disease than their counterparts without NAFLD, independently of traditional CVD risk factors, hemoglobin A1c, medication use and MetS features
Arslan <i>et al</i> ^[33] , 2007	92 consecutive Turkish patients admitted with ACS; NAFLD in 70%	US	CAG (elective)	NAFLD was an independent predictor of CHD (> 50% stenosis of ≥ 1 major coronary artery) after adjustment for traditional CVD risk factors and MetS features
Mirbagheri <i>et al</i> ^[34] , 2007	317 Iranian patients admitted for either ACS, angina or suspected CHD; NAFLD in 54%	US	CAG (elective)	NAFLD was an independent predictor of "clinically relevant" CHD (> 30% stenosis of ≥ 1 major coronary artery) after adjustment for CVD risk factors and MetS features
Alper <i>et al</i> ^[35] , 2008	80 Turkish patients with MS (stable or unstable angina, prognostic reasons); NAFLD in 54%	US	CAG (acute and elective)	NAFLD was the only independent predictor of severe CHD (> 70% stenosis of ≥ 1 major coronary artery) after adjustment for established CVD risk factors and MetS features
Akabame <i>et al</i> ^[36] , 2008	298 consecutive Japanese patients with suspected CHD; NAFLD in 20%	CT	CT (elective)	NAFLD was independently associated with remodeling lesions or lipid core of coronary plaques but not with calcified coronary plaques or stenosis
Açikel <i>et al</i> ^[37] , 2009	355 consecutive Turkish patients admitted for ACS or CHD suspicion; NAFLD in 60%	US	CAG (acute and elective)	NAFLD was an independent predictor of CHD (> 50% stenosis of ≥ 1 major coronary artery) after adjustment for conventional CVD risk factors
Assy <i>et al</i> ^[38] , 2010	29 Israeli patients with low or intermediate risk of CHD and NAFLD and 32 healthy controls matched for age and sex	CT	CT (elective)	NAFLD was associated with greater prevalence of calcified and non-calcified coronary plaques, independently of the MetS and plasma C-reactive protein
Targher <i>et al</i> ^[39] , 2010	250 type 1 diabetic patients; NAFLD in 44.4%	US	Patient history, chart review, ECG, doppler ultrasound of carotid and lower limb arteries	NAFLD was associated with higher prevalence of coronary, cerebrovascular and peripheral vascular disease than their counterparts without NAFLD, independently of traditional CVD risk factors, medication use, hemoglobin A1c, and albuminuria
Sun <i>et al</i> ^[40] , 2011	542 hospitalized Chinese patients with high suspicion of CHD; NAFLD in 46%	CT	CAG (elective)	NAFLD was associated with greater severity of CHD, independently of traditional CVD risk factors
Wong <i>et al</i> ^[41] , 2011	612 Chinese patients with suspicion of CHD; NAFLD in 58%	US	CAG (elective)	NAFLD was associated with CHD, independently of established CVD risk factors and MetS features
Domanski <i>et al</i> ^[42] , 2012	377 patients with NAFLD (retrospective chart review); 219 of these patients had NASH	Biopsy	History of CVD (stroke, unstable angina, myocardial infarction, congestive heart failure, or need for coronary revascularization)	No increased prevalence of CVD in NASH patients compared with those with non-NASH fatty liver
Agaç <i>et al</i> ^[43] , 2013	80 Turkish patients with ACS; NAFLD in 81%	US	CAG (acute)	NAFLD was independently associated with a greater severity of CHD (by Syntax score)
Boddi <i>et al</i> ^[44] , 2013	95 consecutive non-diabetic Italian patients admitted for ACS; NAFLD in 87%	US	CAG (acute)	Presence and severity of NAFLD was independently associated with a three-fold higher risk of multi-vessel CHD
Inci <i>et al</i> ^[45] , 2013	136 consecutive Turkish patients with CHD (stable angina or positive stress test results)	US	CAG (elective)	NAFLD was associated with greater severity of CHD, independently of traditional CVD risk factors

ACS: Acute coronary syndrome; NAFLD: Non-alcoholic fatty liver disease; CAG: Coronary angiography; CT: Computed tomography; CVD: Cardiovascular disease; ECG: Electrocardiogram; MetS: Metabolic syndrome; NASH: Non-alcoholic steatohepatitis; US: Ultrasonography.

with NAFLD had a significantly higher 10-year risk for CHD as calculated by the Framingham risk score (FRS) than the matched control population, and proved the clinical utility of the FRS among these patients, given that

an almost identical number of FRS-predicted and actual new CHD events was registered during the follow-up period of the study. A recent meta-analysis by Musso *et al*^[11] also confirmed that the presence of NAFLD, as detected

Table 2 Main prospective studies relating non-alcoholic fatty liver disease to increased risk of incident coronary heart disease or cardiovascular events, ordered by methodology used for the diagnosis of non-alcoholic fatty liver disease

Ref.	Study characteristics	Years of follow-up	NAFLD diagnosis	Study outcomes	Main findings
Fraser <i>et al</i> ^[47] , 2007	Meta-analysis of 10 population-based cohort studies	7.3	Liver enzymes	Fatal and non-fatal CVD events	Elevated serum GGT level was associated with increased incidence of CVD events, independently of alcohol intake and traditional CVD risk factors
Schindhelm <i>et al</i> ^[48] , 2007	Population-based cohort, <i>n</i> = 1439 subjects (Hoorn Study)	10.0	Liver enzymes	Fatal and non-fatal CHD events	Elevated serum ALT level was associated with CHD events, independently of the MetS and traditional CVD risk factors
Goessling <i>et al</i> ^[49] , 2008	Community-based cohort, <i>n</i> = 2812 (Framingham Offspring Heart Study)	20.0	Liver enzymes	Fatal and non-fatal CVD events	Elevated serum ALT level was not associated with CVD events at multivariate analyses
Dunn <i>et al</i> ^[50] , 2008	Population-based cohort, <i>n</i> = 7574 (NHANES-III)	8.7	Liver enzymes	All-cause and cause-specific mortality	Increased all-cause and CVD mortality rates in NAFLD but only in 45-54 year age group, independently of conventional CVD risk factors and C-reactive protein
Ong <i>et al</i> ^[51] , 2008	Population-based cohort, <i>n</i> = 11285 subjects (NHANES-III)	8.7	Liver enzymes	All-cause and cause-specific mortality	Increased rates of all-cause, CVD and liver-related mortality in NAFLD. Liver disease was the third leading cause of death among persons with NAFLD after CVD and cancer-related mortality
Ruhl <i>et al</i> ^[52] , 2009	Population-based cohort, <i>n</i> = 14950 (NHANES-III)	8.8	Liver enzymes	All-cause and cause-specific mortality	Elevated serum GGT level was associated with mortality from all causes, liver disease but not from CVD causes. Serum ALT level was associated only with liver disease mortality
Yun <i>et al</i> ^[53] , 2009	Community-based cohort, <i>n</i> = 37085 (Health Promotion Center)	5.0	Liver enzymes	CVD or diabetes-related mortality	Elevated serum ALT level was independently associated with increased CVD or diabetes-related mortality
Calori <i>et al</i> ^[54] , 2011	Community based-cohort, <i>n</i> = 2074 (Cremona study)	15.0	FLI index	All-cause and cause-specific mortality	FLI was independently associated with all-cause, hepatic, cancer and CVD mortality. When HOMA-insulin resistance was included in multivariate analyses, FLI retained its statistical association with hepatic-related mortality but not with all-cause, CVD and cancer-related mortality
Lerchbaum <i>et al</i> ^[55] , 2013	Consecutive sample of patients, <i>n</i> = 3270 subjects routinely referred to coronary angiography	7.7	FLI index	All-cause and cause-specific mortality	High FLI was independently associated with increased all-cause, CVD, non-cardiovascular and cancer mortality
Jepsen <i>et al</i> ^[56] , 2003	Population-based cohort, <i>n</i> = 1804 with hospital diagnosis of NAFLD (Danish national registry of patients)	16.0	US	All-cause and cause-specific mortality	Increased rates of all-cause, CVD and liver-related mortality in NAFLD, independently of sex, diabetes, and cirrhosis at baseline
Targher <i>et al</i> ^[57] , 2007	Outpatient cohort, <i>n</i> = 2103 type 2 diabetic subjects (Valpolicella Heart Diabetes Study)	6.5	US	Fatal and non-fatal CVD	Increased rates of fatal and non-fatal CVD events in NAFLD, independently of age, sex, body mass index, smoking, diabetes duration, hemoglobin A1c, LDL-cholesterol, MetS features, medication use
Soler Rodriguez <i>et al</i> ^[58] , 2007	Community-based cohort, <i>n</i> = 1637 healthy Japanese	5.0	US	Non-fatal CVD events	Increased rates of non-fatal CVD events in NAFLD, independently of age, sex, body mass index, alcohol intake, smoking, LDL-cholesterol, MetS features
Lazo <i>et al</i> ^[59] , 2011	Population-based cohort, <i>n</i> = 11371 (NHANES-III)	14.5	US	All-cause and cause-specific mortality	NAFLD was not associated with increased all-cause and cause-specific (CVD, cancer and liver) mortality
Stepanova <i>et al</i> ^[60] , 2012	Population-based cohort, <i>n</i> = 11613 (NHANES-III)	14.2	US	All-cause and cause-specific mortality	NAFLD was associated with increased prevalence of CVD, after adjusting for established CVD risk factors, but not with increased CVD mortality
Zhou <i>et al</i> ^[61] , 2012	Community-based cohort study, <i>n</i> = 3543 adult men and women	4.0	US	All-cause and CVD mortality	Increased rates of all-cause and CVD mortality in NAFLD
Younossi <i>et al</i> ^[62] , 2013	Population-based cohort, <i>n</i> = 1448 with NAFLD (NHANES-III)	14.2	US	All-cause and cause-specific mortality	NAFLD was independently associated with increased all-cause, CVD and liver-related mortality only among NAFLD patients with the MetS
Haring <i>et al</i> ^[63] , 2009	Population-based cohort, <i>n</i> = 4160 German subjects (Study of Health in Pomerania)	7.2	US and liver enzymes	All-cause and CVD mortality	Elevated serum GGT level was independently associated with increased all-cause and CVD mortality in men
Kim <i>et al</i> ^[64] , 2013	Population-based cohort, <i>n</i> = 1154 (NHANES-III)	14.5	US and advanced fibrosis score systems	All-cause and cause-specific mortality	NAFLD was not associated with increased all-cause mortality. However, NAFLD with advanced hepatic fibrosis (defined by NAFLD fibrosis score, APRI index or Fib-4) was independently associated with risk of all-cause mortality, of which the majority of deaths were due to CVD

Treeprasertsuk <i>et al</i> ^[65] , 2012	Community-based cohort, <i>n</i> = 309 patients with NAFLD	11.5	US and CT	Fatal and non-fatal CHD	NAFLD patients had a higher 10-year CHD risk by FRS than the general population of the same age and sex. Almost identical number of FRS-predicted and actual new CHD events
Matteoni <i>et al</i> ^[66] , 1999	Patient-based cohort, <i>n</i> = 132 NAFLD	18.0	Histology	All-cause and cause-specific mortality	Increasing liver-related mortality with the severity of NAFLD histology (according to four different histological subtypes). All-cause mortality and other causes of mortality were not significantly different across histological subtypes
Dam-Larsen <i>et al</i> ^[67] , 2004	Patient-based cohort (Danish national registry of patients), <i>n</i> = 109 subjects with non-alcoholic SS	16.7	Histology	All-cause and cause-specific mortality	All-cause and cause-specific mortality did not significantly differ between patients with non-alcoholic SS and the general population
Adams <i>et al</i> ^[68] , 2005	Community-based cohort, <i>n</i> = 420 patients with NAFLD	7.6	US/CT and histology	All-cause and cause-specific mortality	Increased rate of age- and sex-adjusted all-cause mortality in NAFLD than in the general population with CHD being the second cause of death
Ekstedt <i>et al</i> ^[69] , 2006	Patient-based cohort, <i>n</i> = 129 consecutive patients with NAFLD and elevated serum liver enzymes (55% NASH)	13.7	Histology	All-cause and cause-specific mortality	Increased rates of CVD and liver-related mortality in patients with NASH, but not in those with SS, compared with in the reference population
Rafiq <i>et al</i> ^[70] , 2009	Patient-based cohort, <i>n</i> = 173 patients with NAFLD (41.6% NASH)	13.0	Histology	All-cause and cause-specific mortality	CHD was the first cause of death in NAFLD cohort with no difference between NASH and non-NASH. Liver-related mortality, but not all-cause mortality, was higher in NASH <i>vs</i> non-NASH. No comparison was provided with the general population
Söderberg <i>et al</i> ^[71] , 2010	Patient-based cohort, <i>n</i> = 118 patients with NAFLD and elevated serum liver enzymes (43% NASH)	24.0	Histology	All-cause and cause-specific mortality	Increased mortality rates of CVD, malignancy and liver disease in patients with NASH, but not in those with SS, compared with the matched general population

AST: Alanine aminotransferase; CHD; Coronary heart disease; CT: Computed tomography; US: Ultrasonography; FLI: Fatty liver index; FRS: Framingham risk score; GGT: Gamma-glutamyltransferase; HOMA: Homeostasis model assessment; MetS: Metabolic syndrome; NASH: Non-alcoholic steatohepatitis; SS: Simple steatosis; CVD: Cardiovascular.

by either serum liver enzyme levels or ultrasonography, was strongly associated with an increased risk of fatal and non-fatal CVD events. In contrast, and surprisingly, two recent studies, using the data from the National Health and Examination Survey (NHANES)-III database of over 11000 United States adults, have reported that NAFLD on ultrasonography did not significantly predict the risk of all-cause and cause-specific (CVD, cancer or liver) mortality over 14 years of follow-up period^[59,60]. These two studies, however, were limited by the inclusion of individuals with mild hepatic steatosis within the control arm. Interestingly, the latest analyses of the same NHANES-III cohort found that patients with NAFLD and advanced hepatic fibrosis (as defined by either the NAFLD fibrosis score or Fib-4 score) were indeed at increased risk of CVD mortality after adjustment for established CVD risk factors^[64]. In addition, Younossi *et al*^[62] found that NAFLD was independently associated with increased all-cause, liver-specific and CVD mortality among patients with NAFLD who had the metabolic syndrome but not among those without this syndrome. With regard to biopsy-diagnosed NAFLD (as also shown in Table 2), some retrospective studies with a relatively small sample size but a reasonably long duration of follow-up, that have examined the natural history of patients with biopsy-confirmed NAFLD have consistently shown that the presence and severity of hepatic fibrosis on histology dictates all-cause and liver-related mortality in NAFLD, and that CVD is a common cause of death among such patients^[66-71]. However, only two studies reported spe-

cific data about CHD outcomes rather than dealing with general CVD outcomes. Adams *et al*^[68] found higher all-cause mortality in patients with NAFLD (as detected by radiological imaging or histology) than in the matched control population with CHD being the second cause of death in both populations. Again, Rafiq *et al*^[70] reported that CHD was the first cause of death among patients with NAFLD but did not provide any comparison with the general population. Interestingly, two retrospective studies with a reasonably long duration of follow-up showed that patients with NASH, but not those with simple steatosis, were at substantially higher risk of CVD mortality compared with the reference population^[69,71]. However, it should be noted that a complete adjustment for potentially confounding cardiometabolic factors was not performed in these retrospective studies. In addition, a recent meta-analysis concluded that patients with NAFLD (as detected by histology or ultrasonography) had a significantly greater risk of developing CVD events than the matched control population but that the histological severity of NAFLD did not increase CVD mortality^[70]. However, further larger and longer prospective studies in patients with biopsy-confirmed NAFLD are needed to improve understanding of this issue.

Collectively, the current evidence from the published prospective studies supports that NAFLD, irrespective of the methodology used for diagnosing it, is significantly associated with an increased risk of fatal and non-fatal CHD/CVD events in both nondiabetic and type 2 diabetic individuals. However, uncertainty remains as to

Table 3 Cardiac imaging studies relating -non-alcoholic fatty liver disease to structural and arrhythmogenic cardiac complications

	Ref.	Study characteristics	NAFLD diagnosis	Study measures	Main findings
Abnormalities in myocardial metabolism	Lautamaki <i>et al</i> ^[26] , 2006	55 consecutive type 2 diabetic adults with known CHD	¹ H-MRS	Cardiac PET using [15O]-water and [18F]-2-fluoro-2-deoxy-D-glucose	Decreased coronary functional capacity and myocardial glucose uptake in NAFLD. These abnormalities were worse in those with higher intra-hepatic fat content
	Perseghin <i>et al</i> ^[7] , 2008	Case-control: 21 nondiabetic, nonobese, normotensive, young men with NAFLD and 21 age- and BMI-matched male controls	¹ H-MRS	Cardiac ³¹ P-MRS and MRI	Impaired LV energy metabolism in NAFLD, independently of age, BMI, blood pressure, lipids, fasting glucose. LV mass and function were not different between the groups
	Rijzewijk <i>et al</i> ^[74] , 2008	Case-control: 38 uncomplicated type 2 diabetic men without CHD and 28 age, sex- and BMI-matched healthy controls	¹ H-MRS	Cardiac ¹ H-MRS and MRI	Myocardial fat content, which was much higher in diabetics than in control subjects, was positively associated with intra-hepatic fat content in both groups. Myocardial steatosis was a strong predictor of LV diastolic dysfunction
	Rijzewijk <i>et al</i> ^[75] , 2010	61 uncomplicated type 2 diabetic men without CHD (32 of whom with high intra-hepatic triglyceride content)	¹ H-MRS	Cardiac MRI, ³¹ P-MRS and cardiac PET using [15O]-water, [11C]-palmitate, and [18F]-2-fluoro-2-deoxy-D-glucose	Decreased myocardial perfusion, glucose uptake and impaired LV energy metabolism in NAFLD. Cardiac fatty acid metabolism, LV mass and function were not different between the two groups
Cardiac structure and function in adults	Goland <i>et al</i> ^[76] , 2006	Case-control: 38 non-diabetic, normotensive NAFLD patients and 25 age- and sex-matched healthy controls	US and biopsy (29% of cases)	Echocardiography with TDI	Increased LV mass and increased prevalence of diastolic dysfunction in NAFLD. Reduced E' wave only independent parameter associated with NAFLD on multivariate analysis
	Fallo <i>et al</i> ^[77] , 2009	Case-control: newly-diagnosed untreated hypertensive patients (non-obese, non-diabetic): 48 NAFLD vs 38 controls	US	Echocardiography	Increased prevalence of diastolic dysfunction in NAFLD (according to its severity on ultrasound). LV mass was not different between the groups. Diastolic dysfunction and insulin resistance were independently associated with NAFLD
	Fotbolcu <i>et al</i> ^[78] , 2010	Case-control: 35 nondiabetic, normotensive NAFLD patients and 30 age- and sex-matched healthy controls	US	Echocardiography with TDI	Increased LV mass and early impairment in systolic and diastolic function in NAFLD (no adjustment for potential confounders was made)
	Mantovani <i>et al</i> ^[79] , 2011	116 consecutive older patients with hypertension and type 2 diabetes (53% of whom had NAFLD) without history of CHD and hepatic diseases	US	Echocardiography	Increased prevalence of LV hypertrophy in NAFLD. NAFLD was associated with LV hypertrophy independently of age, sex, BMI, systolic blood pressure, kidney function parameters and other diabetes-related variables
	Bonapace <i>et al</i> ^[8] , 2012	50 consecutive type 2 diabetic patients without CHD and hepatic diseases (32 patients had NAFLD)	US	Echocardiography with TDI (speckle tracking analyses)	Impairment in LV diastolic function (including global longitudinal diastolic strain) in NAFLD, independently of age, sex, BMI, hypertension and other diabetes-related variables. These abnormalities were worse in those with severe NAFLD on ultrasonography. No differences in LV mass and systolic function between the groups
	Hallsworth <i>et al</i> ^[80] , 2013	Case-control: 19 non-diabetic, overweight adults with NAFLD and 19 age-, sex- and BMI-matched healthy controls	¹ H-MRS	Cardiac MRI and ³¹ P-MRS	Early impairment in systolic and diastolic function in NAFLD. Myocardial energy metabolism and LV mass were not altered in NAFLD
	Alp <i>et al</i> ^[81] , 2013	Case-control: 400 obese children (93 with NAFLD) and 150 age- and sex-matched healthy controls	US	Echocardiography with TDI	Increased LV mass and early impairment in systolic and diastolic function in obese children with NAFLD independently of traditional cardiac risk factors. These abnormalities were worse in those with severe NAFLD on ultrasonography
	Singh <i>et al</i> ^[82] , 2013	Case-control: 14 lean adolescents, 15 obese adolescents without NAFLD and 15 obese adolescents with NAFLD	¹ H-MRS	Echocardiography with TDI (speckle tracking analyses)	Decreased rates of LV global longitudinal systolic strain and early diastolic strain in obese adolescents with NAFLD independently of traditional cardiac risk factors. LV mass was not different between the groups
Cardiac structure and function in children or adolescents	Sert <i>et al</i> ^[83] , 2013	Case-control: 108 obese adolescents and 68 healthy controls	US	Echocardiography with TDI (speckle tracking analyses)	Increased LV mass and impaired diastolic function and altered global systolic and diastolic myocardial performance in obese adolescents with NAFLD

	Pacifico <i>et al</i> ^[84] , 2013	Case-control: 108 obese children (54 with NAFLD) and 18 lean healthy controls	MRI and biopsy (in 41 obese children)	Echocardiography with TDI	Early impairment in systolic and diastolic function in obese children with NAFLD independently of traditional cardiac risk factors. These abnormalities were more severe in those with NASH
Risk of atrial fibrillation	Sinner <i>et al</i> ^[87] , 2013	Community-based cohort of 3744 adult individuals free of clinical HF (from the Framingham Heart Study original and Offspring cohorts)	Liver enzymes	Incidence of AF over up 10 yr of follow-up	Mildly elevated serum transaminases were associated with increased incidence of AF, independently of age, sex, BMI, systolic blood pressure, electrocardiographic PR interval, anti-hypertensive treatment, smoking, diabetes, valvular heart disease, alcohol consumption
	Targher <i>et al</i> ^[9] , 2013	Hospital-based sample of 702 patients with type 2 diabetes without a history of hepatic diseases, or excessive alcohol intake (73% of them had NAFLD)	US	Prevalence of persistent or permanent AF	Increased prevalence of AF in those with NAFLD, independently of age, sex, systolic blood pressure, hemoglobin A1c, estimated glomerular filtration rate, total cholesterol, electrocardiographic left ventricular hypertrophy, chronic obstructive pulmonary disease, and prior history of heart failure, valvular heart disease or hyperthyroidism
	Targher <i>et al</i> ^[10] , 2013	Random sample of 400 type 2 diabetic outpatients free from AF, moderate-to-severe heart valve disease and known causes of chronic liver diseases at baseline (70% of them had NAFLD)	US	Incidence of AF over 10 yr of follow-up	Increased incidence of AF in those with NAFLD, independently of age, sex, prior history of HF, BMI, systolic blood pressure, anti-hypertensive treatment, electrocardiographic LV hypertrophy, PR interval

BMI: Body mass index; CHD: Coronary heart disease; LV: Left ventricular; MRI: Magnetic resonance imaging; MRS: Magnetic resonance spectroscopy; TDI: Tissue doppler imaging; US: Ultrasonography; E': Mitral annular tissue doppler early diastolic velocity; NAFLD: Non-alcoholic fatty liver disease; AF: Atrial fibrillation; HF: Heart failure.

whether NAFLD poses an independent risk above and beyond known CVD risk factors. There is a suggestion in that direction, but studies are too few and methodologically not rigorous. Additional large-scale prospective studies of a more extensive panel of known risk factors are needed to draw a firm conclusion about any independent hepatic contribution to the increased risk of CHD/CVD events observed among patients with NAFLD.

NAFLD and abnormalities in cardiac structure and function

Table 3 show the relevant data from the principal cardiac imaging studies that have evaluated the relationship between NAFLD and abnormalities in myocardial metabolism^[7,28,74,75] and cardiac structure and function, both in adults^[8,76-80] and in children or adolescents^[81-84].

Abnormalities in cardiac metabolism: As reported in Table 3, Perseghin *et al*^[7] showed that nondiabetic, non-obese, normotensive, young men with newly diagnosed NAFLD, as detected by proton magnetic resonance spectroscopy (¹H-MRS), had excessive fat accumulation in the epicardial area and impaired left ventricular (LV) energy metabolism [as measured by the phosphocreatine/adenosine triphosphate (PCr/ATP) ratio] compared with age-, sex- and body mass index (BMI)-matched control subjects without NAFLD. These myocardial metabolic alterations were detected despite normal LV morphological features and systolic and diastolic functions^[7].

Similarly, in a study of uncomplicated type 2 diabetic men without CHD, Rijzewijk *et al*^[74] found that compared with those with lower intra-hepatic fat content, patients with higher intra-hepatic fat content on ¹H-MRS

had significantly decreased myocardial perfusion, glucose uptake and high-energy phosphate metabolism (*i.e.*, decreased PCr/ATP ratio) but similar values of myocardial fatty acid metabolism, LV mass and function.

Similar findings were also reported by Lautamäki *et al*^[28] in patients with type 2 diabetes and known CHD. Interestingly, these investigators found that myocardial insulin resistance was more severe among those with higher intra-hepatic fat content on ¹H-MRS.

Again, Rijzewijk *et al*^[75] found that the frequency of myocardial steatosis as diagnosed by cardiac ¹H-MRS was much higher in type 2 diabetic patients than in healthy controls matched for age and BMI, and that higher myocardial fat content was associated with higher intra-hepatic fat content. Notably, although the two groups of subjects did not significantly differ in terms of LV mass and ejection fraction, multivariable regression analyses revealed that myocardial steatosis was associated with LV diastolic dysfunction, independently of diabetic state, age, BMI, visceral adipose tissue, heart rate and blood pressure^[75].

Abnormalities in cardiac structure and function in adults: As reported in Table 3, there is to date an increasing number of case-control studies that have evaluated the effect of NAFLD on cardiac structure and function in adults with or without co-existing established CVD risk factors (*e.g.*, obesity, hypertension or diabetes)^[8,76-80].

Goland *et al*^[76] and Fotbolcu *et al*^[78] have shown a marked LV diastolic dysfunction and mild alterations in LV structure in patients with NAFLD, in the absence of hypertension, diabetes and severe obesity. Again, in a recent study examining cardiac status by high resolu-

tion MRI and ^{31}P -MRS in a small group of NAFLD patients (defined as $> 5\%$ intra-hepatic lipid on ^1H -MRS), Hallsworth *et al*^[80] have demonstrated significant changes in cardiac structure and evidence of early LV diastolic dysfunction compared with age-, sex- and BMI-matched controls, in the absence of cardiac metabolic changes or overt cardiac disease.

In a study of 86 never-treated hypertensive patients, who were subdivided in two subgroups according to the presence or absence of NAFLD on ultrasonography, Fallo *et al*^[77] reported that patients with NAFLD had a three-fold greater prevalence of LV diastolic dysfunction than their counterparts without NAFLD.

In a study of older hypertensive patients with type 2 diabetes, who did not have a pre-existing history of CHD and hepatic diseases, Mantovani *et al*^[79] found that the prevalence of LV hypertrophy on conventional echocardiography was four-fold greater among patients with NAFLD than among those without this disease.

In a recent study, involving 50 consecutive type 2 diabetic adults without history of CHD, excessive alcohol consumption or other known hepatic diseases, we found that early features of LV diastolic dysfunction could be detected by tissue doppler imaging in those with ultrasound-diagnosed NAFLD, even if the LV morphology and systolic function were preserved^[8]. Measurements of LV global longitudinal strain and strain rate by speckle tracking analyses further confirmed these findings. Notably, there was a positive, graded relationship between the ultrasonographic severity of NAFLD and LV diastolic dysfunction, independently of hypertension and other co-existing cardio-metabolic risk factors^[8].

Abnormalities in cardiac structure and function in children and adolescents: As reported in Table 3, some recent published papers have addressed the relationship between NAFLD and changes in cardiac structure and function also in the pediatric population^[81-84].

In a study including 93 obese children with ultrasound-diagnosed NAFLD, 307 obese subjects without liver involvement, and 150 age- and sex-matched healthy controls, Alp *et al*^[81] showed that subclinical systolic and diastolic impairment could be detected by tissue doppler imaging in obese children with NAFLD. Also, cardiac dysfunction progressively increased with ultrasonographic scores of hepatic steatosis.

Recently, Singh *et al*^[82] measured by 2-D speckle tracking echocardiography myocardial function in three small groups of age-, sex- and Tanner-matched adolescents and showed that obese adolescents with NAFLD had greater abnormalities of cardiac function, manifested by decreased systolic and diastolic myocardial strain and strain rate than obese adolescents without NAFLD. These myocardial functional abnormalities were independent of conventional CVD risk factors and insulin resistance^[82]. Similar findings were reported by Sert *et al*^[83] in a larger sample of obese adolescents.

Finally, Pacifico *et al*^[84] found that obese children with

histologically confirmed non-alcoholic steatohepatitis (NASH) had more severe abnormalities in LV systolic and diastolic functions compared with those without NASH, independently of underlying cardio-metabolic abnormalities.

Risk of congestive heart failure: From the data of the available literature, it is plausible to assume that patients with NAFLD have changes in cardiac substrate metabolism (*e.g.*, myocardial insulin resistance, impaired high-energy phosphate metabolism, and reduced mitochondrial ATP production), producing myocardial functional and structural consequences (*e.g.*, LV dysfunction and hypertrophy) that are potentially linked to an increased rate of congestive heart failure (HF) in this patient population.

As regards to this, two recent large population-based cohort studies that used elevated serum liver enzyme levels, as proxy markers of NAFLD (and should therefore be interpreted cautiously) have shown that this disease is associated with an increased risk of incident congestive HF, independently of alcohol consumption and several established CVD risk factors^[85,86].

In the original cohort of the 3544 Framingham Study participants, who were free of HF and myocardial infarction, Dhingra *et al*^[85] reported that higher serum GGT concentrations within the “normal” range were independently associated with greater risk of incident HF (*i.e.*, each SD increase in log-GGT was associated with a 1.4-fold risk of HF) and incrementally improved prediction of HF risk during a mean follow-up period of 24 years.

Similarly, in a population-based cohort study of 3494 British men aged 60 to 79 years with no diagnosed HF or myocardial infarction followed up for a mean period of 9 years, Wannamethee *et al*^[86] reported that elevated serum GGT level (top quartile, ≥ 38 U/L) was associated with significantly increased risk of incident HF, especially in men aged < 70 years. The increased risk of HF associated with elevated serum GGT level persisted after adjustment for a wide range of established and novel risk factors for HF, including also lung function, plasma C-reactive protein and N-terminal pro-brain natriuretic peptide levels. Other liver function markers showed no significant associations with the risk of HF after similar adjustments^[86].

NAFLD and cardiac arrhythmias

Table 3 shows the relevant data from the published studies that have examined the association between NAFLD and the risk of cardiac arrhythmias, specifically AF^[8,9,87].

To date, AF is the most common sustained arrhythmia seen in clinical practice, and its prevalence and incidence are expected to increase substantially over the next few decades because of ageing population and improvements in cardiovascular treatments^[88]. This underscores the urgent need for primary prevention strategies against the development of AF.

Increased risk of AF: As reported in the Table 3, the

investigators of the Framingham Heart Study have shown that elevated serum ALT or aspartate aminotransferase (AST) levels (> 40 U/L for either marker) were closely associated with an increased risk of incident AF over a 10-year follow-up period among 3744 United States white adults, who were free from clinical HF at baseline^[87]. During follow-up, 383 subjects developed AF and both serum transaminases were found to be significantly associated with a greater risk for incident AF (hazard ratio expressed per SD of natural logarithmically transformed biomarker: ALT hazard ratio 1.19, 95%CI: 1.07-1.32, $P = 0.002$; AST hazard ratio 1.12, 95%CI: 1.01-1.24, $P = 0.03$) after adjusting for a broad number of clinical AF risk factors. The association between serum transaminases and incident AF remained consistent even after the exclusion of participants with moderate to heavy alcohol consumption^[87].

More recently, in an observational study, involving 702 hospitalized patients with type 2 diabetes (73% of whom had NAFLD and 12% had persistent or permanent AF), we found that NAFLD on ultrasonography was associated with a about 3-fold higher prevalence of AF, independently of multiple established risk factors for AF^[9].

Additionally, in another recent study, we have shown that type 2 diabetic patients with NAFLD were also more likely to develop incident AF over a 10-year follow-up period than their counterparts without NAFLD. In particular, NAFLD on ultrasonography was strongly associated with an increased risk of incident AF (adjusted OR = 4.96, 95%CI: 1.4-17.0, $P < 0.01$), independently of age, sex, BMI, hypertension and other variables that were included in the 10-year Framingham Heart Study-derived AF risk score^[10].

Increased risk of ventricular arrhythmias: To date, there is a paucity of published data regarding the association between NAFLD and risk of ventricular arrhythmias, which are an established risk factor for sudden cardiac death in the general population.

However, it is plausible that various mechanisms that have been proposed to explain the specific contribution of NAFLD to CVD risk (including hepatic insulin resistance, systemic low-grade inflammation and a pro-thrombotic state)^[9,10,13-16], might be, at least in part, implicated in the pathogenesis of ventricular arrhythmias.

Heart rate variability, which is a measure of the balance of the sympathetic and parasympathetic mediators of heart rate, and QTc interval prolongation on standard electrocardiograms have been proposed as useful tools in identifying patients at risk for sudden cardiac death^[89]. For instance, QTc interval prolongation is a powerful predictor of ventricular tachyarrhythmias, and predicts increased cardiac and all-cause mortality both in patients with type 2 diabetes and in those without diabetes^[90-92].

Recently, in a study of 497 non-diabetic subjects without a history of previous CVD, Liu *et al.*^[93] reported that patients with ultrasound-diagnosed NAFLD had

early cardiac autonomic dysfunction as detected by some parameters of heart rate variability measured during a 5-min Holter monitoring examination compared with those without NAFLD. The reduction in these Holter-derived parameters was independent of conventional cardiovascular risk factors, insulin resistance and circulating leptin levels^[93]. Additionally, in a small study of non-diabetic people comprising a group of people with histologically proven, non-cirrhotic NAFLD and an age-, sex- and BMI-matched control group, there was evidence of cardiac autonomic dysfunction, presenting as orthostatic hypotension, vasovagal syncope (during head up tilt testing) and/or a relative nocturnal hypotension^[94].

More recently, we examined whether NAFLD was associated with longer QTc intervals on standard electrocardiograms in 400 randomly selected patients with type 2 diabetes without a documented history of AF, moderate-to-severe heart valve disease, hepatic diseases or excessive alcohol consumption. Notably, we found that the presence and severity of NAFLD on ultrasonography was associated with prolonged QTc interval (adjusted OR = 2.27, 95%CI: 1.4-3.7, $P < 0.001$), independently of age, sex, hypertension, electrocardiographic LV hypertrophy, hemoglobin A1c and other potential confounders (manuscript under submission).

Collectively, although the arrhythmogenic potential of NAFLD requires further testing and confirmation in larger studies, we believe that this is a promising field of research to explore, and that the pathways that involve the contribution of NAFLD itself to systemic/hepatic insulin resistance and the systemic release of several pro-inflammatory, pro-coagulant and pro-fibrogenic mediators from the steatotic and inflamed liver^[13-16], might provide a potential therapeutic target for the treatment and prevention of cardiac remodelling and electrophysiological abnormalities of the myocardium in people with NAFLD.

NAFLD and aortic valve sclerosis

Until recently, aortic valve sclerosis (AVS), defined as focal or diffuse thickening and calcification of the aortic leaflets without restriction of leaflet motion, was considered an incidental echocardiographic finding of no clinical significance, as it does not obstruct left ventricular outflow.

However, it is known that AVS shows some epidemiologic and histopathologic similarities to coronary atherosclerosis^[95]. In addition, large prospective studies have suggested a strong, positive association between AVS and adverse CVD outcomes, independently of conventional CVD risk factors, both in nondiabetic and diabetic individuals^[96-98]. The prevalence of AVS increases progressively with advancing age and is approximately 20%-30% in individuals aged ≥ 65 years^[96,97].

Notably, Markus *et al.*^[99] have examined for the first time the association between NAFLD and AVS in a community-based cohort study of 2212 German men and women aged ≥ 45 years. In this cross-sectional study,

NAFLD diagnosed by ultrasonography was significantly associated with an increased risk of prevalent AVS on echocardiography (OR = 1.32, 95%CI: 4-66, $P = 0.021$) even after adjusting for several established CVD risk factors, including kidney function parameters, C-reactive protein, serum ferritin, and white blood cells^[99].

Although these results are still unpublished, we have recently confirmed and expanded to patients with type 2 diabetes the interesting observations of the Markus's study, providing further strong evidence that NAFLD and AVS are two inter-related pathologic conditions, in part independent from traditional CVD risk factors and diabetes-related variables. In such preliminary study, involving 180 consecutive type 2 diabetic outpatients without a history of prior CHD, hepatic diseases or excessive alcohol consumption, we found that ultrasound-diagnosed NAFLD was strongly associated with AVS (adjusted OR = 3.04, 95%CI: 1.3-7.3, $P = 0.01$), independently of multiple established CVD risk factors and diabetes-related variables.

However, future research is needed to corroborate these findings in independent samples, to elucidate the responsible mechanisms for this association, and to determine whether NAFLD predicts the development and progression of AVS.

PUTATIVE MECHANISMS LINKING NAFLD WITH STRUCTURAL AND ARRHYTHMOGENIC CARDIAC COMPLICATIONS

The pathophysiological mechanisms that link NAFLD with CHD, AVS, myocardial dysfunction/hypertrophy and cardiac arrhythmias are incompletely understood.

The complex interactions among NAFLD, insulin resistance and visceral obesity make it extremely difficult to dissect out the precise causal relationships responsible for the increased risk of CHD and other cardiac and arrhythmic complications observed in patients with NAFLD. Different pathogenetic theories and mechanisms, not mutually exclusive, may be put forward (as also schematically reported in Figure 1) and some key research questions remain to be addressed.

To date, it remains debatable whether NAFLD is merely a risk marker of co-existing metabolic disorders and ectopic fat deposition in other organs (such as visceral adipose tissue, myocardium and pericardium) in people at increased risk for cardiac and arrhythmic complications, or is an independent risk factor for the development and progression of such cardiac complications. Another unanswered question is whether the risk of cardiac and arrhythmic events is also increased in patients with simple steatosis or whether the hepatic necro-inflammatory milieu of NASH is a necessary pro-atherogenic and pro-thrombotic stimulus.

Accumulating evidence suggests that within the spectrum of disease encapsulated by NAFLD, the presence

of NASH exacerbates systemic and hepatic insulin resistance and causes atherogenic dyslipidemia (typically characterized by high triglycerides, low HDL-cholesterol and increased small, dense LDL particles). In NASH there is also increased production of a variety of pro-inflammatory markers (*e.g.*, C-reactive protein, interleukin-6, tumor necrosis factor- α), pro-coagulant factors (*e.g.*, fibrinogen, factor VIII, plasminogen activator inhibitor-1), pro-oxidant molecules (*e.g.*, oxidized low-density lipoprotein cholesterol, thiobarbituric acid-reacting substances, nitrotyrosine), and pro-fibrogenic mediators (*e.g.*, tumor growth factor- β , insulin-like growth factor-1, endothelin-1)^[13-16,100-109]. Moreover, the release of key components of the renin-angiotensin-aldosterone system, that may contribute to the pathophysiology of hypertension, is also increased in patients with NASH. The experimental findings that NASH is associated with abnormal intra-hepatic messenger RNA expression of these potential mediators of cardiac and vascular injury, further support the conclusion that the increased circulating levels of the aforementioned biomarkers result from the up-regulation of their own synthesis in the steatotic and inflamed liver^[14-16,109]. Some experimental studies have also shown that a number of the genes involved in fatty acid metabolism, lipolysis, monocyte and macrophage recruitment, coagulation, and inflammation are over-expressed in livers of patients with NASH^[104].

It is plausible that the liver-secreted factors, mentioned above, may also play a pathogenic role in the development and progression of AVS (*i.e.*, a condition that shares some epidemiologic and histopathologic similarities with coronary atherosclerosis)^[95] as well as in the development and persistence of AF and other arrhythmias, possibly by inducing cardiac remodelling and electrophysiological abnormalities of the myocardium in people with NAFLD^[9,10]. For instance, some studies reported that increased inflammatory biomarkers, including elevated C-reactive protein levels, are associated with an increased risk of both new-onset AF^[110] and persistence or recurrence of AF after catheter ablation^[111].

Overall, therefore, there is to date a growing body of evidence suggesting that NAFLD is not a simple epiphenomenon but is, at least in part, involved in the pathophysiology of CHD and other cardiac and arrhythmogenic complications, possibly through the contribution of NAFLD itself to systemic and hepatic insulin resistance and atherogenic dyslipidemia, and/or through the hepatic secretion of several pathogenic mediators (as schematically reported in Figure 1)^[13-16].

The primary role of insulin resistance in the development and progression of NAFLD has been recently challenged^[112]. Similarly, some evidence suggests that insulin resistance *per se* does not directly promote atherosclerosis but it does so principally by promoting atherogenic dyslipidemia and other cardiometabolic abnormalities^[113]. Accordingly, perturbed lipid homeostasis could play a key role in accelerated atherogenesis observed in patients with NAFLD/NASH. Further evidence for a specific

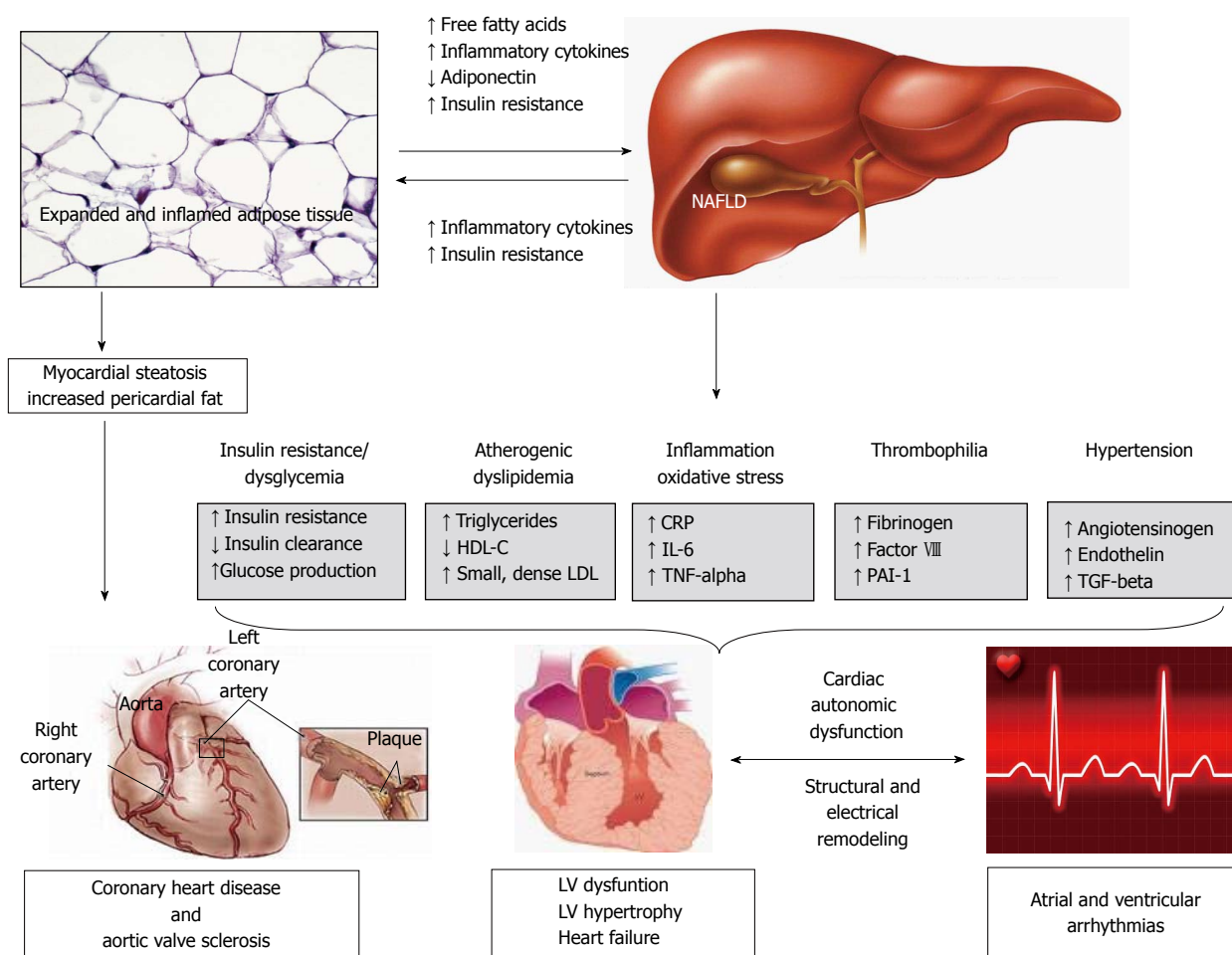


Figure 1 Possible mechanisms leading to cardiac and arrhythmogenic complications in non-alcoholic fatty liver disease. The close and complex interrelationships among non-alcoholic fatty liver disease (NAFLD), visceral obesity and insulin resistance make it extremely difficult to dissect out the specific role of the liver and the underlying mechanisms responsible for the association between NAFLD and the risk of developing coronary heart disease (CHD), aortic valve sclerosis, left ventricular (LV) dysfunction/hypertrophy and arrhythmias. NAFLD might be associated with such complications either as a consequence of shared cardiometabolic risk factors and co-morbidities or as a marker of ectopic fat accumulation in other organs. For instance, myocardial steatosis and increased pericardial fat volume might exert local adverse effects that result in functional and structural derangements of the myocardium. Such myocardial remodelling will likely also result in pro-arrhythmogenic effects. The occurrence of cardiac arrhythmias is likely facilitated in remodeled heart by (local) pro-inflammatory cytokines, chemokines and concurrent cardiac autonomic dysfunction occurring on this dysmetabolic milieu. However, in this dangerous scenario, which may potentially account for premature CHD and increased risk of arrhythmias, NAFLD seems to be not simply a marker of cardiac and arrhythmogenic complications but also may play a part in their pathogenesis possibly via atherogenic dyslipidemia and the hepatic secretion of several pathogenic mediators into the bloodstream. HDL-C: High-density lipoprotein cholesterol; LDL: Low-density lipoprotein; CRP: C reactive protein; IL: Interleukin; TNF: Tumor necrosis factor; PAI-1: Plasminogen activator inhibitor-1; TGF: Transforming growth factor.

role of NAFLD in the development of atherogenic dyslipidemia has been also recently published^[114,115]. Again, post-prandial lipemia might represent an additional, under-diagnosed lipid abnormality that further links NAFLD to accelerated atherogenesis^[102,116,117].

Recent research has also shown that patients with NAFLD exhibit cardiac autonomic dysfunction^[93,94,118,119], a pathophysiological derangement that is, at least in part, reversible following resistance exercise training^[120]. It is plausible that cardiac autonomic dysfunction, resulting from the co-existing dysmetabolic and inflammatory milieu of NAFLD, may contribute together with abnormalities of myocardial structure and function to the development and persistence of AF and other cardiac arrhythmias.

Altered sleep physiology is another emerging risk factor for NAFLD^[121]. Recent epidemiological and experi-

mental data suggest a strong link between disturbed sleep physiology and the histological severity of NAFLD^[121,122]. Obstructive sleep apnea (OSA) is a complex disorder typically characterized by repetitive apnea-hypopnea cycles during sleep, which are associated with chronic intermittent hypoxia and sleep fragmentation^[123]. Symptomatic OSA (also known as obstructive sleep apnea syndrome, or OSAS) is very common in people with severe obesity or type 2 diabetes. Controlled trials have demonstrated that OSAS causes hypertension, and prospective epidemiological studies have indicated that OSAS might be an independent risk factor for incident stroke and CHD^[123].

Collectively, as also mentioned above, it is important to underline that a clear understanding of the pathophysiological pathways that link NAFLD to the development of structural and arrhythmogenic cardiac complications remains lacking because of the complex and intertwined

inter-relationships among NAFLD, visceral obesity and insulin resistance. It is likely that there is a pathogenic cross-talk between the liver and the expanded adipose tissue. As shown in schematic Figure 1, the putative underlying mechanisms that link NAFLD to cardiovascular, cardiac and arrhythmogenic complications might originate from the expanded and inflamed visceral adipose tissue (which increases the rate of free fatty acids and releases multiple adipokines), with the liver functioning as both the target of the resulting systemic abnormalities and the source of several molecular mediators that amplify the cardiac and vascular damage. However, further research is required to define the major sources of some pro-inflammatory and pro-thrombotic mediators (*i.e.*, to determine the relative contributions of visceral adipose tissue and the liver itself), as well as to uncover other specific mechanisms by which NAFLD may contribute to the development and progression of cardiac and arrhythmogenic complications. An improved knowledge of the pathophysiological links of NAFLD with cardiac and arrhythmogenic complications might also provide a potential target for the pharmacological treatment of these diseases.

POTENTIAL IMPACT OF NAFLD TREATMENT ON CARDIAC COMPLICATIONS

Presently, there is no licensed treatment for human NAFLD. Most interventions evaluated for the treatment of NAFLD are those commonly used for the treatment of type 2 diabetes and exert a rather indirect effect through improvement in insulin resistance and glycaemia^[15,16,124-126]. The treatment of NAFLD has also been proposed as a tool to effectively reduce the CVD risk of this group of patients by treating the co-existing features of the metabolic syndrome through a tailored treatment four-step pyramid choice^[12].

The first step to be offered to all patients with NAFLD includes lifestyle modifications such as hypocaloric diet, increased physical activity and smoking cessation^[124-127]. Pharmacotherapy for NAFLD should probably be reserved for patients with NASH or those with co-existing cardiometabolic disorders amenable to specific drug therapy such as obesity, dyslipidemia, hypertension and type 2 diabetes. Of concern, however, there appears to be a dissociation in the actions of some classes of drugs, benefits on the liver side being counter-balanced by (some) important extra-hepatic side effects.

Here, we will briefly discuss whether and how some specific treatment interventions for NAFLD might also beneficially affect the development and progression of cardiac and arrhythmic complications.

Lifestyle modifications

Weight loss obtained through diet alone or combined with physical exercise significantly reduces hepatic ste-

atosis and necro-inflammatory changes of people with NAFLD in proportion to the entity of body weight reduction (5%-10% weight loss reduces hepatic steatosis, while up to a 10% weight loss is needed to improve the degree of hepatic necro-inflammation)^[125,126,128]. However, no study of lifestyle modification has been able to demonstrate an improvement in hepatic fibrosis stage. Interestingly, physical exercise may improve hepatic steatosis and serum liver enzymes in patients with NAFLD, independent of any change in body weight^[129-133]. A recent randomized controlled trial reported that 4 mo of resistance training and aerobic training are equally effective in reducing intra-hepatic fat content among patients with type 2 diabetes and NAFLD^[133].

Lifestyle changes may result in reduced CVD risk *via* improvements in atherogenic risk profile (*e.g.*, blood pressure, glycemia and lipids) and myocardial structure and function^[134-136]. Regular exercise may also exert some of its beneficial health effects by inducing anti-inflammatory actions^[137]. Interestingly, physical activity reduced all-cause and CVD mortality in patients with type 2 diabetes with mildly elevated plasma C-reactive protein levels, whereas this beneficial effect was not observed in those with normal C-reactive protein levels, suggesting that the decrease in CVD mortality in physically active patients may reflect an anti-inflammatory effect of exercise independent of traditional CVD risk factors^[138].

Finally, bariatric surgery, which should be reserved to patients with severe obesity, has been associated with beneficial and sustained improvements of liver histology in people with NAFLD/NASH^[139].

Insulin sensitizers

Indications and limitations for the use of insulin-sensitizing drugs in patients with NAFLD/NASH have been reviewed in detail elsewhere^[112,140]. Although metformin, which is the first-line choice in oral therapy for type 2 diabetes, has been reported to moderately reduce the risk of developing hepatocellular carcinoma^[141-143], it exerts only a marginal beneficial effect on serum aminotransferase levels but does not improve liver histology in NAFLD.

Glitazones (especially pioglitazone) reduce systemic insulin resistance and improve hepatic steatosis and necro-inflammation, but not hepatic fibrosis, in patients with biopsy-proven NASH^[144-146]; unfortunately, however, the hepato-protective effects of glitazones vanish after drug treatment discontinuation^[147]. Of concern, the use of glitazones is limited by their potential serious CVD side effects. Rosiglitazone has been withdrawn from the market because of increased risk of non-fatal myocardial infarction^[144]. Pioglitazone only marginally reduces the risk of major CVD events in people with type 2 diabetes but causes significant weight gain (by increased subcutaneous fat depots), and increases the risk of congestive HF and bone fractures^[148]. A slightly increased risk of bladder cancer has also recently led to pioglitazone being withdrawn from market in France^[149]. Therefore, the potential benefits of glitazones on the liver are counterbal-

anced by their lack of benefits on cardiovascular system, suggesting that the reduction of CVD risk needs a more global approach than just glucose control^[150].

Glucagon-like peptide-1 analogues

Glucagon-like peptide (GLP-1) analogues (exenatide and liraglutide) further to their hypoglycemic effects are potent appetite suppressants and promote body weight reduction^[151]. Animal data suggest that GLP-1 analogues may be useful for the treatment of NAFLD^[152,153]. Adjunctive exenatide treatment for at least 3 years in obese patients with type 2 diabetes resulted in body weight reduction and sustained improvements in glycemic control and serum liver enzyme levels^[154]. Other small intervention trials reported that GLP-1 analogues significantly reduced intra-hepatic fat content on ¹H-MRS and improved serum aminotransferase levels in obese patients with type 2 diabetes^[155,156]. However, further larger and longer randomized clinical trials with histological endpoints are needed to establish a beneficial effect of these drugs for the treatment of NAFLD/NASH. Although still not conclusive, a possible cardio-protective effect of GLP-1 analogues has been recently supported by small animal and human studies^[157].

Statins

Despite their limited benefits on NAFLD, where statins may produce some improvement in serum aminotransferases and hepatic steatosis (hepatic necro-inflammation and fibrosis remaining unaffected), statins represent a unique class of drugs^[125,126]. Such a specificity results from the statin use having been shown to be associated with reduced CVD morbidity in patients with mildly-to-moderately abnormal liver function tests potentially attributable to NAFLD^[158,159]. Moreover, statins are the most effective and widely used class of lipid-lowering drugs for the primary and secondary prevention of CVD, and may also exert a potentially beneficial role in primary and secondary chemoprevention of hepatocellular carcinoma^[160].

Ezetimibe is another lipid-lowering agent, which reduces the intestinal uptake of dietary cholesterol through inhibiting Niemann-Pick C1-like 1 protein, the main transporter of intestinal cholesterol in jejunum, which is also expressed on hepatocytes at the level of canalicular membrane^[161]. Although not tested in randomized clinical trials, preliminary evidence in mice and humans suggests that treatment with ezetimibe may exert some improvement in NAFLD histology^[162,163].

The CVD benefits of the combination of statins and ezetimibe have been largely reported in the literature^[161].

Omega-3 polyunsaturated fatty acids

Omega-3 polyunsaturated fatty acids (PUFA) are useful for the treatment of mild-to-moderate hypertriglyceridemia, which is often associated with insulin resistance and NAFLD. A recent systematic review has shown a significant reduction in hepatic fat content (without any sub-

stantial side effects), although the effect size was relatively small^[164]. However, optimal dose and duration of this therapy need to be addressed in future large clinical trials before recommending omega-3 PUFA supplementation for the treatment of NAFLD.

Although omega-3 PUFAs have also shown to reduce cardiac mortality and sudden cardiac death in patients with previous acute myocardial infarction^[165], however, their role in the primary prevention of CVD in at high-risk patients has recently been challenged by the results of a large randomized clinical trial^[166].

Angiotensin receptor blockers

The renin-angiotensin-aldosterone system is involved in the pathogenesis of insulin resistance, NAFLD and target organ damage^[167]. Angiotensin II increases insulin resistance, exacerbates the systemic inflammatory response by inducing reactive oxygen species and inflammatory cytokines, and stimulates the release of free fatty acids and triglycerides from the liver, thus further increasing systemic insulin resistance^[168].

Some animal and human studies have suggested that angiotensin receptor blockers improve serum liver enzyme levels and histologic features of NAFLD. Specifically, telmisartan attenuated NASH progression in mice by suppressing the macrophage infiltration into the liver. Telmisartan also affected the reduction of adipocyte size and elevation of serum adiponectin in these animals^[169]. Treatment with losartan for 48 wk was associated with some improvement in liver histology in a small sample of hypertensive patients with NASH. However, further larger clinical trials are needed to corroborate these findings^[170].

It is well established that angiotensin receptor blockers reduce blood pressure values and also improve glucose tolerance and insulin sensitivity, thus contributing to further reduce the risk of CVD events even through the prevention of new-onset type 2 diabetes^[171,172].

Vitamin D

Vitamin D₃ has a key role in calcium homeostasis and bone mineralization, and has recently been implicated in the regulation of glucose and lipid metabolism, adipokine production and homeostasis of bile acids^[173]. Vitamin D₃ deficiency is a highly prevalent condition worldwide, present in approximately 30%-60% of the general adult population^[174].

A recent meta-analysis of 17 cross-sectional and case-control studies has shown that patients with NAFLD had about 0.35 ng/mL lower levels of serum 25-hydroxy-vitamin D₃ [25(OH)D₃] and were 1.3 times more likely to be vitamin D deficient than control subjects without NAFLD^[175]. Our group also reported that serum 25(OH)D₃ levels were inversely associated with the histological severity of hepatic steatosis, necro-inflammation and fibrosis, independently of age, sex, season measurement, metabolic syndrome features and kidney function parameters, among patients with histologically proven, non-cirrhotic NAFLD^[176]. Preliminary experimental evi-

dence suggests that *via* effects in both adipose tissue and liver, low serum levels of vitamin D₃ may predispose to hepatic steatosis and necro-inflammation, contributing to the development and progression of NAFLD^[177].

Notably, accumulating evidence from observational, prospective studies suggests that lower serum 25(OH)D₃ levels were strongly associated with higher risks of developing type 2 diabetes, metabolic syndrome and CVD events^[174,178,179]. A recent community-based study also reported that lower serum 25(OH)D₃ levels were significantly associated with abnormalities in cardiac structure and function in elderly patients without a prior history of myocardial infarction, heart failure or valvular heart disease^[180]. However, larger and longer randomized clinical trials are needed to ascertain whether vitamin D₃ supplementation may improve NAFLD and reduce the incidence of adverse CVD outcomes.

Given the increased risk for cardiovascular, cardiac and arrhythmic complications observed in patients with NAFLD and the strong association of NAFLD with the metabolic syndrome, we believe that all cardiometabolic risk factors should be carefully and routinely screened among patients with NAFLD, and that greater emphasis should be placed on both specific lifestyle modifications (*i.e.*, weight loss, increased physical activity and smoking cessation) and aggressive pharmaceutical risk factor modification, which would not only reduce the risk of progressive liver disease, but could also positively impact on the risk of developing structural and arrhythmogenic cardiac complications in this patient population.

CONCLUSION

The relationship between NAFLD and an increased prevalence of clinical CHD appears to be robust, both in adults and in adolescents, and has been consistently replicated across different populations.

However, the specific and independent contribution of NAFLD *per se* to the development and progression of structural and arrhythmogenic cardiac complications is much more controversial. Whether NAFLD is simply a concurrent risk marker in people at increased risk for structural and arrhythmogenic cardiac complications, or is an independent risk factor for the development of such complications remains to be entirely ascertained. Moreover, uncertainty exists about the prognostic value of NAFLD in risk stratification for CHD/CVD. Clearly, more extensive and well-designed prospective studies are needed to answer these key research questions. In theory, such a line of research promises to promote our ability to delay or prevent the development and progression of cardiovascular, cardiac and arrhythmic complications in people with NAFLD.

In conclusion, far from being a benign and “para-physiological” condition, NAFLD should be viewed as a complex and multi-faceted disease often calling for multi-disciplinary intervention. Awareness of NAFLD in general appears to be disappointingly lacking in the

medical community^[15,127]. Data reviewed here strongly support the conclusion that a certain proportion of patients with NAFLD, especially those with NASH, will develop major CVD events and will ultimately die from CVD before developing advanced liver disease. This implies the necessity for specific educational campaigns to be conducted in order to increase awareness of NAFLD as a novel cardiometabolic risk factor, necessitating appropriate diagnostic strategies, aggressive medical management and correct follow-up schedules.

REFERENCES

- 1 Diabetes mellitus; auricular fibrillation; arteriosclerosis obliterans of the legs; gangrene of the 1st and 2d toes of the right foot; fatty degeneration of the liver. *Arq Bras Med* 1952; **42**: 212-216 [PMID: 14953851]
- 2 **Lonardo A**, Bellini M, Tondelli E, Frazzoni M, Grisendi A, Pulvirenti M, Della Casa G. Nonalcoholic steatohepatitis and the “bright liver syndrome”: should a recently expanded clinical entity be further expanded? *Am J Gastroenterol* 1995; **90**: 2072-2074 [PMID: 7485040]
- 3 **Targher G**, Bertolini L, Padovani R, Zenari L, Zoppini G, Falezza G. Relation of nonalcoholic hepatic steatosis to early carotid atherosclerosis in healthy men: role of visceral fat accumulation. *Diabetes Care* 2004; **27**: 2498-2500 [PMID: 15451925 DOI: 10.2337/diacare.27.10.2498]
- 4 **Lonardo A**, Lombardini S, Scaglioni F, Ballestri S, Verrone AM, Bertolotti M, Carulli L, Ganazzi D, Carulli N, Loria P. Fatty liver, carotid disease and gallstones: a study of age-related associations. *World J Gastroenterol* 2006; **12**: 5826-5833 [PMID: 17007049]
- 5 **Sookoian S**, Pirola CJ. Non-alcoholic fatty liver disease is strongly associated with carotid atherosclerosis: a systematic review. *J Hepatol* 2008; **49**: 600-607 [PMID: 18672311 DOI: 10.1016/j.jhep.2008.06.012]
- 6 **Holt LJ**, Krutchinsky AN, Morgan DO. Positive feedback sharpens the anaphase switch. *Nature* 2008; **454**: 353-357 [PMID: 18552837 DOI: 10.1016/j.jhep.2008.05.024]
- 7 **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]
- 8 **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]
- 9 **Targher G**, Mantovani A, Pichiri I, Rigolon R, Dauriz M, Zoppini G, Morani G, Vassanelli C, Bonora E. Non-alcoholic fatty liver disease is associated with an increased prevalence of atrial fibrillation in hospitalized patients with type 2 diabetes. *Clin Sci (Lond)* 2013; **125**: 301-309 [PMID: 23596966 DOI: 10.1042/CS20130036]
- 10 **Targher G**, Valbusa F, Bonapace S, Bertolini L, Zenari L, Rodella S, Zoppini G, Mantovani W, Barbieri E, Byrne CD. Non-alcoholic fatty liver disease is associated with an increased incidence of atrial fibrillation in patients with type 2 diabetes. *PLoS One* 2013; **8**: e57183 [PMID: 23451184 DOI: 10.1371/journal.pone.0057183]
- 11 **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]
- 12 **Maurantonio M**, Ballestri S, Odoardi MR, Lonardo A, Loria P. Treatment of atherogenic liver based on the pathogenesis of nonalcoholic fatty liver disease: a novel approach to reduce cardiovascular risk? *Arch Med Res* 2011; **42**: 337-353 [PMID: 21843565 DOI: 10.1016/j.arcmed.2011.08.004]
 - 13 **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]
 - 14 **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/NEJMr0912063]
 - 15 **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]
 - 16 **Targher G**, Byrne CD. Clinical Review: Nonalcoholic fatty liver disease: a novel cardiometabolic risk factor for type 2 diabetes and its complications. *J Clin Endocrinol Metab* 2013; **98**: 483-495 [PMID: 23293330 DOI: 10.1210/jc.2012-3093]
 - 17 **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 DOI: 10.1002/hep.20781]
 - 18 **Salvi P**, Ruffini R, Agnoletti D, Magnani E, Pagliarani G, Comandini G, Praticò A, Borghi C, Benetos A, Pazzi P. Increased arterial stiffness in nonalcoholic fatty liver disease: the Cardio-GOOSE study. *J Hypertens* 2010; **28**: 1699-1707 [PMID: 20467324 DOI: 10.1097/HJH.0b013e32833a7de6]
 - 19 **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]
 - 20 **Targher G**, Bertolini L, Padovani R, Rodella S, Zoppini G, Zenari L, Cigolini M, Falezza G, Arcaro G. Relations between carotid artery wall thickness and liver histology in subjects with nonalcoholic fatty liver disease. *Diabetes Care* 2006; **29**: 1325-1330 [PMID: 16732016 DOI: 10.2337/dc06-0135]
 - 21 **Colak Y**, Senates E, Yesil A, Yilmaz Y, Ozturk O, Doganay L, Coskunpinar E, Kahraman OT, Mesci B, Ulasoglu C, Tuncer I. Assessment of endothelial function in patients with nonalcoholic fatty liver disease. *Endocrine* 2013; **43**: 100-107 [PMID: 22661277 DOI: 10.1007/s12020-012-9712-1]
 - 22 **Abdulla J**, Asferg C, Kofoed KF. Prognostic value of absence or presence of coronary artery disease determined by 64-slice computed tomography coronary angiography: a systematic review and meta-analysis. *Int J Cardiovasc Imaging* 2011; **27**: 413-420 [PMID: 20549366 DOI: 10.1007/s10554-010-9652-x]
 - 23 **Chen CH**, Nien CK, Yang CC, Yeh YH. Association between nonalcoholic fatty liver disease and coronary artery calcification. *Dig Dis Sci* 2010; **55**: 1752-1760 [PMID: 19688595 DOI: 10.1007/s10620-009-0935-9]
 - 24 **Jung DH**, Lee YJ, Ahn HY, Shim JY, Lee HR. Relationship of hepatic steatosis and alanine aminotransferase with coronary calcification. *Clin Chem Lab Med* 2010; **48**: 1829-1834 [PMID: 20961204 DOI: 10.1515/CCLM.2010.349]
 - 25 **Sung KC**, Wild SH, Kwag HJ, Byrne CD. Fatty liver, insulin resistance, and features of metabolic syndrome: relationships with coronary artery calcium in 10,153 people. *Diabetes Care* 2012; **35**: 2359-2364 [PMID: 22829522 DOI: 10.2337/dc12-0515]
 - 26 **Kim D**, Choi SY, Park EH, Lee W, Kang JH, Kim W, Kim YJ, Yoon JH, Jeong SH, Lee DH, Lee HS, Larson J, Therneau TM, Kim WR. Nonalcoholic fatty liver disease is associated with coronary artery calcification. *Hepatology* 2012; **56**: 605-613 [PMID: 22271511 DOI: 10.1002/hep.25593]
 - 27 **Liu J**, Musani SK, Bidulescu A, Carr JJ, Wilson JG, Taylor HA, Fox CS. Fatty liver, abdominal adipose tissue and atherosclerotic calcification in African Americans: the Jackson Heart Study. *Atherosclerosis* 2012; **224**: 521-525 [PMID: 22902209 DOI: 10.1016/j.atherosclerosis.2012.07.042]
 - 28 **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]
 - 29 **Yilmaz Y**, Kurt R, Yonal O, Polat N, Celikel CA, Gurdal A, Oflaz H, Ozdogan O, Imeryuz N, Kalayci C, Avsar E. Coronary flow reserve is impaired in patients with nonalcoholic fatty liver disease: association with liver fibrosis. *Atherosclerosis* 2010; **211**: 182-186 [PMID: 20181335 DOI: 10.1016/j.atherosclerosis.2010.01.049]
 - 30 **Nakamori S**, Onishi K, Nakajima H, Yoon YE, Nagata M, Kurita T, Yamada T, Kitagawa K, Dohi K, Nakamura M, Sakuma H, Ito M. Impaired myocardial perfusion reserve in patients with fatty liver disease assessed by quantitative myocardial perfusion magnetic resonance imaging. *Circ J* 2012; **76**: 2234-2240 [PMID: 22664721 DOI: 10.1253/circj.CJ-11-1487]
 - 31 **Lin YC**, Lo HM, Chen JD. Sonographic fatty liver, overweight and ischemic heart disease. *World J Gastroenterol* 2005; **11**: 4838-4842 [PMID: 16097054]
 - 32 **Targher G**, Bertolini L, Padovani R, Rodella S, Tessari R, Zenari L, Day C, Arcaro G. Prevalence of nonalcoholic fatty liver disease and its association with cardiovascular disease among type 2 diabetic patients. *Diabetes Care* 2007; **30**: 1212-1218 [PMID: 17277038 DOI: 10.2337/dc06-2247]
 - 33 **Arslan U**, Türkoğlu S, Balcioglu S, Tavil Y, Karakan T, Cengel A. Association between nonalcoholic fatty liver disease and coronary artery disease. *Coron Artery Dis* 2007; **18**: 433-436 [PMID: 17700213 DOI: 10.1097/MCA.0b013e3282583c0d]
 - 34 **Mirbagheri SA**, Rashidi A, Abdi S, Saedi D, Abouzari M. Liver: an alarm for the heart? *Liver Int* 2007; **27**: 891-894 [PMID: 17696926 DOI: 10.1111/j.1478-3231.2007.01531.x]
 - 35 **Alper AT**, Hasdemir H, Sahin S, Ontürk E, Akyol A, Nurekalem Z, Cakmak N, Erdinler I, Gürkan K. The relationship between nonalcoholic fatty liver disease and the severity of coronary artery disease in patients with metabolic syndrome. *Türk Kardiyol Dern Ars* 2008; **36**: 376-381 [PMID: 19155640]
 - 36 **Akabame S**, Hamaguchi M, Tomiyasu K, Tanaka M, Kobayashi-Takenaka Y, Nakano K, Oda Y, Yoshikawa T. Evaluation of vulnerable coronary plaques and non-alcoholic fatty liver disease (NAFLD) by 64-detector multislice computed tomography (MSCT). *Circ J* 2008; **72**: 618-625 [PMID: 18362435 DOI: 10.1253/circj.72.618]
 - 37 **Açikel M**, Sunay S, Koplay M, Gündoğdu F, Karakelleoğlu S. Evaluation of ultrasonographic fatty liver and severity of coronary atherosclerosis, and obesity in patients undergoing coronary angiography. *Anadolu Kardiyol Derg* 2009; **9**: 273-279 [PMID: 19666428]
 - 38 **Assy N**, Djibre A, Farah R, Grosovski M, Marmor A. Presence of coronary plaques in patients with nonalcoholic fatty liver disease. *Radiology* 2010; **254**: 393-400 [PMID: 20093511 DOI: 10.1148/radiol.09090769]
 - 39 **Targher G**, Bertolini L, Padovani R, Rodella S, Zoppini G, Pichiri I, Sorgato C, Zenari L, Bonora E. Prevalence of non-alcoholic fatty liver disease and its association with cardiovascular disease in patients with type 1 diabetes. *J Hepatol* 2010; **53**: 713-718 [PMID: 20619918 DOI: 10.1016/j.jhep.2010.04.030]
 - 40 **Sun L**, Lü SZ. Association between non-alcoholic fatty liver disease and coronary artery disease severity. *Chin Med J*

- (Engl) 2011; **124**: 867-872 [PMID: 21518594 DOI: 10.3760/cma.j.issn.0366-6999.2011.06.012]
- 41 **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]
 - 42 **Domanski JP**, Park SJ, Harrison SA. Cardiovascular disease and nonalcoholic fatty liver disease: does histologic severity matter? *J Clin Gastroenterol* 2012; **46**: 427-430 [PMID: 22469639 DOI: 10.1097/MCG.0b013e31822fb3f7]
 - 43 **Agaç MT**, Korkmaz L, Cavusoglu G, Karadeniz AG, Agaç S, Bektas H, Erkan H, Varol MO, Vatan MB, Acar Z, Mentese U, Celik S. Association between nonalcoholic fatty liver disease and coronary artery disease complexity in patients with acute coronary syndrome: a pilot study. *Angiology* 2013; **64**: 604-608 [PMID: 23439214 DOI: 10.1177/0003319713479155]
 - 44 **Boddi M**, Tarquini R, Chiostrì M, Marra F, Valente S, Giglioli C, Gensini GF, Abbate R. Nonalcoholic fatty liver in nondiabetic patients with acute coronary syndromes. *Eur J Clin Invest* 2013; **43**: 429-438 [PMID: 23480577 DOI: 10.1111/eci.12065]
 - 45 **Inci MF**, Özkan F, Ark B, Vurdem ÜE, Ege MR, Sincer I, Zorlu A. Sonographic evaluation for predicting the presence and severity of coronary artery disease. *Ultrasound Q* 2013; **29**: 125-130 [PMID: 23609339 DOI: 10.1097/RUQ.0b013e318291580e]
 - 46 **Arslan U**, Kocaoglu I, Balci M, Duyuler S, Korkmaz A. The association between impaired collateral circulation and non-alcoholic fatty liver in patients with severe coronary artery disease. *J Cardiol* 2012; **60**: 210-214 [PMID: 22738690 DOI: 10.1016/j.jicc.2012.05.003]
 - 47 **Fraser A**, Harris R, Sattar N, Ebrahim S, Smith GD, Lawlor DA. Gamma-glutamyltransferase is associated with incident vascular events independently of alcohol intake: analysis of the British Women's Heart and Health Study and Meta-Analysis. *Arterioscler Thromb Vasc Biol* 2007; **27**: 2729-2735 [PMID: 17932318 DOI: 10.1161/ATVBAHA.107.152298]
 - 48 **Schindhelm RK**, Dekker JM, Nijpels G, Bouter LM, Stehouwer CD, Heine RJ, Diamant M. Alanine aminotransferase predicts coronary heart disease events: a 10-year follow-up of the Hoorn Study. *Atherosclerosis* 2007; **191**: 391-396 [PMID: 16682043 DOI: 10.1016/j.atherosclerosis.2006.04.006]
 - 49 **Goessling W**, Massaro JM, Vasan RS, D'Agostino RB, Ellison RC, Fox CS. Aminotransferase levels and 20-year risk of metabolic syndrome, diabetes, and cardiovascular disease. *Gastroenterology* 2008; **135**: 1935-1944. e1 [PMID: 19010326 DOI: 10.1053/j.gastro.2008.09.018]
 - 50 **Dunn W**, Xu R, Wingard DL, Rogers C, Angulo P, Younossi ZM, Schwimmer JB. Suspected nonalcoholic fatty liver disease and mortality risk in a population-based cohort study. *Am J Gastroenterol* 2008; **103**: 2263-2271 [PMID: 18684196 DOI: 10.1111/j.1572-0241.2008.02034.x]
 - 51 **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]
 - 52 **Ruhl CE**, Everhart JE. Elevated serum alanine aminotransferase and gamma-glutamyltransferase and mortality in the United States population. *Gastroenterology* 2009; **136**: 477-85. e11 [PMID: 19100265 DOI: 10.1053/j.gastro.2008.10.052]
 - 53 **Yun KE**, Shin CY, Yoon YS, Park HS. Elevated alanine aminotransferase levels predict mortality from cardiovascular disease and diabetes in Koreans. *Atherosclerosis* 2009; **205**: 533-537 [PMID: 19159884 DOI: 10.1016/j.atherosclerosis.2008.12.012]
 - 54 **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]
 - 55 **Leuchbaum E**, Pilz S, Grammer TB, Boehm BO, Stojakovic T, Obermayer-Pietsch B, März W. The fatty liver index is associated with increased mortality in subjects referred to coronary angiography. *Nutr Metab Cardiovasc Dis* 2013; **23**: 1231-1238 [PMID: 23557879 DOI: 10.1016/j.numecd.2013.02.004]
 - 56 **Jepsen P**, Vilstrup H, Møllemejkjaer L, Thulstrup AM, Olsen JH, Baron JA, Sørensen HT. Prognosis of patients with a diagnosis of fatty liver--a registry-based cohort study. *Hepatology* 2003; **50**: 2101-2104 [PMID: 14696473]
 - 57 **Targher G**, Bertolini L, Rodella S, Tessari R, Zenari L, Lippi G, Arcaro G. Nonalcoholic fatty liver disease is independently associated with an increased incidence of cardiovascular events in type 2 diabetic patients. *Diabetes Care* 2007; **30**: 2119-2121 [PMID: 17519430 DOI: 10.2337/dc07-0349]
 - 58 **Soler Rodriguez F**, Miguez Santiyan MP, Pedrera Zamorano JD, Roncero Cordero V. An outbreak of lupinosis in sheep. *Vet Hum Toxicol* 1991; **33**: 492-494 [PMID: 1746145]
 - 59 **Lazo M**, Hernaez R, Bonekamp S, Kamel IR, Brancati FL, Guallar E, Clark JM. Non-alcoholic fatty liver disease and mortality among US adults: prospective cohort study. *BMJ* 2011; **343**: d6891 [PMID: 22102439 DOI: 10.1136/bmj.d6891]
 - 60 **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]
 - 61 **Zhou YJ**, Li YY, Nie YQ, Huang CM, Cao CY. Natural course of nonalcoholic fatty liver disease in southern China: a prospective cohort study. *J Dig Dis* 2012; **13**: 153-160 [PMID: 22356310 DOI: 10.1111/j.1751-2980.2011.00571.x]
 - 62 **Younossi ZM**, Otgonsuren M, Venkatesan C, Mishra A. In patients with non-alcoholic fatty liver disease, metabolically abnormal individuals are at a higher risk for mortality while metabolically normal individuals are not. *Metabolism* 2013; **62**: 352-360 [PMID: 22999011 DOI: 10.1016/j.metabol.2012.08.005]
 - 63 **Haring R**, Wallaschofski H, Nauck M, Dörr M, Baumeister SE, Völzke H. Ultrasonographic hepatic steatosis increases prediction of mortality risk from elevated serum gamma-glutamyl transpeptidase levels. *Hepatology* 2009; **50**: 1403-1411 [PMID: 19670414 DOI: 10.1002/hep.23135]
 - 64 **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]
 - 65 **Treepasertsuk S**, Leverage S, Adams LA, Lindor KD, St Sauver J, Angulo P. The Framingham risk score and heart disease in nonalcoholic fatty liver disease. *Liver Int* 2012; **32**: 945-950 [PMID: 22299674 DOI: 10.1111/j.1478-3231.2011.02753.x]
 - 66 **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]
 - 67 **Dam-Larsen S**, Franzmann M, Andersen IB, Christoffersen P, Jensen LB, Sørensen TI, Becker U, Bendtsen F. Long term prognosis of fatty liver: risk of chronic liver disease and death. *Gut* 2004; **53**: 750-755 [PMID: 15082596 DOI: 10.1136/gut.2003.019984]
 - 68 **Adams LA**, Lymp JF, St Sauver J, Sanderson SO, Lindor KD, Feldstein A, Angulo P. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. *Gastroenterology* 2005; **129**: 113-121 [PMID: 16012941]
 - 69 **Eckstedt M**, Franzén LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, Kechagias S. Long-term follow-up of patients with NAFLD and elevated liver enzymes. *Hepatology* 2006; **44**: 865-873 [PMID: 17006923 DOI: 10.1002/

- hep.21327]
- 70 **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]
 - 71 **Söderberg C**, Stål P, Askling J, Glaumann H, Lindberg G, Marmur J, Hultcrantz R. Decreased survival of subjects with elevated liver function tests during a 28-year follow-up. *Hepatology* 2010; **51**: 595-602 [PMID: 20014114 DOI: 10.1002/hep.23314]
 - 72 **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]
 - 73 **Jiang ZY**, Xu CY, Chang XX, Li WW, Sun LY, Yang XB, Yu LF. Fatty liver index correlates with non-alcoholic fatty liver disease, but not with newly diagnosed coronary artery atherosclerotic disease in Chinese patients. *BMC Gastroenterol* 2013; **13**: 110 [PMID: 23834773 DOI: 10.1186/1471-230X-13-110]
 - 74 **Rijzewijk LJ**, van der Meer RW, Smit JW, Diamant M, Bax JJ, Hammer S, Romijn JA, de Roos A, Lamb HJ. Myocardial steatosis is an independent predictor of diastolic dysfunction in type 2 diabetes mellitus. *J Am Coll Cardiol* 2008; **52**: 1793-1799 [PMID: 19022158 DOI: 10.1016/j.jacc.2008.07.062]
 - 75 **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]
 - 76 **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 DOI: 10.1097/01.mcg.0000225668.53673.e6]
 - 77 **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]
 - 78 **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 non-alcoholic fatty liver disease. *Cardiol J* 2010; **17**: 457-463 [PMID: 20865675]
 - 79 **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]
 - 80 **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]
 - 81 **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]
 - 82 **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, 1168.e1 [PMID: 23260104 DOI: 10.1016/j.jpeds.2012.11.024]
 - 83 **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]
 - 84 **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* 2013 Jul 11; Epub ahead of print [PMID: 23843206 DOI: 10.1002/hep.26610]
 - 85 **Dhingra R**, Gona P, Wang TJ, Fox CS, D'Agostino RB, Vasani RS. Serum gamma-glutamyl transferase and risk of heart failure in the community. *Arterioscler Thromb Vasc Biol* 2010; **30**: 1855-1860 [PMID: 20539015 DOI: 10.1161/ATVBAHA.110.207340]
 - 86 **Wannamethee SG**, Whincup PH, Shaper AG, Lennon L, Sattar N. Γ -glutamyltransferase, hepatic enzymes, and risk of incident heart failure in older men. *Arterioscler Thromb Vasc Biol* 2012; **32**: 830-835 [PMID: 22223732 DOI: 10.1161/ATVBAHA.111.240457]
 - 87 **Sinner MF**, Wang N, Fox CS, Fontes JD, Rienstra M, Magnani JW, Vasani RS, Calderwood AH, Pencina M, Sullivan LM, Ellinor PT, Benjamin EJ. Relation of circulating liver transaminase concentrations to risk of new-onset atrial fibrillation. *Am J Cardiol* 2013; **111**: 219-224 [PMID: 23127690 DOI: 10.1016/j.amjcard.2012.09.021]
 - 88 **Lip GY**, Tse HF, Lane DA. Atrial fibrillation. *Lancet* 2012; **379**: 648-661 [PMID: 22166900 DOI: 10.1016/S0140-6736]
 - 89 **Kleiger RE**, Stein PK, Bigger JT. Heart rate variability: measurement and clinical utility. *Ann Noninvasive Electrocardiol* 2005; **10**: 88-101 [PMID: 15649244 DOI: 10.1111/j.1542-474X.2005.10101.x]
 - 90 **Algra A**, Tijssen JG, Roelandt JR, Pool J, Lubsen J. QTc prolongation measured by standard 12-lead electrocardiography is an independent risk factor for sudden death due to cardiac arrest. *Circulation* 1991; **83**: 1888-1894 [PMID: 2040041 DOI: 10.1161/01.CIR.83.6.1888]
 - 91 **Straus SM**, Kors JA, De Bruin ML, van der Hooft CS, Hofman A, Heeringa J, Deckers JW, Kingma JH, Sturkenboom MC, Stricker BH, Witteman JC. Prolonged QTc interval and risk of sudden cardiac death in a population of older adults. *J Am Coll Cardiol* 2006; **47**: 362-367 [PMID: 16412861 DOI: 10.1016/j.jacc.2005.08.067]
 - 92 **Okin PM**, Devereux RB, Lee ET, Galloway JM, Howard BV. Electrocardiographic repolarization complexity and abnormality predict all-cause and cardiovascular mortality in diabetes: the strong heart study. *Diabetes* 2004; **53**: 434-440 [PMID: 14747295]
 - 93 **Liu YC**, Hung CS, Wu YW, Lee YC, Lin YH, Lin C, Lo MT, Chan CC, Ma HP, Ho YL, Chen CH. Influence of non-alcoholic fatty liver disease on autonomic changes evaluated by the time domain, frequency domain, and symbolic dynamics of heart rate variability. *PLoS One* 2013; **8**: e61803 [PMID: 23626730 DOI: 10.1371/journal.pone.0061803]
 - 94 **Newton JL**, Paiman J, Wilton K, Jones DE, Day C. Fatigue and autonomic dysfunction in non-alcoholic fatty liver disease. *Clin Auton Res* 2009; **19**: 319-326 [PMID: 19768633 DOI: 10.1007/s10286-009-0031-4]
 - 95 **Otto CM**, Kuusisto J, Reichenbach DD, Gown AM, O'Brien KD. Characterization of the early lesion of 'degenerative' valvular aortic stenosis. Histological and immunohistochemical studies. *Circulation* 1994; **90**: 844-853 [PMID: 7519131 DOI: 10.1161/01.CIR.90.2.844]
 - 96 **Otto CM**, Lind BK, Kitzman DW, Gersh BJ, Siscovick DS. Association of aortic-valve sclerosis with cardiovascular mortality and morbidity in the elderly. *N Engl J Med* 1999; **341**: 142-147 [PMID: 10403851 DOI: 10.1056/NEJM199907153410302]
 - 97 **Barasch E**, Gottdiener JS, Marino Larsen EK, Chaves PH, Newman AB. Cardiovascular morbidity and mortality in

- community-dwelling elderly individuals with calcification of the fibrous skeleton of the base of the heart and aortosclerosis (The Cardiovascular Health Study). *Am J Cardiol* 2006; **97**: 1281-1286 [PMID: 16635596 DOI: 10.1016/j.amjcard.2005.11.065]
- 98 **Rossi A**, Targher G, Zoppini G, Ciccoira M, Bonapace S, Negri C, Stoico V, Faggiano P, Vassanelli C, Bonora E. Aortic and mitral annular calcifications are predictive of all-cause and cardiovascular mortality in patients with type 2 diabetes. *Diabetes Care* 2012; **35**: 1781-1786 [PMID: 22699285 DOI: 10.2337/dc12-0134]
 - 99 **Markus MR**, Baumeister SE, Stritzke J, Dörr M, Wallaschofski H, Völzke H, Lieb W. Hepatic steatosis is associated with aortic valve sclerosis in the general population: the Study of Health in Pomerania (SHIP). *Arterioscler Thromb Vasc Biol* 2013; **33**: 1690-1695 [PMID: 23685558 DOI: 10.1161/ATVBAHA.112.300556]
 - 100 **Verrijken A**, Francque S, Mertens I, Prawitt J, Caron S, Hubens G, Van Marck E, Staels B, Michielsen P, Gaal LV. Prothrombotic factors in histologically proven NAFLD and NASH. *Hepatology* 2013 May 23; Epub ahead of print [PMID: 23703589 DOI: 10.1002/hep.26510]
 - 101 **Simonen M**, Männistö V, Leppänen J, Kaminska D, Kärjä V, Venesmaa S, Käkälä P, Kuusisto J, Gylling H, Laakso M, Pihlajamäki J. Desmosterol in human nonalcoholic steatohepatitis. *Hepatology* 2013; **58**: 976-982 [PMID: 23447451 DOI: 10.1002/hep.26342]
 - 102 **Musso G**, Cassader M, De Micheli F, Rosina F, Orlandi F, Gambino R. Nonalcoholic steatohepatitis versus steatosis: adipose tissue insulin resistance and dysfunctional response to fat ingestion predict liver injury and altered glucose and lipoprotein metabolism. *Hepatology* 2012; **56**: 933-942 [PMID: 22684858 DOI: 10.1002/hep.25739]
 - 103 **Musso G**, Cassader M, Bo S, De Micheli 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]
 - 104 **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]
 - 105 **Ndumele CE**, Nasir K, Conceicao RD, Carvalho JA, Blumenthal RS, Santos RD. Hepatic steatosis, obesity, and the metabolic syndrome are independently and additively associated with increased systemic inflammation. *Arterioscler Thromb Vasc Biol* 2011; **31**: 1927-1932 [PMID: 21546603 DOI: 10.1161/ATVBAHA.111.228262]
 - 106 **Papathodoridis GV**, Chrysanthos N, Cholongitas E, Pavlou E, Apergis G, Tiniakos DG, Andrioti E, Theodosiades G, Archimandritis AJ. Thrombotic risk factors and liver histologic lesions in non-alcoholic fatty liver disease. *J Hepatol* 2009; **51**: 931-938 [PMID: 19726097 DOI: 10.1016/j.jhep.2009.06.023]
 - 107 **Assy N**, Bekirov I, Mejritsky Y, Solomon L, Szvalb S, Hussein O. Association between thrombotic risk factors and extent of fibrosis in patients with non-alcoholic fatty liver diseases. *World J Gastroenterol* 2005; **11**: 5834-5839 [PMID: 16270394]
 - 108 **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]
 - 109 **Targher G**, Byrne CD. Diagnosis and management of non-alcoholic fatty liver disease and its hemostatic/thrombotic and vascular complications. *Semin Thromb Hemost* 2013; **39**: 214-228 [PMID: 23397556 DOI: 10.1055/s-0033-1334866]
 - 110 **Nyrnes A**, Njølstad I, Mathiesen EB, Wilsgaard T, Hansen JB, Skjelbakken T, Jørgensen L, Løchen ML. Inflammatory biomarkers as risk factors for future atrial fibrillation. An eleven-year follow-up of 6315 men and women: the Tromsø study. *Gen Med* 2012; **9**: 536-547.e2 [PMID: 23046763 DOI: 10.1016/j.genm.2012.09.001]
 - 111 **Liu T**, Li G, Li L, Korantzopoulos P. Association between C-reactive protein and recurrence of atrial fibrillation after successful electrical cardioversion: a meta-analysis. *J Am Coll Cardiol* 2007; **49**: 1642-1648 [PMID: 17433956 DOI: 10.1016/j.jacc.2006.12.042]
 - 112 **Lonardo A**, Bellentani S, Ratzu 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]
 - 113 **Razani B**, Chakravarthy MV, Semenkovich CF. Insulin resistance and atherosclerosis. *Endocrinol Metab Clin North Am* 2008; **37**: 603-621, viii [PMID: 18775354 DOI: 10.1016/j.jec.2008.05.001]
 - 114 **DeFilippis AP**, Blaha MJ, Martin SS, Reed RM, Jones SR, Nasir K, Blumenthal RS, Budoff MJ. Nonalcoholic fatty liver disease and serum lipoproteins: the Multi-Ethnic Study of Atherosclerosis. *Atherosclerosis* 2013; **227**: 429-436 [PMID: 23419204 DOI: 10.1016/j.atherosclerosis.2013.01.022]
 - 115 **Liu J**, Fox CS, Hickson D, Bidulescu A, Carr JJ, Taylor HA. Fatty liver, abdominal visceral fat, and cardiometabolic risk factors: the Jackson Heart Study. *Arterioscler Thromb Vasc Biol* 2011; **31**: 2715-2722 [PMID: 21885852 DOI: 10.1161/ATVBAHA.111.234062]
 - 116 **Pastromas S**, Terzi AB, Tousoulis D, Koulouris S. Postprandial lipemia: an under-recognized atherogenic factor in patients with diabetes mellitus. *Int J Cardiol* 2008; **126**: 3-12 [PMID: 17689745 DOI: 10.1016/j.ijcard.2007.04.172]
 - 117 **Goldberg IJ**, Eckel RH, McPherson R. Triglycerides and heart disease: still a hypothesis? *Arterioscler Thromb Vasc Biol* 2011; **31**: 1716-1725 [PMID: 21527746 DOI: 10.1161/ATVBAHA.111.226100]
 - 118 **Newton JL**, Jones DE, Henderson E, Kane L, Wilton K, Burt AD, Day CP. Fatigue in non-alcoholic fatty liver disease (NAFLD) is significant and associates with inactivity and excessive daytime sleepiness but not with liver disease severity or insulin resistance. *Gut* 2008; **57**: 807-813 [PMID: 18270241 DOI: 10.1136/gut.2007.139303]
 - 119 **Newton JL**. Systemic symptoms in non-alcoholic fatty liver disease. *Dig Dis* 2010; **28**: 214-219 [PMID: 20460914 DOI: 10.1159/000282089]
 - 120 **Jakovljevic DG**, Hallsworth K, Zalewski P, Thoma C, Klawe JJ, Day CP, Newton J, Trenell MI. Resistance exercise improves autonomic regulation at rest and haemodynamic response to exercise in non-alcoholic fatty liver disease. *Clin Sci (Lond)* 2013; **125**: 143-149 [PMID: 23458257 DOI: 10.1042/CS20120684]
 - 121 **Ahmed MH**, Byrne CD. Obstructive sleep apnea syndrome and fatty liver: association or causal link? *World J Gastroenterol* 2010; **16**: 4243-4252 [PMID: 20818807 DOI: 10.3748/wjg.v16.i34.4243]
 - 122 **Musso G**, Cassader M, Olivetti C, Rosina F, Carbone G, Gambino R. Association of obstructive sleep apnoea with the presence and severity of non-alcoholic fatty liver disease. A systematic review and meta-analysis. *Obes Rev* 2013; **14**: 417-431 [PMID: 23387384 DOI: 10.1111/obr.12020]
 - 123 **Kohler M**, Stradling JR. Mechanisms of vascular damage in obstructive sleep apnea. *Nat Rev Cardiol* 2010; **7**: 677-685 [PMID: 21079639 DOI: 10.1038/nrcardio.2010.145]
 - 124 **Ratzu 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]
 - 125 **Loria P**, Adinolfi LE, Bellentani S, Bugianesi E, Grieco A, Fargion S, Gasbarrini A, Loguercio C, Lonardo A, Marchesini G, Marra F, Persico M, Prati D, Baroni GS. Practice guide-

- lines for the diagnosis and management of nonalcoholic fatty liver disease. A decalogue from the Italian Association for the Study of the Liver (AISF) Expert Committee. *Dig Liver Dis* 2010; **42**: 272-282 [PMID: 20171943 DOI: 10.1016/j.dld.2010.01.021]
- 126 **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]
 - 127 **Nascimbeni F**, Pais R, Bellentani S, Day CP, Ratziu V, Loria P, Lonardo A. From NAFLD in clinical practice to answers from guidelines. *J Hepatol* 2013; **59**: 859-871 [PMID: 23751754 DOI: 10.1016/j.jhep.2013.05.044]
 - 128 **Thoma C**, Day CP, Trenell MI. Lifestyle interventions for the treatment of non-alcoholic fatty liver disease in adults: a systematic review. *J Hepatol* 2012; **56**: 255-266 [PMID: 21723839 DOI: 10.1016/j.jhep.2011.06.010]
 - 129 **Johnson NA**, George J. Fitness versus fatness: moving beyond weight loss in nonalcoholic fatty liver disease. *Hepatology* 2010; **52**: 370-381 [PMID: 20578153 DOI: 10.1002/hep.23711]
 - 130 **Hallsworth K**, Fattakhova G, Hollingsworth KG, Thoma C, Moore S, Taylor R, Day CP, Trenell MI. Resistance exercise reduces liver fat and its mediators in non-alcoholic fatty liver disease independent of weight loss. *Gut* 2011; **60**: 1278-1283 [PMID: 21708823 DOI: 10.1136/gut.2011.242073]
 - 131 **Bae JC**, Suh S, Park SE, Rhee EJ, Park CY, Oh KW, Park SW, Kim SW, Hur KY, Kim JH, Lee MS, Lee MK, Kim KW, Lee WY. Regular exercise is associated with a reduction in the risk of NAFLD and decreased liver enzymes in individuals with NAFLD independent of obesity in Korean adults. *PLoS One* 2012; **7**: e46819 [PMID: 23110056 DOI: 10.1371/journal.pone.0046819]
 - 132 **Keating SE**, Hackett DA, George J, Johnson NA. Exercise and non-alcoholic fatty liver disease: a systematic review and meta-analysis. *J Hepatol* 2012; **57**: 157-166 [PMID: 22414768 DOI: 10.1016/j.jhep.2012.02.023]
 - 133 **Bacchi E**, Negri C, Targher G, Faccioli N, Lanza M, Zoppini G, Zanolin E, Schena F, Bonora E, Moghetti P. Both resistance training and aerobic training reduce hepatic fat content in type 2 diabetic subjects with nonalcoholic fatty liver disease (the RAED2 Randomized Trial). *Hepatology* 2013; **58**: 1287-1295 [PMID: 23504926 DOI: 10.1002/hep.26393]
 - 134 **Bhupathiraju SN**, Tucker KL. Coronary heart disease prevention: nutrients, foods, and dietary patterns. *Clin Chim Acta* 2011; **412**: 1493-1514 [PMID: 21575619 DOI: 10.1016/j.cca.2011.04.038]
 - 135 **Kardassis D**, Bech-Hanssen O, Schönander M, Sjöström L, Karason K. The influence of body composition, fat distribution, and sustained weight loss on left ventricular mass and geometry in obesity. *Obesity* (Silver Spring) 2012; **20**: 605-611 [PMID: 21566562 DOI: 10.1038/oby.2011.101]
 - 136 **Frohlich J**, Al-Sarraf A. Cardiovascular risk and atherosclerosis prevention. *Cardiovasc Pathol* 2013; **22**: 16-18 [PMID: 22502868 DOI: 10.1016/j.carpath.2012.03.001]
 - 137 **Mathur N**, Pedersen BK. Exercise as a mean to control low-grade systemic inflammation. *Mediators Inflamm* 2008; **2008**: 109502 [PMID: 19148295 DOI: 10.1155/2008/109502]
 - 138 **Vepsäläinen T**, Soinio M, Marniemi J, Lehto S, Juutilainen A, Laakso M, Rönkämaa T. Physical activity, high-sensitivity C-reactive protein, and total and cardiovascular disease mortality in type 2 diabetes. *Diabetes Care* 2011; **34**: 1492-1496 [PMID: 21602429 DOI: 10.2337/dc11-0469]
 - 139 **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]
 - 140 **Carulli L**, Maurantonio M, Hebbard L, Baldelli E, Loria P, George J. Classical and innovative insulin sensitizing drugs for the prevention and treatment of NAFLD. *Curr Pharm Des* 2013; **19**: 5280-5296 [PMID: 23394096 DOI: 10.2174/1381612811319290009]
 - 141 **Singh S**, Singh PP, Singh AG, Murad MH, Sanchez W. Anti-diabetic medications and the risk of hepatocellular cancer: a systematic review and meta-analysis. *Am J Gastroenterol* 2013; **108**: 881-91; quiz 892 [PMID: 23381014 DOI: 10.1038/ajg.2013.5]
 - 142 **Zhang H**, Gao C, Fang L, Zhao HC, Yao SK. Metformin and reduced risk of hepatocellular carcinoma in diabetic patients: a meta-analysis. *Scand J Gastroenterol* 2013; **48**: 78-87 [PMID: 23137049 DOI: 10.3109/00365521.2012.719926]
 - 143 **Chen HP**, Shieh JJ, Chang CC, Chen TT, Lin JT, Wu MS, Lin JH, Wu CY. Metformin decreases hepatocellular carcinoma risk in a dose-dependent manner: population-based and in vitro studies. *Gut* 2013; **62**: 606-615 [PMID: 22773548]
 - 144 **Ratziu V**, Caldwell S, Neuschwander-Tetri BA. Therapeutic trials in nonalcoholic steatohepatitis: insulin sensitizers and related methodological issues. *Hepatology* 2010; **52**: 2206-2215 [PMID: 21105109 DOI: 10.1002/hep.24042]
 - 145 **Rakoski MO**, Singal AG, Rogers MA, Conjeevaram H. Meta-analysis: insulin sensitizers for the treatment of non-alcoholic steatohepatitis. *Aliment Pharmacol Ther* 2010; **32**: 1211-1221 [PMID: 20955440 DOI: 10.1111/j.1365-2036.2010.04467.x]
 - 146 **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]
 - 147 **Lutchman G**, Modi A, Kleiner DE, Promrat K, Heller T, Ghany M, Borg B, Loomba R, Liang TJ, Premkumar A, Hoofnagle JH. The effects of discontinuing pioglitazone in patients with nonalcoholic steatohepatitis. *Hepatology* 2007; **46**: 424-429 [PMID: 17559148 DOI: 10.1002/hep.21661]
 - 148 **Lincoff AM**, Wolski K, Nicholls SJ, Nissen SE. Pioglitazone and risk of cardiovascular events in patients with type 2 diabetes mellitus: a meta-analysis of randomized trials. *JAMA* 2007; **298**: 1180-1188 [PMID: 17848652 DOI: 10.1001/jama]
 - 149 **Neumann A**, Weill A, Ricordeau P, Fagot JP, Alla F, Allemand H. Pioglitazone and risk of bladder cancer among diabetic patients in France: a population-based cohort study. *Diabetologia* 2012; **55**: 1953-1962 [PMID: 22460763 DOI: 10.1007/s00125-012-2538-9]
 - 150 **Hiatt WR**, Kaul S, Smith RJ. The cardiovascular safety of diabetes drugs--insights from the rosiglitazone experience. *N Engl J Med* 2013; **369**: 1285-1287 [PMID: 23992603 DOI: 10.1056/NEJMp1309610]
 - 151 **Wajsborg E**, Tavarua A. Exenatide: clinical aspects of the first incretin-mimetic for the treatment of type 2 diabetes mellitus. *Expert Opin Pharmacother* 2009; **10**: 135-142 [PMID: 19236187 DOI: 10.1517/14656560802611832]
 - 152 **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]
 - 153 **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 Acip30 knockdown by inhibiting c-JNK. *Liver Int* 2013; **33**: 794-804 [PMID: 23432843 DOI: 10.1111/liv.12120]
 - 154 **Klonoff DC**, Buse JB, Nielsen LL, Guan X, Bowls CL, Holcombe JH, Wintle ME, Maggs DG. Exenatide effects on diabetes, obesity, cardiovascular risk factors and hepatic biomarkers in patients with type 2 diabetes treated for at least 3 years. *Curr Med Res Opin* 2008; **24**: 275-286 [PMID:

- 18053320]
- 155 **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]
 - 156 **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]
 - 157 **Mundil D**, Cameron-Vendrig A, Husain M. GLP-1 receptor agonists: a clinical perspective on cardiovascular effects. *Diab Vasc Dis Res* 2012; **9**: 95-108 [PMID: 22496442 DOI: 10.1177/14791641112441526]
 - 158 **Athyros VG**, Tziomalos K, Gossios TD, Griva T, Anagnostis P, Kargiotis K, Pagourelias ED, Theocharidou E, Karagiannis A, Mikhailidis DP. Safety and efficacy of long-term statin treatment for cardiovascular events in patients with coronary heart disease and abnormal liver tests in the Greek Atorvastatin and Coronary Heart Disease Evaluation (GREACE) Study: a post-hoc analysis. *Lancet* 2010; **376**: 1916-1922 [PMID: 21109302 DOI: 10.1016/S0140-6736(10)61272-x]
 - 159 **Tikkanen MJ**, Fayyad R, Faergeman O, Olsson AG, Wun CC, Laskey R, Kastelein JJ, Holme I, Pedersen TR. Effect of intensive lipid lowering with atorvastatin on cardiovascular outcomes in coronary heart disease patients with mild-to-moderate baseline elevations in alanine aminotransferase levels. *Int J Cardiol* 2013; **168**: 3846-3852 [PMID: 24001698 DOI: 10.1016/j.ijcard.2013.06.024]
 - 160 **Lonardo A**, Loria P. Potential for statins in the chemoprevention and management of hepatocellular carcinoma. *J Gastroenterol Hepatol* 2012; **27**: 1654-1664 [PMID: 22849701 DOI: 10.1111/j.1440-1746.2012.07232.x]
 - 161 **Phan BA**, Dayspring TD, Toth PP. Ezetimibe therapy: mechanism of action and clinical update. *Vasc Health Risk Manag* 2012; **8**: 415-427 [PMID: 22910633 DOI: 10.2147/VHRM.S33664]
 - 162 **Van Rooyen DM**, Gan LT, Yeh MM, Haigh WG, Larter CZ, Ioannou G, Teoh NC, Farrell GC. Pharmacological cholesterol lowering reverses fibrotic NASH in obese, diabetic mice with metabolic syndrome. *J Hepatol* 2013; **59**: 144-152 [PMID: 23500152 DOI: 10.1016/j.jhep.2013.02.024]
 - 163 **Park H**, Shima T, Yamaguchi K, Mitsuyoshi H, Minami M, Yasui K, Itoh Y, Yoshikawa T, Fukui M, Hasegawa G, Nakamura N, Ohta M, Obayashi H, Okanoue T. Efficacy of long-term ezetimibe therapy in patients with nonalcoholic fatty liver disease. *J Gastroenterol* 2011; **46**: 101-107 [PMID: 20658156 DOI: 10.1007/s00535-010-0291-8]
 - 164 **Parker HM**, Johnson NA, Burdon CA, Cohn JS, O'Connor HT, George J. Omega-3 supplementation and non-alcoholic fatty liver disease: a systematic review and meta-analysis. *J Hepatol* 2012; **56**: 944-951 [PMID: 22023985 DOI: 10.1016/j.jhep.2011.08.018]
 - 165 **Marik PE**, Varon J. Omega-3 dietary supplements and the risk of cardiovascular events: a systematic review. *Clin Cardiol* 2009; **32**: 365-372 [PMID: 19609891 DOI: 10.1002/clc.20604]
 - 166 **Roncaglioni MC**, Tombesi M, Avanzini F, Barlera S, Caimi V, Longoni P, Marzona I, Milani V, Silletta MG, Tognoni G, Marchioli R. n-3 fatty acids in patients with multiple cardiovascular risk factors. *N Engl J Med* 2013; **368**: 1800-1808 [PMID: 23656645 DOI: 10.1056/NEJMoa1205409]
 - 167 **Hsueh WA**, Wyne K. Renin-Angiotensin-aldosterone system in diabetes and hypertension. *J Clin Hypertens* (Greenwich) 2011; **13**: 224-237 [PMID: 21466617 DOI: 10.1111/j.1751-7176.2011.00449.x]
 - 168 **Schuppan D**, Gorrell MD, Klein T, Mark M, Afdhal NH. The challenge of developing novel pharmacological therapies for non-alcoholic steatohepatitis. *Liver Int* 2010; **30**: 795-808 [PMID: 20624207 DOI: 10.1111/j.1478-3231.2010.02264.x]
 - 169 **Kudo H**, Yata Y, Takahara T, Kawai K, Nakayama Y, Kanayama M, Oya T, Morita S, Sasahara M, Mann DA, Sugiyama T. Telmisartan attenuates progression of steatohepatitis in mice: role of hepatic macrophage infiltration and effects on adipose tissue. *Liver Int* 2009; **29**: 988-996 [PMID: 19386026 DOI: 10.1111/j.1478-3231.2009.02006.x]
 - 170 **Yokohama S**, Yoneda M, Haneda M, Okamoto S, Okada M, Aso K, Hasegawa T, Tokusashi Y, Miyokawa N, Nakamura K. Therapeutic efficacy of an angiotensin II receptor antagonist in patients with nonalcoholic steatohepatitis. *Hepatology* 2004; **40**: 1222-1225 [PMID: 15382153 DOI: 10.1002/hep.20420]
 - 171 **Scheen AJ**. Prevention of type 2 diabetes mellitus through inhibition of the Renin-Angiotensin system. *Drugs* 2004; **64**: 2537-2565 [PMID: 15516153]
 - 172 **Mancia G**, Fagard R, Narkiewicz K, Redon J, Zanchetti A, Böhm M, Christiaens T, Cifkova R, De Backer G, Dominiczak A, Galderisi M, Grobbee DE, Jaarsma T, Kirchhof P, Kjeldsen SE, Laurent S, Manolis AJ, Nilsson PM, Ruilope LM, Schmieder RE, Sirnes PA, Sleight P, Viigimaa M, Waeber B, Zannad F, Redon J, Dominiczak A, Narkiewicz K, Nilsson PM, Burnier M, Viigimaa M, Ambrosioni E, Caulfield M, Coca A, Olsen MH, Schmieder RE, Tsioufis C, van de Borne P, Zamorano JL, Achenbach S, Baumgartner H, Bax JJ, Bueno H, Dean V, Deaton C, Erol C, Fagard R, Ferrarini R, Hasdai D, Hoes AW, Kirchhof P, Knuuti J, Kolh P, Lancellotti P, Linhart A, Nihoyannopoulos P, Piepoli MF, Ponikowski P, Sirnes PA, Tamargo JL, Tendera M, Torbicki A, Wijns W, Windecker S, Clement DL, Coca A, Gillebert TC, Tendera M, Rosei EA, Ambrosioni E, Anker SD, Bauersachs J, Hitij JB, Caulfield M, De Buyzere M, De Geest S, Derumeaux GA, Erdine S, Farsang C, Funck-Brentano C, Gerc V, Germano G, Gielen S, Haller H, Hoes AW, Jordan J, Kahan T, Komajda M, Lovic D, Mahrholdt H, Olsen MH, Ostergren J, Parati G, Perk J, Polonia J, Popescu BA, Reiner Z, Rydén L, Sirenko Y, Stanton A, Struijker-Boudier H, Tsioufis C, van de Borne P, Vlachopoulos C, Volpe M, Wood DA. ESH/ESC guidelines for the management of arterial hypertension: the Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *Eur Heart J* 2013; **34**: 2159-2219 [PMID: 23771844 DOI: 10.1093/eurheartj/ehf151]
 - 173 **Makishima M**, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR, Mangelsdorf DJ. Vitamin D receptor as an intestinal bile acid sensor. *Science* 2002; **296**: 1313-1316 [PMID: 12016314]
 - 174 **Targher G**, Pichiri I, Lippi G. Vitamin D, thrombosis, and hemostasis: more than skin deep. *Semin Thromb Hemost* 2012; **38**: 114-124 [PMID: 22314609 DOI: 10.1055/s-0031-1300957]
 - 175 **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]
 - 176 **Targher G**, Bertolini L, Scala L, Cigolini M, Zenari L, Falezza G, Arcaro G. Associations between serum 25-hydroxyvitamin D3 concentrations and liver histology in patients with non-alcoholic fatty liver disease. *Nutr Metab Cardiovasc Dis* 2007; **17**: 517-524 [PMID: 16928437 DOI: 10.1016/j.numecd.2006.04.002]
 - 177 **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]
 - 178 **Lee JH**, O'Keefe JH, Bell D, Hensrud DD, Holick MF. Vita-

- min D deficiency an important, common, and easily treatable cardiovascular risk factor? *J Am Coll Cardiol* 2008; **52**: 1949-1956 [PMID: 19055985 DOI: 10.1016/j.jacc.2008.08.050]
- 179 **Lutsey PL**, Michos ED. Vitamin D, calcium, and atherosclerotic risk: evidence from serum levels and supplementation studies. *Curr Atheroscler Rep* 2013; **15**: 293 [PMID: 23232985 DOI: 10.1007/s11883-012-0293-5]
- 180 **Fall T**, Shiue I, Bergeå af Geijerstam P, Sundström J, Ärnlöv J, Larsson A, Melhus H, Lind L, Ingelsson E. Relations of circulating vitamin D concentrations with left ventricular geometry and function. *Eur J Heart Fail* 2012; **14**: 985-991 [PMID: 22723659 DOI: 10.1093/eurjhf/hfs091]

P- Reviewers: Basar O, Guerrero-Romero F, Vos MB

S- Editor: Qi Y **L- Editor:** A **E- Editor:** Wu HL



WJG 20th Anniversary Special Issues (12): Fatty liver

Modulation of hepatic steatosis by dietary fatty acids

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Received: September 13, 2013 Revised: October 13, 2013

Accepted: November 3, 2013

Published online: February 21, 2014

Abstract

Non-alcoholic fatty liver disease (NAFLD) describes a range of conditions caused by fat deposition within liver cells. Liver fat content reflects the equilibrium between several metabolic pathways involved in triglyceride synthesis and disposal, such as lipolysis in adipose tissue and *de novo* lipogenesis, triglyceride esterification, fatty acid oxidation and very-low-density lipoprotein synthesis/secretion in hepatic tissue. In particular, it has been demonstrated that hepatic *de novo* lipogenesis plays a significant role in NAFLD pathogenesis. It is widely known that the fatty acid composition of the diet influences hepatic lipogenesis along with other metabolic pathways. Therefore, dietary fat may not only be involved in the pathogenesis of hepatic steatosis, but may also prevent and/or reverse hepatic fat accumulation. In this review, major data from the literature about the role of some dietary fats as a potential cause of hepatic fat accumulation or as a potential treatment for NAFLD are described. Moreover, biochemical mechanisms responsible for an increase or decrease in hepatic lipid content are critically analyzed. It is noteworthy that both quantitative and qualitative aspects of dietary fat influence triglyceride deposition in the liver. A high-fat diet or the dietary administration of conjugated linoleic acids induced hepatic steatosis. In con-

trast, supplementation of the diet with krill oil or pine nut oil helped in the prevention and/or in the treatment of steatotic liver. Quite interesting is the "case" of olive oil, since several studies have often provided different and/or conflicting results in animal models.

Key words: Hepatic steatosis; Non-alcoholic fatty liver; Fatty acids; Lipogenesis

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Core tip: Dietary fats may not only influence the pathogenesis of liver diseases, but may also prevent and/or reverse their expression. This manuscript reviews the molecular mechanisms responsible for the regulation of hepatic lipogenesis, through which some fatty acids may be beneficial or detrimental to non-alcoholic fatty liver disease (NAFLD). We believe that an understanding of the biochemical mechanisms underlying fat accumulation in the liver will lead to more targeted and effective therapeutics for hepatic steatosis. This is a particularly important topic because NAFLD is an increasingly prevalent disease which, to date, has no proven pharmacologic treatment to prevent or reverse its course.

Ferramosca A, Zara V. Modulation of hepatic steatosis by dietary fatty acids. *World J Gastroenterol* 2014; 20(7): 1746-1755 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1746.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1746>

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a condition that is caused by the pathological accumulation of fat in liver. NAFLD affects 10%-35% of the current world population. In the great majority of patients, NAFLD develops in association with obesity, type 2 diabetes, insu-

lin resistance and other metabolic abnormalities, including hypertension and dyslipidemia, collectively termed as “metabolic syndrome”^[1,2].

The severity of the disease ranges from simple steatosis to acute steatohepatitis, but the pathogenesis and the molecular mechanisms controlling its progression are poorly understood. The classical pathogenesis of NAFLD is based on the “two-hit hypothesis”^[3,4]. According to this hypothesis, hepatic triglyceride accumulation^[5], or steatosis, represents the “first hit”, which then sensitizes the liver to injury mediated by a “second hit”, such as the secretion of proinflammatory and prothrombotic adipocytokines and the reduced production of the adipocytokine adiponectin, a potent anti-inflammatory and insulin-sensitizing agent^[6-8]. In addition, mitochondrial dysfunction and oxidative stress trigger an inflammatory and fibrogenic cascade in the primed liver^[3]. These events lead to steatohepatitis and fibrosis. More recently, Serviddio *et al.*^[9] underlined the importance of mitochondria in NAFLD prevention since these organelles play fundamental roles in fat metabolism and energy homeostasis, thereby counteracting the excessive accumulation of liver triglycerides.

Indeed, the main feature of NAFLD pathogenesis, both histologically and metabolically, is the accumulation of triglycerides in the liver. Although the increased mobilization of free fatty acids from adipose tissue mainly contributes to fatty liver, the specific origin of the lipids that accumulate in the liver remains unknown. Therefore, the understanding of the molecular mechanisms leading to the accumulation of lipids into the liver of NAFLD patients is of importance for the prevention and/or the reversal of this condition.

In this review, we focus our attention on the *de novo* lipogenesis which plays a significant role in the pathogenesis of NAFLD^[10-13]. It is widely known that hepatic lipogenesis is strictly regulated by several nutritional factors^[14,15], such as the fatty acid composition of the diet^[16]. Such knowledge will eventually translate into the development of novel treatment strategies for NAFLD.

ROLE OF *DE NOVO* LIPOGENESIS IN THE DEVELOPMENT OF HEPATIC STEATOSIS

Steatosis occurs when there is an imbalance between lipid availability through fatty acid uptake and *de novo* lipogenesis, and lipid secretion and disposal *via* free fatty acid oxidation^[17,18]. Figure 1 depicts the main pathways involved in fatty acid metabolism in liver and shows that a perturbed balance between triglyceride synthesis and triglyceride disposal leads to hepatic steatosis.

As shown in Figure 1, the potential sources of fatty acids contributing to fatty liver are the non-esterified fatty acid pool from adipose tissue, dietary fatty acids and newly made fatty acids within the liver through *de novo* lipogenesis^[13,18]. Modulation of any of the multiple mechanisms involved in lipid accumulation in the liver could provide useful targets to prevent the development of NAFLD.

To establish the relative contribution of lipid accumulation in patients with NAFLD, Donnelly *et al.*^[10] used a multiple-stable-isotope approach. These authors demonstrated that approximately 60% of liver triglyceride content was derived from free fatty acid influx from adipose tissue, 26% from *de novo* lipogenesis, and 15% from the diet. Other studies, carried out in animal models, provided further evidence that lipogenesis plays a key role in the development of hepatic steatosis^[12]. In particular, the use of genetically engineered mice have helped to clarify that knockdown of enzymes involved in fatty acid synthesis was able to reverse NAFLD^[11].

De novo fatty acid synthesis implies a complex series of reactions starting in the mitochondrial matrix and continuing in the cytosol of hepatocytes (Figure 2). The main fuel for fatty acid synthesis is acetyl-CoA derived from carbohydrate or amino acid catabolism. Since acetyl-CoA is formed in the mitochondrion, and fatty acid synthesis occurs in the cytosol, the acetyl group must be exported from the intra-mitochondrial to the extra-mitochondrial compartment of the cell before its conversion into fatty acids. Actually, in the mitochondrial matrix, acetyl-CoA is at first condensed with oxaloacetate, thereby forming the tricarboxylate citrate, an intermediate of the Krebs cycle. When this intermediate cannot be burned in the Krebs cycle because of an excess of cellular energy, it is exported from the mitochondrial matrix into the cytosol by the mitochondrial tricarboxylate carrier or citrate carrier (CIC). This carrier protein is firmly inserted into the inner mitochondrial membrane, where it catalyzes the efflux of citrate from the matrix towards the cytosol, thus playing an important role in intermediary metabolism^[19]. In fact, in the cytosol the transported citrate generates acetyl-CoA, which now represents the primer for *de novo* fatty acid and cholesterol biosyntheses^[20]. As shown in Figure 2, cytosolic fatty acid synthesis begins with the conversion of acetyl-CoA to malonyl-CoA in the reaction catalyzed by acetyl-CoA carboxylase (ACC). Next, the sequential extension of an alkanolic chain, two carbons at a time, is catalyzed by fatty acid synthase (FAS) which eventually leads to palmitic acid (16:0), the main product of *de novo* fatty acid synthesis.

The high rate of lipogenesis, observed in hepatic steatosis, seems to be associated with hyperglycemia and hyperinsulinemia^[21,22]. Induction of lipogenic genes is under the combined actions of sterol regulatory element binding protein-1c (SREBP-1c) in response to insulin, and ChREBP (carbohydrate responsive element binding protein) in response to glucose^[23,24]. These events also result in a shift in cellular metabolism from lipid oxidation to triglyceride esterification, thereby increasing the production of liver triglycerides^[12,25].

MODULATION OF HEPATIC LIPID METABOLISM BY DIETARY FATTY ACIDS

It is widely known that the fatty acid composition of

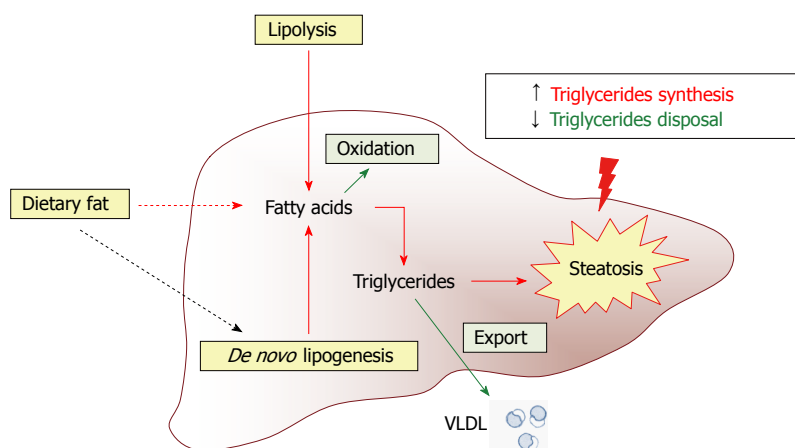


Figure 1 Contribution of various metabolic pathways to hepatic steatosis. Liver fat content reflects the equilibrium between several metabolic pathways involved in triglyceride synthesis (red arrows) and clearance (green arrows). Dietary fat is an important factor capable of influencing *de novo* lipogenesis (black dotted arrow).

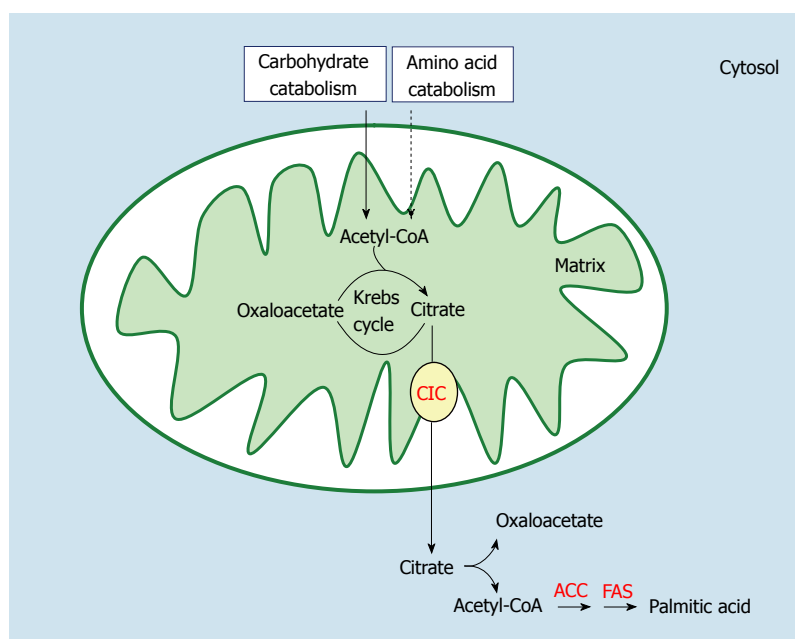


Figure 2 Pathway of *de novo* fatty acid synthesis in the liver. CIC: Citrate carrier; ACC: Acetyl-CoA carboxylase; FAS: Fatty acid synthase.

the diet is an important factor capable of influencing hepatic lipid metabolism. Indeed, dietary fatty acids are able to regulate various metabolic pathways involved in lipid metabolism mainly through a fine modulation of gene transcription of specific enzymes^[16,26]. Since the liver plays a key role in lipid metabolism, dietary fats and their oxidized metabolites may not only influence the pathogenesis of liver diseases^[27-31], but may also prevent and/or reverse disease manifestations^[32]. In particular, it has been reported that n-3 polyunsaturated fatty acids (PUFA) are able to limit triglyceride deposition in the liver^[33,34], whereas a diet deficient in n-3 PUFA with a high n-6/n-3 ratio could induce fatty liver^[28] and chronic diseases^[35-37].

Modulation of hepatic lipid metabolism by fatty acids is quite complex, involving a sequence of molecular events which are interdependent and cross-regulated. Because of the complexity of this topic, in the present review we focus our attention on dietary fat modulation of hepatic fatty acid synthesis, a pathway which plays a key role in the pathogenesis of liver fat accumulation. It is known from the literature that dietary PUFA of the

n-3 and n-6 series are potent inhibitors of hepatic lipogenesis^[16]. However, most of these studies investigated the variations in the activity and in the expression of the cytosolic lipogenic enzymes (ACC and FAS).

In recent years, attention has also been gradually directed towards the effects of dietary PUFA of the n-3 and n-6 series on the activity of the mitochondrial CIC, which, as stated before, transports citrate outside mitochondria for cytosolic fatty acid biosynthesis. It has been found that PUFA significantly decrease the activity and the expression of the mitochondrial CIC^[38-43]. Very interestingly, parallel reductions in the activities of mitochondrial CIC and of cytosolic lipogenic enzymes were found, thereby highlighting a close coordination between mitochondrial and cytosolic reactions^[38-44]. In contrast, a diet enriched in monounsaturated fatty acids (MUFA) or saturated fatty acids (SFA) did not exert any appreciable effect on mitochondrial CIC activity and expression, and therefore did not influence *de novo* fatty acid synthesis^[41,42]. This mitochondrial carrier, therefore, acts as a sensor for changes occurring in hepatic lipogenesis^[39-44] which, in turn, may influence fat deposition in liver.

Table 1 Use of dietary supplement in the prevention of hepatic steatosis

Oil source	Oil fatty acid composition (%)		Experimental design	Results	Ref.
Krill	C14:0	2.5	C57BL/6	↓ Hepatomegaly	Tandy <i>et al</i> ^[50]
	C16:0	18.2	mice fed for	↓ Hepatic	
	C18:0	2.8	8 wk with a	steatosis	
	C18:1	25.8	high-fat diet	↓ <i>De novo</i>	
	C18:2	54.4	containing	lipogenesis	
	C18:3	4.9	1.25%-5%	↓ Blood glucose	Ferramosca <i>et al</i> ^[43]
	C20:5	5.3	krill oil		
	C22:5	2.3	Wistar rats	↓ Hepatic	
	C22:6	3.0	fed for 6 wk	steatosis	
			with a diet	↓ <i>De novo</i>	
			containing	lipogenesis	
			2.5% krill oil	↓ Haematic triglycerides	
				↓ Haematic cholesterol	
			Sprague-Dawley rats	↓ Hepatic	Ferramosca <i>et al</i> ^[42]
			fed for 12	steatosis	
			wk with a	↓ <i>De novo</i>	
			high-fat diet	lipogenesis	
			containing	↑ Fatty acid oxidation	
Pine nut			2.5% krill oil	↓ Mitochondrial uncoupling	Ferramosca <i>et al</i> ^[40]
				↓ Haematic glucose	
				↓ Haematic insulin	
				↓ Haematic triglycerides	
	C16:0	8.0	Mice fed	↓ Liver weight	
	C18:0	2.2	for 8 wk	↓ Hepatic	
	C18:1	23.2	with a diet	steatosis	
	C18:2	51.1	containing	↓ <i>De novo</i>	
	C18:3	2.1	7.5% pine	lipogenesis	
	C18:3	8.8	nut oil	↓ Haematic triglycerides	
	(5,9,12)		↓ Haematic cholesterol		

DIETARY FATTY ACIDS AND PREVENTION OF HEPATIC STEATOSIS: NEW INSIGHTS FROM KRILL AND PINE NUT OIL

In recent years, the use of dietary supplements has rapidly increased. For instance, krill oil, a novel dietary supplement of n-3 PUFA, has become increasingly popular as a food supplement during the last decade. This oil is extracted from Antarctic krill (*Euphausia superba*), a shrimp-like zooplankton at the bottom of the food chain. Krill oil contains two n-3 PUFA, eicosapentaenoic acid (EPA, 20:5) and docosahexanoic acid (DHA, 22:6), in amounts similar to those present in fish oil. However, these long-chain n-3 fatty acids are present in krill oil in the form of phospholipids rather than triglycerides. Furthermore, the ratio of EPA to DHA is different in the two oils, with EPA prevailing in krill oil and DHA prevailing in

fish oil^[45,46]. The health-promoting effects of krill oil have been reported both in humans and in animal models by several authors^[47-50]. Moreover, a higher potency of krill oil in comparison to fish oil has also been proposed^[43,51]. This may be biologically and therapeutically significant, since it has been found that krill oil supplementation showed beneficial effects on hepatomegaly and hepatic steatosis in mice fed a high-fat diet (Table 1). Indeed, krill oil supplementation of the diet caused a significant reduction in liver weight (hepatomegaly) and total liver fat (steatosis)^[42,50]. Recently, investigation of the molecular mechanisms responsible for the action of krill oil revealed that the beneficial effects of this fat were due to a favourable combination of several elements^[42]. First, diet supplementation with 2.5% krill oil in rats fed a high-fat diet reduced the activity and expression of mitochondrial CIC, thereby decreasing the amount of substrate available for hepatic fatty acid synthesis. The concomitant and concerted reduction of the lipogenic enzymes ACC and FAS resulted in a strong inhibition of hepatic fatty acid synthesis. A similar effect was also observed when animals were fed with a standard diet, suggesting that krill oil has an intrinsic capability to reduce hepatic lipogenesis^[43]. Second, besides the inhibition of *de novo* lipogenesis, a marked increase in fatty acid oxidation was observed in animals fed with a diet enriched in krill oil. Third, dietary krill oil was also able to retain efficient mitochondrial oxidative phosphorylation in treated rats, thus preventing the possible uncoupling effects of a high-fat diet. Overall, krill oil stimulated the catabolization of excess fat introduced by a hypercaloric diet, while inhibiting *de novo* fatty acid synthesis and therefore preventing the onset of fatty liver.

It is also important to underline that surplus of energy supplied by fat often leads to reduced tissue utilization of glucose, thus causing hyperglycemia and hyperinsulinemia^[52]. Very interestingly, krill oil was also able to reverse the increase in the levels of blood glucose and insulin, normally observed in steatotic animals, thus preventing insulin resistance^[42]. On the other hand, lower levels of triglycerides were found in plasma of animals fed with krill oil in comparison to those detected in rats fed a high-fat diet, and this may also be significant in preventing cardiovascular diseases. All these results suggest that krill oil has the ability to improve lipid and glucose metabolism and highlight the possible protective effects of krill oil against hepatic steatosis.

In the last few years, vegetable oils extracted from the seeds of some conifers^[53] have been also under investigation for their use as dietary supplements^[54-56]. The oil from the seeds of *Pinus koraiensis* is a dietary fat that contains, along with various fatty acids, pinolenic acid or all-*cis*-5,9,12-octadecatrienoic acid. This is a quite unusual n-6 PUFA which is characterized by polymethylene-interrupted double bonds. Preliminary studies carried out in rats indicated that pine nut oil exerted some beneficial effects on lipid metabolism, such as a decrease in plasma triglycerides and in circulating very-low-density lipoprotein^[54,56]. Further studies demonstrated that diet supple-

mentation with pine nut oil caused a significant reduction in liver weight and liver lipids^[40]. These results are noteworthy because this dietary fat might be of interest in the case of hepatic steatosis (Table 1). A concomitant reduction in the mitochondrial CIC activity and in the cytosolic ACC and FAS activities was observed in animals fed pine nut oil^[40]. However, a similar decrease in *de novo* fatty acid synthesis was also found in control mice which were fed with a diet enriched with maize oil. This latter diet has a fatty acid composition similar to that of the pine nut oil diet, except for the absence of pinolenic acid. Therefore, the specific capability of decreasing hepatic and plasma lipids, shown by pine nut oil, is probably due to pinolenic acid, or to some of its possible metabolites^[40].

DIETARY FATTY ACIDS AND DEVELOPMENT OF HEPATIC STEATOSIS

Fatty liver is diet-inducible in rodent animal models, in which high-fat diets are able to cause an increase in the liver fat levels. Indeed, an increase in the level of liver lipids was observed in rats fed for 12 wk with a diet containing a high content of fat (35% lard)^[42] (Table 2). It must be underlined that the approximate fatty acid profile of the high-fat diet used in this study was kept low in PUFA, with the aim of preventing the inhibitory effect of hepatic fatty acid synthesis by high levels of these unsaturated fatty acids. The excess dietary fat anyhow inhibited hepatic lipogenesis at the beginning of dietary treatment. This inhibition progressively decreased over time and was completely abolished at longer feeding times. The high level of triglycerides found in the liver at the beginning of this dietary treatment was therefore not due to an increased fatty acid synthesis, since this anabolic pathway was inhibited at that time. Interestingly, a decrease in fatty acid oxidation, as well as a strong decrease in mitochondrial respiratory efficiency, was clearly observed in animals fed a high-fat diet^[42]. This last observation suggests that the excess of fat in the diet most probably induced a partial uncoupling between respiration and phosphorylation in the mitochondria^[57]. A concomitant increase in the plasma levels of glucose and insulin was also observed in animals fed a high-fat diet^[42].

In the last decade, the attention of some authors has been focused on the fatty acid composition of the diet in the induction of hepatic steatosis. In this context, a recent review reports that an increase in free fatty acids, especially SFA, may play an important role in the development of hepatic steatosis^[29]. It has been demonstrated that SFA caused liver dysfunction by promoting endoplasmic reticulum stress and apoptosis^[58-60]. In contrast to SFA, an increase in MUFA induced steatotic liver, but did not initiate apoptosis^[61].

Conjugated linoleic acids (CLA) have also been linked to the development of hepatic steatosis. CLA is the acronym for a class of positional and geometric isomers of linoleic acid^[62]. These compounds are naturally present in food derived from ruminant animals, such as bovine and

ovine meat and dairy products. The main CLA isomer in natural products is the *cis*-9,*trans*-11-octadecadienoic acid, but the commercially available CLA, currently used as a food supplement, contains a 1:1 mixture of this isomer and the *trans*-10,*cis*-12 isomer. Several authors have indicated that CLA has beneficial effects in the case of cardiovascular diseases, obesity and diabetes^[63]. These beneficial effects, however, are in some instances associated with adverse effects, such as liver steatosis^[64].

Several studies (Table 2) suggested that *de novo* fatty acid synthesis may play a role in the onset of hepatic steatosis produced by CLA administration^[44,65,66]. Interestingly, in CLA-fed mice, a time-dependent increase in the enzymatic activities involved in hepatic lipogenesis was clearly found. Indeed, at the 16th week of CLA feeding, an approximate doubling of the activities of mitochondrial CIC and of cytosolic lipogenic enzymes (ACC and FAS) was detected^[59,44]. It is important to underline that, in the first period of CLA feeding, liver enlargement and hepatic triglyceride accumulation occurred independently of the fatty acid synthesis stimulation. At longer times (weeks 12-16), the level of hepatic triglycerides in CLA-fed mice increased dramatically. The concomitant strong increase in the levels of plasma insulin suggested that this hormone, possibly in addition to other factors, could play an important role in the development of hepatic steatosis after longer durations of dietary treatment^[39].

Several authors reported that *trans*-10,*cis*-12 CLA was the isomer responsible for the development of fatty liver in mice in which a loss of adipose tissue was concomitantly observed^[64,67,68]. However, the mechanisms by which the liver becomes steatotic in response to *trans*-10,*cis*-12 CLA are not well understood and appear to be puzzling since this isomer also induces a concomitant increase in cellular fatty acid oxidation^[68].

"CASE" OF OLIVE OIL

Olive oil, a basic component of the Mediterranean diet, mainly contains oleic acid, a MUFA fatty acid of the n-9 series. One of the most intriguing aspects regarding olive oil is its effect on hepatic lipid metabolism. In some studies (Table 3) carried out in rodents, an olive oil-enriched diet induced fat accumulation in the liver^[41,69-72]. Furthermore, in olive oil-treated animals, an increase in hepatic lipogenesis was surprisingly found^[71,72]. However, the animals used as control group in these studies were fed with a diet enriched in PUFA. Therefore, the observed increase in hepatic lipogenesis in olive oil-fed animals was only apparent and probably due to the comparison with the PUFA-enriched diet fed to control animals.

Nevertheless, the molecular mechanisms of fat accumulation in the liver of olive oil fed animals are not clear. In the organism, the liver plays a fundamental role in lipid metabolism, because it is involved in many different processes, such as fatty acid uptake, storage, conversion, oxidation, synthesis and secretion. Therefore, a clear definition of the molecular events leading to lipid accumulation in the liver, consequent to olive oil administration,

Table 2 Role of dietary fat in the development of hepatic steatosis

Dietary fat	Fatty acid composition (%)		Experimental design	Results	Ref.
Lard	C14:0	0.50	Sprague-Dawley rats fed for 12 wk with a high-fat diet (35% fat)	↑ Hepatic steatosis	Ferramosca <i>et al</i> ^[42]
	C16:0	8.70		↓ Fatty acid oxidation	
	C18:0	4.30		↑ Haematic triglycerides	
	C18:1	15.80		↑ Mitochondrial uncoupling	
	C18:2	3.50		↑ Haematic insulin	
CLA	CLA	0.004	C57Bl/6J mice fed were fed for 4 wk with a diet containing 0.4% CLA	↑ Haematic glucose	Clément <i>et al</i> ^[64]
	c-9, t-11			↑ Hepatic steatosis	
	CLA	0.40		↑ <i>De novo</i> lipogenesis	
	t-10, c-12			↑ Haematic insulin	
	C16:0	5.91	C57BL/6J mice were fed for 21 days with a diet containing 1.5% CLA	↑ Hepatomegaly	Takahashi <i>et al</i> ^[65]
	C18:0	0.60		↑ Hepatic steatosis	
	C18:1	5.27		↑ <i>De novo</i> lipogenesis	
	C18:2	1.22		↑ Fatty acid oxidation	
	CLA	0.49			
	c-9, t-11/ t-9, c-11				
	CLA	0.51			
	t-10, c-12				
	CLA	0.03			
	c-9, c-11/c10, c12				
	CLA	0.02			
	t-9, t-11/t10, t12				
	CLA	1.00	C57BL/6J mice fed for 4 wk with a diet supplemented with 1% CLA	↑ Liver weight	Degrace <i>et al</i> ^[68]
	t-10, c-12			↓ Haematic triglycerides	
				↓ Haematic FFA	
				↑ Fatty acid oxidation	
	C16:0	5.94	ICR mice fed for 22 d with a diet containing 1.0% CLA	↑ Liver weight	Ide ^[66]
	C16:1	0.01		↑ Hepatic steatosis	
	C18:0	0.60		↑ <i>De novo</i> lipogenesis	
	C18:1	5.30		↑ Fatty acid oxidation	
	C18:2	1.27		↑ Haematic insulin	
	CLA	0.49			
	c-9, t-11				
	CLA	0.51			
	t-10, c-12				
	CLA	0.03			
	c-9, c-11/c10, c12				
	CLA	0.02			
	t-9, t-11/t10, t12				
	C16:0	12.00	ICR mice fed for 16 wk with a diet containing 1% CLA	↑ Liver weight	Ferramosca <i>et al</i> ^[44]
	C18:0	2.70		↑ Hepatic steatosis	
	C18:1	42.50		↑ <i>De novo</i> lipogenesis	
	C18:2	30.50		↑ Fatty acid oxidation	
	C18:3	2.90		↓ Haematic triglycerides	
	CLA	3.50		↓ Haematic FFA	
	c-9, t-11				
	CLA	3.70			
	t-10, c-12				
	C16:0	12.00	ICR mice fed for 16 wk with a diet containing 1% CLA	↑ Liver weight	Ferramosca <i>et al</i> ^[39]
	C18:0	2.70		↑ Hepatic steatosis	
	C18:1	42.50		↑ <i>De novo</i> lipogenesis	
	C18:2	30.50		↑ Fatty acid oxidation	
	C18:3	2.90		↓ Haematic triglycerides	
	CLA	3.50		↓ Haematic cholesterol	
	c-9, t-11			↑ Haematic insulin	
	CLA	3.70			
	t-10, c-12				

CLA: Conjugated linoleic acids.

is quite difficult. However, it has been proposed that the increase in the hepatic triglyceride content of olive oil-fed mice was due to an impairment of mitochondrial fatty acid oxidation^[41].

On the other hand, an interesting study^[73], carried

out in rats with NAFLD, demonstrated that olive oil decreased the accumulation of liver triglycerides. In particular, it has been suggested that olive oil may improve insulin resistance, increase the release of triglyceride from liver and decrease the lipolytic flux from peripheral adi-

Table 3 Effect of olive oil on liver lipid profile

Fatty acid composition (%)		Experimental design	Results	Ref.
C16:0	11.80	Wistar rats fed for 12 wk with a diet containing 10% olive oil	↑ Hepatic lipids	Ruiz-Gutiérrez <i>et al</i> ^[69]
C16:1	0.90			
C17:0	0.40			
C18:0	2.80			
C18:1	79.20			
C18:2	3.50			
C18:3	0.60			
C20:0	0.30			
C20:1	0.20			
C24:0	0.40			
C16:0	11.79	Spontaneously hypertensive rats fed for 12 wk with a diet containing 10% of energy as olive oil	↑ Hepatic lipids	Perona <i>et al</i> ^[70]
C16:1	0.86			
C17:0	0.37			
C18:0	2.79			
C18:1	79.22			
C18:2	3.45			
C18:3	0.60			
C20:0	0.28			
C20:1	0.20			
C24:0	0.44			
C16:0	11.87	Wistar rats fed for 4 wk with a diet containing 40% of energy as olive oil	↑ Hepatic lipids ↑ <i>De novo</i> lipogenesis	Portillo <i>et al</i> ^[71]
C16:1	0.94			
C18:0	2.92			
C18:1	77.26			
C18:2	5.69			
C18:3	0.50			
C20:0	0.39			
C16:0	10.70			
C18:0	2.00			
C18:1	74.80			
C18:2	10.40	Sprague-Dawley rats fed for 2 mo with a methionine choline-deficient diet containing olive oil (0.45 mg/g rat weight)	↑ Hepatic lipids ↑ <i>De novo</i> lipogenesis	Takeuchi <i>et al</i> ^[72]
C18:3	0.70			
C16:0	11.29			
C18:1	71.26			
C18:2	9.76			
C16:0	12.10			
C18:0	3.00			
C18:1	27.40			
C18:2	51.90			
C18:3	2.70			
C16:0	12.10	ICR mice fed for 8 wk with a diet containing 7.5% olive oil	↑ Hepatic lipids ↓ Fatty acid oxidation	Ferramosca <i>et al</i> ^[41]
C18:0	3.00			
C18:1	27.40			
C18:2	51.90			

pose tissue in steatotic animals^[73]. Further studies, carried out in humans, demonstrated that MUFA were able to increase lipid oxidation and to decrease insulin resistance, suggesting that olive oil should be included in the diet of NAFLD patients^[74,75].

SPECIFIC COMBINATIONS OF PUFA CAN MODULATE HEPATIC STEATOSIS

Some attempts have been made to overcome the adverse effects of CLA, such as fatty liver, by using appropriate mixtures of CLA and other dietary fatty acids. Nakaniishi *et al*^[76] found that the addition of gamma-linolenic acid to a CLA-enriched diet was able to prevent fatty liver in mice, even if the antiobesity properties of CLA were

only partially retained. Another study^[77] demonstrated that 0.5% supplementation of DHA to the CLA diet attenuated CLA-induced fatty liver through the reduction of hepatic fatty acid synthesis. In agreement with these results, Ide^[66] demonstrated that different amounts of fish oil added to a CLA-enriched diet downregulated *de novo* lipogenesis in a dose-dependent manner, concomitantly reducing hepatic triglyceride levels. Indeed, both dietary CLA and fish oil strongly affect hepatic lipogenic activities: CLA enhances, whereas fish oil inhibits the activities and the expression of the enzymes involved in hepatic lipogenesis^[43,44]. Therefore, the simultaneous ingestion of fish oil and CLA may represent a “dietary trick” to retain the positive effects exerted by CLA, such as its anti-obesity property, while avoiding the negative consequences mainly consisting of excessive fat deposition in the liver.

In line with this concept, a further study^[39] investigated the effects of a dietary combination of CLA and pine nut oil on lipid metabolism in mice. Previous studies demonstrated that CLA greatly increased *de novo* fatty acid synthesis in mouse hepatocytes, thus leading to hepatic steatosis^[44]. In contrast, pine nut oil decreased liver lipid concentration^[40]. Starting with these observations, it has been found that the co-administration of CLA and pine nut oil in the diet exerted a series of positive effects in treated animals: (1) the CLA-mediated body fat reduction was preserved; (2) the onset of hepatic steatosis was effectively prevented; and (3) the liver and plasma lipid content was normalized. The detailed analysis of metabolic changes occurring in mice under this dietary treatment revealed that the enzymatic activities involved in fatty acid synthesis had time-dependent biphasic behavior. In fact, hepatic lipogenesis showed a biphasic trend in CLA + pine nut oil-fed animals, consisting of a moderate increase within the first 6–8 wk of dietary treatment, followed by a progressive decrease from the 8th week onward. A strong decrease in hepatic fatty synthesis was indeed detected at the 16th week of feeding. Furthermore, whereas a sharp increase in plasma insulin levels occurred in CLA-fed animals at the 8th week, insulinemia remained stable in CLA + pine nut oil-fed mice. It was also found that a CLA + pine nut oil diet positively influenced plasma lipid levels. In fact, this dietary association reinforced the capability of CLA in decreasing plasma triglyceride levels, reducing at the same time plasma levels of cholesterol and phospholipids. The healthy beneficial effects promoted by a CLA + pine nut oil diet are probably due to the peculiar fatty acid composition of the mixture. In fact, the CLA + pine nut oil diet has a lower amount of SFA and MUFA and a higher amount of PUFA in comparison to the CLA diet. Among PUFA, the specific presence of 1.12% pinolenic acid may have a fundamental impact on the observed hypolipidemic effects.

CONCLUSION

NAFLD is characterized by abnormal fat deposition in the liver, where lipids are mainly stored as triglycerides.

Hepatic fat accumulation results from an imbalance between lipid supply (uptake or *de novo* lipogenesis) and lipid clearance (fatty-acid oxidation or triglyceride-rich lipoprotein secretion). Among the mechanisms involved in triglyceride accumulation, uptake of fatty acids consequent to adipose tissue lipolysis and *de novo* lipogenesis seem to be the major sources of lipid in the steatotic liver^[13].

In recent years, several studies carried out in animal models focused their attention on the role of dietary fats in the modulation of *de novo* lipogenesis, which plays a significant role in the pathogenesis of NAFLD. It has been demonstrated that a high-fat diet is able to induce a condition of hepatic steatosis. A similar effect was observed after the dietary administration of CLA which, at the same time, are able to strongly prevent fat accumulation in adipose tissue. While the effects of olive oil on hepatic lipid content are not completely clear, novel dietary supplements, such as krill oil or pine nut oil, seem to have a protective effect against hepatic steatosis. Nevertheless, further studies in humans are needed to ascertain whether the consumption of these dietary fats may be helpful in NAFLD patients.

REFERENCES

- Lewis JR, Mohanty SR. Nonalcoholic fatty liver disease: a review and update. *Dig Dis Sci* 2010; **55**: 560-578 [PMID: 20101463 DOI: 10.1007/s10620-009-1081-0]
- Petersen KF, Dufour S, Savage DB, Bilz S, Solomon G, Yonemitsu S, Cline GW, Befroy D, Zeman L, Kahn BB, Papademetris X, Rothman DL, Shulman GI. The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. *Proc Natl Acad Sci USA* 2007; **104**: 12587-12594 [PMID: 17640906 DOI: 10.1073/pnas.0705408104]
- Day CP, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998; **114**: 842-845 [PMID: 9547102 DOI: 10.1016/S0016-5085(98)70599-2]
- Dowman JK, Tomlinson JW, Newsome PN. Pathogenesis of non-alcoholic fatty liver disease. *QJM* 2010; **103**: 71-83 [PMID: 19914930 DOI: 10.1093/qjmed/hcp158]
- Puri P, Baillie RA, Wiest MM, Mirshahi F, Choudhury J, Cheung O, Sargeant C, Contos MJ, Sanyal AJ. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology* 2007; **46**: 1081-1090 [PMID: 17654743 DOI: 10.1002/hep.21763]
- 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]
- Pagano C, Soardo G, Esposito W, Fallo F, Basan L, Donnini D, Federspil G, Sechi LA, Vettor R. Plasma adiponectin is decreased in nonalcoholic fatty liver disease. *Eur J Endocrinol* 2005; **152**: 113-118 [PMID: 15762194 DOI: 10.1530/eje.1.01821]
- Tilg H, Moschen AR. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nat Rev Immunol* 2006; **6**: 772-783 [PMID: 16998510 DOI: 10.1038/nri1937]
- 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]
- 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]
- Postic C, Girard J. Contribution of *de novo* fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. *J Clin Invest* 2008; **118**: 829-838 [PMID: 18317565 DOI: 10.1172/JCI34275]
- Postic C, Girard J. The role of the lipogenic pathway in the development of hepatic steatosis. *Diabetes Metab* 2008; **34**: 643-648 [PMID: 19195625 DOI: 10.1016/S1262-3636(08)74599-3]
- Ferré P, Foulfelle F. Hepatic steatosis: a role for *de novo* lipogenesis and the transcription factor SREBP-1c. *Diabetes Obes Metab* 2010; **12** Suppl 2: 83-92 [PMID: 21029304 DOI: 10.1111/j.1463-1326.2010.01275.x]
- Girard J, Perdureau D, Foulfelle F, Prip-Buus C, Ferré P. Regulation of lipogenic enzyme gene expression by nutrients and hormones. *FASEB J* 1994; **8**: 36-42 [PMID: 7905448]
- Strable MS, Ntambi JM. Genetic control of *de novo* lipogenesis: role in diet-induced obesity. *Crit Rev Biochem Mol Biol* 2010; **45**: 199-214 [PMID: 20218765 DOI: 10.3109/10409231003667500]
- Jump DB. Fatty acid regulation of hepatic lipid metabolism. *Curr Opin Clin Nutr Metab Care* 2011; **14**: 115-120 [PMID: 21178610 DOI: 10.1097/MCO.0b013e328342991c]
- Lou-Bonafante JM, Arnal C, Osada J. New genes involved in hepatic steatosis. *Curr Opin Lipidol* 2011; **22**: 159-164 [PMID: 21494144 DOI: 10.1097/MOL.0b013e3283462288]
- Barrows BR, Timlin MT, Parks EJ. Spillover of dietary fatty acids and use of serum nonesterified fatty acids for the synthesis of VLDL-triacylglycerol under two different feeding regimens. *Diabetes* 2005; **54**: 2668-2673 [PMID: 16123356 DOI: 10.2337/diabetes.54.9.2668]
- Palmieri F, Bisaccia F, Iacobazzi V, Indiveri C, Zara V. Mitochondrial substrate carriers. *Biochim Biophys Acta* 1992; **1101**: 223-227 [PMID: 1633189]
- Watson JA, Lowenstein JM. Citrate and the conversion of carbohydrate into fat. Fatty acid synthesis by a combination of cytoplasm and mitochondria. *J Biol Chem* 1970; **245**: 5993-6002 [PMID: 5484459]
- Kumashiro N, Erion DM, Zhang D, Kahn M, Beddow SA, Chu X, Still CD, Gerhard GS, Han X, Dziura J, Petersen KF, Samuel VT, Shulman GI. Cellular mechanism of insulin resistance in nonalcoholic fatty liver disease. *Proc Natl Acad Sci USA* 2011; **108**: 16381-16385 [PMID: 21930939 DOI: 10.1073/pnas.1113359108]
- Foulfelle F, Ferré P. New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. *Biochem J* 2002; **366**: 377-391 [PMID: 12061893 DOI: 10.1042/BJ20020430]
- Flannery C, Dufour S, Rabøl R, Shulman GI, Petersen KF. Skeletal muscle insulin resistance promotes increased hepatic *de novo* lipogenesis, hyperlipidemia, and hepatic steatosis in the elderly. *Diabetes* 2012; **61**: 2711-2717 [PMID: 22829450 DOI: 10.2337/db12-0206]
- Dentin R, Girard J, Postic C. Carbohydrate responsive element binding protein (ChREBP) and sterol regulatory element binding protein-1c (SREBP-1c): two key regulators of glucose metabolism and lipid synthesis in liver. *Biochimie* 2005; **87**: 81-86 [PMID: 15733741 DOI: 10.1016/j.biochi.2004.11.008]
- Parks EJ, Skokan LE, Timlin MT, Dingfelder CS. Dietary sugars stimulate fatty acid synthesis in adults. *J Nutr* 2008; **138**: 1039-1046 [PMID: 18492831]
- Nguyen P, Leray V, Diez M, Serisier S, Le Bloc'h J, Siliart B, Dumon H. Liver lipid metabolism. *J Anim Physiol Anim Nutr (Berl)* 2008; **92**: 272-283 [PMID: 18477307 DOI: 10.1111/j.1439-0396.2007.00752.x]
- Lee S, Gura KM, Puder M. Omega-3 fatty acids and liver disease. *Hepatology* 2007; **45**: 841-845 [PMID: 17393527 DOI: 10.1002/hep.21645]
- El-Badry AM, Graf R, Clavien PA. Omega 3 - Omega 6: What is right for the liver? *J Hepatol* 2007; **47**: 718-725 [PMID: 17869370 DOI: 10.1016/j.jhep.2007.08.005]

- 29 **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]
- 30 **Feldstein AE**, Lopez R, Tamimi TA, Yerian L, Chung YM, Berk M, Zhang R, McIntyre TM, Hazen SL. Mass spectrometric profiling of oxidized lipid products in human nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *J Lipid Res* 2010; **51**: 3046-3054 [PMID: 20631297 DOI: 10.1194/jlr.M007096]
- 31 **Santoro N**, Caprio S, Giannini C, Kim G, Kursawe R, Pierpont B, Shaw MM, Feldstein AE. Oxidized Fatty acids: a potential pathogenic link between Fatty liver and type 2 diabetes in obese adolescents? *Antioxid Redox Signal* 2014; **20**: 383-389 [PMID: 23815500 DOI: 10.1089/ars.2013.5466]
- 32 **Masterton GS**, Plevris JN, Hayes PC. Review article: omega-3 fatty acids - a promising novel therapy for non-alcoholic fatty liver disease. *Aliment Pharmacol Ther* 2010; **31**: 679-692 [PMID: 20415840 DOI: 10.1111/j.1365-2036.2009.04230.x]
- 33 **Xin YN**, Xuan SY, Zhang JH, Zheng MH, Guan HS. Omega-3 polyunsaturated fatty acids: a specific liver drug for non-alcoholic fatty liver disease (NAFLD). *Med Hypotheses* 2008; **71**: 820-821 [PMID: 18752901 DOI: 10.1016/j.mehy.2008.07.008]
- 34 **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]
- 35 **Hansen HS**. Dietary essential fatty acids and in vivo prostaglandin production in mammals. *World Rev Nutr Diet* 1983; **42**: 102-134 [PMID: 6375159]
- 36 **Zampelas A**, Paschos G, Rallidis L, Yiannakouris N. Linoleic acid to alpha-linolenic acid ratio. From clinical trials to inflammatory markers of coronary artery disease. *World Rev Nutr Diet* 2003; **92**: 92-108 [PMID: 14579686 DOI: 10.1159/000073795]
- 37 **Schmitz G**, Ecker J. The opposing effects of n-3 and n-6 fatty acids. *Prog Lipid Res* 2008; **47**: 147-155 [PMID: 18198131 DOI: 10.1016/j.plipres.2007.12.004]
- 38 **Zara V**, Giudetti AM, Siculella L, Palmieri F, Gnoni GV. Covariance of tricarboxylate carrier activity and lipogenesis in liver of polyunsaturated fatty acid (n-6) fed rats. *Eur J Biochem* 2001; **268**: 5734-5739 [PMID: 11722557 DOI: 10.1046/j.0014-2956.2001.02508.x]
- 39 **Ferramosca A**, Savy V, Conte L, Zara V. Dietary combination of conjugated linoleic acid (CLA) and pine nut oil prevents CLA-induced fatty liver in mice. *J Agric Food Chem* 2008; **56**: 8148-8158 [PMID: 18702470 DOI: 10.1021/jf8010728]
- 40 **Ferramosca A**, Savy V, Einerhand AWC, Zara V. Pinus koraiensis seed oil (PinnoThin™) supplementation reduces body weight gain and lipid concentration of liver and plasma in mice. *J Animal Feed Sci* 2008; **17**: 621-630
- 41 **Ferramosca A**, Savy V, Zara V. Olive oil increases the hepatic triacylglycerol content in mice by a distinct influence on the synthesis and oxidation of fatty acids. *Biosci Biotechnol Biochem* 2008; **72**: 62-69 [PMID: 18175925 DOI: 10.1271/bbb.70369]
- 42 **Ferramosca A**, Conte A, Burri L, Berge K, De Nuccio F, Giudetti AM, Zara V. A krill oil supplemented diet suppresses hepatic steatosis in high-fat fed rats. *PLoS One* 2012; **7**: e38797 [PMID: 22685607 DOI: 10.1371/journal.pone.0038797]
- 43 **Ferramosca A**, Conte L, Zara V. A krill oil supplemented diet reduces the activities of the mitochondrial tricarboxylate carrier and of the cytosolic lipogenic enzymes in rats. *J Anim Physiol Anim Nutr (Berl)* 2012; **96**: 295-306 [PMID: 21429045 DOI: 10.1111/j.1439-0396.2011.01135.x]
- 44 **Ferramosca A**, Savy V, Conte L, Colombo S, Einerhand AW, Zara V. Conjugated linoleic acid and hepatic lipogenesis in mouse: role of the mitochondrial citrate carrier. *J Lipid Res* 2006; **47**: 1994-2003 [PMID: 16816327 DOI: 10.1194/jlr.M600138-JLR200]
- 45 **Kolakowska A**, Kolakowski E, Szczygieski M. Winter season krill (*Euphausia superba* Dana) as a source of n-3 polyunsaturated fatty acids. *Food/Nahrung* 1994; **38**: 128-134 [DOI: 10.1002/food.19940380204]
- 46 **Burri L**, Hoem N, Banni S, Berge K. Marine omega-3 phospholipids: metabolism and biological activities. *Int J Mol Sci* 2012; **13**: 15401-15419 [PMID: 23203133 DOI: 10.3390/ijms131115401]
- 47 **Bunea R**, El Farrah K, Deutsch L. Evaluation of the effects of Neptune Krill Oil on the clinical course of hyperlipidemia. *Altern Med Rev* 2004; **9**: 420-428 [PMID: 15656713]
- 48 **Deutsch L**. Evaluation of the effect of Neptune Krill Oil on chronic inflammation and arthritic symptoms. *J Am Coll Nutr* 2007; **26**: 39-48 [PMID: 17353582 DOI: 10.1080/07315724.2007.10719584]
- 49 **Zhu JJ**, Shi JH, Qian WB, Cai ZZ, Li D. Effects of krill oil on serum lipids of hyperlipidemic rats and human SW480 cells. *Lipids Health Dis* 2008; **7**: 30 [PMID: 18755044 DOI: 10.1186/1476-511X-7-30]
- 50 **Tandy S**, Chung RW, Wat E, Kamili A, Berge K, Griinari M, Cohn JS. Dietary krill oil supplementation reduces hepatic steatosis, glycemia, and hypercholesterolemia in high-fat-fed mice. *J Agric Food Chem* 2009; **57**: 9339-9345 [PMID: 19761211 DOI: 10.1021/jf9016042]
- 51 **Burri L**, Berge K, Wibrand K, Berge RK, Barger JL. Differential effects of krill oil and fish oil on the hepatic transcriptome in mice. *Front Genet* 2011; **2**: 45 [PMID: 22303341 DOI: 10.3389/fgene.2011.00045]
- 52 **Ikemoto S**, Takahashi M, Tsunoda N, Maruyama K, Itakura H, Ezaki O. High-fat diet-induced hyperglycemia and obesity in mice: differential effects of dietary oils. *Metabolism* 1996; **45**: 1539-1546 [PMID: 8969289 DOI: 10.1016/S0026-0495(96)90185-7]
- 53 **Wolff RL**, Pédrone F, Pasquier E, Marpeau AM. General characteristics of Pinus spp. seed fatty acid compositions, and importance of delta5-olefinic acids in the taxonomy and phylogeny of the genus. *Lipids* 2000; **35**: 1-22 [PMID: 10695919 DOI: 10.1007/s11745-000-0489-y]
- 54 **Sugano M**, Ikeda I, Wakamatsu K, Oka T. Influence of Korean pine (*Pinus koraiensis*)-seed oil containing cis-5,cis-9,cis-12-octadecatrienoic acid on polyunsaturated fatty acid metabolism, eicosanoid production and blood pressure of rats. *Br J Nutr* 1994; **72**: 775-783 [PMID: 7826999 DOI: 10.1079/BJN19940079]
- 55 **Matsuo N**, Osada K, Kodama T, Lim BO, Nakao A, Yamada K, Sugano M. Effects of gamma-linolenic acid and its positional isomer pinolenic acid on immune parameters of brown-Norway rats. *Prostaglandins Leukot Essent Fatty Acids* 1996; **55**: 223-229 [PMID: 8951990 DOI: 10.1016/S0952-3278(96)90002-2]
- 56 **Asset G**, Staels B, Wolff RL, Baugé E, Madj Z, Fruchart JC, Dallongeville J. Effects of Pinus pinaster and Pinus koraiensis seed oil supplementation on lipoprotein metabolism in the rat. *Lipids* 1999; **34**: 39-44 [PMID: 10188595 DOI: 10.1007/s11745-999-335-2]
- 57 **Vial G**, Dubouchaud H, Couturier K, Cottet-Rousselle C, Taleux N, Athias A, Galinier A, Casteilla L, Leverve XM. Effects of a high-fat diet on energy metabolism and ROS production in rat liver. *J Hepatol* 2011; **54**: 348-356 [PMID: 21109325 DOI: 10.1016/j.jhep.2010.06.044]
- 58 **Wang D**, Wei Y, Pagliassotti MJ. Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology* 2006; **147**: 943-951 [PMID: 16269465 DOI: 10.1210/en.2005-0570]
- 59 **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]

- 60 **Pfaffenbach KT**, Gentile CL, Nivala AM, Wang D, Wei Y, Pagliassotti MJ. Linking endoplasmic reticulum stress to cell death in hepatocytes: roles of C/EBP homologous protein and chemical chaperones in palmitate-mediated cell death. *Am J Physiol Endocrinol Metab* 2010; **298**: E1027-E1035 [PMID: 20159858 DOI: 10.1152/ajpendo.00642.2009]
- 61 **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]
- 62 **Kelly GS**. Conjugated linoleic acid: a review. *Altern Med Rev* 2001; **6**: 367-382 [PMID: 11578253]
- 63 **Vyas D**, Kadegowda AK, Erdman RA. Dietary conjugated linoleic Acid and hepatic steatosis: species-specific effects on liver and adipose lipid metabolism and gene expression. *J Nutr Metab* 2012; **2012**: 932928 [PMID: 21869929]
- 64 **Clément L**, Poirier H, Niot I, Bocher V, Guerre-Millo M, Krief S, Staels B, Besnard P. Dietary trans-10,cis-12 conjugated linoleic acid induces hyperinsulinemia and fatty liver in the mouse. *J Lipid Res* 2002; **43**: 1400-1409 [PMID: 12235171 DOI: 10.1194/jlr.M20008-JLR200]
- 65 **Takahashi Y**, Kushiro M, Shinohara K, Ide T. Activity and mRNA levels of enzymes involved in hepatic fatty acid synthesis and oxidation in mice fed conjugated linoleic acid. *Biochim Biophys Acta* 2003; **1631**: 265-273 [PMID: 12668178 DOI: 10.1016/S1388-1981(03)00038-6]
- 66 **Ide T**. Interaction of fish oil and conjugated linoleic acid in affecting hepatic activity of lipogenic enzymes and gene expression in liver and adipose tissue. *Diabetes* 2005; **54**: 412-423 [PMID: 15677499 DOI: 10.2337/diabetes.54.2.412]
- 67 **Park Y**, Storkson JM, Albright KJ, Liu W, Pariza MW. Evidence that the trans-10,cis-12 isomer of conjugated linoleic acid induces body composition changes in mice. *Lipids* 1999; **34**: 235-241 [PMID: 10230716 DOI: 10.1007/s11745-999-0358-8]
- 68 **Degrace P**, Demizieux L, Gresti J, Chardigny JM, Sébédio JL, Clouet P. Hepatic steatosis is not due to impaired fatty acid oxidation capacities in C57BL/6J mice fed the conjugated trans-10,cis-12-isomer of linoleic acid. *J Nutr* 2004; **134**: 861-867 [PMID: 15051838]
- 69 **Ruiz-Gutiérrez V**, Pérez-Espinosa A, Vázquez CM, Santa-María C. Effects of dietary fats (fish, olive and high-oleic-acid sunflower oils) on lipid composition and antioxidant enzymes in rat liver. *Br J Nutr* 1999; **82**: 233-241 [PMID: 10655970]
- 70 **Perona JS**, Ruiz-Gutiérrez V. Effect of two high-oleic oils on the liver lipid composition of spontaneously hypertensive rats. *Life Sci* 2000; **66**: 521-531 [PMID: 10794069 DOI: 10.1016/S0024-3205(99)00622-0]
- 71 **Portillo MP**, Chávarri M, Durán D, Rodríguez VM, Macarulla MT. Differential effects of diets that provide different lipid sources on hepatic lipogenic activities in rats under ad libitum or restricted feeding. *Nutrition* 2001; **17**: 467-473 [PMID: 11399405 DOI: 10.1016/S0899-9007(01)00513-5]
- 72 **Takeuchi H**, Nakamoto T, Mori Y, Kawakami M, Mabuchi H, Ohishi Y, Ichikawa N, Koike A, Masuda K. Comparative effects of dietary fat types on hepatic enzyme activities related to the synthesis and oxidation of fatty acid and to lipogenesis in rats. *Biosci Biotechnol Biochem* 2001; **65**: 1748-1754 [PMID: 11577713 DOI: 10.1271/bbb.65.1748]
- 73 **Hussein O**, Grosowski M, Lasri E, Svalb S, Ravid U, Assy N. Monounsaturated fat decreases hepatic lipid content in non-alcoholic fatty liver disease in rats. *World J Gastroenterol* 2007; **13**: 361-368 [PMID: 17230603]
- 74 **Soriguer F**, Morcillo S, Cardona F, Rojo-Martínez G, de la Cruz Almaráz M, Ruiz de Adana Mde L, Oliveira G, Tinahones F, Esteva I. Pro12Ala polymorphism of the PPARG2 gene is associated with type 2 diabetes mellitus and peripheral insulin sensitivity in a population with a high intake of oleic acid. *J Nutr* 2006; **136**: 2325-2330 [PMID: 16920849]
- 75 **Assy N**, Nassar F, Nasser G, Grosowski M. Olive oil consumption and non-alcoholic fatty liver disease. *World J Gastroenterol* 2009; **15**: 1809-1815 [PMID: 19370776 DOI: 10.3748/wjg.15.1809]
- 76 **Nakanishi T**, Oikawa D, Koutoku T, Hirakawa H, Kido Y, Tachibana T, Furuse M. Gamma-linolenic acid prevents conjugated linoleic acid-induced fatty liver in mice. *Nutrition* 2004; **20**: 390-393 [PMID: 15043857 DOI: 10.1016/j.nut.2003.12.014]
- 77 **Yanagita T**, Wang YM, Nagao K, Ujino Y, Inoue N. Conjugated linoleic acid-induced fatty liver can be attenuated by combination with docosahexaenoic acid in C57BL/6N mice. *J Agric Food Chem* 2005; **53**: 9629-9633 [PMID: 16302788 DOI: 10.1021/jf052203i]

P- Reviewers: Akcam M, Caprio S **S- Editor:** Zhai HH

L- Editor: Cant MR **E- Editor:** Wu HL



WJG 20th Anniversary Special Issues (12): Fatty liver

Dietary habits and behaviors associated with nonalcoholic fatty liver disease

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Received: September 11, 2013 Revised: November 11, 2013

Accepted: December 3, 2013

Published online: February 21, 2014

Abstract

Nonalcoholic fatty liver disease (NAFLD) is one of the most frequent causes of health problems in Western (industrialized) countries. Moreover, the incidence of infantile NAFLD is increasing, with some of these patients progressing to nonalcoholic steatohepatitis. These trends depend on dietary habits and life-style. In particular, overeating and its associated obesity affect the development of NAFLD. Nutritional problems in patients with NAFLD include excess intake of energy, carbohydrates, and lipids, and shortages of polyunsaturated fatty acids, vitamins, and minerals. Although nutritional therapeutic approaches are required for prophylaxis and treatment of NAFLD, continuous nutri-

tion therapy is difficult for many patients because of their dietary habits and lifestyle, and because the motivation for treatment differs among patients. Thus, it is necessary to assess the nutritional background and to identify nutritional problems in each patient with NAFLD. When assessing dietary habits, it is important to individually evaluate those that are consumed excessively or insufficiently, as well as inappropriate eating behaviors. Successful nutrition therapy requires patient education, based on assessments of individual nutrients, and continuing the treatment. In this article, we update knowledge about NAFLD, review the important aspects of nutritional assessment targeting treatment success, and present some concrete nutritional care plans which can be applied generally.

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Key words: Nonalcoholic fatty liver disease; Nutritional therapy; Carbohydrates; Fatty acids; Cholesterol

Core tip: The onset and development of nonalcoholic fatty liver disease (NAFLD) are closely associated with dietary habits and lifestyle; therefore, nutritional therapeutic approaches are required for these patients and those at risk of developing NAFLD. This article reviewed current nutritional status of NAFLD patients and the important aspects of nutritional assessment targeting treatment success.

Yasutake K, Kohjima M, Kotoh K, Nakashima M, Nakamuta M, Enjoji M. Dietary habits and behaviors associated with nonalcoholic fatty liver disease. *World J Gastroenterol* 2014; 20(7): 1756-1767 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1756.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1756>

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a major health problem in Western countries, affecting 30% of the adult population and 60%-80% of patients with diabetes mellitus and/or obesity^[1,2]. The 2011 National Health and Nutrition Examination Survey reported that the rates of NAFLD, obesity, and type 2 diabetes have increased coordinately over time since the 1988-1994 survey^[2], indicating that NAFLD is associated with obesity and type 2 diabetes.

In addition, the prevalence of NAFLD in children and adolescents is increasing, and has been reported to be about 10%^[3-5]. Although genetic factors have been associated with the onset of pediatric NAFLD^[6], the most important risk factor in children, as in adults, is overweight, with the prevalence of NAFLD higher in obese than in non-obese children^[3,7-9]. Moreover, nonalcoholic steatohepatitis (NASH) has been diagnosed in 3% of children and adolescents^[5].

About 20%-25% of adults with NASH have been reported to develop liver cirrhosis within 10 years^[1], with hepatocellular carcinoma occurring in 8.6% of cirrhotic NASH patients within 12 years^[10] or in 11.3% within 5 years^[11]. A recent meta-analysis showed that, compared with patients with simple steatosis, those with NASH have higher liver-related mortality rates, with an OR for patients with NASH of 5.71 and an OR for patients with NASH and advanced fibrosis of 10.06^[12]. In addition, NAFLD is considered as a risk factor for cardiovascular disease (CVD), because many patients with NAFLD develop metabolic disorders^[13]. A longitudinal study of 129 patients with biopsy-proven NAFLD who were followed for a mean of 13.7 years found that mortality from cardiovascular events was higher than liver-related mortality, with the overall mortality of patients with NASH being twice that of a matched reference population^[14]. Similarly, a cohort study of Swedish patients with NAFLD who were followed-up for a mean of 28 years showed that mortality risks were higher for patients with NAFLD (OR = 1.69) and NASH (OR = 1.86), compared with the general Swedish population, and that CVD is the most frequent cause of death^[15]. Another prospective, nested, case-control study in 2103 patients with type 2 diabetes without diagnosed CVD at baseline who were followed-up for a mean 5 years found that the presence of NAFLD was significantly associated with an increased CVD risk (OR = 1.84) and that this relationship was independent of classical risk factors^[16].

Because NAFLD develops as early as childhood and was found to exacerbate other conditions and worsen patient prognosis, treatment methods are urgently needed. Nutrition therapy is the basic form of treatment for patients with NAFLD and those at risk of developing this disorder. Therefore, all clinical staff involved in NAFLD prevention or treatment should understand nutritional strategies for dealing with NAFLD.

BEHAVIORAL SCIENCE AND MULTIDISCIPLINARY NUTRITIONAL CARE FOR SUCCESSFUL NUTRITION THERAPY

NAFLD is closely associated with obesity. Put simply, obesity results from greater energy intake than consumption, with excessive energy accumulated as fat. NAFLD patients with excess energy intake have shown improvements following weight loss resulting from restricted energy intake; *e.g.*, 600-800 kcal/d, 25-30 kcal/kg (standard weight) per day, or baseline minus 500-1000 kcal/d (Table 1)^[17-24]. Although restricted diets are clinically effective in the short-term, long-term energy and weight control is very difficult for many patients^[25]. For example, a 6-mo nutritional intervention was successful in only 54.8% of patients with NAFLD^[24], perhaps because patients differ in grade of motivation and preparation for the therapy.

Generally, a desirable health behavior is attained by changes that progress through five stages evaluated by the transtheoretical model: (1) a precontemplation stage, in which a patient has no intention of changing in the foreseeable future; (2) a contemplation stage, in which a patient intends to change, but not soon; (3) a preparation stage, in which a patient intends to change during the next month; (4) an action stage, in which a patient changes; and (5) a maintenance stage, in which a patient has maintains the change for at least 6 mo^[26,27]. The transtheoretical model, a popular concept in the area of health psychology, has been applied in patients with smoking, obesity, human immunodeficiency virus infection, and so on. The answers to the different questions are summed up to evaluate motivation to change according to the transtheoretical model of upper five stages of change, using 10 statements; two for each stage. The different stages of change have been theorized to predict treatment participation to programs and dropout, as well as efficacy and long-term maintenance of improvement. An evaluation of the intake of low-fat health food diets by obese patients with diabetes using these five stages found that 48.2% of male patients and 25.0% of female patients were at the precontemplation and contemplation stages^[28,29]. This trend was similar in patients with NAFLD. Dietary habits and physical activity in NAFLD patients were reported to be associated with the stages of change evaluated by the transtheoretical model, in which highest 36.0% of patients were at the contemplation stages^[30]. Therefore, although all NAFLD patients require nutrition therapy, more than 50% will not readily accept the need for or practice nutrition therapy. Thus, prior to initiating nutrition therapy, it is important to assess whether an individual patient is at a receptive stage for it. Behavioral counselors should therefore work flexibly with patients. For example, motivation by raising a patient's consciousness level is important during the precontemplation stage. During the contemplation stage, it is necessary for the

Table 1 Effect of nutritional intervention on nonalcoholic fatty liver disease

Study design	No. of cases	Duration (mo)	Improved items	Ref.
Balanced hypocaloric diet, exercise	9	9-30	ALT, fatty change	[19]
Very-low calorie diet	41	4-23	fatty change	[20]
Weight reduction (retrospective)	39	-	ALT	[21]
Diet (25 cal/kg•ibw), exercise	25	3	AST, ALT, ChE, TC, FPG fatty change	[22]
Diet (add 300 mg/d)	22	12	ALT, TGF- β , fatty change inflammation, fibrosis	[23]
Diet (baseline minus 500-1000 cal/d)	31	6	ALT, GGT, HDL-C, HOMA-IR WHR, fatty change, visceral fat	[24]

Ibw: Ideal body weight; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ChE: Choline esterase; TC: Total cholesterol; FPG: Fasting plasma glucose; TGF: Transforming growth factor; GGT: γ -glutamyl transpeptidase; HDL-C: High density lipoprotein-cholesterol; HOMA-IR: Homeostasis model assessment for insulin resistance; WHR: Waist-to-hip ratio.

patient to evaluate the effects of behavior modification, strengthening each patient's motivation and supporting his/her decision making. A recent randomized controlled trial in patients with NASH found that 48-wk-long life-style intervention, using a combination of diet, exercise, and behavior modification, significantly improved patient histologic activity score, body weight, body mass index (BMI), and serum alanine aminotransferase (ALT) levels^[31]. Patients discussed dietary and health problems during weekly group sessions, and their nutritional education employed several techniques of behavioral science, including self-monitoring of food eaten, body weight, and exercise; stimulus control techniques; and education to prevent relapse^[31-33].

Similarly, in patients with pediatric NAFLD, a 6-mo-long lifestyle intervention, consisting of physical exercise, dietary counseling, and behavioral counseling, improved steatosis and serum ALT levels^[34]. In another study on obese pediatric NAFLD patients, lifestyle intervention, consisting of physical activity, nutritional education, and behavioral therapy, for 1 year decreased BMI and serum ALT levels, with improvements maintained 1 year after the completion of this intervention^[35]. Reductions in BMI and ALT not only improved the grade of NAFLD in these patients but prevented its progression to steatohepatitis^[36]. These findings indicate that nutritional education employing behavioral methods conducted by a multidisciplinary nutritional care team is extremely useful and effective.

ASSESSMENT OF THE MAIN CAUSE OF EXCESS ENERGY INTAKE

For nutritional therapy to yield better outcomes, more detailed assessments of excess energy intake are needed. Some eating patterns are closely associated with excessive intake, such as increased dietary volume, high energy-dense diets, inappropriate mealtimes and manner of eating, and excessive intake of specific nutrients. It is important to determine the factor(s) crucial for each patient and to supply each patient with individual knowledge for appropriate dietary interventions.

Increased dietary volume

Increased dietary volume may be due to, for example, a

high frequency of eating outside the home, larger food portions, and the diffusion of all-you-can-eat style. Energy intake during a meal is usually larger while eating out than while eating at home. Eating out was reported to increase overall energy intake by 14% in 1977-1978, a rate that increased to 32% during 1994-1996^[37]. In addition, portion sizes of salty snacks, hamburger, soft drinks, fried potatoes, and Mexican food eaten outside the home in 1977-1978, 1989-1991, and 1994-1998 increased over time in almost all examined subjects^[38]. Enlarged meal volume increases energy intake, resulting in obesity and NAFLD^[39]. Energy intake also tends to be higher at all-you-can-eat restaurants because various kinds of foods are displayed. Actually, an increase in the variety of dishes at a meal has been found to enhance food intake by at least 25%, because of the variety of sensory properties of the foods, such as taste, palatability, and flavor^[40-43]. Food intake may be reduced by reducing the frequency of eating out and of eating at all-you-can-eat establishments. Moreover, when eating at home, the food/energy requirement in a meal should be habitually arranged beforehand, by, for example, the distribution of individual portions.

High energy-dense diets

Fast-foods, meals eaten out, and fried foods are representative of a high energy-dense diet. A study assessing the influence of fast-foods on liver function found that young adults with a daily energy intake of 2273 ± 558 kcal (fat: $36\% \pm 5.7\%$, sugar: 95 ± 42 g) given fast-food-based hyperalimentation of 5753 ± 1495 kcal (fat: $43\% \pm 6.8\%$, sugar: 285 ± 117 g) for 4 wk showed an increase in body weight from 67.6 ± 9.1 to 74.0 ± 11.0 kg and an increase in serum ALT levels from 22.1 ± 11.4 to 97 ± 103 U/L^[44]. These findings indicated that a high energy-dense diet can increase energy intake easily and markedly, resulting in obesity and NAFLD. In the CARDIO study, the habitual eating of fast-foods was assessed in young adults at baseline and 15 years later, and the association of a fast-food diet with weight gain and insulin resistance was analyzed^[45]. A higher frequency of fast-foods at baseline and at the end of the 15-year follow-up resulted in greater weight gain, independent of race or ethnicity, with the frequency of eating fast-foods positively correlated with mean energy intake^[45,46].

Official rules for the fast-food industry, such as energy restriction, increasing quantities of vegetables, and non-inclusion in children's meals of toy lagniappes, have been introduced in several countries. Thus, prior to starting a patients with NAFLD on nutrition therapy, the frequency of eating fast-foods, fried foods, and eating out should be assessed beforehand. Decreasing all of these may prevent the development and/or progression of NAFLD. It is recommended that these individuals eat at home more frequently and that they consume a low energy-dense diet, with higher quantities of vegetables.

Inappropriate mealtimes and eating manners

Inappropriate patterns of food intake, including the habit of eating too much at evening meals, eating at night, missing breakfast, and eating too rapidly, are often seen in patients with obesity and NAFLD. The night-eating syndrome is frequently observed in obese patients^[47]. Night workers and shift workers were recently shown to be at high risks of obesity, metabolic syndrome, and fatty liver disease^[48-50]. Food intake at unusual times by shift workers induces chronic sleep disorder and increased desire for fats, resulting in obesity and diabetes^[51]. This phenomenon may be due to the activity of the clock genes. Male Period gene-mutant mice gain significantly more body mass than wild-type controls on high-fat diet^[52].

Missing breakfast, especially by children and adolescents, has been associated with obesity^[53]. Missing breakfast usually increases food intake at other mealtimes. Mice with a greater energy intake in the evening meal had a higher body weight, more visceral fat, and higher fasting blood glucose levels, whereas all of these were lowest when the breakfast:evening meal energy ratio was 3:1^[54]. Whenever possible, therefore, patients on nutrition therapy for NAFLD should be started on a diet in which energy intake in the evening and night-time is restricted and intake at breakfast should be enhanced. However, the problem of shift work cannot be resolved easily.

Individuals who eat more quickly eat more food and have a lessened feeling of satiety than those who eat more slowly (20-30 chews per mouthful)^[55]. Persons who eat faster have a higher mean BMI and an increased rate of BMI^[56,57]. Increased mastication of each mouthful has been reported to prevent overeating and promote general and oral health^[58]. Similarly, more than 20 chews per mouthful should be recommended during nutritional education for NAFLD patients to prevent overeating.

OVER-INGESTION OF CARBOHYDRATES

Carbohydrates are classified as simple and complex, with over-ingestion of simple carbohydrates, such as sucrose and fructose, being a major cause of NAFLD. Consumption of soft drinks, including those containing sucrose, is significantly increasing worldwide^[59]. In comparison between NAFLD and non-NAFLD cases, mean daily consumption and mean frequency of soft

drinks is at least two fold higher in patients with than without NAFLD^[60-62]. The degree of ultrasonography-evaluated hepatic fatty changes was found to correlate with the increase in the number of consumed bottles of soft drinks, indicating that soft drink consumption is strongly predictive of fatty liver^[62]. Moreover, the rates of consumption of simple and total carbohydrates were found to be higher in patients with NASH than in those with simple steatosis^[17]. Excess intake of simple carbohydrates was found to rapidly induce elevated serum glucose levels and reactive hypoglycemia, resulting in a sensation of hunger, increasing appetite, and finally resulting in hyperphagia^[63]. Excess intake of simple carbohydrates is closely associated with obesity and steatosis, perhaps through the activation of sterol regulatory element-binding protein-1c (SREBP-1c), a transcription factor that enhances the expression of enzymes associating with fatty acid synthesis^[64].

The basic strategy in nutritional care is to understand each patient's habits of consuming foods and soft drinks, including simple carbohydrates, and to restrict the intake of these foods and drinks, if excessive amounts have been ingested^[65]. Restricting the intake of soft drinks requires patient motivation, although governments can also act by restricting these items. Taxation of soft drinks in the United States has been proposed to decrease their consumption and to provide revenue for national health programs^[59,66,67].

In contrast, appropriate intake of complex carbohydrates, especially that of whole grains, may prevent the development and/or progression of NAFLD, because these grains contain antioxidative vitamins, minerals, and dietary fibers, in addition to carbohydrates^[68]. Indeed, intake of whole grains may decrease visceral fat and improve obesity, dyslipidemia, and metabolic syndrome^[69,70]. Moreover, a meta-analysis showed that whole grains reduced the risks of heart disease and type 2 diabetes; serum levels of fasting insulin, fasting glucose, and lipids; and body weight, all of which are associated with the pathogenesis of NAFLD^[71-73]. Thus, paradoxically, a nutritional care plan for patients with NAFLD should seek to restrict carbohydrates, while increasing ingestion of whole grains.

OVER-INGESTION OF LIPIDS

Lipid over-ingestion results in excess energy intake and body fat accumulation. Increased visceral fat increase the inflow of free fatty acids into the liver, resulting in hepatic steatosis^[76]. Over-ingestion of saturated fatty acids is thought to induce insulin resistance and type 2 diabetes^[77-80]. A 7-d nutritional survey of diet showed that ingestion of saturated fatty acids was significantly greater in NAFLD patients than in healthy controls^[81]. Moreover, intake of saturated fatty acids, as well as of lipids, was reported significantly greater in NAFLD and NASH patients than in healthy individuals^[17]. When patients with NAFLD were randomly allocated an isoen-

ergetic low-fat/low-saturated fat/low-glycemic index (GI) diet (LSAT: 23% fat/7% saturated fat/GI < 55) or a high-fat/high-saturated fat/high-GI diet (HSAT: 43% fat/24% saturated fat/GI > 70), with liver fat quantitated by magnetic resonance spectroscopy before and after 4 wk on the LSAT and HSAT diets, those in the LSAT, but not those in the HSAT group showed significant reductions in liver fat^[82]. In other animal studies, a high-fat diet induced hepatic steatosis and inflammation, insulin resistance, and tumor necrosis factor α (TNF α) elevation^[83-85]. These changes may be associated with the activation of peroxisome proliferators-activated receptor γ (PPAR γ)^[64].

Thus, over-ingestion of lipids, especially saturated fatty acids, is one of the most important risk factors for NAFLD onset and development. Therefore, before addressing NAFLD, it is recommended that patients' eating habits be assessed, including patient intake of dairy products; fats in meat, butter and margarine; chocolate, and snack foods. If any of these foods are consumed excessively, its quantity should be reduced. Clinically, more concrete nutritional care plans are necessary; *e.g.*, bacon and sirloin, which contain considerable quantities of fat, should be switched to leg meat, fillet, or, if appropriate, to fish containing polyunsaturated fatty acids (PUFAs); and butter and margarine should be switched to the calorie-half products.

OVER-INGESTION OF CHOLESTEROL

Over-ingestion of cholesterol has been regarded as a critical cause of NAFLD^[86-89]. For example, a 7-d nutritional survey found that dietary cholesterol intake was significantly greater in NASH patients than in healthy subjects^[81]. In addition, our investigation of the dietary records of obese and non-obese NAFLD patients found that cholesterol ingestion was significantly greater in NAFLD patients than in healthy controls^[90]. Interestingly, non-obese NAFLD patients ingested more cholesterol than obese NAFLD patients^[90], indicating that cholesterol intake is dietetically essential for NAFLD onset/progression independent of obesity. These findings are supported by animal experiments, in which a high-cholesterol diet within the normal energy range induced the onset of non-obese NAFLD in mice^[91-93]. Although the mechanisms have not been determined, the hepatic metabolic products of cholesterol, oxysterols, are ligands of liver X receptor α , which activates SREBP-1c and the *de novo* synthesis of fatty acids^[94,95]. Although several studies reported no significant differences in cholesterol intake levels between NAFLD patients and healthy subjects, those studies did not assess dietary records but used food frequency questionnaires^[96,97]. Future studies are needed to assess cholesterol intake levels and the clinical effects of cholesterol restriction in larger populations of patients with NAFLD. Over-ingestion of dietary cholesterol should be suspected in non-obese patients with NAFLD, and their dietary intake of food high in chole-

sterol, such as eggs, fish eggs, liver, and cakes, should be assessed. Reduction of cholesterol intake has also been recommended to prevent the development of CVD, regardless of the presence of obesity^[98,99].

DEFICIENCY OF POLYUNSATURATED FATTY ACIDS

PUFA intake is lower in patients with NAFLD than in healthy individuals, regardless of excessive lipid intake^[17,81]. Moreover, we found that PUFA intake was significantly lower in non-obese than in obese NAFLD patients^[90]. These findings suggest that dietary contents are unbalanced in patients with NAFLD and that PUFA deficiency is involved in the onset and progression of NAFLD. PUFAs can improve insulin sensitivity by decreasing hepatic TNF α , can repress fatty acid synthesis by negatively controlling SREBP-1c, and can enhance fatty acid oxidation by positively controlling PPAR α ^[100,101]. In some studies in animal models, administration of n-3 PUFAs reduced liver fat and improved hepatic inflammation^[102,103]. These results are supported by nutritional interventions in patients with NAFLD. Although saturated fatty acids increase the likelihood of NAFLD development, PUFAs may be beneficial for these patients. For example, treatment of NAFLD patients for 12 mo with 1 g/d of n-3 PUFAs decreased ultrasonography-detected liver fat^[104]. Moreover, in a study of patients receiving diet therapy with or without 2 g/d n-3 PUFAs for 6 mo, those administrated n-3 PUFA showed greater decreases in liver fat^[105,106]. Furthermore, an 8-wk-long, double blind, crossover trial of 4 g/d n-3 PUFA and placebo showed that the accumulated liver fat decreased significantly in the n-3 PUFA group^[107]. In none of these studies did n-3 PUFA show any adverse effects. Moreover, n-3 PUFAs improved risk factors for CVD, including markers of insulin resistance and inflammation, as well as serum levels of ALT, lipids and glucose. Deliberate supplementation with PUFA, especially n-3 PUFA, may be an effective form of nutrition therapy in patients with NAFLD, and may also prevent CVD^[108,109]. However, combination with appropriate energy intake is a prerequisite, with the basic nutritional approach consisting of increasing the frequency of eating n-3 PUFA-rich fish in place of meat containing high quantities of saturated fatty acids.

DEFICIENCY OF VITAMIN E

Progression from NAFLD to NASH is commonly explained by the two-hit theory^[110], with oxidative stress considered as a second hit factor^[111-113]. Intake of vitamin E has been reported deficient in NAFLD and NASH patients compared with healthy subjects^[81,114]. Moreover, in children, vitamin E intake was negatively correlated with the grade of liver fat. Total peroxide level and oxidative stress index were found to be positively correlated, and total antioxidant status negatively correlated,

with fibrosis scores in patients with NAFLD^[115]. These results are supported by other studies, which found that serum markers of oxidative stress were independent prognostic indicators of hepatic fibrosis^[116-118]. Patients with NAFLD and NASH require more vitamin E to counteract increases in oxidative stress. Even if NAFLD patients take amounts of vitamin E equivalent to those taken by healthy individuals, patients may experience a net shortage, with reduced serum levels. However, many foods with high vitamin E contents, including some oils and fats, liver, and fish eggs, also contain large amounts of cholesterol, and greater quantities of these foods are ingested by NAFLD patients than by healthy individuals^[18]. Although vegetables, especially green and yellow vegetables, do not have high vitamin E contents, they are important sources of this nutrient. Because vegetable intake by NAFLD patients is generally reduced^[18], NAFLD patients with vitamin E deficiency should ingest higher quantities of green and yellow vegetables.

Patients who have dietary compliance problems should be administered vitamin E supplements. High-dose vitamin E supplementation has been reported to lower serum ALT levels in patients with NAFLD^[23], and a randomized control study of vitamin E supplementation for 2 years to NASH patients without diabetes found that histologic activity score improved in a significantly higher percentage of the vitamin E than of the placebo group (43% *vs* 19%, $P = 0.001$)^[119], demonstrating that vitamin E has clinical antioxidative effects. However, administration of high amounts of vitamin E may induce cerebral vascular disease and increase all-cause mortality^[120,121]. A recent meta-analysis found that vitamin E treatment for 2 years of patients with NAFLD improved histology score, but led to a deterioration in insulin resistance and an increase in serum triglyceride levels^[12]. In the United States practice guideline for the diagnosis and management of NAFLD, (1) Vitamin E (α -tocopherol) administered at daily dose of 800 IU/d improves liver histology in non-diabetic adults with biopsy-proven NASH and therefore it should be considered as a first-line pharmacotherapy for this patient population, and (2) Until further data supporting its effectiveness become available, vitamin E is not recommended to treat NASH in diabetic patients, NAFLD without liver biopsy, NASH cirrhosis, or cryptogenic cirrhosis. Thus, while vitamin E is an important nutrient that can improve disease activity in patients with NASH, it also has the potential to cause other clinical problems. Patients receiving high-dose vitamin E supplementation should be closely monitored. According to recent studies, supplementation of 300 mg/d vitamin E seems to be safe and effective even in patients with fibrosis and/or impaired fasting glucose^[23,122,123].

DEFICIENCY OF VITAMIN D

Vitamin D plays an important part in the processes of inflammation and autoimmunity. Deficiencies in vitamin

D can result in insulin resistance, metabolic syndrome, and NAFLD^[124]. Many obese children ingest a high-energy diet with low vitamin and mineral content, and are not sufficiently exposed to sunlight^[125]. For example, serum vitamin D levels were low in 55% of young Americans^[126]. Rats fed a Westernized diet (WD: high-fat/high-fructose corn syrup) with vitamin D depletion (29% compared with controls) had significantly poorer liver fat, lobular inflammation, and NAFLD activity scores than rats fed a WD, a low-fat diet (LFD), or a LFD with vitamin D depletion^[127]. In humans, deficiency of vitamin D has been correlated with the severity of NAFLD activity score and hepatic fibrosis^[128], perhaps owing to the greater oxidative stress resulting from vitamin D deficiency^[129]. Hepatic expression of vitamin D receptors, CYP2R1 and CYP 27A1, has been negatively correlated with the severity of steatosis, inflammation, and NAFLD score in patients with NAFLD^[130].

Taken together, these findings indicate that excess energy intake accompanied by vitamin D deficiency enhances the onset and progression of NAFLD/NASH. Thus, the status of vitamin D intake and serum vitamin D levels should be ascertained prior to beginning nutritional therapy for NAFLD. Patients with vitamin D deficiency should ingest foods with a high vitamin D content, such as fishes and mushrooms, at least once per day.

NUTRITIONAL THERAPY WITH PROBIOTICS

Probiotics are live bacteria or foods containing them that may confer a health benefit on the host by regulating intestinal microbial flora. Intestinal microbial flora change with BMI and eating habits^[131,132]. Alteration of the enteral environment by probiotics has been shown to improve the pathology of NAFLD^[133,134]. In animal models, administration of probiotics had desirable clinical effects, including decreases in liver fat and serum ALT and lipid levels, and improvements in inflammation, liver fibrosis, oxidative stress, and insulin resistance^[132-142]. Serum levels of ALT, malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), and TNF α have been reported to be decreased in NAFLD patients administered probiotics (*Lactobacillus acidophilus*, *bifidus*, *rhamnosus*, *plantarum*, *salivarius*, *bulgaricus*, *lactis*, *casei*, *breve* mixed with prebiotic fructooligosaccharide and vitamins as B₂, B₁₂, B₆, D₃, C, and folate) for 2 mo^[143]. In addition, the probiotic VSL#3 had beneficial effects on lipid peroxidation markers (MDA, 4-HNE) in NAFLD patients^[144]. A randomized, double-blind, placebo-controlled clinical trial found that administration of probiotics (*Lactobacillus bulgaricus* and *Streptococcus thermophiles*) for 3 mo significantly decreased serum aspartate aminotransferase, ALT, and γ -glutamyl transpeptidase levels in patients with NAFLD^[145], in good agreement with results in animal models. Probiotic treatment can be included in nutrition therapy for NAFLD. Future studies investigating

the effects of probiotics on other outcomes in patients with NAFLD, such as inhibition of hepatic fat accumulation and inflammation, as well as studies investigating foods containing probiotics, such as yogurt and lactic acid drinks, are expected.

CONCLUSION

Because the onset and development of NAFLD are closely associated with dietary habits and lifestyle, nutritional therapeutic approaches are required for these patients and those at risk of developing NAFLD. This article reviewed current nutritional strategies and their effects and problems.

REFERENCES

- 1 **Ratzliff 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]
- 2 **Younossi ZM**, Stepanova M, Afendy M, Fang Y, Younossi Y, Mir H, Srisord M. Changes in the prevalence of the most common causes of chronic liver diseases in the United States from 1988 to 2008. *Clin Gastroenterol Hepatol* 2011; **9**: 524-530. e1; quiz e60 [PMID: 21440669 DOI: 10.1016/j.cgh.2011.03.020]
- 3 **Patton HM**, Sirlin C, Behling C, Middleton M, Schwimmer JB, Lavine JE. Pediatric nonalcoholic fatty liver disease: a critical appraisal of current data and implications for future research. *J Pediatr Gastroenterol Nutr* 2006; **43**: 413-427 [PMID: 17033514 DOI: 10.4103/1319-3767.74476]
- 4 **Papandreou D**, Rousso I, Mavromichalis I. Update on non-alcoholic fatty liver disease in children. *Clin Nutr* 2007; **26**: 409-415 [PMID: 17449148 DOI: 10.1016/j.clnu.2007.02.002]
- 5 **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]
- 6 **Younossi ZM**, Baranova A, Ziegler K, Del Giacco L, Schlauch K, Born TL, Elariny H, Gorreta F, VanMeter A, Younoszai A, Ong JP, Goodman Z, Chandhoke V. A genomic and proteomic study of the spectrum of nonalcoholic fatty liver disease. *Hepatology* 2005; **42**: 665-674 [PMID: 16116632 DOI: 10.1002/hep.20838]
- 7 **Alisi A**, Manco M, Panera N, Nobili V. Association between type two diabetes and non-alcoholic fatty liver disease in youth. *Ann Hepatol* 2009; **8** Suppl 1: S44-S50 [PMID: 19381124]
- 8 **Huang SC**, Yang YJ. Serum retinol-binding protein 4 is independently associated with pediatric NAFLD and fasting triglyceride level. *J Pediatr Gastroenterol Nutr* 2013; **56**: 145-150 [PMID: 22983378 DOI: 10.1097/MPG.0b013e3182722aee]
- 9 **Wiegand S**, Keller KM, Röbl M, L'Allemand D, Reinehr T, Widhalm K, Holl RW. Obese boys at increased risk for non-alcoholic liver disease: evaluation of 16,390 overweight or obese children and adolescents. *Int J Obes (Lond)* 2010; **34**: 1468-1474 [PMID: 20531349 DOI: 10.1038/ijo.2010.106]
- 10 **Krawczyk K**, Szczesniak P, Kumor A, Jasinska A, Omulecka A, Pietruczuk M, Orszulak-Michalak D, Sporny S, Malecka-Panas E. Adipohormones as prognostic markers in patients with nonalcoholic steatohepatitis (NASH). *J Physiol Pharmacol* 2009; **60** Suppl 3: 71-75 [PMID: 19996485]
- 11 **Yatsuji S**, Hashimoto E, Tobari M, Taniai M, Tokushige K, Shiratori K. Clinical features and outcomes of cirrhosis due to non-alcoholic steatohepatitis compared with cirrhosis caused by chronic hepatitis C. *J Gastroenterol Hepatol* 2009; **24**: 248-254 [PMID: 19032450 DOI: 10.1111/j.1440-1746.2008.05640.x]
- 12 **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]
- 13 **Angulo P**. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231 [PMID: 11961152 DOI: 10.1056/NEJM-ra011775]
- 14 **Ekstedt M**, Franzén LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, Kechagias S. Long-term follow-up of patients with NAFLD and elevated liver enzymes. *Hepatology* 2006; **44**: 865-873 [PMID: 17006923 DOI: 10.1002/hep.21327]
- 15 **Söderberg C**, Stål P, Askling J, Glaumann H, Lindberg G, Marmur J, Hultcrantz R. Decreased survival of subjects with elevated liver function tests during a 28-year follow-up. *Hepatology* 2010; **51**: 595-602 [PMID: 20014114 DOI: 10.1002/hep.23314]
- 16 **Targher G**, Bertolini L, Poli F, Rodella S, Scala L, Tessari R, Zenari L, Falezza G. Nonalcoholic fatty liver disease and risk of future cardiovascular events among type 2 diabetic patients. *Diabetes* 2005; **54**: 3541-3546 [PMID: 16306373 DOI: 10.2337/diabetes.54.12.3541]
- 17 **Toshimitsu K**, Matsuura B, Ohkubo I, Niiya T, Furukawa S, Hiasa Y, Kawamura M, Ebihara K, Onji M. Dietary habits and nutrient intake in non-alcoholic steatohepatitis. *Nutrition* 2007; **23**: 46-52 [PMID: 17140767 DOI: 10.1016/j.nut.2006.09.004]
- 18 **Shi L**, Liu ZW, Li Y, Gong C, Zhang H, Song LJ, Huang CY, Li M. The prevalence of nonalcoholic fatty liver disease and its association with lifestyle/dietary habits among university faculty and staff in Chengdu. *Biomed Environ Sci* 2012; **25**: 383-391 [PMID: 23026517 DOI: 10.3967/0895-3988.2012.04.002]
- 19 **Vajro P**, Fontanella A, Perna C, Orso G, Tedesco M, De Vincenzo A. Persistent hyperamino-transferasemia resolving after weight reduction in obese children. *J Pediatr* 1994; **125**: 239-241 [PMID: 8040771 DOI: 10.1016/S0022-3476(94)70202-0]
- 20 **Andersen T**, Gluud C, Franzmann MB, Christoffersen P. Hepatic effects of dietary weight loss in morbidly obese subjects. *J Hepatol* 1991; **12**: 224-229 [PMID: 2051001 DOI: 10.1016/0168-8278(91)90942-5]
- 21 **Palmer M**, Schaffner F. Effect of weight reduction on hepatic abnormalities in overweight patients. *Gastroenterology* 1990; **99**: 1408-1413 [PMID: 2210247]
- 22 **Ueno T**, Sugawara H, Sujaku K, Hashimoto O, Tsuji R, Tamaki S, Torimura T, Inuzuka S, Sata M, Tanikawa K. Therapeutic effects of restricted diet and exercise in obese patients with fatty liver. *J Hepatol* 1997; **27**: 103-107 [PMID: 9252081 DOI: 10.1016/S0168-8278(97)80287-5]
- 23 **Hasegawa T**, Yoneda M, Nakamura K, Makino I, Terano A. Plasma transforming growth factor-beta1 level and efficacy of alpha-tocopherol in patients with non-alcoholic steatohepatitis: a pilot study. *Aliment Pharmacol Ther* 2001; **15**: 1667-1672 [PMID: 11564008 DOI: 10.1046/j.1365-2036.2001.01083.x]
- 24 **Elias MC**, Parise ER, de Carvalho L, Szejnfeld D, Netto JP. Effect of 6-month nutritional intervention on non-alcoholic fatty liver disease. *Nutrition* 2010; **26**: 1094-1099 [PMID: 20022466 DOI: 10.1016/j.nut.2009.09.001]
- 25 **Bugianesi E**, Marzocchi R, Villanova N, Marchesini G. Non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD/NASH): treatment. *Best Pract Res Clin Gastroenterol* 2004; **18**: 1105-1116 [PMID: 15561641 DOI: 10.1016/j.bpg.2004.06.025]
- 26 **Prochaska JO**, Velicer WF. The transtheoretical model of health behavior change. *Am J Health Promot* 1997; **12**: 38-48 [PMID: 10170434 DOI: 10.4278/0890-1171-12.1.38]
- 27 **Prochaska JO**, DiClemente CC. Transtheoretical therapy: toward a more integrative model of change. *Psychotherapy* 1982; **19**: 276-288 [DOI: 10.1037/h0088437]
- 28 **Greene GW**, Rossi SR. Stages of change for reducing dietary fat intake over 18 months. *J Am Diet Assoc* 1998; **98**: 529-534; quiz

- 535-536 [PMID: 9597025 DOI: 10.1016/S0002-8223(98)00120-5]
- 29 **Vallis M**, Ruggiero L, Greene G, Jones H, Zinman B, Rossi S, Edwards L, Rossi JS, Prochaska JO. Stages of change for healthy eating in diabetes: relation to demographic, eating-related, health care utilization, and psychosocial factors. *Diabetes Care* 2003; **26**: 1468-1474 [PMID: 12716806 DOI: 10.2337/diacare.26.5.1468]
 - 30 **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]
 - 31 **Promrat K**, Kleiner DE, Niemeier HM, Jackvony E, Kearns M, Wands JR, Fava JL, Wing RR. Randomized controlled trial testing the effects of weight loss on nonalcoholic steatohepatitis. *Hepatology* 2010; **51**: 121-129 [PMID: 19827166 DOI: 10.1002/hep.23276]
 - 32 **D'Zurilla TJ**, Goldfried MR. Problem solving and behavior modification. *J Abnorm Psychol* 1971; **78**: 107-126 [PMID: 4938262 DOI: 10.1037/h0031360]
 - 33 **Collins RL**, Parks GA, Marlatt GA. Social determinants of alcohol consumption: the effects of social interaction and model status on the self-administration of alcohol. *J Consult Clin Psychol* 1985; **53**: 189-200 [PMID: 3998247 DOI: 10.1037/0022-006X.53.2.189]
 - 34 **Koot BG**, van der Baan-Slootweg OH, Tamminga-Smeulders CL, Rijcken TH, Korevaar JC, van Aalderen WM, Jansen PL, Benninga MA. Lifestyle intervention for non-alcoholic fatty liver disease: prospective cohort study of its efficacy and factors related to improvement. *Arch Dis Child* 2011; **96**: 669-674 [PMID: 21518734 DOI: 10.1136/adc.2010.199760]
 - 35 **Reinehr T**, Schmidt C, Toschke AM, Andler W. Lifestyle intervention in obese children with non-alcoholic fatty liver disease: 2-year follow-up study. *Arch Dis Child* 2009; **94**: 437-442 [PMID: 19224892 DOI: 10.1136/adc.2008.143594]
 - 36 **Nobili V**, Manco M, Devito R, Di Ciommo V, Comparcola D, Sartorelli MR, Piemonte F, Marcellini M, Angulo P. Lifestyle intervention and antioxidant therapy in children with non-alcoholic fatty liver disease: a randomized, controlled trial. *Hepatology* 2008; **48**: 119-128 [PMID: 18537181 DOI: 10.1002/hep.22336]
 - 37 **Guthrie JF**, Lin BH, Frazao E. Role of food prepared away from home in the American diet, 1977-78 versus 1994-96: changes and consequences. *J Nutr Educ Behav* 2002; **34**: 140-150 [PMID: 12047838 DOI: 10.1016/S1499-4046(06)60083-3]
 - 38 **Nielsen SJ**, Popkin BM. Patterns and trends in food portion sizes, 1977-1998. *JAMA* 2003; **289**: 450-453 [PMID: 12533124 DOI: 10.1001/jama.289.4.450]
 - 39 **Ledikwe JH**, Ello-Martin JA, Rolls BJ. Portion sizes and the obesity epidemic. *J Nutr* 2005; **135**: 905-909 [PMID: 15795457]
 - 40 **Bellisle F**, Le Magnen J. The structure of meals in humans: eating and drinking patterns in lean and obese subjects. *Physiol Behav* 1981; **27**: 649-658 [PMID: 7323168 DOI: 10.1016/0031-9384(81)90237-7]
 - 41 **Rolls BJ**, Rowe EA, Rolls ET, Kingston B, Megson A, Gunary R. Variety in a meal enhances food intake in man. *Physiol Behav* 1981; **26**: 215-221 [PMID: 7232526 DOI: 10.1016/0031-9384(81)90014-7]
 - 42 **Rolls BJ**, Rowe EA, Rolls ET. How sensory properties of foods affect human feeding behavior. *Physiol Behav* 1982; **29**: 409-417 [PMID: 7178247 DOI: 10.1016/0031-9384(82)90259-1]
 - 43 **Berry SL**, Beatty WW, Klesges RC. Sensory and social influences on ice cream consumption by males and females in a laboratory setting. *Appetite* 1985; **6**: 41-45 [PMID: 3994354 DOI: 10.1016/S0195-6663(85)80049-0]
 - 44 **Kechagias S**, Ernersson A, Dahlqvist O, Lundberg P, Lindström T, Nystrom FH. Fast-food-based hyper-alimentation can induce rapid and profound elevation of serum alanine aminotransferase in healthy subjects. *Gut* 2008; **57**: 649-654 [PMID: 18276725 DOI: 10.1136/gut.2007.131797]
 - 45 **Pereira MA**, Kartashov AI, Ebbeling CB, Van Horn L, Slatery ML, Jacobs DR, Ludwig DS. Fast-food habits, weight gain, and insulin resistance (the CARDIA study): 15-year prospective analysis. *Lancet* 2005; **365**: 36-42 [PMID: 15639678 DOI: 10.1016/S0140-6736(04)17663-0]
 - 46 **Bowman SA**, Gortmaker SL, Ebbeling CB, Pereira MA, Ludwig DS. Effects of fast-food consumption on energy intake and diet quality among children in a national household survey. *Pediatrics* 2004; **113**: 112-118 [PMID: 14702458 DOI: 10.1542/peds.113.1.112]
 - 47 **STUNKARD AJ**, GRACE WJ, WOLFF HG. The night-eating syndrome; a pattern of food intake among certain obese patients. *Am J Med* 1955; **19**: 78-86 [PMID: 14388031]
 - 48 **Sookoian S**, Gemma C, Fernández Gianotti T, Burgueño A, Alvarez A, González CD, Pirola CJ. Effects of rotating shift work on biomarkers of metabolic syndrome and inflammation. *J Intern Med* 2007; **261**: 285-292 [PMID: 17305651 DOI: 10.1111/j.1365-2796.2007.01766.x]
 - 49 **Wang XS**, Armstrong ME, Cairns BJ, Key TJ, Travis RC. Shift work and chronic disease: the epidemiological evidence. *Occup Med (Lond)* 2011; **61**: 78-89 [PMID: 21355031 DOI: 10.1093/occmed/kqr001]
 - 50 **Lin YC**, Chen PC. Persistent Rotating Shift Work Exposure Is a Tough Second Hit Contributing to Abnormal Liver Function Among On-Site Workers Having Sonographic Fatty Liver. *Asia Pac J Public Health* 2012; Epub ahead of print [PMID: 23239752 DOI: 10.1038/gene.2011.74]
 - 51 **Antunes LC**, Levandovski R, Dantas G, Caumo W, Hidalgo MP. Obesity and shift work: chronobiological aspects. *Nutr Res Rev* 2010; **23**: 155-168 [PMID: 20122305 DOI: 10.1017/S0954422410000016]
 - 52 **Dallmann R**, Weaver DR. Altered body mass regulation in male mPeriod mutant mice on high-fat diet. *Chronobiol Int* 2010; **27**: 1317-1328 [PMID: 20653457 DOI: 10.3109/07420528.2010.489166]
 - 53 **de Castro JM**. When, how much and what foods are eaten are related to total daily food intake. *Br J Nutr* 2009; **102**: 1228-1237 [PMID: 19650955 DOI: 10.1017/S0007114509371640]
 - 54 **Fuse Y**, Hirao A, Kuroda H, Otsuka M, Tahara Y, Shibata S. Differential roles of breakfast only (one meal per day) and a bigger breakfast with a small dinner (two meals per day) in mice fed a high-fat diet with regard to induced obesity and lipid metabolism. *J Circadian Rhythms* 2012; **10**: 4 [PMID: 22587351 DOI: 10.1186/1740-3391-10-4]
 - 55 **Andrade AM**, Greene GW, Melanson KJ. Eating slowly led to decreases in energy intake within meals in healthy women. *J Am Diet Assoc* 2008; **108**: 1186-1191 [PMID: 18589027 DOI: 10.1016/j.jada.2008.04.026]
 - 56 **Sasaki S**, Katagiri A, Tsuji T, Shimoda T, Amano K. Self-reported rate of eating correlates with body mass index in 18-y-old Japanese women. *Int J Obes Relat Metab Disord* 2003; **27**: 1405-1410 [PMID: 14574353 DOI: 10.1038/sj.ijo.0802425]
 - 57 **Otsuka R**, Tamakoshi K, Yatsuya H, Murata C, Sekiya A, Wada K, Zhang HM, Matsushita K, Sugiura K, Takefuji S, Ouyang P, Nagasawa N, Kondo T, Sasaki S, Toyoshima H. Eating fast leads to obesity: findings based on self-administered questionnaires among middle-aged Japanese men and women. *J Epidemiol* 2006; **16**: 117-124 [PMID: 16710080 DOI: 10.2188/jea.16.117]
 - 58 **Christen AG**, Christen JA. Horace Fletcher (1849-1919): "The Great Masticator.". *J Hist Dent* 1997; **45**: 95-100 [PMID: 9693596]
 - 59 **Brownell KD**, Farley T, Willett WC, Popkin BM, Chaloupka FJ, Thompson JW, Ludwig DS. The public health and economic benefits of taxing sugar-sweetened beverages. *N Engl J Med* 2009; **361**: 1599-1605 [PMID: 19759377 DOI: 10.1056/NEJMp0905723]
 - 60 **Assy N**, Nasser G, Kamayse I, Nseir W, Beniashvili Z, Djibre A, Grosovski M. Soft drink consumption linked with fatty liver in the absence of traditional risk factors. *Can J Gastroen-*

- terol 2008; **22**: 811-816 [PMID: 18925303]
- 61 **Ouyang X**, Cirillo P, Sautin Y, McCall S, Bruchette JL, Diehl AM, Johnson RJ, Abdelmalek MF. Fructose consumption as a risk factor for non-alcoholic fatty liver disease. *J Hepatol* 2008; **48**: 993-999 [PMID: 18395287 DOI: 10.1016/j.jhep.2008.02.011]
 - 62 **Abid A**, Taha O, Nseir W, Farah R, Grosovski M, Assy N. Soft drink consumption is associated with fatty liver disease independent of metabolic syndrome. *J Hepatol* 2009; **51**: 918-924 [PMID: 19765850 DOI: 10.1016/j.jhep.2009.05.033]
 - 63 **Melanson KJ**, Westerterp-Plantenga MS, Saris WH, Smith FJ, Campfield LA. Blood glucose patterns and appetite in time-blinded humans: carbohydrate versus fat. *Am J Physiol* 1999; **277**: R337-R345 [PMID: 10444538]
 - 64 **Yamazaki T**, Nakamori A, Sasaki E, Wada S, Ezaki O. Fish oil prevents sucrose-induced fatty liver but exacerbates high-safflower oil-induced fatty liver in ddy mice. *Hepatology* 2007; **46**: 1779-1790 [PMID: 17935225 DOI: 10.1002/hep.21934]
 - 65 **Schulze MB**, Manson JE, Ludwig DS, Colditz GA, Stampfer MJ, Willett WC, Hu FB. Sugar-sweetened beverages, weight gain, and incidence of type 2 diabetes in young and middle-aged women. *JAMA* 2004; **292**: 927-934 [PMID: 15328324 DOI: 10.1001/jama.292.8.927]
 - 66 **Jacobson MF**, Brownell KD. Small taxes on soft drinks and snack foods to promote health. *Am J Public Health* 2000; **90**: 854-857 [PMID: 10846500]
 - 67 **Powell LM**, Chaloupka FJ. Food prices and obesity: evidence and policy implications for taxes and subsidies. *Milbank Q* 2009; **87**: 229-257 [PMID: 19298422 DOI: 10.1111/j.1468-0009.2009.00554.x]
 - 68 **Ross AB**, Godin JP, Minehira K, Kirwan JP. Increasing whole grain intake as part of prevention and treatment of nonalcoholic Fatty liver disease. *Int J Endocrinol* 2013; **2013**: 585876 [PMID: 23762052 DOI: 10.1155/2013/585876]
 - 69 **McKeown NM**, Yoshida M, Shea MK, Jacques PF, Lichtenstein AH, Rogers G, Booth SL, Saltzman E. Whole-grain intake and cereal fiber are associated with lower abdominal adiposity in older adults. *J Nutr* 2009; **139**: 1950-1955 [PMID: 19726588 DOI: 10.3945/jn.108.103762]
 - 70 **Katcher HI**, Legro RS, Kunselman AR, Gillies PJ, Demers LM, Bagshaw DM, Kris-Etherton PM. The effects of a whole grain-enriched hypocaloric diet on cardiovascular disease risk factors in men and women with metabolic syndrome. *Am J Clin Nutr* 2008; **87**: 79-90 [PMID: 18175740]
 - 71 **Ye EQ**, Chacko SA, Chou EL, Kugizaki M, Liu S. Greater whole-grain intake is associated with lower risk of type 2 diabetes, cardiovascular disease, and weight gain. *J Nutr* 2012; **142**: 1304-1313 [PMID: 22649266 DOI: 10.3945/jn.111.155325]
 - 72 **Mellen PB**, Walsh TF, Herrington DM. Whole grain intake and cardiovascular disease: a meta-analysis. *Nutr Metab Cardiovasc Dis* 2008; **18**: 283-290 [PMID: 17449231 DOI: 10.1016/j.numecd.2006.12.008]
 - 73 **de Munter JS**, Hu FB, Spiegelman D, Franz M, van Dam RM. Whole grain, bran, and germ intake and risk of type 2 diabetes: a prospective cohort study and systematic review. *PLoS Med* 2007; **4**: e261 [PMID: 17760498 DOI: 10.1371/journal.pmed.0040261]
 - 74 **Nettleton JA**, McKeown NM, Kanoni S, Lemaitre RN, Hivert MF, Ngwa J, van Rooij FJ, Sonestedt E, Wojczynski MK, Ye Z, Tanaka T, Garcia M, Anderson JS, Follis JL, Djousse L, Mukamal K, Papoutsakis C, Mozaffarian D, Zillikens MC, Bandinelli S, Bennett AJ, Borecki IB, Feitosa MF, Ferrucci L, Forouhi NG, Groves CJ, Hallmans G, Harris T, Hofman A, Houston DK, Hu FB, Johansson I, Kritchevsky SB, Langenberg C, Launer L, Liu Y, Loos RJ, Nalls M, Orho-Melander M, Renstrom F, Rice K, Riserus U, Rolandsson O, Rotter JL, Saylor G, Sijbrands EJ, Sjogren P, Smith A, Steingrimsdottir L, Uitterlinden AG, Wareham NJ, Prokopenko I, Pankow JS, van Duijn CM, Florez JC, Witteman JC, Dupuis J, Dedousis GV, Ordovas JM, Ingelsson E, Cupples L, Siscovick DS, Franks PW, Meigs JB. Interactions of dietary whole-grain intake with fasting glucose- and insulin-related genetic loci in individuals of European descent: a meta-analysis of 14 cohort studies. *Diabetes Care* 2010; **33**: 2684-2691 [PMID: 20693352 DOI: 10.2337/dc10-1150]
 - 75 **Mozaffarian D**, Hao T, Rimm EB, Willett WC, Hu FB. Changes in diet and lifestyle and long-term weight gain in women and men. *N Engl J Med* 2011; **364**: 2392-2404 [PMID: 21696306 DOI: 10.1056/NEJMoa1014296]
 - 76 **Pagano G**, Pacini G, Musso G, Gambino R, Mecca F, Depe- tris N, Cassader M, David E, Cavallo-Perin P, Rizzetto M. Nonalcoholic steatohepatitis, insulin resistance, and meta- bolic syndrome: further evidence for an etiologic association. *Hepatology* 2002; **35**: 367-372 [PMID: 11826410 DOI: 10.1053/ jhep.2002.30690]
 - 77 **Maron DJ**, Fair JM, Haskell WL. Saturated fat intake and insulin resistance in men with coronary artery disease. The Stanford Coronary Risk Intervention Project Investigators and Staff. *Circulation* 1991; **84**: 2020-2027 [PMID: 1934376 DOI: 10.1161/01.CIR.84.5.2020]
 - 78 **Feskens EJ**, Loeber JG, Kromhout D. Diet and physical activ- ity as determinants of hyperinsulinemia: the Zutphen Elderly Study. *Am J Epidemiol* 1994; **140**: 350-360 [PMID: 8059770]
 - 79 **Marshall JA**, Bessesen DH, Hamman RF. High saturated fat and low starch and fibre are associated with hyperinsulinae- mia in a non-diabetic population: the San Luis Valley Diabe- tes Study. *Diabetologia* 1997; **40**: 430-438 [PMID: 9112020 DOI: 10.1007/s001250050697]
 - 80 **Vessby B**, Uusitupa M, Hermansen K, Riccardi G, Rivel- lese AA, Tapsell LC, Nälsén C, Berglund L, Louheranta A, Rasmussen BM, Calvert GD, Maffetone A, Pedersen E, Gus- tafsson IB, Storlien LH. Substituting dietary saturated for monounsaturated fat impairs insulin sensitivity in healthy men and women: The KANWU Study. *Diabetologia* 2001; **44**: 312-319 [PMID: 11317662 DOI: 10.1007/s001250051620]
 - 81 **Musso G**, Gambino R, De Micheli F, Cassader M, Rizzetto M, Durazzo M, Fagà E, Silli B, Pagano G. Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. *Hepatology* 2003; **37**: 909-916 [PMID: 12668986 DOI: 10.1053/jhep.2003.50132]
 - 82 **Utzschneider KM**, Bayer-Carter JL, Arbuckle MD, Tidwell JM, Richards TL, Craft S. Beneficial effect of a weight-stable, low-fat/low-saturated fat/low-glycaemic index diet to re- duce liver fat in older subjects. *Br J Nutr* 2013; **109**: 1096-1104 [PMID: 22849970 DOI: 10.1017/S0007114512002966]
 - 83 **Ha SK**, Chae C. Inducible nitric oxide distribution in the fatty liver of a mouse with high fat diet-induced obesity. *Exp Anim* 2010; **59**: 595-604 [PMID: 21030787 DOI: 10.1538/ex- panim.59.595]
 - 84 **Longato L**, Tong M, Wands JR, de la Monte SM. High fat diet induced hepatic steatosis and insulin resistance: Role of dys- regulated ceramide metabolism. *Hepatol Res* 2012; **42**: 412-427 [PMID: 22176347 DOI: 10.1111/j.1872-034X.2011.00934.x]
 - 85 **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]
 - 86 **Zelber-Sagi S**, Ratzin V, Oren R. Nutrition and physical activ- ity in NAFLD: an overview of the epidemiological evidence. *World J Gastroenterol* 2011; **17**: 3377-3389 [PMID: 21876630 DOI: 10.3748/wjg.v17.i29.3377]
 - 87 **Musso G**, Cassader M, Rosina F, Gambino R. Impact of cur- rent treatments on liver disease, glucose metabolism and car- diovascular 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]
 - 88 **Papandreou D**, Karaboula Z, Rouso I. Are dietary chole- sterol intake and serum cholesterol levels related to nonalco- holic Fatty liver disease in obese children? *Cholesterol* 2012; **2012**: 572820 [PMID: 22811894 DOI: 10.1155/2012/572820]
 - 89 **Enjoji M**, Yasutake K, Kohjima M, Nakamuta M. Nutri-

- tion and nonalcoholic Fatty liver disease: the significance of cholesterol. *Int J Hepatol* 2012; **2012**: 925807 [PMID: 22550592 DOI: 10.1155/2012/925807]
- 90 **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]
 - 91 **Kainuma M**, Fujimoto M, Sekiya N, Tsuneyama K, Cheng C, Takano Y, Terasawa K, Shimada Y. Cholesterol-fed rabbit as a unique model of nonalcoholic, nonobese, non-insulin-resistant fatty liver disease with characteristic fibrosis. *J Gastroenterol* 2006; **41**: 971-980 [PMID: 17096066 DOI: 10.1007/s00535-006-1883-1]
 - 92 **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]
 - 93 **Wouters K**, van Gorp PJ, Bieghs V, Gijbels MJ, Duimel H, Lütjohann D, Kerksiek A, van Kruchten R, Maeda N, Staels B, van Bilsen M, Shiri-Sverdlov R, Hofker MH. Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. *Hepatology* 2008; **48**: 474-486 [PMID: 18666236 DOI: 10.1002/hep.22363]
 - 94 **Higuchi N**, Kato M, Shundo Y, Tajiri H, Tanaka M, Yamashita N, Kohjima M, Kotoh K, Nakamuta M, Takayanagi R, Enjoji M. Liver X receptor in cooperation with SREBP-1c is a major lipid synthesis regulator in nonalcoholic fatty liver disease. *Hepatol Res* 2008; **38**: 1122-1129 [PMID: 18684130 DOI: 10.1111/j.1872-034X.2008.00382.x]
 - 95 **Nakamuta M**, Fujino T, Yada R, Yada M, Yasutake K, Yoshimoto T, Harada N, Higuchi N, Kato M, Kohjima M, Taketomi A, Maehara Y, Nakashima M, Kotoh K, Enjoji M. Impact of cholesterol metabolism and the LXRalpha-SREBP-1c pathway on nonalcoholic fatty liver disease. *Int J Mol Med* 2009; **23**: 603-608 [PMID: 19360318]
 - 96 **Cortez-Pinto H**, Jesus L, Barros H, Lopes C, Moura MC, Camilo ME. How different is the dietary pattern in non-alcoholic steatohepatitis patients? *Clin Nutr* 2006; **25**: 816-823 [PMID: 16677739 DOI: 10.1016/j.clnu.2006.01.027]
 - 97 **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]
 - 98 **U.S. Department of Agriculture**, U.S. Department of Health and Human Services. Dietary Guidelines for Americans. 7th ed. Washington, D.C.: U.S. Government Printing Office, 2010. Available from: URL: <http://www.health.gov/dietaryguidelines/>
 - 99 **Teramoto T**, Sasaki J, Ishibashi S, Birou S, Daida H, Dohi S, Egusa G, Hiro T, Hirobe K, Iida M, Kihara S, Kinoshita M, Maruyama C, Ohta T, Okamura T, Yamashita S, Yokode M, Yokote K. Executive summary of the Japan Atherosclerosis Society (JAS) guidelines for the diagnosis and prevention of atherosclerotic cardiovascular diseases in Japan -2012 version. *J Atheroscler Thromb* 2013; **20**: 517-523 [PMID: 23665881 DOI: 10.5551/jat.15792]
 - 100 **Ghafoorunnisa A**, Rajkumar L, Acharya V. Dietary (n-3) long chain polyunsaturated fatty acids prevent sucrose-induced insulin resistance in rats. *J Nutr* 2005; **135**: 2634-2638 [PMID: 16253960]
 - 101 **Teran-Garcia M**, Adamson AW, Yu G, Rufo C, Suchankova G, Dreesen TD, Tekle M, Clarke SD, Gettys TW. Polyunsaturated fatty acid suppression of fatty acid synthase (FASN): evidence for dietary modulation of NF-Y binding to the Fasn promoter by SREBP-1c. *Biochem J* 2007; **402**: 591-600 [PMID: 17313375]
 - 102 **Sekiya M**, Yahagi N, Matsuzaka T, Najima Y, Nakakuki M, Nagai R, Ishibashi S, Osuga J, Yamada N, Shimano H. Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. *Hepatology* 2003; **38**: 1529-1539 [PMID: 14647064 DOI: 10.1053/jhep.2003.09028]
 - 103 **Levy JR**, Clore JN, Stevens W. Dietary n-3 polyunsaturated fatty acids decrease hepatic triglycerides in Fischer 344 rats. *Hepatology* 2004; **39**: 608-616 [PMID: 14999679 DOI: 10.1002/hep.20093]
 - 104 **Capanni M**, Calella F, Biagini MR, Genise S, Raimondi L, Bedogni G, Svegliati-Baroni G, Sofi F, Milani S, Abbate R, Surrenti C, Casini A. Prolonged n-3 polyunsaturated fatty acid supplementation ameliorates hepatic steatosis in patients with non-alcoholic fatty liver disease: a pilot study. *Aliment Pharmacol Ther* 2006; **23**: 1143-1151 [PMID: 16611275 DOI: 10.1111/j.1365-2036.2006.02885.x]
 - 105 **Zhu FS**, Liu S, Chen XM, Huang ZG, Zhang DW. Effects of n-3 polyunsaturated fatty acids from seal oils on nonalcoholic fatty liver disease associated with hyperlipidemia. *World J Gastroenterol* 2008; **14**: 6395-6400 [PMID: 19009658 DOI: 10.3748/wjg.14.6395]
 - 106 **Spadaro L**, Magliocco O, Spampinato D, Piro S, Oliveri C, Alagona C, Papa G, Rabuazzo AM, Purrello F. Effects of n-3 polyunsaturated fatty acids in subjects with nonalcoholic fatty liver disease. *Dig Liver Dis* 2008; **40**: 194-199 [PMID: 18054848 DOI: 10.1016/j.dld.2007.10.003]
 - 107 **Cussons AJ**, Watts GF, Mori TA, Stuckey BG. Omega-3 fatty acid supplementation decreases liver fat content in polycystic ovary syndrome: a randomized controlled trial employing proton magnetic resonance spectroscopy. *J Clin Endocrinol Metab* 2009; **94**: 3842-3848 [PMID: 19622617 DOI: 10.1210/jc.2009-0870]
 - 108 **Iso H**, Kobayashi M, Ishihara J, Sasaki S, Okada K, Kita Y, Kokubo Y, Tsugane S. Intake of fish and n3 fatty acids and risk of coronary heart disease among Japanese: the Japan Public Health Center-Based (JPHC) Study Cohort I. *Circulation* 2006; **113**: 195-202 [PMID: 16401768 DOI: 10.1161/CIRCULATIONAHA.105.581355]
 - 109 **Yamagishi K**, Iso H, Date C, Fukui M, Wakai K, Kikuchi S, Inaba Y, Tanabe N, Tamakoshi A. Fish, omega-3 polyunsaturated fatty acids, and mortality from cardiovascular diseases in a nationwide community-based cohort of Japanese men and women the JACC (Japan Collaborative Cohort Study for Evaluation of Cancer Risk) Study. *J Am Coll Cardiol* 2008; **52**: 988-996 [PMID: 18786479 DOI: 10.1016/j.jacc.2008.06.018]
 - 110 **Day CP**, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998; **114**: 842-845 [PMID: 9547102 DOI: 10.1016/S0016-5085(98)70599-2]
 - 111 **Albano E**, Mottaran E, Vidali M, Reale E, Saksena S, Occhino G, Burt AD, Day CP. Immune response towards lipid peroxidation products as a predictor of progression of non-alcoholic fatty liver disease to advanced fibrosis. *Gut* 2005; **54**: 987-993 [PMID: 15951547 DOI: 10.1136/gut.2004.057968]
 - 112 **Seki S**, Kitada T, Yamada T, Sakaguchi H, Nakatani K, Wakasa K. In situ detection of lipid peroxidation and oxidative DNA damage in non-alcoholic fatty liver diseases. *J Hepatol* 2002; **37**: 56-62 [PMID: 12076862 DOI: 10.1016/S0168-8278(02)00073-9]
 - 113 **Begriffe K**, Igoudjil A, Pessayre D, Fromenty B. Mitochondrial dysfunction in NASH: causes, consequences and possible means to prevent it. *Mitochondrion* 2006; **6**: 1-28 [PMID: 16406828 DOI: 10.1016/j.mito.2005.10.004]
 - 114 **Erhardt A**, Stahl W, Sies H, Lirussi F, Donner A, Häussinger D. Plasma levels of vitamin E and carotenoids are decreased in patients with Nonalcoholic Steatohepatitis (NASH). *Eur J Med Res* 2011; **16**: 76-78 [PMID: 21463986 DOI: 10.1186/2047-783X-16-2-76]

- 115 **Horoz M**, Bolukbas C, Bolukbas FF, Sabuncu T, Aslan M, Sarifakiogullari S, Gunaydin N, Erel O. Measurement of the total antioxidant response using a novel automated method in subjects with nonalcoholic steatohepatitis. *BMC Gastroenterol* 2005; **5**: 35 [PMID: 16283935]
- 116 **Koruk M**, Taysi S, Savas MC, Yilmaz O, Akcay F, Karakok M. Oxidative stress and enzymatic antioxidant status in patients with nonalcoholic steatohepatitis. *Ann Clin Lab Sci* 2004; **34**: 57-62 [PMID: 15038668]
- 117 **Fierbințeanu-Braticevici C**, Bengus A, Neamțu M, Usvat R. The risk factors of fibrosis in nonalcoholic steatohepatitis. *Rom J Intern Med* 2002; **40**: 81-88 [PMID: 15526543]
- 118 **Videla LA**, Rodrigo R, Orellana M, Fernandez V, Tapia G, Quiñones L, Varela N, Contreras J, Lazarte R, Csendes A, Rojas J, Maluenda F, Burdiles P, Diaz JC, Smok G, Thielemann L, Poniachik J. Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients. *Clin Sci (Lond)* 2004; **106**: 261-268 [PMID: 14556645 DOI: 10.1042/CS20030285]
- 119 **Sanyal AJ**, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, Neuschwander-Tetri BA, Lavine JE, Tonascia J, Unalp A, Van Natta M, Clark J, Brunt EM, Kleiner DE, Hoofnagle JH, Robuck PR. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 2010; **362**: 1675-1685 [PMID: 20427778 DOI: 10.1056/NEJMoa0907929]
- 120 **Sesso HD**, Buring JE, Christen WG, Kurth T, Belanger C, MacFadyen J, Bubes V, Manson JE, Glynn RJ, Gaziano JM. Vitamins E and C in the prevention of cardiovascular disease in men: the Physicians' Health Study II randomized controlled trial. *JAMA* 2008; **300**: 2123-2133 [PMID: 18997197 DOI: 10.1001/jama.2008.600]
- 121 **Bjelakovic G**, Nikolova D, Simonetti RG, Gluud C. Antioxidant supplements for preventing gastrointestinal cancers. *Cochrane Database Syst Rev* 2008; (3): CD004183 [PMID: 18677777 DOI: 10.1002/14651858.CD004183.pub3]
- 122 **Sumida Y**, Naito Y, Tanaka S, Sakai K, Inada Y, Taketani H, Kanemasa K, Yasui K, Itoh Y, Okanoue T, Yoshikawa T. Long-term (<gt;=2 yr) efficacy of vitamin e for non-alcoholic steatohepatitis. *Hepatology* 2013; **60**: 1445-1450 [PMID: 23933938 DOI: 10.5754/hge11421]
- 123 **Han Y**, Shi JP, Ma AL, Xu Y, Ding XD, Fan JG. Randomized, vitamin e-controlled trial of bicyclol plus metformin in non-alcoholic fatty liver disease patients with impaired fasting glucose. *Clin Drug Investig* 2014; **34**: 1-7 [PMID: 24081374 DOI: 10.1007/s40261-013-0136-3]
- 124 **Alvarez JA**, Ashraf A. Role of vitamin d in insulin secretion and insulin sensitivity for glucose homeostasis. *Int J Endocrinol* 2010; **2010**: 351385 [PMID: 20011094 DOI: 10.1155/2010/351385]
- 125 **Bradlee ML**, Singer MR, Qureshi MM, Moore LL. Food group intake and central obesity among children and adolescents in the Third National Health and Nutrition Examination Survey (NHANES III). *Public Health Nutr* 2010; **13**: 797-805 [PMID: 19772691 DOI: 10.1017/S1368980009991546]
- 126 **Smotkin-Tangorra M**, Purushothaman R, Gupta A, Nejadi G, Anhalt H, Ten S. Prevalence of vitamin D insufficiency in obese children and adolescents. *J Pediatr Endocrinol Metab* 2007; **20**: 817-823 [PMID: 17849744 DOI: 10.1515/JPEM.2007.20.7.817]
- 127 **Roth CL**, Elfers CT, Figlewicz DP, Melhorn SJ, Morton GJ, Hoofnagle A, Yeh MM, Nelson JE, Kowdley KV. Vitamin D deficiency in obese rats exacerbates nonalcoholic fatty liver disease and increases hepatic resistin and Toll-like receptor activation. *Hepatology* 2012; **55**: 1103-1111 [PMID: 21994008 DOI: 10.1002/hep.24737]
- 128 **Manco M**, Ciampalini P, Nobili V. Low levels of 25-hydroxyvitamin D(3) in children with biopsy-proven nonalcoholic fatty liver disease. *Hepatology* 2010; **51**: 2229; author reply 2230 [PMID: 20513013 DOI: 10.1002/hep.23724]
- 129 **Wu CC**, Chang JH, Chen CC, Su SB, Yang LK, Ma WY, Zheng CM, Diang LK, Lu KC. Calcitriol treatment attenuates inflammation and oxidative stress in hemodialysis patients with secondary hyperparathyroidism. *Tohoku J Exp Med* 2011; **223**: 153-159 [PMID: 21350317 DOI: 10.1620/tjem.223.153]
- 130 **Barchetta I**, Carotti S, Labbadia G, Gentilucci UV, Muda AO, Angelico F, Silecchia G, Leonetti F, Fraioli A, Picardi A, Morini S, Cavallo MG. Liver vitamin D receptor, CYP2R1, and CYP27A1 expression: relationship with liver histology and vitamin D3 levels in patients with nonalcoholic steatohepatitis or hepatitis C virus. *Hepatology* 2012; **56**: 2180-2187 [PMID: 22753133 DOI: 10.1002/hep.25930]
- 131 **Ley RE**, Turnbaugh PJ, Klein S, Gordon JL. Microbial ecology: human gut microbes associated with obesity. *Nature* 2006; **444**: 1022-1023 [PMID: 17183309 DOI: 10.1038/4441022a]
- 132 **Ma X**, Hua J, Li Z. Probiotics improve high fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells. *J Hepatol* 2008; **49**: 821-830 [PMID: 18674841 DOI: 10.1016/j.jhep.2008.05.025]
- 133 **Iacono A**, Raso GM, Canani RB, Calignano A, Meli R. Probiotics as an emerging therapeutic strategy to treat NAFLD: focus on molecular and biochemical mechanisms. *J Nutr Biochem* 2011; **22**: 699-711 [PMID: 21292470 DOI: 10.1016/j.jnutbio.2010.10.002]
- 134 **Kelishadi R**, Farajian S, Mirlohi M. Probiotics as a novel treatment for non-alcoholic fatty liver disease; a systematic review on the current evidences. *Hepat Mon* 2013; **13**: e7233 [PMID: 23885277 DOI: 10.5812/hepatmon.7233]
- 135 **Li Z**, Yang S, Lin H, Huang J, Watkins PA, Moser AB, Desimone C, Song XY, Diehl AM. Probiotics and antibodies to TNF inhibit inflammatory activity and improve nonalcoholic fatty liver disease. *Hepatology* 2003; **37**: 343-350 [PMID: 12540784 DOI: 10.1053/jhep.2003.50048]
- 136 **Velayudham A**, Dolganiuc A, Ellis M, Petrasko J, Kodys K, Mandrekar P, Szabo G. VSL#3 probiotic treatment attenuates fibrosis without changes in steatohepatitis in a diet-induced nonalcoholic steatohepatitis model in mice. *Hepatology* 2009; **49**: 989-997 [PMID: 19115316 DOI: 10.1002/hep.22711]
- 137 **Lee HY**, Park JH, Seok SH, Baek MW, Kim DJ, Lee KE, Paek KS, Lee Y, Park JH. Human originated bacteria, *Lactobacillus rhamnosus* PL60, produce conjugated linoleic acid and show anti-obesity effects in diet-induced obese mice. *Biochim Biophys Acta* 2006; **1761**: 736-744 [PMID: 16807088 DOI: 10.1016/j.bbalip.2006.05.007]
- 138 **Esposito E**, Iacono A, Bianco G, Autore G, Cuzzocrea S, Vajro P, Canani RB, Calignano A, Raso GM, Meli R. Probiotics reduce the inflammatory response induced by a high-fat diet in the liver of young rats. *J Nutr* 2009; **139**: 905-911 [PMID: 19321579 DOI: 10.3945/jn.108.101808]
- 139 **Karahan N**, İşler M, Koyu A, Karahan AG, Başyığıt Kiliç G, Çırış İM, Sütçü R, Onaran I, Cam H, Keskin M. Effects of probiotics on methionine choline deficient diet-induced steatohepatitis in rats. *Turk J Gastroenterol* 2012; **23**: 110-121 [PMID: 22706738]
- 140 **Wang Y**, Xu N, Xi A, Ahmed Z, Zhang B, Bai X. Effects of *Lactobacillus plantarum* MA2 isolated from Tibet kefir on lipid metabolism and intestinal microflora of rats fed on high-cholesterol diet. *Appl Microbiol Biotechnol* 2009; **84**: 341-347 [PMID: 19444443 DOI: 10.1007/s00253-009-2012-x]
- 141 **Paik HD**, Park JS, Park E. Effects of *Bacillus polyfermenticus* SCD on lipid and antioxidant metabolisms in rats fed a high-fat and high-cholesterol diet. *Biol Pharm Bull* 2005; **28**: 1270-1274 [PMID: 15997112 DOI: 10.1248/bpb.28.1270]
- 142 **Yadav H**, Jain S, Sinha PR. Antidiabetic effect of probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei* in high fructose fed rats. *Nutrition* 2007; **23**: 62-68 [PMID: 17084593 DOI: 10.1016/j.nut.2006.09.002]
- 143 **Loguercio C**, De Simone T, Federico A, Terracciano F, Tuccillo C, Di Chicco M, Carotenì M. Gut-liver axis: a new point of attack to treat chronic liver damage? *Am J Gastroenterol* 2002; **97**: 2144-2146 [PMID: 12190198 DOI: 10.1111/j.1572-0241.2002.05942.x]

- 144 **Loguercio C**, Federico A, Tuccillo C, Terracciano F, D'Auria MV, De Simone C, Del Vecchio Blanco C. Beneficial effects of a probiotic VSL#3 on parameters of liver dysfunction in chronic liver diseases. *J Clin Gastroenterol* 2005; **39**: 540-543 [PMID: 15942443 DOI: 10.1097/01.mcg.0000165671.25272.0f]
- 145 **Aller R**, De Luis DA, Izaola O, Conde R, Gonzalez Sagrado M, Primo D, De La Fuente B, Gonzalez J. Effect of a probiotic on liver aminotransferases in nonalcoholic fatty liver disease patients: a double blind randomized clinical trial. *Eur Rev Med Pharmacol Sci* 2011; **15**: 1090-1095 [PMID: 22013734]

P- Reviewers: Faintuch J, Fan JG, Gong ZJ

S- Editor: Song XX **L- Editor:** A **E- Editor:** Liu XM



WJG 20th Anniversary Special Issues (12): Fatty liver

Role of endoplasmic reticulum stress in the pathogenesis of nonalcoholic fatty liver disease

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Supported by National Key Basic Research Development Program, No. 2012CB524905; National Science and Technology Support Plan Project, No. 2012BAI06B04; National Natural Science Foundation of China, No. 30900677, No. 81070315, No. 81070366, No. 81100278, No. 81170378, No. 81230012 and No. 81270487; Zhejiang Provincial Natural Science Foundation of China, No. Y2090463 and No. Y2110026; International Science and Technology Cooperation Projects of Zhejiang Province, No. 2013C24010; Science Foundation of Health Bureau of Zhejiang Province, No. 2009A070 and No. 2012RCA026; and Specialized Research Fund for the Doctoral Program of Higher Education, No. 20090101120110

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Received: August 29, 2013 Revised: November 20, 2013

Accepted: December 5, 2013

Published online: February 21, 2014

Abstract

Nonalcoholic fatty liver disease (NAFLD) has emerged as a common public health problem in recent decades. However, the underlying mechanisms leading to the development of NAFLD are not fully understood. The endoplasmic reticulum (ER) stress response has recently been proposed to play a crucial role in both the development of steatosis and progression to nonalcoholic steatohepatitis. ER stress is activated to regulate protein synthesis and restore homeostatic equilibrium when the cell is stressed due to the accumulation of unfolded or misfolded proteins. However, delayed or

insufficient responses to ER stress may turn physiological mechanisms into pathological consequences, including fat accumulation, insulin resistance, inflammation, and apoptosis, all of which play important roles in the pathogenesis of NAFLD. Therefore, understanding the role of ER stress in the pathogenesis of NAFLD has become a topic of intense investigation. This review highlights the recent findings linking ER stress signaling pathways to the pathogenesis of NAFLD.

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Key words: Endoplasmic reticulum stress; Unfolded protein response; Nonalcoholic fatty liver disease; Non-alcoholic steatohepatitis

Core tip: Nonalcoholic fatty liver disease (NAFLD) is a progressive disorder that can lead to impaired liver function and, ultimately, liver failure. Chronic endoplasmic reticulum stress induces numerous intracellular pathways that can lead to hepatic steatosis, systemic inflammation, and hepatocyte cell death, all of which are keystones of NAFLD. This review highlights the recent findings linking ER stress signaling pathways with the pathogenesis of NAFLD, which may help identify novel therapeutic targets for the prevention and treatment of NAFLD.

Zhang XQ, Xu CF, Yu CH, Chen WX, Li YM. Role of endoplasmic reticulum stress in the pathogenesis of nonalcoholic fatty liver disease. *World J Gastroenterol* 2014; 20(7): 1768-1776 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1768.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1768>

INTRODUCTION

In recent decades, nonalcoholic fatty liver disease

(NAFLD) has increasingly been recognized as one of the most common chronic liver diseases. The reported prevalence of NAFLD in Western countries ranges from 30% to 46%^[1,2]. This disease has also become prevalent in Eastern countries and has become a significant public health concern in these areas^[3,4]. NAFLD encompasses a spectrum of liver damage ranging from steatosis to nonalcoholic steatohepatitis (NASH), which can progress to fibrosis, cirrhosis, and ultimately to liver failure or hepatocellular carcinoma^[5,6]. Individuals with NAFLD are also at an increased risk of cardiovascular disease, type 2 diabetes, and obesity-related mortality^[7-9].

The precise mechanisms of NAFLD remain poorly understood. The “multiple-hits hypothesis” is currently the most recognized theory to explain disease development and progression. The initial hit leads to the development of simple steatosis, while the following hits, including mitochondrial dysfunction, oxidative stress, adipocytokine alteration, lipid peroxidation, Kupffer cell activation, *etc.* results in liver cell inflammation and apoptosis, which finally leads from simple steatosis to NASH. Recently, accumulating data have implicated disruption of endoplasmic reticulum (ER) homeostasis, or ER stress, in both the development of steatosis and progression to NASH^[10-12]. ER stress may lead to activation of various intracellular stress pathways that can initiate or exacerbate insulin resistance (IR) and inflammation and, in some cases, culminate in hepatocyte cell death and liver damage, all of which are important in the pathogenesis of NAFLD. Therefore, there is an urgent need to better understand the mechanisms that disrupt ER homeostasis and lead to activation of the unfolded protein response (UPR) in NAFLD. The aim of this review is to highlight the recent findings linking activation of ER stress and NAFLD development.

UPR AND ER STRESS

The ER is a membrane-bound organelle that provides a specialized environment for the production and post-translational modification of secretory and membrane proteins, lipid biosynthesis, and homeostasis of intracellular Ca^{2+} . In the ER lumen, newly synthesized proteins undergo post-translational modifications such as N-glycosylation, disulfide bond formation, and oligomerization, which require the presence of chaperone proteins. The ER is thus considered as a quality control checkpoint, and only correctly folded proteins can exit the ER and go through the secretory pathway.

Any event that disturbs ER folding capacity, such as excessive protein synthesis, accumulation of mutant misfolded proteins, ER calcium depletion, or a change in redox status, will induce a physiological reaction referred to as the UPR. These homeostatic responses trigger the production of additional chaperones to increase the folding capacity of the ER, enhance endoplasmic reticulum-associated protein degradation (ERAD), and reduce protein entry by altering the translation and synthesis of

new proteins, thus bringing the organelle and the cell into a state of equilibrium^[13-15].

The UPR is mediated by three integral proteins of the ER^[16,17]: protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme-1 (IRE1), and activating transcription factor-6 (ATF6). These proteins are maintained in an inactive state as long as they are bound to binding immunoglobulin protein (Bip), which is an intraluminal chaperone. When the ER is stressed, Bip is displaced from these stress sensors, leading to their activation. Once activated, PERK phosphorylates and inhibits eukaryotic translation initiation factor 2 α (eIF2 α), resulting in a global decrease in protein translation. Moreover, p-eIF2 α selectively promotes the translation of a growing number of mRNAs, including activating transcription factor 4 (ATF4) mRNA. Activation of IRE1 promotes the splicing of X-box-binding protein-1 (XBP1) mRNA and the subsequent transcription of molecular chaperones and genes involved in ERAD. Finally, activated ATF6 undergoes proteolytic cleavage in the Golgi, allowing its mature form to enter the nucleus and transactivate ER stress-related genes such as ER chaperones and foldases (Figure 1).

Thus, the UPR is primarily a cytoprotective survival response that aims to regulate protein folding and restore homeostatic balance. However, when the activation of the UPR fails to promote cell survival, the cell is directed down the pro-apoptotic ER stress response pathway, which can ultimately lead to apoptotic cell death^[18-20].

ER STRESS AND NAFLD

Similar to other secretory cells, hepatocytes are rich in ER. Due to its high capacity for protein synthesis, the UPR/ER stress response plays important roles in both preventing and mediating pathological changes in various liver diseases^[21,22]. The signaling pathways activated by ER stress have been linked to lipotoxicity, IR, inflammation, and apoptotic cell death, which are common to both obesity and NAFLD. Therefore, the role of ER stress in NAFLD has become a subject of considerable interest in recent years. The induction of ER stress was first described in the livers of genetic and diet-induced models of NASH^[23]. Since then, these findings have been confirmed in other obese animal models^[24-26] and in mice fed a methionine-choline-deficient (MCD) diet exhibiting hepatic steatosis without obesity^[27]. Later, the activation of several UPR components was reported in the livers of patients with NAFLD or NASH^[10,28]. Despite rapid growth in the field of ER stress research in the context of NAFLD, the exact contribution of the ER stress response to the pathogenesis of NAFLD remains to be fully elucidated. Here, we review and update the well-established associations between the ER stress response and NAFLD (Figure 2).

ER stress and lipid metabolism

The first step in the development of NAFLD is hepatic

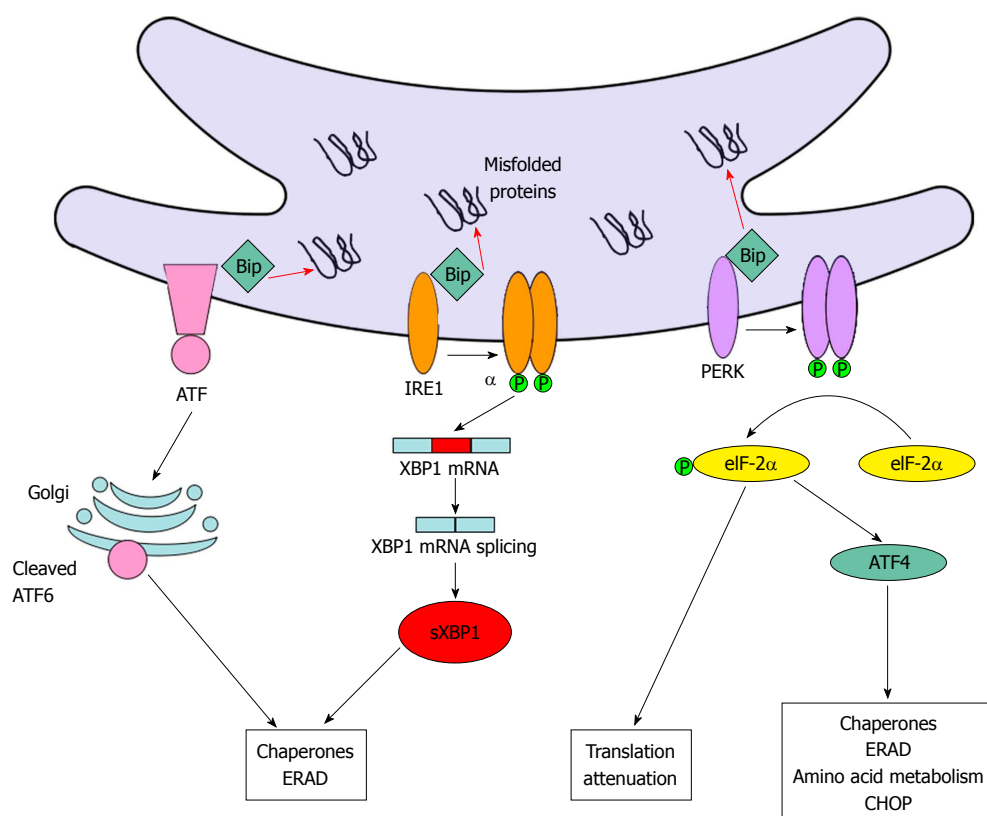


Figure 1 The unfolded protein response pathway. When the unfolded protein response is activated, the first event is the dissociation of the chaperone Bip from the three integral proteins PERK, IRE1, and ATF6, leading to their activation. When activated, PERK phosphorylates and inhibits eIF2 α , leading to a global decrease in protein translation. Moreover, p-eIF2 α activates ATF4, which induces the expression of several genes, including amino acid transporters, chaperones, and CHOP. Activation of IRE1 promotes the splicing of XBP1 mRNA and the subsequent transcription of molecular chaperones and genes involved in ERAD. Finally, activated ATF6 undergoes proteolytic cleavage in the Golgi, transactivating genes such as endoplasmic reticulum (ER) chaperones and foldases. Bip: Binding immunoglobulin protein; ATF6: Activating transcription factor-6; IRE1: Inositol requiring enzyme-1; PERK: Protein kinase RNA-like ER kinase; XBP1: X-box-binding protein-1; eIF2 α : Eukaryotic translation initiation factor 2 α ; ATF4: Transcription factor 4; ERAD: Endoplasmic reticulum associated protein degradation; CHOP: C/EBP-homologous protein.

steatosis, which is characterized by macrovesicular accumulation of triglycerides in the cytoplasm of hepatocytes. Sources of increased hepatic lipids in NAFLD include (1) excess dietary chylomicron remnants; (2) increased *de novo* lipogenesis; (3) excess free fatty acids released from the lipolysis of adipose tissue; (4) diminished very-low-density lipoprotein (VLDL) secretion; and (5) reduced oxidation of fatty acids^[29-31].

For approximately one decade, it has been known that ER stress can lead to altered lipid metabolism and hepatic steatosis. Recently, specific arms of the UPR and their downstream signaling molecules have been examined in cell culture and animal models to decipher their functions and roles in lipid metabolism. It is now well established that various components of the UPR signaling network play roles in the regulation of lipid metabolism.

First, the PERK-eIF2 α -ATF4 pathway was reported to regulate lipogenesis and hepatic steatosis. PERK-dependent signaling has been found to contribute to lipogenic differentiation in the mammary epithelium, and deletion of PERK inhibits the sustained expression of the lipogenic enzymes fatty acid synthase (FAS), ATP-citrate lyase, and stearoyl-CoA desaturase-1 (SCD1)^[32].

Oyadomari *et al.*^[33] reported that selectively compromising eIF2 α -mediated signaling under ER stress conditions using GADD34, which acts to dephosphorylate eIF2 α , results in diminished hepatosteatosis in animals fed a high-fat diet. Attenuated eIF2 α phosphorylation is correlated with decreased expression of the adipogenic nuclear receptor peroxisome proliferator-activated receptor γ (PPAR γ) and its upstream regulators, the transcription factors CCAAT/enhancer-binding proteins α and β (C/EBP α , C/EBP β)^[33]. Protein kinase-mediated p-eIF2 α increases ATF4 translation. ATF4-knockout mice are protected from diet-induced obesity, hypertriglyceridemia, and hepatic steatosis, as ATF4 depletion results in significantly decreased liver and white adipose tissue expression of lipogenic genes, such as PPAR γ , sterol regulatory element-binding protein-1c (SREBP-1c), acetyl-CoA carboxylase (ACC), and FAS^[34-36]. Taken together, these results suggest that the PERK-eIF2 α -ATF4 pathway plays an important role in promoting lipogenesis, both in the liver and other tissues.

Second, the IRE1 α -XBP1 pathway has been reported to be required for the maintenance of hepatic lipid homeostasis under ER stress conditions. Mice with a hepa-

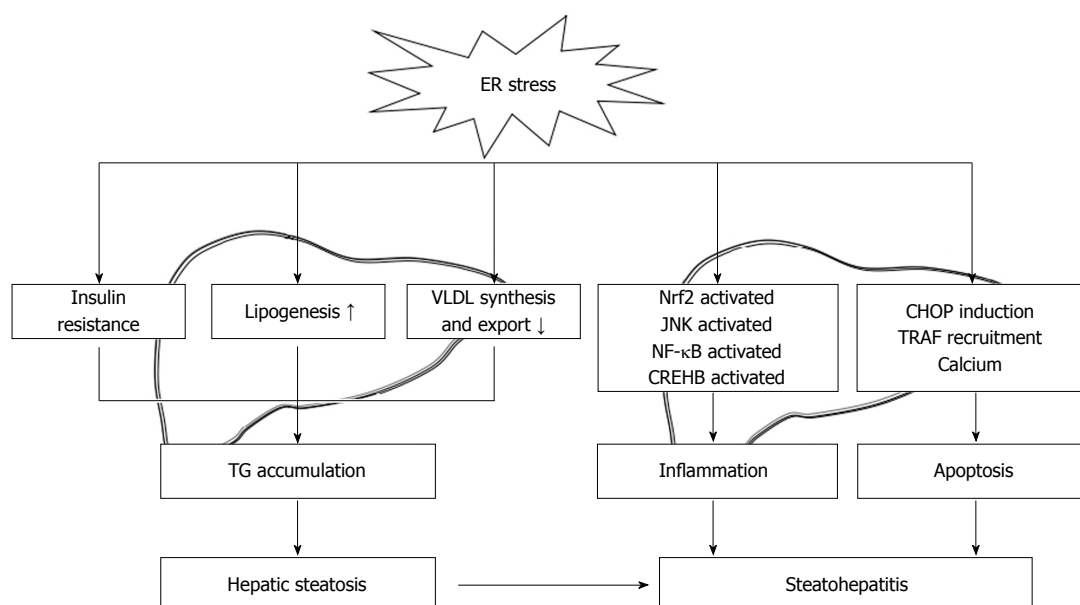


Figure 2 Roles of endoplasmic reticulum stress in the development of steatosis and progression to nonalcoholic steatohepatitis. ER stress interferes with hepatic lipid metabolism by activating lipogenesis and limiting VLDL formation and secretion. ER stress also acts indirectly on liver triglyceride accumulation by promoting insulin resistance in both the liver and adipose tissue. Furthermore, ER stress promotes the activation of Nrf2, JNK, NF- κ B, CREBH, and CHOP, which actively participate in the inflammatory process and cell death. ER: Endoplasmic reticulum; VLDL: Very-low-density lipoprotein; Nrf2: Nuclear factor-erythroid-derived 2-related factor 2; JNK: c-Jun N-terminal kinase; NF- κ B: Nuclear factor- κ B; CREBH: Cyclic-AMP responsive element-binding protein H; CHOP: C/EBP-homologous protein; TRAF: Tumor-necrosis factor α -receptor-associated factor; TG: Triglyceride.

toocyte-specific deletion of IRE1 α develop severe hepatic steatosis after treatment with an ER stress inducer due to the repressed expression of key metabolic transcriptional regulators, including C/EBP β , C/EBP δ , and PPAR γ , and enzymes involved in triglyceride biosynthesis^[37]. IRE1 α is also reportedly required for efficient apolipoprotein secretion upon disruption of ER homeostasis^[37]. Moreover, the IRE1 α -XBP1 pathway plays an essential role in the regulation of hepatic VLDL assembly and secretion, which is at least in part due to decreased microsomal triglyceride-transfer protein activity resulting from reduced protein disulfide isomerase expression^[38]. In hepatocytes, XBP1 regulates hepatic lipogenesis by directly binding to the promoters of a subset of lipogenic genes, including SCD1, diacylglycerol acyltransferase-2 (DGAT2), and ACC2, and activating their expression^[39]. Therefore, *de novo* lipid biosynthesis is reduced in the livers of mice with an XBP1 deletion^[39].

Third, the ATF6 pathway also plays a role in stress-induced lipid accumulation. A close examination of the relationship between ATF6 activity and SREBP2-mediated lipogenesis has revealed that nuclear ATF6 interacts with the nuclear form of SREBP2, thereby antagonizing SREBP2-regulated transcription of lipogenic genes and lipid accumulation in cultured liver and kidney cells^[40]. Moreover, ATF6 α -knockout mice develop hepatic steatosis in response to an ER stress inducer as a result of reduced fatty acid β -oxidation and attenuated VLDL formation^[41]. When fed a high-fat diet, ATF6 α -deficient mice show a tendency to develop greater degrees of hepatic steatosis and glucose intolerance in association with

increased expression of SREBP1c^[42].

Taken together, all three proximal UPR sensors, including PERK, IRE1 α , and ATF6 α , can regulate lipid stores in the liver. Although the evidence summarized above provides strong support for ER stress response-induced steatosis, it remains uncertain whether a stressed UPR contributes to hepatic steatosis, or conversely, whether it is an adaptive response to restore hepatocyte function. Future studies should address this important issue.

ER stress and IR

IR is a disruption in insulin signaling in organs including the liver, fat, and muscle, and is a major characteristic of obesity, type 2 diabetes, and NAFLD. In the past decade, ER stress has emerged as an important factor that contributes to IR.

Several mechanisms can account for the impact of ER stress and UPR signaling on hepatic IR. Ozcan *et al.*^[23] have demonstrated that ER stress induces hepatic IR through IRE1 α -mediated activation of c-Jun N-terminal kinase (JNK), which impairs insulin signaling through the serine phosphorylation of insulin receptor substrate-1. Moreover, tribbles 3 (TRB3), an ER stress-induced protein, was suggested to induce IR *via* inhibition of Akt/PKB signaling^[43]. Furthermore, PERK-mediated FOXO phosphorylation is also involved in ER stress-induced IR. FOXO is the Forkhead transcription factor that mediates many of the effects of insulin on the phosphatidylinositol 3-kinase (PI3-kinase) \rightarrow Akt cascade. Inhibiting FOXO1 activity has been shown to improve insulin sensitivity in genetic and diet-induced models of IR in

mice^[44,45]. Inhibition of PERK also improves cellular insulin responsiveness at the level of FOXO activity^[46]. Further support is derived from *in vivo* studies in XBP^{+/-} mice fed a high-fat diet, which exhibit IR and type 2 diabetes in conjunction with an increased ER stress response due to impaired UPR protection^[23]. Db/db diabetic obese mice, which overexpress the ER chaperone oxygen-regulated protein 150 (ORP150), demonstrate improved IR, whereas silencing ORP150 in normal mice decreases insulin sensitivity^[47].

NAFLD is strongly associated with hepatic IR as well as reduced whole-body insulin sensitivity^[48-50]. Despite mounting evidence indicating that ER stress is responsible for hepatic IR, the contribution of ER stress to IR in NAFLD remains uncertain. Few studies have comprehensively examined these potential mechanisms in humans. One study reported that phosphorylation of eIF2 α and C/EBP-homologous protein (CHOP) protein expression increased with worsening IR in 37 obese, non-diabetic individuals. However, there was no significant relationship between HOMA-IR and the expression of other ER stress factors, including spliced XBP1 mRNA and JNK phosphorylation, arguing against a causal role for ER stress in IR^[48].

ER stress and inflammation

Approximately 10%-20% of patients who have NAFLD develop inflammation and fibrosis, termed NASH, which is a more progressive, inflammatory disease phenotype of NAFLD. ER stress and related signaling networks are emerging as central pathways that regulate the key features of NASH.

Several signaling pathways connect ER stress to inflammation, including (1) the production of reactive oxygen species (ROS); (2) the activation of the transcription factors nuclear factor- κ B (NF- κ B) and JNK; and (3) the induction of the acute-phase response.

ROS are important mediators of inflammation. Protein folding in the ER is linked to the generation of ROS and oxidative stress^[51]. An increase in the protein-folding load in the ER can lead to the accumulation of ROS, which might initiate an inflammatory response. However, the PERK pathway of the UPR can activate an antioxidant program to limit the accumulation of ROS in response to ER stress *via* phosphorylation of nuclear factor-erythroid-derived 2-related factor 2 (Nrf2). Phosphorylated Nrf2 translocates to the nucleus and activates the transcription of a set of antioxidant and oxidant detoxifying enzymes^[52,53]. Therefore, Nrf2 deletion results in the rapid onset and progression of steatohepatitis in mice fed an MCD diet or a high-fat diet^[54,55]. Nrf2 activators can attenuate oxidative stress-induced liver injury in nutritional steatohepatitis^[56-58]. Thus, Nrf2 activation by pharmaceutical intervention could be a new option for the prevention and treatment of steatohepatitis.

Activation of NF- κ B and JNK is also involved in ER stress-induced inflammation. In response to ER stress, IRE1 α binds to the adaptor protein tumor-necrosis

factor- α (TNF α) receptor-associated factor 2 (TRAF2)^[59]. The IRE1 α -TRAF2 complex activates NF- κ B and JNK, which in turn induce the synthesis of proinflammatory cytokines. Thus, IRE1 α might provide a link between ER stress and inflammation^[59]. Sustained ER stress can also activate NF- κ B through the PERK and ATF6 branches^[60-62]. Although the activation of NF- κ B is detected in the MCD dietary model of steatohepatitis^[63], it is presently unclear whether and how the ER stress-mediated activation of NF- κ B is linked to NAFLD. Further studies will be needed to determine how ER-stress-induced signaling involving NF- κ B and JNK might regulate inflammation, metabolism, cell survival, and apoptosis in NAFLD.

CREBH is another example of a protein that links ER stress to inflammatory processes. CREBH is a liver-enriched transcription factor that is activated by a regulated intramembrane proteolysis (RIP) process upon ER stress. This transcription factor transactivates genes of the acute phase response, such as C-reactive protein (CRP) and serum amyloid P-component (SAP)^[64]. CREBH expression is itself strongly induced by inflammatory cytokines such as TNF α and interleukin 6 (IL-6) or by lipopolysaccharide (LPS)^[64], which also contributes to the maintenance of an inflammatory state during ER stress. Thus, ER stress in the liver may be linked to systemic inflammation *via* the RIP-mediated mobilization of CREBH.

ER stress and apoptosis

Hepatocyte apoptosis is increased in patients with NASH, is correlated with disease severity^[65], and has been proposed as a component of disease progression in NAFLD^[66]. Chronic or unresolved ER stress can lead to apoptosis. Alteration in several signaling pathways are involved in ER stress-induced cell death, including (1) the induction of CHOP pathway; (2) the activation of the JNK pathway by IRE1-mediated recruitment of TRAF2; and (3) the disruption of the calcium homeostasis pathway.

CHOP, an ER-resident transcription factor that functions downstream of the transmembrane proteins PERK and ATF6, is perhaps the most well-characterized mediator of ER stress-induced cell death. Silencing CHOP reduces hepatocyte apoptosis in alcohol-induced liver disease and attenuates cholestasis-induced liver fibrosis^[67,68]. However, the role of CHOP in NAFLD is paradoxical. One study demonstrated that CHOP depletion could reduce palmitate-induced apoptosis in hepatocyte cell lines, whereas MCD diet-induced liver injury was not reduced in CHOP knockout mice^[69]. Moreover, another study showed that MCD diet-induced liver injury was even greater in CHOP^{-/-} mice than in wild-type mice due to decreased cell death of activated macrophages^[70]. Therefore, future studies in mice with tissue-specific CHOP deletions are needed to delineate the contribution of CHOP to the onset and progression of NASH.

The IRE1 branch of the UPR also plays an essential role in ER stress-induced apoptosis. Phosphorylated,

activated mammalian IRE1 interacts with the adaptor protein TRAF2 and promotes a cascade of phosphorylation events that ultimately activates JNK^[59]. Caspase-12 is also recruited by the IRE1-TRAF2 complex in ER stress-induced apoptosis in mice^[71]. Moreover, IRE1 physically interacts with Bax and Bak, two proapoptotic Bcl-2 family members that promote mitochondrial-dependent cell death^[72].

Perturbations in ER calcium levels are also linked to apoptosis effectors. The ER lumen is a major site of calcium storage, and calcium homeostasis is critical for maintaining both ER folding capacity and cell viability. A disruption of ER calcium homeostasis inhibits the sarco/endoplasmic reticulum ATPase (SERCA) uptake pump, reduces the folding capacity of the ER, induces ER stress, and is an important mediator of ER-associated apoptosis^[73]. Moreover, sustained accumulation of calcium in the mitochondrial matrix induced by ER stress triggers mitochondrial membrane permeability and activates the apoptotic pathway^[74].

Despite all of these potential and emerging mediators, the exact role of ER-induced hepatocyte apoptosis in the pathogenesis of NAFLD is not well defined. Further studies are needed to elucidate the exact pathways that mediate ER stress-induced apoptosis in the progression of NASH.

CONCLUSION

Despite the high prevalence of NAFLD in recent years, the mechanisms responsible for disease progression remains poorly understood. Chronic ER stress induces numerous intracellular pathways that can lead to NAFLD development and progression, including hepatic steatosis, systemic inflammation, and hepatocyte cell death. ER stress interferes with hepatic lipid metabolism by activating lipogenesis and limiting VLDL formation and secretion. ER stress also acts indirectly on liver triglyceride accumulation by promoting insulin resistance in both the liver and adipose tissue. Furthermore, ER stress promotes the activation of Nrf2, JNK, NF- κ B, CREBH, and CHOP, which actively participate in the inflammatory process and cell death and provoke disease progression in NAFLD (Figure 2). Despite the many advances made in recent years, important questions remain. For example, is ER stress solely an adaptive response? Which of the UPR mediators are key players during the onset and progression of NAFLD? Which UPR pathway is linked with which specific cellular response? How does ER stress-induced signaling involving JNK, NF- κ B, and CHOP regulate inflammation, metabolism, cell survival, and apoptosis in NAFLD? Studies that answer these questions could identify novel therapeutic strategies for the prevention and treatment of NAFLD.

REFERENCES

1 Williams CD, Stengel J, Asike MI, Torres DM, Shaw J, Con-

- terras 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]
- 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. *Aliment Pharmacol Ther* 2011; **34**: 274-285 [PMID: 21623852 DOI: 10.1111/j.1365-2036.2011.04724.x]
- 3 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]
- 4 Farrell GC, Wong VW, Chitturi S. NAFLD in Asia—as common and important as in the West. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 307-318 [PMID: 23458891 DOI: 10.1038/nrgastro.2013.34]
- 5 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 DOI: 10.1016/S0016-5085(99)70506-8]
- 6 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 DOI: 10.1053/gast.2002.34168]
- 7 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/NEJMr0912063]
- 8 Targher G, Byrne CD. Clinical Review: Nonalcoholic fatty liver disease: a novel cardiometabolic risk factor for type 2 diabetes and its complications. *J Clin Endocrinol Metab* 2013; **98**: 483-495 [PMID: 23293330 DOI: 10.1210/jc.2012-3093]
- 9 Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. *Hepatology* 2010; **51**: 679-689 [PMID: 20041406 DOI: 10.1002/hep.23280]
- 10 Puri P, Mirshahi F, Cheung O, Natarajan R, Maher JW, Kellum JM, Sanyal AJ. Activation and dysregulation of the unfolded protein response in nonalcoholic fatty liver disease. *Gastroenterology* 2008; **134**: 568-576 [PMID: 18082745 DOI: 10.1053/j.gastro.2007.10.039]
- 11 Rinella ME, Siddiqui MS, Gardikiotes K, Gottstein J, Elias M, Green RM. Dysregulation of the unfolded protein response in db/db mice with diet-induced steatohepatitis. *Hepatology* 2011; **54**: 1600-1609 [PMID: 21748768 DOI: 10.1002/hep.24553]
- 12 Fang DL, Wan Y, Shen W, Cao J, Sun ZX, Yu HH, Zhang Q, Cheng WH, Chen J, Ning B. Endoplasmic reticulum stress leads to lipid accumulation through upregulation of SREBP-1c in normal hepatic and hepatoma cells. *Mol Cell Biochem* 2013; **381**: 127-137 [PMID: 23703028 DOI: 10.1007/s11010-013-1694-7]
- 13 Bravo R, Parra V, Gatica D, Rodriguez AE, Torrealba N, Paredes F, Wang ZV, Zorzano A, Hill JA, Jaimovich E, Quest AF, Lavandro S. Endoplasmic reticulum and the unfolded protein response: dynamics and metabolic integration. *Int Rev Cell Mol Biol* 2013; **301**: 215-290 [PMID: 23317820 DOI: 10.1016/B978-0-12-407704-1.00005-1]
- 14 Diehl JA, Fuchs SY, Koumenis C. The cell biology of the unfolded protein response. *Gastroenterology* 2011; **141**: 38-41, 41.e1-2 [PMID: 21620842 DOI: 10.1053/j.gastro.2011.05.018]
- 15 Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 2011; **334**: 1081-1086 [PMID: 22116877 DOI: 10.1126/science.1209038]
- 16 Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol*

- 2007; **8**: 519-529 [PMID: 17565364 DOI: 10.1038/nrm2199]
- 17 **Hetz C.** The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol* 2012; **13**: 89-102 [PMID: 22251901 DOI: 10.1038/nrm3270]
- 18 **Tabas I, Ron D.** Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol* 2011; **13**: 184-190 [PMID: 21364565 DOI: 10.1038/ncb0311-184]
- 19 **Xu C, Bailly-Maitre B, Reed JC.** Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest* 2005; **115**: 2656-2664 [PMID: 16200199 DOI: 10.1172/JCI26373]
- 20 **Kim I, Xu W, Reed JC.** Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nat Rev Drug Discov* 2008; **7**: 1013-1030 [PMID: 19043451 DOI: 10.1038/nrd2755]
- 21 **Malhi H, Kaufman RJ.** Endoplasmic reticulum stress in liver disease. *J Hepatol* 2011; **54**: 795-809 [PMID: 21145844 DOI: 10.1016/j.jhep.2010.11.005]
- 22 **Kaplowitz N, Than TA, Shinohara M, Ji C.** Endoplasmic reticulum stress and liver injury. *Semin Liver Dis* 2007; **27**: 367-377 [PMID: 17979073 DOI: 10.1055/s-2007-991513]
- 23 **Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Görgün C, Glimcher LH, Hotamisligil GS.** Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 2004; **306**: 457-461 [PMID: 15486293 DOI: 10.1126/science.1103160]
- 24 **Wang D, Wei Y, Pagliassotti MJ.** Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology* 2006; **147**: 943-951 [PMID: 16269465 DOI: 10.1210/en.2005-0570]
- 25 **Yang L, Jhaveri R, Huang J, Qi Y, Diehl AM.** Endoplasmic reticulum stress, hepatocyte CD1d and NKT cell abnormalities in murine fatty livers. *Lab Invest* 2007; **87**: 927-937 [PMID: 17607300 DOI: 10.1038/labinvest.3700603]
- 26 **Sreejayan N, Dong F, Kandadi MR, Yang X, Ren J.** Chromium alleviates glucose intolerance, insulin resistance, and hepatic ER stress in obese mice. *Obesity* (Silver Spring) 2008; **16**: 1331-1337 [PMID: 18388893 DOI: 10.1038/oby.2008.217]
- 27 **Rahman SM, Schroeder-Glockler JM, Janssen RC, Jiang H, Qadri I, Maclean KN, Friedman JE.** CCAAT/enhancing binding protein beta deletion in mice attenuates inflammation, endoplasmic reticulum stress, and lipid accumulation in diet-induced nonalcoholic steatohepatitis. *Hepatology* 2007; **45**: 1108-1117 [PMID: 17464987 DOI: 10.1002/hep.21614]
- 28 **Gregor MF, Yang L, Fabbrini E, Mohammed BS, Eagon JC, Hotamisligil GS, Klein S.** Endoplasmic reticulum stress is reduced in tissues of obese subjects after weight loss. *Diabetes* 2009; **58**: 693-700 [PMID: 19066313 DOI: 10.2337/db08-1220]
- 29 **Cusi K.** Role of obesity and lipotoxicity in the development of nonalcoholic steatohepatitis: pathophysiology and clinical implications. *Gastroenterology* 2012; **142**: 711-725.e6 [PMID: 22326434 DOI: 10.1053/j.gastro.2012.02.003]
- 30 **Sozio MS, Liangpunsakul S, Crabb D.** The role of lipid metabolism in the pathogenesis of alcoholic and nonalcoholic hepatic steatosis. *Semin Liver Dis* 2010; **30**: 378-390 [PMID: 20960377 DOI: 10.1055/s-0030-1267538]
- 31 **Reddy JK, Rao MS.** Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G852-G858 [PMID: 16603729 DOI: 10.1152/ajpgi.00521.2005]
- 32 **Bobrovnikova-Marjon E, Hatzivassiliou G, Grigoriadou C, Romero M, Caverer DR, Thompson CB, Diehl JA.** PERK-dependent regulation of lipogenesis during mouse mammary gland development and adipocyte differentiation. *Proc Natl Acad Sci USA* 2008; **105**: 16314-16319 [PMID: 18852460 DOI: 10.1073/pnas.0808517105]
- 33 **Oyadomari S, Harding HP, Zhang Y, Oyadomari M, Ron D.** Dephosphorylation of translation initiation factor 2alpha enhances glucose tolerance and attenuates hepatosteatosis in mice. *Cell Metab* 2008; **7**: 520-532 [PMID: 18522833 DOI: 10.1016/j.cmet.2008.04.011]
- 34 **Seo J, Fortuno ES, Suh JM, Stenesen D, Tang W, Parks EJ, Adams CM, Townes T, Graff JM.** Atf4 regulates obesity, glucose homeostasis, and energy expenditure. *Diabetes* 2009; **58**: 2565-2573 [PMID: 19690063 DOI: 10.2337/db09-0335]
- 35 **Wang C, Huang Z, Du Y, Cheng Y, Chen S, Guo F.** ATF4 regulates lipid metabolism and thermogenesis. *Cell Res* 2010; **20**: 174-184 [PMID: 20066008 DOI: 10.1038/cr.2010.4]
- 36 **Xiao G, Zhang T, Yu S, Lee S, Calabuig-Navarro V, Yamachi J, Ringquist S, Dong HH.** ATF4 protein deficiency protects against high fructose-induced hypertriglyceridemia in mice. *J Biol Chem* 2013; **288**: 25350-25361 [PMID: 23888053 DOI: 10.1074/jbc.M113.470526]
- 37 **Zhang K, Wang S, Malhotra J, Hassler JR, Back SH, Wang G, Chang L, Xu W, Miao H, Leonardi R, Chen YE, Jackowski S, Kaufman RJ.** The unfolded protein response transducer IRE1α prevents ER stress-induced hepatic steatosis. *EMBO J* 2011; **30**: 1357-1375 [PMID: 21407177 DOI: 10.1038/emboj.2011.52]
- 38 **Wang S, Chen Z, Lam V, Han J, Hassler J, Finck BN, Davidson NO, Kaufman RJ.** IRE1α-XBPs induces PDI expression to increase MTP activity for hepatic VLDL assembly and lipid homeostasis. *Cell Metab* 2012; **16**: 473-486 [PMID: 23040069 DOI: 10.1016/j.cmet.2012.09.003]
- 39 **Lee AH, Scapa EF, Cohen DE, Glimcher LH.** Regulation of hepatic lipogenesis by the transcription factor XBP1. *Science* 2008; **320**: 1492-1496 [PMID: 18556558 DOI: 10.1126/science.1158042]
- 40 **Zeng L, Lu M, Mori K, Luo S, Lee AS, Zhu Y, Shyy JY.** ATF6 modulates SREBP2-mediated lipogenesis. *EMBO J* 2004; **23**: 950-958 [PMID: 14765107 DOI: 10.1038/sj.emboj.7600106]
- 41 **Yamamoto K, Takahara K, Oyadomari S, Okada T, Sato T, Harada A, Mori K.** Induction of liver steatosis and lipid droplet formation in ATF6α-knockout mice burdened with pharmacological endoplasmic reticulum stress. *Mol Biol Cell* 2010; **21**: 2975-2986 [PMID: 20631254 DOI: 10.1091/mbc.E09-02-0133]
- 42 **Usui M, Yamaguchi S, Tanji Y, Tominaga R, Ishigaki Y, Fukumoto M, Katagiri H, Mori K, Oka Y, Ishihara H.** Atf6α-null mice are glucose intolerant due to pancreatic β-cell failure on a high-fat diet but partially resistant to diet-induced insulin resistance. *Metabolism* 2012; **61**: 1118-1128 [PMID: 22386934 DOI: 10.1016/j.metabol.2012.01.004]
- 43 **Du K, Herzig S, Kulkarni RN, Montminy M.** TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. *Science* 2003; **300**: 1574-1577 [PMID: 12791994 DOI: 10.1126/science.1079817]
- 44 **Nakae J, Biggs WH, Kitamura T, Cavenee WK, Wright CV, Arden KC, Accili D.** Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. *Nat Genet* 2002; **32**: 245-253 [PMID: 12219087 DOI: 10.1038/ng890]
- 45 **Kim JJ, Li P, Huntley J, Chang JP, Arden KC, Olefsky JM.** FoxO1 haploinsufficiency protects against high-fat diet-induced insulin resistance with enhanced peroxisome proliferator-activated receptor gamma activation in adipose tissue. *Diabetes* 2009; **58**: 1275-1282 [PMID: 19289458 DOI: 10.2337/db08-1001]
- 46 **Zhang W, Hietakangas V, Wee S, Lim SC, Gunaratne J, Cohen SM.** ER stress potentiates insulin resistance through PERK-mediated FOXO phosphorylation. *Genes Dev* 2013; **27**: 441-449 [PMID: 23431056 DOI: 10.1101/gad.201731.112]
- 47 **Nakatani Y, Kaneto H, Kawamori D, Yoshiuchi K, Hatazaki M, Matsuoka TA, Ozawa K, Ogawa S, Hori M, Yamasaki Y, Matsuhisa M.** Involvement of endoplasmic reticulum stress in insulin resistance and diabetes. *J Biol Chem* 2005; **280**: 847-851 [PMID: 15509553 DOI: 10.1074/jbc.M411860200]
- 48 **Kumashiro N, Erion DM, Zhang D, Kahn M, Beddo SA,**

- Chu X, Still CD, Gerhard GS, Han X, Dziura J, Petersen KF, Samuel VT, Shulman GI. Cellular mechanism of insulin resistance in nonalcoholic fatty liver disease. *Proc Natl Acad Sci USA* 2011; **108**: 16381-16385 [PMID: 21930939 DOI: 10.1073/pnas.1113359108]
- 49 Bugianesi E, Moscatiello S, Ciaravella MF, Marchesini G. Insulin resistance in nonalcoholic fatty liver disease. *Curr Pharm Des* 2010; **16**: 1941-1951 [PMID: 20370677 DOI: 10.2174/138161210791208875]
- 50 Utzschneider KM, Kahn SE. Review: The role of insulin resistance in nonalcoholic fatty liver disease. *J Clin Endocrinol Metab* 2006; **91**: 4753-4761 [PMID: 16968800 DOI: 10.1210/jc.2006-0587]
- 51 Santos CX, Tanaka LY, Wosniak J, Laurindo FR. Mechanisms and implications of reactive oxygen species generation during the unfolded protein response: roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase. *Antioxid Redox Signal* 2009; **11**: 2409-2427 [PMID: 19388824 DOI: 10.1089/ARS.2009.2625]
- 52 Cullinan SB, Diehl JA. Coordination of ER and oxidative stress signaling: the PERK/Nrf2 signaling pathway. *Int J Biochem Cell Biol* 2006; **38**: 317-332 [PMID: 16290097 DOI: 10.1016/j.biocel.2005.09.018]
- 53 Cullinan SB, Diehl JA. PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *J Biol Chem* 2004; **279**: 20108-20117 [PMID: 14978030 DOI: 10.1074/jbc.M314219200]
- 54 Okada K, Warabi E, Sugimoto H, Horie M, Gotoh N, Tokushige K, Hashimoto E, Utsunomiya H, Takahashi H, Ishii T, Yamamoto M, Shoda J. Deletion of Nrf2 leads to rapid progression of steatohepatitis in mice fed atherogenic plus high-fat diet. *J Gastroenterol* 2013; **48**: 620-632 [PMID: 22972520 DOI: 10.1007/s00535-012-0659-z]
- 55 Chowdhry S, Nazmy MH, Meakin PJ, Dinkova-Kostova AT, Walsh SV, Tsujita T, Dillon JF, Ashford ML, Hayes JD. Loss of Nrf2 markedly exacerbates nonalcoholic steatohepatitis. *Free Radic Biol Med* 2010; **48**: 357-371 [PMID: 19914374 DOI: 10.1016/j.freeradbiomed.2009.11.007]
- 56 Shimozono R, Asaoka Y, Yoshizawa Y, Aoki T, Noda H, Yamada M, Kaino M, Mochizuki H. Nrf2 activators attenuate the progression of nonalcoholic steatohepatitis-related fibrosis in a dietary rat model. *Mol Pharmacol* 2013; **84**: 62-70 [PMID: 23592516 DOI: 10.1124/mol.112.084269]
- 57 Okada K, Warabi E, Sugimoto H, Horie M, Tokushige K, Ueda T, Harada N, Taguchi K, Hashimoto E, Itoh K, Ishii T, Utsunomiya H, Yamamoto M, Shoda J. Nrf2 inhibits hepatic iron accumulation and counteracts oxidative stress-induced liver injury in nutritional steatohepatitis. *J Gastroenterol* 2012; **47**: 924-935 [PMID: 22367278 DOI: 10.1007/s00535-012-0552-9]
- 58 Gupte AA, Lyon CJ, Hsueh WA. Nuclear factor (erythroid-derived 2)-like-2 factor (Nrf2), a key regulator of the antioxidant response to protect against atherosclerosis and nonalcoholic steatohepatitis. *Curr Diab Rep* 2013; **13**: 362-371 [PMID: 23475581 DOI: 10.1007/s11892-013-0372-1]
- 59 Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 2000; **287**: 664-666 [PMID: 10650002 DOI: 10.1126/science.287.5453.664]
- 60 Yamazaki H, Hiramatsu N, Hayakawa K, Tagawa Y, Okamura M, Ogata R, Huang T, Nakajima S, Yao J, Paton AW, Paton JC, Kitamura M. Activation of the Akt-NF-kappaB pathway by subtilase cytotoxin through the ATF6 branch of the unfolded protein response. *J Immunol* 2009; **183**: 1480-1487 [PMID: 19561103 DOI: 10.4049/jimmunol.0900017]
- 61 Deng J, Lu PD, Zhang Y, Scheuner D, Kaufman RJ, Sonenberg N, Harding HP, Ron D. Translational repression mediates activation of nuclear factor kappa B by phosphorylated translation initiation factor 2. *Mol Cell Biol* 2004; **24**: 10161-10168 [PMID: 15542827 DOI: 10.1128/MCB.24.23.10161-10168.2004]
- 62 Jiang HY, Wek SA, McGrath BC, Scheuner D, Kaufman RJ, Cavener DR, Wek RC. Phosphorylation of the alpha subunit of eukaryotic initiation factor 2 is required for activation of NF-kappaB in response to diverse cellular stresses. *Mol Cell Biol* 2003; **23**: 5651-5663 [PMID: 12897138 DOI: 10.1128/MC.B.23.16.5651-5663.2003]
- 63 Dela Peña A, Leclercq I, Field J, George J, Jones B, Farrell G. NF-kappaB activation, rather than TNF, mediates hepatic inflammation in a murine dietary model of steatohepatitis. *Gastroenterology* 2005; **129**: 1663-1674 [PMID: 16285964 DOI: 10.1053/j.gastro.2005.09.004]
- 64 Zhang K, Shen X, Wu J, Sakaki K, Saunders T, Rutkowski DT, Back SH, Kaufman RJ. Endoplasmic reticulum stress activates cleavage of CREB to induce a systemic inflammatory response. *Cell* 2006; **124**: 587-599 [PMID: 16469704 DOI: 10.1016/j.cell.2005.11.040]
- 65 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 DOI: 10.1016/S0016-5085(03)00907-7]
- 66 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 DOI: 10.1002/hep.21223]
- 67 Ji C, Mehrian-Shai R, Chan C, Hsu YH, Kaplowitz N. Role of CHOP in hepatic apoptosis in the murine model of intragastric ethanol feeding. *Alcohol Clin Exp Res* 2005; **29**: 1496-1503 [PMID: 16131858 DOI: 10.1097/01.alc.0000174691.03751.11]
- 68 Tamaki N, Hatano E, Taura K, Tada M, Kodama Y, Nitta T, Iwaisako K, Seo S, Nakajima A, Ikai I, Uemoto S. CHOP deficiency attenuates cholestasis-induced liver fibrosis by reduction of hepatocyte injury. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G498-G505 [PMID: 18174271 DOI: 10.1152/ajpgi.00482.2007]
- 69 Pfaffenbach KT, Gentile CL, Nivala AM, Wang D, Wei Y, Pagliassotti MJ. Linking endoplasmic reticulum stress to cell death in hepatocytes: roles of C/EBP homologous protein and chemical chaperones in palmitate-mediated cell death. *Am J Physiol Endocrinol Metab* 2010; **298**: E1027-E1035 [PMID: 20159858 DOI: 10.1152/ajpendo.00642.2009]
- 70 Malhi H, Kropp EM, Clavo VF, Kobrossi CR, Han J, Mauer AS, Yong J, Kaufman RJ. C/EBP homologous protein-induced macrophage apoptosis protects mice from steatohepatitis. *J Biol Chem* 2013; **288**: 18624-18642 [PMID: 23720735 DOI: 10.1074/jbc.M112.442954]
- 71 Yoneda T, Imaizumi K, Oono K, Yui D, Gomi F, Katayama T, Tohyama M. Activation of caspase-12, an endoplasmic reticulum (ER) resident caspase, through tumor necrosis factor receptor-associated factor 2-dependent mechanism in response to the ER stress. *J Biol Chem* 2001; **276**: 13935-13940 [PMID: 11278723 DOI: 10.1074/jbc.M010677200]
- 72 Hetz C, Bernasconi P, Fisher J, Lee AH, Bassik MC, Antonsson B, Brandt GS, Iwakoshi NN, Schinzel A, Glimcher LH, Korsmeyer SJ. Proapoptotic BAX and BAK modulate the unfolded protein response by a direct interaction with IRE1alpha. *Science* 2006; **312**: 572-576 [PMID: 16645094 DOI: 10.1126/science.1123480]
- 73 Luciani DS, Gwiazda KS, Yang TL, Kalynyak TB, Bychkivska Y, Frey MH, Jeffrey KD, Sampaio AV, Underhill TM, Johnson JD. Roles of IP3R and RyR Ca2+ channels in endoplasmic reticulum stress and beta-cell death. *Diabetes* 2009; **58**: 422-432 [PMID: 19033399 DOI: 10.2337/db07-1762]

- 74 **Deniaud A**, Sharaf el dein O, Maillier E, Poncet D, Kroemer G, Lemaire C, Brenner C. Endoplasmic reticulum stress induces calcium-dependent permeability transition, mito-

chondrial outer membrane permeabilization and apoptosis. *Oncogene* 2008; **27**: 285-299 [PMID: 17700538 DOI: 10.1038/sj.onc.1210638]

P- Reviewers: Assy N, Jun DW, Lu MY, Nowicki M, Pei ZH, Tiniakos DG, Zhang Y
S- Editor: Ma YJ **L- Editor:** A **E- Editor:** Ma S



WJG 20th Anniversary Special Issues (15): Laparoscopic resection of gastrointestinal

Laparoscopic approach to gastrointestinal malignancies: Toward the future with caution

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Author contributions: Bencini L ideated and designed the research; Bencini L, Bernini M and Farsi M performed the research and contributed to the final draft of the paper.

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Received: October 12, 2013 Revised: November 7, 2013

Accepted: November 28, 2013

Published online: February 21, 2014

Abstract

After the rapid acceptance of laparoscopy to manage multiple benign diseases arising from gastrointestinal districts, some surgeons started to treat malignancies by the same way. However, if the limits of laparoscopy for benign diseases are mainly represented by technical issues, oncologic outcomes remain the foundation of any procedures to cure malignancies. Cancerous patients represent an important group with peculiar aspects including reduced survival expectancy, worsened quality of life due to surgery itself and adjuvant therapies, and challenging psychological impact. All these issues could, potentially, receive a better management with a laparoscopic surgical approach. In order to confirm such aspects, similarly to testing the newest weapons (surgical or pharmacologic) against cancer, long-term follow-up is always recommendable to assess the real benefits in terms of overall survival, cancer-free survival and quality of life. Furthermore, it seems of crucial importance that surgeons will be correctly trained in specific oncologic principles of surgical oncology as well as in modern miniinvasive technologies. Therefore, laparoscopic treatment of gastrointestinal malignancies requires more caution and deep

analysis of published evidences, as compared to those achieved for inflammatory bowel diseases, gastroesophageal reflux disease or diverticular disease. This review tries to examine the evidence available to date for the use of laparoscopy and robotics in malignancies arising from the gastrointestinal district.

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Key words: Gastrointestinal cancer; Laparoscopic; Oncology; Laparoscopic surgery; Robotic surgery; Surgical outcomes; Oncologic outcomes

Core tip: Laparoscopic treatment of gastrointestinal malignancies requires more caution and deep analysis of published evidences, as compared to those achieved for benign diseases. Oncologic outcomes remain the foundation of any procedures to cure malignancies, hence a long-term follow-up is always recommendable in order to assess overall survival, cancer-free survival and quality of life.

Bencini L, Bernini M, Farsi M. Laparoscopic approach to gastrointestinal malignancies: Toward the future with caution. *World J Gastroenterol* 2014; 20(7): 1777-1789 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1777.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1777>

INTRODUCTION

From an epidemiologic point of view, gastrointestinal malignancies represent a vast share of both incidence and mortality for cancer worldwide (Figure 1)^[1]. Therefore, the widespread adoption of the minimally invasive (endoscopic, laparoscopic and robotic) approach to cure these malignancies was an attractive and valuable consequence, and many surgeons reported tangible benefits of

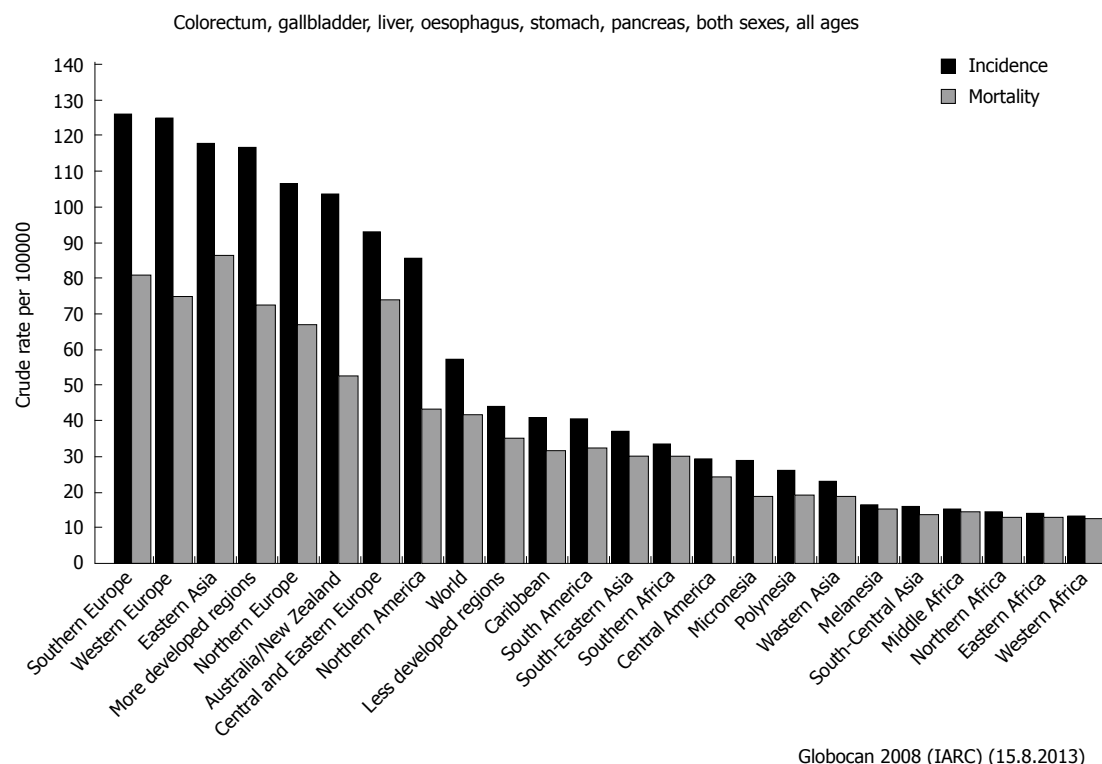


Figure 1 Incidence and mortality from the most important gastrointestinal malignancies worldwide^[1].

this technique in terms of morbidity, return to normal activities and mortality.

Nevertheless, the popularity of miniinvasive surgery (MIS) among both surgeons and patients, mixed with some industrial pressure, could have also played a role in the worldwide diffusion and explosion of new technologies.

The proven advantages of laparoscopy are mainly represented by better short-term outcomes including fewer wound complications, less pulmonary impairment, reduction of postoperative pain, shorter length of postoperative stay and, eventually, better cosmetic result. Moreover, there is a well-recognized role of laparoscopy in decreasing the pro-inflammatory and immunologic response to surgery that are, hypothetically, related to an improved immediate or even long-term oncologic result^[2]. For all these issues, the laparoscopic technique in oncology seem to be very promising^[3].

However, if the limits of MIS are mainly represented by technical issues and patients conformation for benign diseases, oncologic outcomes remain the foundation of any procedure to cure malignancies. Mostly, any laparoscopic procedure should follow the same standard of care of open surgery, including oncologic principles, such as wide margins of resection and extended lymphadenectomy. Such prerequisites often require very good skills that are generally limited to high volume centers with subspecialized teams.

Historically, the first concerns about MIS for cancer were represented by the possibility of port-site metastasis and lower number of lymphnodes retrieved, but none of these issue has been confirmed successively^[4,5].

Moreover, advanced laparoscopy for gastrointestinal malignancy requires the whole specimen extraction (often with regional nodes) through a minilaparotomy, thus flawing one of the strongest advantages of MIS represented by short incisions.

Most of the reviewers concluded that, although slowly, colorectal oncologic resections had been recognized to have a non-inferior cancer-free survival and superior short-term results, while the laparoscopic approach to gastric and solid organs malignancies will require further studies with longer follow-up^[3,6].

The role of laparoscopy is known to be important in staging gastrointestinal malignancies, limiting the number of unnecessary laparotomies for carcinosis or occult metastasis. Many palliative procedures such as gastrointestinal by-pass, gastrostomy, jejunostomy and colostomy or radiofrequency ablations are easily carried out with MIS^[6].

The pure endoscopic treatments of both esophago-gastric and colorectal small malignancies (precancerous or T1 lesions) are well accepted among cancer professionals for being as safe and as curative as traditional resections, and they will not be considered further in this review. Therefore, we focused on the full laparoscopic (and robotic) treatment of gastrointestinal malignancies, including those arising from the esophagus, stomach, liver and biliary system, pancreas, small bowel and colorectum. If not otherwise specified, malignancies are intended to be carcinomas: other histopathological subtypes, such as neuroendocrine or sarcomas, will not be considered.

A Pubmed, Embase and Cochrane databases bibliography search was conducted until September 2013, including important cross-matched manual references. References to historical reports and older articles were limited to the minimum. A particular attention was reserved to data arising from randomized controlled clinical trials (RCTs) (or meta-analyses) with long-term follow-up. Oncologic results will be considered of utmost interest, in order to assess overall survival, cancer-free survivals and quality of life, rather than feasibility of the procedure itself and short-term outcomes.

ESOPHAGUS

The three-field (Mc Keown procedure) has been the treatment of choice for esophageal cancer for many years. This procedure, as well as the so-called Ivor-Lewis (two-field esophagectomy with esophagogastric intra-thoracic anastomosis) and the Orringer procedure (transhiatal esophagectomy) are all feasible by laparoscopy (thoracoscopy) or hybrid (with open surgery combined) technique^[7]. MIS for esophageal cancer has spread worldwide, reducing the significative perioperative complications (mainly respiratory). However, many debates still exist on the real efficacy and cost-effectiveness of minimally invasive esophagectomy (MIE). If these complex operations should be performed by the open traditional approaches or carried out by the laparoscopic and thoracoscopic route is still far to be demonstrated by the surgical community^[2].

Several technical variables, such as the role of laparoscopic, thoracoscopic or combined steps, the usefulness of the prone or supine position, the choice of stapled or hand-sawn anastomosis and the route of stapled suture are under study. Last, the general poor quality of studies published leads to a great caution, when dealing with outcomes and oncologic results^[8]. All of these issues contribute to jeopardize the surgical results and perioperative complications data reporting. Moreover, it should be considered how technical demanding this kind of surgery is, even in the traditional open way, with very few centers having sufficient case-load to gain adequate specific proficiency.

A single-center review^[7] of more than 1000 patients (thoraco-laparoscopic McKeown and Ivor-Lewis operations compared) reported global excellent results, with a morbidity and mortality rate of less than 2% and 1%, respectively; with the best approach being the thoraco-laparoscopic Ivor-Lewis. A well-conducted review on MIE concluded that both laparoscopy and thoracoscopy are at least comparable to open surgery in terms of outcomes for non-locally advanced cancers, but the open transthoracic route is superior when considering field exposure^[9].

The only prospective, multicentric RCT including few patients^[10], and one large retrospective cohort study also confirmed the superiority of MIE in terms of postoperative pulmonary complications (13% in the thoraco-

laparoscopic MIE, 38% in the thoracoscopic MIE, and 39% in the open group)^[11]. Another ongoing trial was designed to evaluate the benefits of laparoscopic gastric mobilization during Ivor-Lewis intervention in terms of postoperative complications^[12]. Moreover, a recent review failed to find any important differences between the two classic stapled anastomosis techniques (transoral anvil introduction and transthoracic) during Ivor-Lewis esophagectomy for cancer^[13].

If many review articles report how MIE statistically decreases blood loss, length of stay, and perioperative morbidity at the price of increased operative time and costs, large-scale multicentric trials are still lacking, and few studies had long-term follow-up^[14]. Hanna *et al.*^[15] selected thirty of the largest and best designed trials concerning MIE for cancer (including only 1 RCT). The author concluded that in most studies a suboptimal lymphadenectomy was carried out, with an average number of nodes retrieved below the standard (over 23), while no homogeneous complications reporting was available. However, the final oncologic outcomes for each stage (disease-free survival and overall survival) were comparable to those achieved by the open traditional surgery.

Lastly, robotic-assisted MIE was also employed in the treatment of esophago-gastric malignancies, but very few studies, even of poor quality, failed to demonstrate real advantages of this method as compared to open surgery^[16,17]. A monocentric trial targeted to robotic MIE started recently^[18].

In conclusions, due to the relative low frequency of esophageal cancer (especially in Western countries), the technical difficulties, the debated approach (two-field, three-field, transhiatal) and lack of literature evidence, the MIE, although promising, should be reserved to specialized centers within controlled trials.

STOMACH

The standard of care is open gastric resection with complete D2 lymphadenectomy for curable gastric cancer in both Western and Eastern countries, although a debate lasting decades on the extent of lymphadenectomy has been carried on. However, some endoscopic techniques (such as submucosal dissection) are recognized to be appropriate for selected patients with T1 cancers^[2]. More controversies still persist regarding laparoscopic gastric resection (LGR) and sentinel node mapping, in those patients who are unfit for endoscopic resection or who have more advanced tumors.

Almost every gastric procedure is feasible by the laparoscopic route, including distal and the more challenging total gastrectomy with intracorporeal anastomosis^[19,20] and formal lymphadenectomy^[21], but also gastric resections and sentinel node sampling are recommended in selected cases.

The limit of the widespread adoption of the LGR is represented by the technical difficulties (mainly anastomosis) and the oncologic safety. Indeed, the standard D2

lymphadectomy, although feasible with few complications^[22,23] is technically demanding, but mandatory for all advanced tumors.

Several review articles and meta-analysis reported that laparoscopy was a safe alternative, if not superior (perioperative outcomes), to open surgery for the treatment of early and advanced gastric cancer^[24-30], but data on long-term survival, quality of life and cost effectiveness are still lacking^[31,32].

One of the most updated review, including only comparative trials with a pooled cohort of more than 1000 patients^[33], and another^[34], that included 8 RCTs (more than 700 patients) reached the same conclusions. LGR is better or comparable in the early perioperative results with similar long-term outcomes respect to open surgery, although at the price of longer duration of surgery and technical difficulties. Other two meta-analysis by Wang *et al*^[35] and Chen *et al*^[36] reached the same conclusions in terms of oncologic effectiveness (node dissection) and outcomes. Many benefits of LGR are also confirmed in elderly people suffering for comorbidities according an enormous Chinese database^[37].

Large Asian trials with longer follow-up are still ongoing, and only one European study reported data on 10-years follow-up^[38]. To the present, one recent RCT (KLASS trial) reported early results: the authors confirmed equivalent outcomes of laparoscopic and open approach to gastrectomy for cancer^[39].

A crucial point of concern is represented by the steep learning curve, although a paper reported encouraging results of LGR initiated by experienced surgeons in open gastrectomy and laparoscopy who received adequate training^[40].

Most of the papers on LGR come from Eastern countries due to the high volumes of disease, high rates of early cancers and perhaps less diffusion of obesity that can obstacle laparoscopy. Therefore, the reported proportions of LGR rises to more than 20% in Japan in a recent review article^[41]. However, some good results are also reported from many Western countries including Europe^[42,43] and an international panel published some guidelines for the introduction and diffusion of the technique^[44].

More recently, the introduction of the robotic approach to perform very complex operation, including gastric surgery, seems to be promising in order to reduce some of the technical difficulties of laparoscopy^[41]. However, very few rigorous studies were published on robotic approach for gastric cancer and a recent meta-analysis^[45] ended up selecting only 3 RCTs comparing robotic and laparoscopy. The pooled results showed no significant differences between the two approaches in terms of complications, mortality, conversion, length of stay and number of nodes retrieved. On the other hand, blood loss resulted inferior by robotics, at the price of an increased operative time and costs. If laparoscopic treatment of gastric cancer is still debated, this is even more for robotics, especially in terms of real benefits for patients.

Recent systematic review and meta-analysis of few

retrospective comparative trials seem to confirm superiority of LGR as compared to open surgery also when dealing with gastrointestinal stromal tumours (GISTs)^[46,47].

LIVER, GALLBLADDER AND BILIARY TRACT

Since the advent of advanced laparoscopic techniques and availability of efficacious transection devices, many authors reported the feasibility of liver resections by the key-hole approach, both for malignancies and benign disease. Some retrospective and review studies (including very few comparative trials) provided relative evidence to support further development of case-load and research, to assess safety of laparoscopic hepatectomy for cancer patients or, if any, superiority as compared to standard surgery^[48-50].

In 2007, the most acknowledged hepatobiliary surgeons worldwide met in Luisville (United States) to find an international common position on laparoscopic liver surgery (LLS): although few relevant data was available, the experts concluded that this kind of surgery (or hybrid technique, including hand-assisting) is safe and effective, in the hand of trained surgeons and under the control of societies and government. The preferred indications (despite for malignancy) were represented by solitary lesions of less than 5 cm in maximum diameter located in segments 2 to 6^[51]. On the other hand, many surgeons began LLS dealing with benign diseases involving left lateral segments^[52], while others brought the indications toward upper limits^[53]. An international multi-institutional review article proposed the laparoscopic approach to left-sided hepatectomies as the future gold-standard of care^[54].

LLS for cancer [including both hepatocellular carcinoma (HCC) and colorectal metastasis (CRM)] seems to offer oncologic results similar to those of laparotomy^[55]. Excellent results were also achieved from 3 specialized European centers with large experience in HCC^[56].

Laparoscopy seems to add also some benefits in terms of reducing early complications in the subset of patients affected by HCC and cirrhosis^[57]. In a case-matched analysis published by Lee *et al*^[58] LLS for HCC showed similar long-term outcomes but some early clinical advantages (complications and hospital stay) as compared to open surgery. Feasibility, less morbidity and shorter hospital stay were also found in patients after hepatectomies carried out for CRM^[59,60].

Another very large (300 patients, 103 cancerous) single-center case-matched experience from Chicago (United States)^[61] concluded that miniinvasive hepatectomy (including major resections) compared favourably with contemporaneous controls operated by the open approach without any oncological detriment. Positive parameters included blood loss, transfusion requirement, overall complications, postoperative stay and, surprisingly, operative time.

In the most recent and rigorous review of available studies, carried out by Rao *et al*^[62] for the Cochrane

Library, the author reported that no conclusion can be drawn on the benefits or harm of laparoscopy versus open technique for liver resection. These unsatisfactory data are consequence of lacking of any published RCT that met strong scientific criteria, although some are still ongoing.

R0 resection represents the main goal of treatment when dealing with hilar cholangiocarcinoma (Klatskin tumor), gallbladder cancer or extrahepatic bile duct cancer. Regional lymphadenectomy should be also performed in order to reduce recurrences^[63,64]. Therefore, the laparoscopic approach to hilar structures is very challenging, even for a skilled laparoscopist, although MIS is highly accepted to confirm resectability and avoid unnecessary laparotomies. Some recent retrospective multicentric studies reported encouraging and oncologically acceptable laparoscopic procedures for hilar and gallbladder malignancies, but in the hands of very experienced surgeons working in highly subspecialized surgical units^[65].

With the widespread adoption of laparoscopic cholecystectomy, it seems that an increased number of incidental gallbladder cancer could be diagnosed nowadays. However, no difference in survival was demonstrated, if the surgeon decides to perform a more aggressive resection immediately or during a second look intervention^[66,67]. Theoretically, this fact leads to correctly plan the adequate operation and to reach maximum oncologic results by both open delayed resection or immediate laparoscopy. Nevertheless, the experiences of laparoscopic second look resections and lymphadenectomy for gallbladder cancer are almost anecdotal^[68]. Some authors reported initial experiences with the use of single-port laparoscopic technique for specific group of selected patients with malignancy and liver dysfunction^[69].

Robotics could play a role in development of minimally invasive techniques for hepato-biliary malignancies due to easier dissection in deep and narrow spaces and for the possibility of knot-tying of vascular structures. Good short-term results were reported for robotic-assisted liver resections for HCC and CRM^[70,71], while robot-assisted radical resection for gallbladder cancer is both feasible and safe^[72] in specialized environments.

A recent paper targeted to a matched comparison of robotic and laparoscopic liver resections failed to show significative differences between the two techniques^[73]. Long-term outcomes, larger patient records and comparative studies (with open surgery and pure laparoscopy) are not available yet.

PANCREAS

Pancreatic cancer still represents one of the major challenge for the oncologic surgeons due to complex reconstruction, high perioperative morbidity and mortality and poor overall survival. Thus, the advent of laparoscopy was advisable and exciting, in order to minimize operative complications and maximize the early recovery of the patients. On the other hand, the specific technical

difficulties and the relative low incidence of pancreatic cancer, have limited the laparoscopic approach to few specialized centers with great experience in both pancreatic surgery and advanced laparoscopy^[74]. Moreover, the problem of pancreatic remnant fistula is the same that in open surgery, while some initial and more recent sporadic port-site recurrences were reported in literature^[75].

Historically, the first procedure carried out by laparoscopy was distal pancreatectomy for benign disease, because it does not require any anastomosis. However, the preservation of the spleen, when dealing with benign or neuroendocrine tumor, remains challenging^[76,77].

A very comprehensive review of the literature by Iacobone *et al.*^[78] found more than 300 articles regarding laparoscopic left or distal pancreatectomies (LDP), but most were case-series, with short-term follow-up, different techniques and confused data reporting. Similar findings were reported by Borja-Cacho *et al.*^[79]. In addition, the experiences with pancreatic adenocarcinoma or Intraductal Papillary Mucinous Neoplasm (IPMN) were much more limited^[80].

One of the largest single-center case series on LDP was published from the Memorial Sloan Kettering Center^[81] on more than 300 cases over a 7-year period, resulting in excellent outcomes (27% *vs* 40% of postoperative complication, $P = 0.03$, as compared to standard surgery). LDP seems to be almost the standard of care for many centers, in order to achieve a systematic reduction of blood loss and postoperative stay, although a careful patients selection is often advocated^[82].

A comparative study demonstrated the cost-effectiveness of LDP as compared to open surgery, when considering the reduction of hospital stay (5 d *vs* 7 d, $P < 0.001$)^[83], while another^[84] reported increasing experience and more complex patients selection although maintaining the same morbidity over a 11-years period.

When limiting literature search to case-matched study, Pericleous^[85] identified only 4 articles that fit for quality assessment (but none was a RCT): results were that LDP had a longer operative time, but reduced length of postoperative stay without any differences in perioperative morbidity and mortality, as compared to open surgery. Another similar and more recent meta-analysis^[86] found 18 comparative studies including more than 1800 patients. LDP reduced blood loss, length of hospital stay, and overall complications, without increasing the duration of surgery significantly. However, no definitive conclusions were drawn regarding the oncologic safety, although the rate of margins positivity was comparable between open and laparoscopic resection.

As the numbers of laparoscopic advanced procedures increase, some centers began to perform also laparoscopic pancreatodudenectomies (LPD) for malignancies. Some advantages over traditional surgery and comparable oncologic outcomes are reported, although long learning curves limit these initial experiences to subspecialized surgical teams^[87-92].

A single center case series (from United Kingdom)^[93]

with a final review, identified an increasing number of LDP and LPD performed, but almost all were reported in poor quality studies and limited number of patients. The authors concluded that laparoscopic pancreatic procedures should be reserved to selected cases with benign to low grade malignancies. Nonetheless, major vessels resection for malignant involvement have also been reported to be completed by laparoscopy^[94].

Few articles reported the oncologic main outcomes, including numbers of nodes harvested, margins of resection, disease-free survival and overall survival. A review by Fischer *et al*^[95] was specifically targeted to laparoscopic pancreatectomies for malignancies to assess those issues. Early results seemed to be oncologically adequate for LDP, while literature, in general, was highly insufficient for LPD. Another recent review of Kudsi *et al*^[96] concluded that, although becoming highly popular, LDP for aggressive tumors may not be appropriate due to the lack of oncologic safety studies.

A very recent single-center series of 200 consecutive laparoscopic pancreatic resections (including LDP, LPD and other more limited procedures) reported excellent result with the use of a robotic controlled laparoscope holder^[97].

Some surgeons argue that full robotic surgery could ease many difficult technical maneuvers of the laparoscopic approach, including biliary and pancreatic anastomosis or preservation of the spleen^[98-101]. Others^[102-104] reported more encouraging early experiences with robotic-assisted pancreatectomies as compared to open approach. A meta-analysis of Zhang *et al*^[105] including 7 trials suggested that robotic pancreatectomy is as safe and efficient as, if not superior to, open surgery but the evidence is highly insufficient.

To the present, those excellent results with robotics are far to be reproducible in most centers worldwide.

SMALL BOWEL

Due to the relative low incidence of small bowel carcinomas, most of the laparoscopic resections carried out for malignancy include gastrointestinal stromal tumours (GISTs). According to the well-known peculiar biologic tumoral behaviour, very wide margin and formal lymphadenectomy are unnecessary for GISTs^[106]. Therefore, it seems that laparoscopy could be particularly indicated to manage these neoplasms and a variety of endoscopic, laparoscopic, and hybrid techniques are described to surgically excise GISTs of different anatomic locations^[107].

However, few papers are specifically targeted to small bowel resections and quality of studies is generally poor (no randomization). Nevertheless, initial experiences reported the laparoscopic treatment of small bowel GISTs to be safe and effective, without oncologic outcome impairment^[108,109].

A retrospective comparative study^[110] including 9 and 11 patients each arm only, analyzed laparoscopic approach

to small bowel tumors compared to open surgery. Despite the insignificant number of patients and the statistical insufficiency of the sample, the authors found how laparoscopic resection favoured short-term outcomes in selected cases. Other similar results were also published^[111].

In conclusions, although many of the results advocated for small bowel GISTs are extrapolated from gastric series, it seems that laparoscopic resections of GISTs lead to excellent outcomes in term of perioperative and oncologic outcomes.

COLON AND RECTUM

To the present, laparoscopic treatment of colorectal cancer has becoming the gold standard of care, and has gained large diffusion worldwide^[112-114]. The main reasons are represented by the highest number of good quality studies published, including many RCTs with long follow-up and meta-analysis, the high incidence of colorectal cancer, that permits adequate case-load and the acceptable technical challenge^[115,116].

Although laparoscopic colorectal resection (LCR) is feasible in around 90% of elective cancer patients^[117] and excellent results are achieved also outside clinical trials^[118], many smaller centers still continue to perform routine colorectal operation using the traditional open approach due to the lack of laparoscopic expertise or devices and, probably, some socio-economic disparities^[119]. The widespread acceptance of laparoscopic rectal resections, in which some surgeon have demonstrated advantages of robotics, has been slower compared to colon resections.

The most important multicenter RCTs were published in the early 2000's from the Clinical Outcomes of Surgical Therapy Study Group (COST trial)^[120], led by Nelson of the Mayo Clinic (Rochester, MN, United States), the Colon Cancer Laparoscopic or Open Resection Study Group (COLOR trial)^[121] arisen in Europe, the United Kingdom Medical Research Council (MRC CLASSIC trial)^[122], and the Barcellona^[123] and the Australasian (ALCCAS Trial)^[124] groups.

All these trials confirmed, in the short-term period, that the use of laparoscopic colon resection maximizes the outcomes without compromising oncological results. Surprisingly, the Barcellona^[123] trial showed an increased survival in the stage III patients treated laparoscopically, while the CLASSIC trial^[122] reported inferior results for laparoscopic anterior rectal resection that lead the authors to advise against the adoption of this specific procedure.

A meta-analysis^[125] of the first four randomized trials (COST, COLOR, CLASSIC and Barcellona, involving 1765 patients overall), with at least 3 years of complete follow-up, confirmed that laparoscopy for colon cancer was oncologically safe (3-year disease-free survival rates in the laparoscopically assisted and open arms were 75.8% and 75.3%, respectively; the 3-year overall survival rates 82.2% and 83.5%; without any difference between stages).

In addition, a very comprehensive review and meta-analysis from the Cochrane Group^[126] including the best 25 RCTs (3526 patients) stated that, although operative time was longer in the laparoscopic surgery, many parameters such as the intraoperative blood loss, postoperative pain and ileus, hospital stay and quality of life at the 30th day were superior in comparison to open surgery. Therefore, total morbidity and local (surgical) morbidity were decreased in the laparoscopic groups. General (non-surgical) morbidity and mortality were not different between both groups. Some benefits of LCR for cancer rather than the oncologic outcome, could be stronger in the elderly people^[127] or in the long-term period, including reduction of adhesions and incisional hernias^[128].

On the other hand, when considering the absolute values (rather than statistic difference) of some of the short-term advantages of the trials, only very few benefits were detectable (for example 5-9 *vs* 6-12 postoperative days)^[126], with comparable overall morbidity and mortality, while some trials reported increased duration of surgery for LCR^[129].

The oncologic long-term results were also tested in the COST trial, demonstrating the non-inferiority of LCR in terms of disease-free 5-year survival, overall 5-year survival and sites of recurrence^[130]. Similarly, the long-term outcomes of the COLOR^[131] trial found a statistically insignificant difference in favour of open colectomy, while the Barcellona^[132] trial confirmed how LCR was associated with a reduced risk of tumor relapse. Also the CLASSIC trial^[133] confirmed, after a 5-year analysis, the oncological safety of laparoscopic surgery for both colonic and rectal cancer. A more recent Australasian RCT reported similar long-term oncologic outcomes (recurrence and survival) between open and LCR, although it found some short-term surrogate oncological markers (smaller distal resection margin) to be worst in the laparoscopic group^[134].

Another specific meta-analysis from the Cochrane Group^[4], including 33 RCTs and 3346 patients, concluded that laparoscopic resection of carcinoma of the colon is associated with a long-term outcome not different from that of open colectomy, although more RCTs are needed to confirm long-term outcomes for rectal cancer and the real incidence of incisional hernias and adhesions.

Recent pioneeristic experiences begin to report the application of NOTES (Natural Orifice Transluminal Endoscopic Surgery)^[135,136] or SILC (Single Incision Laparoscopic Colectomy)^[137] for colon cancer, but they should be considered absolutely insufficient to be proposed in routine clinical practice.

Data supporting the routine laparoscopic approach to rectal cancer are still incomplete, and the first experiences failed to confirm oncologic safety (CLASSIC trial, not statistically significant increase of positive margins)^[122], while a specific Cochrane review^[138] including 80 poor quality studies and only 1 RCT did not assess safety of the procedure. However, many data come from patients

operated at the end of the nineties or beginning of the twenties, thus justifying some technical mistake in the hands of surgeons without great experience. Moreover, there is also a generalized scientific confusion in the definition of rectal cancer, the distinction between low and medium rectal cancer, the standardization of total mesorectal excision (TME) and the need of perioperative radiochemotherapy. All these issues contribute to jeopardize results and increase difficulties in data reporting.

A very recent RCT from the same group (European centers) of COLOR trial^[139] was targeted to laparoscopic treatment of rectal cancer (LRR) with more encouraging results in terms of similar safety, excision margins, and completeness of resection to that of open surgery. Indeed, completeness of the resection was not different between laparoscopy and open surgery (88% *vs* 92%; *P* = 0.250), while a positive circumferential resection margin (< 2 mm) and a median distal resection margin were of 10% and 3 cm in both groups respectively. Recovery was confirmed to be improved after laparoscopic surgery, although the results for the primary endpoint - locoregional recurrence - are expected by the end of 2013.

Similar early good results were reached by another recent Korean trial^[140] (170 patients each arm of study), when considering blood loss, pain and recovery, that were superior in the laparoscopic group without differences in the margin of resection. Moreover, a very large (more than 4000 patients) non-randomised Spanish trial^[141] concluded that laparoscopic surgery is the best option for the surgical treatment of rectal cancer, with similar rates of local recurrence and survival.

Despite the lack of any evidence to support its use, some surgeons began to perform colorectal surgery by the robotic-assisted technique^[142-144]. A large retrospective review of colorectal operation in the United States found the percentage of robotic operations to be less than 3%, without any tangible advantages over conventional laparoscopy (except for decreased conversion rates) and higher rate of postoperative bleeding^[145].

To resolve the intrinsic difficulty of performing a formal laparoscopic TME, many centers with the available technology and expertise, introduced the use of robot to perform LRR^[146]. However, robotic rectal surgery is at least more expensive than laparoscopy^[147] and probably equivalent in terms of short term results^[148]. Nevertheless, oncologic early results (number of harvested nodes, distal and circumferential margins, port-site recurrence) lead to consider robotic rectal resections safe^[149,150]. A prospective, international, multicenter, RCT was recently designed to test robotic versus standard LRR^[151].

CONCLUSION

The dramatic widespread popularity of laparoscopy has significantly changed the surgical approach to gastrointestinal malignancy toward less invasive, miniinvasive, laparoscopic, hybrid and robotic interventions. Excellent

Table 1 Recommended approach to gastrointestinal malignancies

District	Open surgery	Laparoscopic surgery	Robotic	Level of Evidence ¹
Esophagus	Standard	Accepted	Pioneeristic	LE 2
Stomach (proximal)	Standard	Being accepted	Pioneeristic	LE 3
Stomach (distal)	Standard	Accepted	Pioneeristic	LE 2
Liver (major resection)	Standard	Pioneeristic	Pioneeristic	LE 4
Liver (minor resection)	Standard	Being standard	Pioneeristic	LE 3
Gallbladder	Standard	Pioneeristic	Pioneeristic	LE 5
Biliary tree	Standard	Pioneeristic	Pioneeristic	LE 5
Pancreas (head)	Standard	Pioneeristic	Pioneeristic	LE 4
Pancreas (body-tail)	Standard	Being standard	Pioneeristic	LE 3
Small bowel	Standard	Being standard	Pioneeristic	LE 5
Colon	Accepted	Standard	Pioneeristic	LE 1
Rectum	Standard	Being standard	Pioneeristic	LE 2

¹Oxford Centre for Evidence-Based Medicine. Levels of Evidence Working Group. "The Oxford 2011 Levels of Evidence". Available from: URL: <http://www.cebm.net/index.aspx?o=5653>.

results in terms of reduction of postoperative "stress" (including immunologic response, pain, overall morbidity, length of stay, self-corporeal appearance and mortality) have been reported from North America, Europe and Eastern countries.

Laparoscopy is now accepted and, probably, recognized as the gold standard in the management of colorectal malignancy in most of hospitals worldwide. GISTs should also be treated by laparoscopy whenever feasible, and very good results have recently been reported for gastric (mainly distal stomach), esophageal and pancreatic (mostly tail) cancers as well. Total gastrectomy, pancreaticoduodenectomy and major hepatic resections (except for left lateral segments) should be considered pioneeristic operations, reserved to few surgeons within rigorous clinical protocol studies (Table 1).

However, the lack (and the intrinsic difficulty of techniques) of RCTs still leaves many important unresolved issues. The cornerstone of oncologic safety, the real benefits for the cancerous patients and the cost-effectiveness, in the setting of limiting health care resources, are the principal ones. It is also well established that advanced laparoscopic techniques, especially for malignant disease, should be initiated and carried out only in selected tertiary centers with the greatest surgical experience in both laparoscopy and surgical oncology. Moreover, every new laparoscopic program should be tutored, monitored and validated by a final and a long-term oncologic follow-up.

REFERENCES

- 1 Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. GLOBOCAN 2008, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. Lyon: International Agency for Research on Cancer, 2010. Available from: URL: <http://globocan.iarc.fr>, accessed on day/month/year
- 2 Goldfarb M, Brower S, Schwaitzberg SD. Minimally invasive surgery and cancer: controversies part 1. *Surg Endosc* 2010; **24**: 304-334 [PMID: 19572178 DOI: 10.1007/s00464-009-0583-3]
- 3 Sharma B, Baxter N, Grantcharov T. Outcomes after laparoscopic techniques in major gastrointestinal surgery. *Curr Opin Crit Care* 2010; **16**: 371-376 [PMID: 20613501 DOI: 10.1097/MCC.0b013e32833b0480]
- 4 Kuhry E, Schwenk WF, Gaupset R, Romild U, Bonjer HJ. Long-term results of laparoscopic colorectal cancer resection. *Cochrane Database Syst Rev* 2008; (2): CD003432 [PMID: 18425886 DOI: 10.1002/14651858.CD003432.pub2]
- 5 Zanghi A, Cavallaro A, Piccolo G, Fisichella R, Di Vita M, Spartà D, Zanghi G, Berretta S, Palermo F, Cappellani A. Dissemination metastasis after laparoscopic colorectal surgery versus conventional open surgery for colorectal cancer: a metaanalysis. *Eur Rev Med Pharmacol Sci* 2013; **17**: 1174-1184 [PMID: 23690186]
- 6 Torab FC, Bokobza B, Branicki F. Laparoscopy in gastrointestinal malignancies. *Ann N Y Acad Sci* 2008; **1138**: 155-161 [PMID: 18837896 DOI: 10.1196/annals.1414.022]
- 7 Luketich JD, Pennathur A, Awais O, Levy RM, Keeley S, Shende M, Christie NA, Weksler B, Landreneau RJ, Abbas G, Schuchert MJ, Nason KS. Outcomes after minimally invasive esophagectomy: review of over 1000 patients. *Ann Surg* 2012; **256**: 95-103 [PMID: 22668811 DOI: 10.1097/SLA.0b013e3182590603]
- 8 Utley L, Campbell F, Rhodes M, Cantrell A, Stegenga H, Lloyd-Jones M. Minimally invasive oesophagectomy versus open surgery: is there an advantage? *Surg Endosc* 2013; **27**: 724-731 [PMID: 23052523]
- 9 Butler N, Collins S, Memon B, Memon MA. Minimally invasive oesophagectomy: current status and future direction. *Surg Endosc* 2011; **25**: 2071-2083 [PMID: 21298548 DOI: 10.1007/s00464-010-1511-2]
- 10 Biere SS, van Berge Henegouwen MI, Maas KW, Bonavina L, Rosman C, Garcia JR, Gisbertz SS, Klinkenbijn JH, Hollmann MW, de Lange ES, Bonjer HJ, van der Peet DL, Cuesta MA. Minimally invasive versus open oesophagectomy for patients with oesophageal cancer: a multicentre, open-label, randomised controlled trial. *Lancet* 2012; **379**: 1887-1892 [PMID: 22552194 DOI: 10.1016/S0140-6736(12)60516-9]
- 11 Kinjo Y, Kurita N, Nakamura F, Okabe H, Tanaka E, Kataoka Y, Itami A, Sakai Y, Fukuhara S. Effectiveness of combined thoracoscopic-laparoscopic esophagectomy: comparison of postoperative complications and midterm oncological outcomes in patients with esophageal cancer. *Surg Endosc* 2012; **26**: 381-390 [PMID: 21898014 DOI: 10.1007/s00464-011-1883-y]
- 12 Briez N, Piessen G, Bonnetain F, Brigand C, Carrere N, Collet D, Doddoli C, Flamein R, Mabrut JY, Meunier B, Msika S, Perniceni T, Peschard F, Prudhomme M, Triboulet JP, Mariette C. Open versus laparoscopically-assisted oesophagectomy for cancer: a multicentre randomised controlled phase III trial - the MIRO trial. *BMC Cancer* 2011; **11**: 310 [PMID: 21781337 DOI: 10.1186/1471-2407-11-310]

- 13 **Maas KW**, Biere SS, Scheepers JJ, Gisbertz SS, Turrado Rodriguez VT, van der Peet DL, Cuesta MA. Minimally invasive intrathoracic anastomosis after Ivor Lewis esophagectomy for cancer: a review of transoral or transthoracic use of staplers. *Surg Endosc* 2012; **26**: 1795-1802 [PMID: 22294057 DOI: 10.1007/s00464-012-2149-z]
- 14 **Schumer E**, Perry K, Melvin WS. Minimally invasive esophagectomy for esophageal cancer: evolution and review. *Surg Laparosc Endosc Percutan Tech* 2012; **22**: 383-386 [PMID: 23047377 DOI: 10.1097/SLE.0b013e31826295a4]
- 15 **Hanna GB**, Arya S, Markar SR. Variation in the standard of minimally invasive esophagectomy for cancer--systematic review. *Semin Thorac Cardiovasc Surg* 2012; **24**: 176-187 [PMID: 23200072 DOI: 10.1053/j.semtcvs.2012.10.004]
- 16 **Clark J**, Sodergren MH, Purkayastha S, Mayer EK, James D, Athanasiou T, Yang GZ, Darzi A. The role of robotic assisted laparoscopy for oesophagogastric oncological resection; an appraisal of the literature. *Dis Esophagus* 2011; **24**: 240-250 [PMID: 21073622 DOI: 10.1111/j.1442-2050.2010.01129.x]
- 17 **de la Fuente SG**, Weber J, Hoffe SE, Shridhar R, Karl R, Meredith KL. Initial experience from a large referral center with robotic-assisted Ivor Lewis esophagogastric resection for oncologic purposes. *Surg Endosc* 2013; **27**: 3339-3347 [PMID: 23549761 DOI: 10.1007/s00464-013-2915-6]
- 18 **van der Sluis PC**, Ruurda JP, van der Horst S, Verhage RJ, Besselink MG, Prins MJ, Haverkamp L, Schippers C, Rinkes IH, Joore HC, Ten Kate FJ, Koffijberg H, Kroese CC, van Leeuwen MS, Lolkema MP, Reerink O, Schipper ME, Steenhagen E, Vleggaar FP, Voest EE, Siersema PD, van Hillegersberg R. Robot-assisted minimally invasive thoraco-laparoscopic esophagectomy versus open transthoracic esophagectomy for resectable esophageal cancer, a randomized controlled trial (ROBOT trial). *Trials* 2012; **13**: 230 [PMID: 23199187 DOI: 10.1186/1745-6215-13-230]
- 19 **Liao GQ**, Ou XW, Liu SQ, Zhang SR, Huang W. Laparoscopy-assisted total gastrectomy with trans-orally inserted anvil (OrVil™): a single institution experience. *World J Gastroenterol* 2013; **19**: 755-760 [PMID: 23431026 DOI: 10.3748/wjg.v19.i5.755]
- 20 **Chen K**, Xu XW, Zhang RC, Pan Y, Wu D, Mou YP. Systematic review and meta-analysis of laparoscopy-assisted and open total gastrectomy for gastric cancer. *World J Gastroenterol* 2013; **19**: 5365-5376 [PMID: 23983442 DOI: 10.3748/wjg.v19.i32.5365]
- 21 **Cui M**, Xing JD, Yang W, Ma YY, Yao ZD, Zhang N, Su XQ. D2 dissection in laparoscopic and open gastrectomy for gastric cancer. *World J Gastroenterol* 2012; **18**: 833-839 [PMID: 22371644 DOI: 10.3748/wjg.v18.i8.833]
- 22 **Shinohara T**, Kanaya S, Taniguchi K, Fujita T, Yanaga K, Uyama I. Laparoscopic total gastrectomy with D2 lymph node dissection for gastric cancer. *Arch Surg* 2009; **144**: 1138-1142 [PMID: 20026832 DOI: 10.1001/archsurg.2009.223]
- 23 **Zhao XF**, Jeong O, Jung MR, Ryu SY, Park YK. A propensity score-matched case-control comparative study of laparoscopic and open extended (D2) lymph node dissection for distal gastric carcinoma. *Surg Endosc* 2013; **27**: 2792-2800 [PMID: 23389075 DOI: 10.1007/s00464-013-2809-7]
- 24 **Yakoub D**, Athanasiou T, Tekkis P, Hanna GB. Laparoscopic assisted distal gastrectomy for early gastric cancer: is it an alternative to the open approach? *Surg Oncol* 2009; **18**: 322-333 [PMID: 18922689 DOI: 10.1016/j.suronc.2008.08.006]
- 25 **Kodera Y**, Fujiwara M, Ohashi N, Nakayama G, Koike M, Morita S, Nakao A. Laparoscopic surgery for gastric cancer: a collective review with meta-analysis of randomized trials. *J Am Coll Surg* 2010; **211**: 677-686 [PMID: 20869270 DOI: 10.1016/j.jamcollsurg.2010.07.013]
- 26 **Wei HB**, Wei B, Qi CL, Chen TF, Huang Y, Zheng ZH, Huang JL, Fang JF. Laparoscopic versus open gastrectomy with D2 lymph node dissection for gastric cancer: a meta-analysis. *Surg Laparosc Endosc Percutan Tech* 2011; **21**: 383-390 [PMID: 22146158 DOI: 10.1097/SLE.0b013e31822d02dc]
- 27 **Sun J**, Li J, Wang J, Pan T, Zhou J, Fu X, Zhang S. Meta-analysis of randomized controlled trials on laparoscopic gastrectomy vs. open gastrectomy for distal gastric cancer. *Hepatogastroenterology* 2012; **59**: 1699-1705 [PMID: 22626787 DOI: 10.5754/hge12259]
- 28 **Bracale U**, Rovani M, Bracale M, Pignata G, Corcione F, Pecchia L. Totally laparoscopic gastrectomy for gastric cancer: meta-analysis of short-term outcomes. *Minim Invasive Ther Allied Technol* 2012; **21**: 150-160 [PMID: 21619505 DOI: 10.3109/13645706.2011.588712]
- 29 **Pavlidis TE**, Pavlidis ET, Sakantamis AK. The role of laparoscopic surgery in gastric cancer. *J Minim Access Surg* 2012; **8**: 35-38 [PMID: 22623823 DOI: 10.4103/0972-9941.95524]
- 30 **Phillips JD**, Nagle AP, Soper NJ. Laparoscopic gastrectomy for cancer. *Surg Oncol Clin N Am* 2013; **22**: 39-57, v-vi [PMID: 23158084 DOI: 10.1016/j.soc.2012.08.004]
- 31 **Lee JH**, Son SY, Lee CM, Ahn SH, Park do J, Kim HH. Morbidity and mortality after laparoscopic gastrectomy for advanced gastric cancer: results of a phase II clinical trial. *Surg Endosc* 2013; **27**: 2877-2885 [PMID: 23404155 DOI: 10.1007/s00464-013-2848-0]
- 32 **Wang W**, Chen K, Xu XW, Pan Y, Mou YP. Case-matched comparison of laparoscopy-assisted and open distal gastrectomy for gastric cancer. *World J Gastroenterol* 2013; **19**: 3672-3677 [PMID: 23801871 DOI: 10.3748/wjg.v19.i23.3672]
- 33 **Lee HJ**, Yang HK. Laparoscopic gastrectomy for gastric cancer. *Dig Surg* 2013; **30**: 132-141 [PMID: 23867590 DOI: 10.1159/000350884]
- 34 **Liang Y**, Li G, Chen P, Yu J, Zhang C. Laparoscopic versus open gastrectomy for early distal gastric cancer: a meta-analysis. *ANZ J Surg* 2011; **81**: 673-680 [PMID: 22295306 DOI: 10.1111/j.1445-2197.2010.05599.x]
- 35 **Wang W**, Li Z, Tang J, Wang M, Wang B, Xu Z. Laparoscopic versus open total gastrectomy with D2 dissection for gastric cancer: a meta-analysis. *J Cancer Res Clin Oncol* 2013; **139**: 1721-1734 [PMID: 23990014 DOI: 10.1007/s00432-013-1462-9]
- 36 **Chen K**, Xu XW, Mou YP, Pan Y, Zhou YC, Zhang RC, Wu D. Systematic review and meta-analysis of laparoscopic and open gastrectomy for advanced gastric cancer. *World J Surg Oncol* 2013; **11**: 182 [PMID: 23927773 DOI: 10.1186/1477-7819-11-182]
- 37 **Yu J**, Hu J, Huang C, Ying M, Peng X, Wei H, Jiang Z, Du X, Liu Z, Liu H, Li G. The impact of age and comorbidity on postoperative complications in patients with advanced gastric cancer after laparoscopic D2 gastrectomy: results from the Chinese laparoscopic gastrointestinal surgery study (CLASS) group. *Eur J Surg Oncol* 2013; **39**: 1144-1149 [PMID: 23850088]
- 38 **Huscher CG**, Mingoli A, Sgarzini G, Brachini G, Binda B, Di Paola M, Ponzano C. Totally laparoscopic total and subtotal gastrectomy with extended lymph node dissection for early and advanced gastric cancer: early and long-term results of a 100-patient series. *Am J Surg* 2007; **194**: 839-844; discussion 844 [PMID: 18005781 DOI: 10.1016/j.amjsurg.2007.08.037]
- 39 **Kim HH**, Hyung WJ, Cho GS, Kim MC, Han SU, Kim W, Ryu SW, Lee HJ, Song KY. Morbidity and mortality of laparoscopic gastrectomy versus open gastrectomy for gastric cancer: an interim report--a phase III multicenter, prospective, randomized Trial (KLASS Trial). *Ann Surg* 2010; **251**: 417-420 [PMID: 20160637 DOI: 10.1097/SLA.0b013e3181cc8f6b]
- 40 **Yoshikawa T**, Cho H, Rino Y, Yamamoto Y, Kimura M, Fukunaga T, Hasegawa S, Yamada T, Aoyama T, Tsuburaya A. A prospective feasibility and safety study of laparoscopy-assisted distal gastrectomy for clinical stage I gastric cancer initiated by surgeons with much experience of open gastrectomy and laparoscopic surgery. *Gastric Cancer* 2013; **16**: 126-132 [PMID: 22527185 DOI: 10.1007/s10120-012-0157-2]
- 41 **Uyama I**, Suda K, Satoh S. Laparoscopic surgery for advanced gastric cancer: current status and future perspectives

- tives. *J Gastric Cancer* 2013; **13**: 19-25 [PMID: 23610715 DOI: 10.5230/jgc.2013.13.1.19]
- 42 **Huscher CG**, Mingoli A, Sgarzini G, Sansonetti A, Di Paola M, Recher A, Ponzano C. Laparoscopic versus open subtotal gastrectomy for distal gastric cancer: five-year results of a randomized prospective trial. *Ann Surg* 2005; **241**: 232-237 [PMID: 15650632 DOI: 10.1097/01.sla.0000151892.35922.f2]
 - 43 **Rosin D**, Goldes Y, Bar Zakai B, Shabtai M, Ayalon A, Zmora O. Laparoscopic subtotal gastrectomy for gastric cancer. *JSLs* 2009; **13**: 318-322 [PMID: 19793469]
 - 44 **Bracale U**, Pignata G, Lirici MM, Hüscher CG, Pugliese R, Sgroi G, Romano G, Spinoglio G, Gualtierotti M, Maglione V, Azagra S, Kanehira E, Kim JG, Song KY. Laparoscopic gastrectomies for cancer: The ACOI-IHTSC national guidelines. *Minim Invasive Ther Allied Technol* 2012; **21**: 313-319 [PMID: 22793780 DOI: 10.3109/13645706.2012.704877]
 - 45 **Xiong B**, Ma L, Zhang C. Robotic versus laparoscopic gastrectomy for gastric cancer: a meta-analysis of short outcomes. *Surg Oncol* 2012; **21**: 274-280 [PMID: 22789391 DOI: 10.1016/j.suronc.2012.05.004]
 - 46 **Koh YX**, Chok AY, Zheng HL, Tan CS, Chow PK, Wong WK, Goh BK. A systematic review and meta-analysis comparing laparoscopic versus open gastric resections for gastrointestinal stromal tumors of the stomach. *Ann Surg Oncol* 2013; **20**: 3549-3560 [PMID: 23793362 DOI: 10.1245/s10434-013-3051-1]
 - 47 **Liang JW**, Zheng ZC, Zhang JJ, Zhang T, Zhao Y, Yang W, Liu YQ. Laparoscopic versus open gastric resections for gastric gastrointestinal stromal tumors: a meta-analysis. *Surg Laparosc Endosc Percutan Tech* 2013; **23**: 378-387 [PMID: 23917593 DOI: 10.1097/SLE.0b013e31828e3e9d]
 - 48 **Laurence JM**, Lam VW, Langcake ME, Hollands MJ, Crawford MD, Pleass HC. Laparoscopic hepatectomy, a systematic review. *ANZ J Surg* 2007; **77**: 948-953 [PMID: 17931255 DOI: 10.1111/j.1445-2197.2007.04288.x]
 - 49 **Pilgrim CH**, To H, Usatoff V, Evans PM. Laparoscopic hepatectomy is a safe procedure for cancer patients. *HPB (Oxford)* 2009; **11**: 247-251 [PMID: 19590655 DOI: 10.1111/j.1477-2574.2009.00045.x]
 - 50 **Mizuguchi T**, Kawamoto M, Meguro M, Shibata T, Nakamura Y, Kimura Y, Furuhashi T, Sonoda T, Hirata K. Laparoscopic hepatectomy: a systematic review, meta-analysis, and power analysis. *Surg Today* 2011; **41**: 39-47 [PMID: 21191689 DOI: 10.1007/s00595-010-4337-6]
 - 51 **Buell JF**, Cherqui D, Geller DA, O'Rourke N, Iannitti D, Dagher I, Koffron AJ, Thomas M, Gayet B, Han HS, Wakabayashi G, Belli G, Kaneko H, Ker CG, Scatton O, Laurent A, Abdalla EK, Chaudhury P, Dutson E, Gamblin C, D'Angelica M, Nagorney D, Testa G, Labow D, Manas D, Poon RT, Nelson H, Martin R, Clary B, Pinson WC, Martinie J, Vauthier JN, Goldstein R, Roayaie S, Barlet D, Espat J, Abecassis M, Rees M, Fong Y, McMasters KM, Broelsch C, Busuttil R, Belghiti J, Strasberg S, Chari RS. The international position on laparoscopic liver surgery: The Louisville Statement, 2008. *Ann Surg* 2009; **250**: 825-830 [PMID: 19916210 DOI: 10.1097/SLA.0b013e3181b3b2d8]
 - 52 **Hasegawa Y**, Nitta H, Sasaki A, Takahara T, Ito N, Fujita T, Kanno S, Nishizuka S, Wakabayashi G. Laparoscopic left lateral sectionectomy as a training procedure for surgeons learning laparoscopic hepatectomy. *J Hepatobiliary Pancreat Sci* 2013; **20**: 525-530 [PMID: 23430054 DOI: 10.1007/s00534-012-0591-x]
 - 53 **Doughtie CA**, Egger ME, Cannon RM, Martin RC, McMasters KM, Scoggins CR. Laparoscopic hepatectomy is a safe and effective approach for resecting large colorectal liver metastases. *Am Surg* 2013; **79**: 566-571 [PMID: 23711264]
 - 54 **Belli G**, Gayet B, Han HS, Wakabayashi G, Kim KH, Cannon R, Kaneko H, Gamblin C, Koffron A, Dagher I, Buell JF. Laparoscopic left hemihepatectomy: a consideration for acceptance as standard of care. *Surg Endosc* 2013; **27**: 2721-2726 [PMID: 23436090 DOI: 10.1007/s00464-013-2840-8]
 - 55 **Vibert E**, Perniceni T, Levard H, Denet C, Shahri NK, Gayet B. Laparoscopic liver resection. *Br J Surg* 2006; **93**: 67-72 [PMID: 16273531 DOI: 10.1002/bjs.5150]
 - 56 **Dagher I**, Belli G, Fantini C, Laurent A, Tayar C, Lainas P, Tranchart H, Franco D, Cherqui D. Laparoscopic hepatectomy for hepatocellular carcinoma: a European experience. *J Am Coll Surg* 2010; **211**: 16-23 [PMID: 20610244 DOI: 10.1016/j.jamcollsurg.2010.03.012]
 - 57 **Kaneko H**, Tsuchiya M, Otsuka Y, Yajima S, Minagawa T, Watanabe M, Tamura A. Laparoscopic hepatectomy for hepatocellular carcinoma in cirrhotic patients. *J Hepatobiliary Pancreat Surg* 2009; **16**: 433-438 [PMID: 19458892 DOI: 10.1007/s00534-009-0123-5]
 - 58 **Lee KF**, Chong CN, Wong J, Cheung YS, Wong J, Lai P. Long-term results of laparoscopic hepatectomy versus open hepatectomy for hepatocellular carcinoma: a case-matched analysis. *World J Surg* 2011; **35**: 2268-2274 [PMID: 21842300 DOI: 10.1007/s00268-011-1212-6]
 - 59 **Qiu J**, Chen S, Pankaj P, Wu H. Laparoscopic hepatectomy for hepatic colorectal metastases -- a retrospective comparative cohort analysis and literature review. *PLoS One* 2013; **8**: e60153 [PMID: 23555908 DOI: 10.1371/journal.pone.0060153]
 - 60 **Zhang L**, Chen YJ, Shang CZ, Zhang HW, Huang ZJ. Total laparoscopic liver resection in 78 patients. *World J Gastroenterol* 2009; **15**: 5727-5731 [PMID: 19960572 DOI: 10.3748/wjg.15.5727]
 - 61 **Koffron AJ**, Auffenberg G, Kung R, Abecassis M. Evaluation of 300 minimally invasive liver resections at a single institution: less is more. *Ann Surg* 2007; **246**: 385-392; discussion 392-394 [PMID: 17717442 DOI: 10.1097/SLA.0b013e318146996c]
 - 62 **Rao AM**, Ahmed I. Laparoscopic versus open liver resection for benign and malignant hepatic lesions in adults. *Cochrane Database Syst Rev* 2013; **5**: CD010162 [PMID: 23728700 DOI: 10.1002/14651858.CD010162.pub2]
 - 63 **Ito F**, Cho CS, Rikkers LF, Weber SM. Hilar cholangiocarcinoma: current management. *Ann Surg* 2009; **250**: 210-218 [PMID: 19638920 DOI: 10.1097/SLA.0b013e3181afe0ab]
 - 64 **Lau SH**, Lau WY. Current therapy of hilar cholangiocarcinoma. *Hepatobiliary Pancreat Dis Int* 2012; **11**: 12-17 [PMID: 22251465 DOI: 10.1016/S1499-3872(11)60119-7]
 - 65 **Gumbs AA**, Jarufe N, Gayet B. Minimally invasive approaches to extrapancreatic cholangiocarcinoma. *Surg Endosc* 2013; **27**: 406-414 [PMID: 22926892 DOI: 10.1007/s00464-012-2489-8]
 - 66 **Shih SP**, Schulick RD, Cameron JL, Lillemoe KD, Pitt HA, Choti MA, Campbell KA, Yeo CJ, Talamini MA. Gallbladder cancer: the role of laparoscopy and radical resection. *Ann Surg* 2007; **245**: 893-901 [PMID: 17522515 DOI: 10.1097/SLA.0b013e31806beec2]
 - 67 **Goetze TO**, Paolucci V. Prognosis of incidental gallbladder carcinoma is not influenced by the primary access technique: analysis of 837 incidental gallbladder carcinomas in the German Registry. *Surg Endosc* 2013; **27**: 2821-2828 [PMID: 23404149 DOI: 10.1007/s00464-013-2819-5]
 - 68 **de Aretxabala X**, Leon J, Hepp J, Maluenda F, Roa I. Gallbladder cancer: role of laparoscopy in the management of potentially resectable tumors. *Surg Endosc* 2010; **24**: 2192-2196 [PMID: 20177932 DOI: 10.1007/s00464-010-0925-1]
 - 69 **Aikawa M**, Miyazawa M, Okamoto K, Toshimitsu Y, Okada K, Ueno Y, Yamaguchi S, Koyama I. Single-port laparoscopic hepatectomy: technique, safety, and feasibility in a clinical case series. *Surg Endosc* 2012; **26**: 1696-1701 [PMID: 22179479 DOI: 10.1007/s00464-011-2095-1]
 - 70 **Lai EC**, Yang GP, Tang CN. Robot-assisted laparoscopic liver resection for hepatocellular carcinoma: short-term outcome. *Am J Surg* 2013; **205**: 697-702 [PMID: 23561638 DOI: 10.1016/j.amjsurg.2012.08.015]
 - 71 **Ho CM**, Wakabayashi G, Nitta H, Ito N, Hasegawa Y, Takahara T. Systematic review of robotic liver resection. *Surg Endosc* 2013; **27**: 732-739 [PMID: 23232988 DOI: 10.1007/s00464-012-2547-2]
 - 72 **Shen BY**, Zhan Q, Deng XX, Bo H, Liu Q, Peng CH, Li HW.

- Radical resection of gallbladder cancer: could it be robotic? *Surg Endosc* 2012; **26**: 3245-3250 [PMID: 22648103 DOI: 10.1007/s00464-012-2330-4]
- 73 **Tsung A**, Geller DA, Sukato DC, Sabbaghian S, Tohme S, Steel J, Marsh W, Reddy SK, Bartlett DL. Robotic Versus Laparoscopic Hepatectomy: A Matched Comparison. *Ann Surg* 2013; Epub ahead of print [PMID: 24045442 DOI: 10.1097/SLA.0000000000000250]
 - 74 **Rosales-Velderrain A**, Bowers SP, Goldberg RF, Clarke TM, Buchanan MA, Stauffer JA, Asbun HJ. National trends in resection of the distal pancreas. *World J Gastroenterol* 2012; **18**: 4342-4349 [PMID: 22969197 DOI: 10.3748/wjg.v18.i32.4342]
 - 75 **Young S**, Abbott P, Hughes SJ. Port-site recurrence of pancreatic adenocarcinoma following laparoscopic pancreaticoduodenectomy. *J Gastrointest Surg* 2012; **16**: 2294-2296 [PMID: 23093448 DOI: 10.1007/s11605-012-2050-z]
 - 76 **Salky BA**, Edye M. Laparoscopic pancreatectomy. *Surg Clin North Am* 1996; **76**: 539-545 [PMID: 8669013 DOI: 10.1016/S0039-6109(05)70460-6]
 - 77 **Mabrut JY**, Fernandez-Cruz L, Azagra JS, Bassi C, Delvaux G, Weerts J, Fabre JM, Boulez J, Baulieux J, Peix JL, Gigot JF. Laparoscopic pancreatic resection: results of a multicenter European study of 127 patients. *Surgery* 2005; **137**: 597-605 [PMID: 15962401 DOI: 10.1016/j.surg.2005.02.002]
 - 78 **Iacobone M**, Citton M, Nitti D. Laparoscopic distal pancreatectomy: up-to-date and literature review. *World J Gastroenterol* 2012; **18**: 5329-5337 [PMID: 23082049 DOI: 10.3748/wjg.v18.i38.5329]
 - 79 **Borja-Cacho D**, Al-Refaie WB, Vickers SM, Tuttle TM, Jensen EH. Laparoscopic distal pancreatectomy. *J Am Coll Surg* 2009; **209**: 758-765; quiz 800 [PMID: 19959046 DOI: 10.1016/j.jamcollsurg.2009.08.021]
 - 80 **Gumbs AA**, Chouillard EK. Laparoscopic distal pancreatectomy and splenectomy for malignant tumors. *J Gastrointest Cancer* 2012; **43**: 83-86 [PMID: 22090189 DOI: 10.1007/s12029-011-9347-0]
 - 81 **Jayaraman S**, Gonen M, Brennan MF, D'Angelica MI, DeMatteo RP, Fong Y, Jarnagin WR, Allen PJ. Laparoscopic distal pancreatectomy: evolution of a technique at a single institution. *J Am Coll Surg* 2010; **211**: 503-509 [PMID: 20868976 DOI: 10.1016/j.jamcollsurg.2010.06.010]
 - 82 **Nakamura M**, Nakashima H. Laparoscopic distal pancreatectomy and pancreaticoduodenectomy: is it worthwhile? A meta-analysis of laparoscopic pancreatectomy. *J Hepatobiliary Pancreat Sci* 2013; **20**: 421-428 [PMID: 23224732 DOI: 10.1007/s00534-012-0578-7]
 - 83 **Fox AM**, Pitzul K, Bhojani F, Kaplan M, Moulton CA, Wei AC, McGilvray I, Cleary S, Okrainec A. Comparison of outcomes and costs between laparoscopic distal pancreatectomy and open resection at a single center. *Surg Endosc* 2012; **26**: 1220-1230 [PMID: 22179451 DOI: 10.1007/s00464-011-2061-y]
 - 84 **Kneuert PJ**, Patel SH, Chu CK, Fisher SB, Maithel SK, Sarmiento JM, Weber SM, Staley CA, Kooby DA. Laparoscopic distal pancreatectomy: trends and lessons learned through an 11-year experience. *J Am Coll Surg* 2012; **215**: 167-176 [PMID: 22632910 DOI: 10.1016/j.jamcollsurg.2012.03.023]
 - 85 **Pericleous S**, Middleton N, McKay SC, Bowers KA, Hutchins RR. Systematic review and meta-analysis of case-matched studies comparing open and laparoscopic distal pancreatectomy: is it a safe procedure? *Pancreas* 2012; **41**: 993-1000 [PMID: 22836858 DOI: 10.1097/MPA.0b013e31824f3669]
 - 86 **Venkat R**, Edil BH, Schulick RD, Lidor AO, Makary MA, Wolfgang CL. Laparoscopic distal pancreatectomy is associated with significantly less overall morbidity compared to the open technique: a systematic review and meta-analysis. *Ann Surg* 2012; **255**: 1048-1059 [PMID: 22511003 DOI: 10.1097/SLA.0b013e318251ee09]
 - 87 **Dulucq JL**, Wintringer P, Mahajna A. Laparoscopic pancreaticoduodenectomy for benign and malignant diseases. *Surg Endosc* 2006; **20**: 1045-1050 [PMID: 16736311 DOI: 10.1007/s00464-005-0474-1]
 - 88 **Palanivelu C**, Jani K, Senthilnathan P, Parthasarathi R, Rajapandian S, Madhankumar MV. Laparoscopic pancreaticoduodenectomy: technique and outcomes. *J Am Coll Surg* 2007; **205**: 222-230 [PMID: 17660068 DOI: 10.1016/j.jamcollsurg.2007.04.004]
 - 89 **Pugliese R**, Scandroglio I, Sansonna F, Maggioni D, Costanzi A, Citterio D, Ferrari GC, Di Lernia S, Magistro C. Laparoscopic pancreaticoduodenectomy: a retrospective review of 19 cases. *Surg Laparosc Endosc Percutan Tech* 2008; **18**: 13-18 [PMID: 18287976 DOI: 10.1097/SLE.0b013e3181581609]
 - 90 **Cho A**, Yamamoto H, Nagata M, Takiguchi N, Shimada H, Kainuma O, Souda H, Gunji H, Miyazaki A, Ikeda A, Tohma T, Matsumoto I. Comparison of laparoscopy-assisted and open pylorus-preserving pancreaticoduodenectomy for periampullary disease. *Am J Surg* 2009; **198**: 445-449 [PMID: 19342003 DOI: 10.1016/j.amjsurg.2008.12.025]
 - 91 **Kendrick ML**, Cusati D. Total laparoscopic pancreaticoduodenectomy: feasibility and outcome in an early experience. *Arch Surg* 2010; **145**: 19-23 [PMID: 20083750 DOI: 10.1001/archsurg.2009.243]
 - 92 **Corcione F**, Pirozzi F, Cuccurullo D, Piccolboni D, Caracino V, Galante F, Cusano D, Sciuto A. Laparoscopic pancreaticoduodenectomy: experience of 22 cases. *Surg Endosc* 2013; **27**: 2131-2136 [PMID: 23355144 DOI: 10.1007/s00464-012-2728-z]
 - 93 **Ammori BJ**, Ayiomamitis GD. Laparoscopic pancreaticoduodenectomy and distal pancreatectomy: a UK experience and a systematic review of the literature. *Surg Endosc* 2011; **25**: 2084-2099 [PMID: 21298539 DOI: 10.1007/s00464-010-1538-4]
 - 94 **Kendrick ML**, Sclabas GM. Major venous resection during total laparoscopic pancreaticoduodenectomy. *HPB (Oxford)* 2011; **13**: 454-458 [PMID: 21689228 DOI: 10.1111/j.1477-2574.2011.00323.x]
 - 95 **Fisher SB**, Kooby DA. Laparoscopic pancreatectomy for malignancy. *J Surg Oncol* 2013; **107**: 39-50 [PMID: 22991263 DOI: 10.1002/jso.23253]
 - 96 **Kudsi OY**, Gagner M, Jones DB. Laparoscopic distal pancreatectomy. *Surg Oncol Clin N Am* 2013; **22**: 59-73, vi [PMID: 23158085 DOI: 10.1016/j.soc.2012.08.003]
 - 97 **Moore PS**, Porter PE. Nursing deans in small, liberal arts colleges and universities: roles, challenges, and opportunities. *J Prof Nurs* 1987; **3**: 20-27 [PMID: 3644837 DOI: 10.1007/s00464-013-2969-5]
 - 98 **Giulianotti PC**, Sbrana F, Bianco FM, Elli EF, Shah G, Addeo P, Caravaglios G, Coratti A. Robot-assisted laparoscopic pancreatic surgery: single-surgeon experience. *Surg Endosc* 2010; **24**: 1646-1657 [PMID: 20063016 DOI: 10.1007/s00464-009-0825-4]
 - 99 **Choi SH**, Kang CM, Lee WJ, Chi HS. Robot-assisted spleen-preserving laparoscopic distal pancreatectomy. *Ann Surg Oncol* 2011; **18**: 3623 [PMID: 21667330 DOI: 10.1245/s10434-011-1816-y]
 - 100 **Horiguchi A**, Uyama I, Miyakawa S. Robot-assisted laparoscopic pancreaticoduodenectomy. *J Hepatobiliary Pancreat Sci* 2011; **18**: 287-291 [PMID: 20811915 DOI: 10.1007/s00534-010-0325-x]
 - 101 **Zeh HJ**, Zureikat AH, Secrest A, Dauoudi M, Bartlett D, Moser AJ. Outcomes after robot-assisted pancreaticoduodenectomy for periampullary lesions. *Ann Surg Oncol* 2012; **19**: 864-870 [PMID: 21947670 DOI: 10.1245/s10434-011-2045-0]
 - 102 **Narula VK**, Mikami DJ, Melvin WS. Robotic and laparoscopic pancreaticoduodenectomy: a hybrid approach. *Pancreas* 2010; **39**: 160-164 [PMID: 19910835 DOI: 10.1097/MPA.0b013e3181bd604e]
 - 103 **Lai EC**, Yang GP, Tang CN. Robot-assisted laparoscopic pancreaticoduodenectomy versus open pancreaticoduodenectomy--a comparative study. *Int J Surg* 2012; **10**: 475-479 [PMID: 22732431 DOI: 10.1016/j.jisu.2012.06.003]
 - 104 **Daouadi M**, Zureikat AH, Zenati MS, Choudry H, Tsung A, Bartlett DL, Hughes SJ, Lee KK, Moser AJ, Zeh HJ. Robot-as-

- sisted minimally invasive distal pancreatectomy is superior to the laparoscopic technique. *Ann Surg* 2013; **257**: 128-132 [PMID: 22868357 DOI: 10.1097/SLA.0b013e31825fff08]
- 105 **Zhang J**, Wu WM, You L, Zhao YP. Robotic versus open pancreatectomy: a systematic review and meta-analysis. *Ann Surg Oncol* 2013; **20**: 1774-1780 [PMID: 23504140 DOI: 10.1245/s10434-012-2823-3]
 - 106 **Grover S**, Ashley SW, Raut CP. Small intestine gastrointestinal stromal tumors. *Curr Opin Gastroenterol* 2012; **28**: 113-123 [PMID: 22157511 DOI: 10.1097/MOG.0b013e3182834ec154]
 - 107 **Dholakia C**, Gould J. Minimally invasive resection of gastrointestinal stromal tumors. *Surg Clin North Am* 2008; **88**: 1009-118, vi [PMID: 18790151 DOI: 10.1016/j.suc.2008.05.006]
 - 108 **Tabrizian P**, Nguyen SQ, Divino CM. Laparoscopic management and longterm outcomes of gastrointestinal stromal tumors. *J Am Coll Surg* 2009; **208**: 80-86 [PMID: 19228508 DOI: 10.1016/j.jamcollsurg.2008.08.028]
 - 109 **Arolfo S**, Teggie PM, Nano M. Gastrointestinal stromal tumors: thirty years experience of an institution. *World J Gastroenterol* 2011; **17**: 1836-1839 [PMID: 21528056 DOI: 10.3748/wjg.v17.i14.1836]
 - 110 **Tsui DK**, Tang CN, Ha JP, Li MK. Laparoscopic approach for small bowel tumors. *Surg Laparosc Endosc Percutan Tech* 2008; **18**: 556-560 [PMID: 19098659 DOI: 10.1097/SLE.0b013e3181889d25]
 - 111 **Chen YH**, Liu KH, Yeh CN, Hsu JT, Liu YY, Tsai CY, Chiu CT, Jan YY, Yeh TS. Laparoscopic resection of gastrointestinal stromal tumors: safe, efficient, and comparable oncologic outcomes. *J Laparoendosc Adv Surg Tech A* 2012; **22**: 758-763 [PMID: 22957924 DOI: 10.1089/lap.2012.0115]
 - 112 **Lee SW**. Laparoscopic procedures for colon and rectal cancer surgery. *Clin Colon Rectal Surg* 2009; **22**: 218-224 [PMID: 21037812 DOI: 10.1055/s-00029-1242461]
 - 113 **Martel G**, Crawford A, Barkun JS, Boushey RP, Ramsay CR, Fergusson DA. Expert opinion on laparoscopic surgery for colorectal cancer parallels evidence from a cumulative meta-analysis of randomized controlled trials. *PLoS One* 2012; **7**: e35292 [PMID: 22532846 DOI: 10.1371/journal.pone.0035292]
 - 114 **Aly EH**. Colorectal surgery: current practice & future developments. *Int J Surg* 2012; **10**: 182-186 [PMID: 22406541 DOI: 10.1016/j.ijsu.2012.02.016]
 - 115 **Stocchi L**, Nelson H. Laparoscopic colon resection for cancer. *Adv Surg* 2006; **40**: 59-76 [PMID: 17163095 DOI: 10.1016/j.yasu.2006.05.004]
 - 116 **Martel G**, Boushey RP. Laparoscopic colon surgery: past, present and future. *Surg Clin North Am* 2006; **86**: 867-897 [PMID: 16905414 DOI: 10.1016/j.suc.2006.05.006]
 - 117 **Buchanan GN**, Malik A, Parvaiz A, Sheffield JP, Kennedy RH. Laparoscopic resection for colorectal cancer. *Br J Surg* 2008; **95**: 893-902 [PMID: 18551725 DOI: 10.1002/bjs.6019]
 - 118 **Aslani N**, Lobo-Prabhu K, Heidary B, Phang T, Raval MJ, Brown CJ. Outcomes of laparoscopic colon cancer surgery in a population-based cohort in British Columbia: are they as good as the clinical trials? *Am J Surg* 2012; **204**: 411-415 [PMID: 22607740 DOI: 10.1016/j.amjsurg.2011.11.015]
 - 119 **Robinson CN**, Balentine CJ, Sangsriy S, Berger DH. Disparities in the use of minimally invasive surgery for colorectal disease. *J Gastrointest Surg* 2012; **16**: 897-903; discussion 903-904 [PMID: 22411487 DOI: 10.1007/s11605-012-1844-3]
 - 120 **Clinical Outcomes of Surgical Therapy Study Group**. A comparison of laparoscopically assisted and open colectomy for colon cancer. *N Engl J Med* 2004; **350**: 2050-2059 [PMID: 15141043 DOI: 10.1056/NEJMoa032651]
 - 121 **Veldkamp R**, Kuhry E, Hop WC, Jeekel J, Kazemier G, Bonjer HJ, Haglind E, Pahlman L, Cuesta MA, Msika S, Morino M, Lacy AM. Laparoscopic surgery versus open surgery for colon cancer: short-term outcomes of a randomised trial. *Lancet Oncol* 2005; **6**: 477-484 [PMID: 15992696 DOI: 10.1016/S1470-2045(05)70221-7]
 - 122 **Guillou PJ**, Quirke P, Thorpe H, Walker J, Jayne DG, Smith AM, Heath RM, Brown JM. Short-term endpoints of conventional versus laparoscopic-assisted surgery in patients with colorectal cancer (MRC CLASICC trial): multicentre, randomised controlled trial. *Lancet* 2005; **365**: 1718-1726 [PMID: 15894098 DOI: 10.1016/S0140-6736(05)66545-2]
 - 123 **Lacy AM**, García-Valdecasas JC, Delgado S, Castells A, Taurá P, Piqué JM, Visa J. Laparoscopy-assisted colectomy versus open colectomy for treatment of non-metastatic colon cancer: a randomised trial. *Lancet* 2002; **359**: 2224-2229 [PMID: 12103285 DOI: 10.1016/S0140-6736(02)09290-5]
 - 124 **Hewett PJ**, Allardyce RA, Bagshaw PF, Frampton CM, Frizelle FA, Rieger NA, Smith JS, Solomon MJ, Stephens JH, Stevenson AR. Short-term outcomes of the Australasian randomized clinical study comparing laparoscopic and conventional open surgical treatments for colon cancer: the ALCCaS trial. *Ann Surg* 2008; **248**: 728-738 [PMID: 18948799 DOI: 10.1097/SLA.0b013e31818b7595]
 - 125 **Bonjer HJ**, Hop WC, Nelson H, Sargent DJ, Lacy AM, Castells A, Guillou PJ, Thorpe H, Brown J, Delgado S, Kuhrij E, Haglind E, Pahlman L. Laparoscopically assisted vs open colectomy for colon cancer: a meta-analysis. *Arch Surg* 2007; **142**: 298-303 [PMID: 17372057 DOI: 10.1001/archsurg.142.3.298]
 - 126 **Schwenk W**, Haase O, Neudecker J, Müller JM. Short term benefits for laparoscopic colorectal resection. *Cochrane Database Syst Rev* 2005; **(3)**: CD003145 [PMID: 16034888 DOI: 10.1002/14651858.CD003145.pub2]
 - 127 **Allardyce RA**, Bagshaw PF, Frampton CM, Frizelle FA, Hewett PJ, Rieger NA, Smith JS, Solomon MJ, Stevenson AR. Australasian Laparoscopic Colon Cancer Study shows that elderly patients may benefit from lower postoperative complication rates following laparoscopic versus open resection. *Br J Surg* 2010; **97**: 86-91 [PMID: 19937975 DOI: 10.1002/bjs.6785]
 - 128 **Taylor GW**, Jayne DG, Brown SR, Thorpe H, Brown JM, Dewberry SC, Parker MC, Guillou PJ. Adhesions and incisional hernias following laparoscopic versus open surgery for colorectal cancer in the CLASICC trial. *Br J Surg* 2010; **97**: 70-78 [PMID: 20013936 DOI: 10.1002/bjs.6742]
 - 129 **Neudecker J**, Klein F, Bittner R, Carus T, Stroux A, Schwenk W. Short-term outcomes from a prospective randomized trial comparing laparoscopic and open surgery for colorectal cancer. *Br J Surg* 2009; **96**: 1458-1467 [PMID: 19918852 DOI: 10.1002/bjs.6782]
 - 130 **Fleshman J**, Sargent DJ, Green E, Anvari M, Stryker SJ, Beart RW, Hellinger M, Flanagan R, Peters W, Nelson H. Laparoscopic colectomy for cancer is not inferior to open surgery based on 5-year data from the COST Study Group trial. *Ann Surg* 2007; **246**: 655-662; discussion 662-664 [PMID: 17893502 DOI: 10.1097/SLA.0b013e318155a762]
 - 131 **Buunen M**, Veldkamp R, Hop WC, Kuhry E, Jeekel J, Haglind E, Pahlman L, Cuesta MA, Msika S, Morino M, Lacy A, Bonjer HJ. Survival after laparoscopic surgery versus open surgery for colon cancer: long-term outcome of a randomised clinical trial. *Lancet Oncol* 2009; **10**: 44-52 [PMID: 19071061 DOI: 10.1016/S1470-2045(08)70310-3]
 - 132 **Lacy AM**, Delgado S, Castells A, Prins HA, Arroyo V, Ibarzabal A, Piqué JM. The long-term results of a randomized clinical trial of laparoscopy-assisted versus open surgery for colon cancer. *Ann Surg* 2008; **248**: 1-7 [PMID: 18580199 DOI: 10.1097/SLA.0b013e31816a9d65]
 - 133 **Jayne DG**, Thorpe HC, Copeland J, Quirke P, Brown JM, Guillou PJ. Five-year follow-up of the Medical Research Council CLASICC trial of laparoscopically assisted versus open surgery for colorectal cancer. *Br J Surg* 2010; **97**: 1638-1645 [PMID: 20629110 DOI: 10.1002/bjs.7160]
 - 134 **Bagshaw PF**, Allardyce RA, Frampton CM, Frizelle FA, Hewett PJ, McMurrick PJ, Rieger NA, Smith JS, Solomon MJ, Stevenson AR. Long-term outcomes of the australasian randomized clinical trial comparing laparoscopic and conventional open surgical treatments for colon cancer: the

- Australasian Laparoscopic Colon Cancer Study trial. *Ann Surg* 2012; **256**: 915-919 [PMID: 23154392 DOI: 10.1097/SLA.0b013e3182765ff8]
- 135 **Leroy J**, Diana M, Wall J, Costantino F, D'Agostino J, Marescaux J. Laparo-endoscopic single-site (LESS) with transanal natural orifice specimen extraction (NOSE) sigmoidectomy: a new step before pure colorectal natural orifices transluminal endoscopic surgery (NOTES®). *J Gastrointest Surg* 2011; **15**: 1488-1492 [PMID: 21584823 DOI: 10.1007/s11605-011-1557-z]
- 136 **Diana M**, Perretta S, Wall J, Costantino FA, Leroy J, Demartines N, Marescaux J. Transvaginal specimen extraction in colorectal surgery: current state of the art. *Colorectal Dis* 2011; **13**: e104-e111 [PMID: 21564461 DOI: 10.1111/j.1463-1318.2011.02599]
- 137 **Egi H**, Hattori M, Hinoi T, Takakura Y, Kawaguchi Y, Shimomura M, Tokunaga M, Adachi T, Urushihara T, Itamoto T, Ohdan H. Single-port laparoscopic colectomy versus conventional laparoscopic colectomy for colon cancer: a comparison of surgical results. *World J Surg Oncol* 2012; **10**: 61 [PMID: 22531017 DOI: 10.1186/1477-7819-10-61]
- 138 **Breukink S**, Pierie J, Wiggers T. Laparoscopic versus open total mesorectal excision for rectal cancer. *Cochrane Database Syst Rev* 2006; **(4)**: CD005200 [PMID: 17054246 DOI: 10.1002/14651858.CD005200.pub2]
- 139 **van der Pas MH**, Haglind E, Cuesta MA, Fürst A, Lacy AM, Hop WC, Bonjer HJ. Laparoscopic versus open surgery for rectal cancer (COLOR II): short-term outcomes of a randomised, phase 3 trial. *Lancet Oncol* 2013; **14**: 210-218 [PMID: 23395398 DOI: 10.1016/S1470-2045(13)70016-0]
- 140 **Kang SB**, Park JW, Jeong SY, Nam BH, Choi HS, Kim DW, Lim SB, Lee TG, Kim DY, Kim JS, Chang HJ, Lee HS, Kim SY, Jung KH, Hong YS, Kim JH, Sohn DK, Kim DH, Oh JH. Open versus laparoscopic surgery for mid or low rectal cancer after neoadjuvant chemoradiotherapy (COREAN trial): short-term outcomes of an open-label randomised controlled trial. *Lancet Oncol* 2010; **11**: 637-645 [PMID: 20610322 DOI: 10.1016/S1470-2045(10)70131-5]
- 141 **Lujan J**, Valero G, Biondo S, Espin E, Parrilla P, Ortiz H. Laparoscopic versus open surgery for rectal cancer: results of a prospective multicentre analysis of 4,970 patients. *Surg Endosc* 2013; **27**: 295-302 [PMID: 22736289 DOI: 10.1007/s00464-012-2444-8]
- 142 **Patel CB**, Ragupathi M, Ramos-Valadez DI, Haas EM. A three-arm (laparoscopic, hand-assisted, and robotic) matched-case analysis of intraoperative and postoperative outcomes in minimally invasive colorectal surgery. *Dis Colon Rectum* 2011; **54**: 144-150 [PMID: 21228660 DOI: 10.1007/DCR.0b013e3181fec377]
- 143 **Deutsch GB**, Sathyanarayana SA, Gunabushanam V, Mishra N, Rubach E, Zemon H, Klein JD, Denoto G. Robotic vs. laparoscopic colorectal surgery: an institutional experience. *Surg Endosc* 2012; **26**: 956-963 [PMID: 22044968 DOI: 10.1007/s00464-011-1977-6]
- 144 **Fung AK**, Aly EH. Robotic colonic surgery: is it advisable to commence a new learning curve? *Dis Colon Rectum* 2013; **56**: 786-796 [PMID: 23652755 DOI: 10.1097/DCR.0b013e318285b810]
- 145 **Halabi WJ**, Kang CY, Jafari MD, Nguyen VQ, Carmichael JC, Mills S, Stamos MJ, Pigazzi A. Robotic-assisted colorectal surgery in the United States: a nationwide analysis of trends and outcomes. *World J Surg* 2013; **37**: 2782-2790 [PMID: 23564216 DOI: 10.1007/s00268-013-2024-7]
- 146 **Kim SH**, Kwak JM. Robotic total mesorectal excision: operative technique and review of the literature. *Tech Coloproctol* 2013; **17** Suppl 1: S47-S53 [PMID: 23307506 DOI: 10.1007/s10151-012-0939-x]
- 147 **Baek SJ**, Kim SH, Cho JS, Shin JW, Kim J. Robotic versus conventional laparoscopic surgery for rectal cancer: a cost analysis from a single institute in Korea. *World J Surg* 2012; **36**: 2722-2729 [PMID: 22855217 DOI: 10.1007/s00268-012-1728-4]
- 148 **Helvind NM**, Eriksen JR, Mogensen A, Tas B, Olsen J, Bundgaard M, Jakobsen HL, Gögenür I. No differences in short-term morbidity and mortality after robot-assisted laparoscopic versus laparoscopic resection for colonic cancer: a case-control study of 263 patients. *Surg Endosc* 2013; **27**: 2575-2580 [PMID: 23389069 DOI: 10.1007/s00464-013-2792-z]
- 149 **Fernandez R**, Anaya DA, Li LT, Orcutt ST, Balentine CJ, Awad SA, Berger DH, Albo DA, Artinyan A. Laparoscopic versus robotic rectal resection for rectal cancer in a veteran population. *Am J Surg* 2013; **206**: 509-517 [PMID: 23809672 DOI: 10.1016/j.amjsurg.2013.01.036]
- 150 **Baek JH**, McKenzie S, Garcia-Aguilar J, Pigazzi A. Oncologic outcomes of robotic-assisted total mesorectal excision for the treatment of rectal cancer. *Ann Surg* 2010; **251**: 882-886 [PMID: 20395863 DOI: 10.1097/SLA.0b013e3181c79114]
- 151 **Collinson FJ**, Jayne DG, Pigazzi A, Tsang C, Barrie JM, Edlin R, Garbett C, Guillou P, Holloway I, Howard H, Marshall H, McCabe C, Pavitt S, Quirke P, Rivers CS, Brown JM. An international, multicentre, prospective, randomised, controlled, unblinded, parallel-group trial of robotic-assisted versus standard laparoscopic surgery for the curative treatment of rectal cancer. *Int J Colorectal Dis* 2012; **27**: 233-241 [PMID: 21912876 DOI: 10.1007/s00384-011-1313-6]

P- Reviewers: Hsiao KCW, Jani K

S- Editor: Ma YJ L- Editor: A E- Editor: Liu XM



Role of prophylactic antibiotics in cirrhotic patients with variceal bleeding

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Author contributions: All the authors were involved in the design, analysis and writing of manuscript.

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Received: September 18, 2013 Revised: November 11, 2013

Accepted: January 6, 2014

Published online: February 21, 2014

Abstract

Bacterial infections are common in cirrhotic patients with acute variceal bleeding, occurring in 20% within 48 h. Outcomes including early rebleeding and failure to control bleeding are strongly associated with bacterial infection. However, mortality from variceal bleeding is largely determined by the severity of liver disease. Besides a higher Child-Pugh score, patients with hepatocellular carcinoma are particularly susceptible to infections. Despite several hypotheses that include increased use of instruments, greater risk of aspiration pneumonia and higher bacterial translocation, it remains debatable whether variceal bleeding results in infection or vice versa but studies suggest that antibiotic prophylaxis prior to endoscopy and up to 8 h is useful in reducing bacteremia and spontaneous bacterial peritonitis. Aerobic gram negative bacilli of enteric origin are most commonly isolated from cultures, but more recently, gram positives and quinolone-resistant organisms are increasingly seen, even though their clinical significance is unclear. Fluoroquinolones (including ciprofloxacin and norfloxacin) used for short term (7 d)

have the most robust evidence and are recommended in most expert guidelines. Short term intravenous cephalosporin (especially ceftriaxone), given in a hospital setting with prevalent quinolone-resistant organisms, has been shown in studies to be beneficial, particularly in high risk patients with advanced cirrhosis.

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Key words: Antibiotics; Prophylaxis; Cirrhosis; Variceal bleeding; Infection

Core tip: Bacterial infections are common in cirrhotics with variceal bleeding and can influence its outcomes that include early rebleeding, failure to control bleeding and mortality. It remains unsure whether infection or bleeding is the initiating event but prophylactic antibiotics have been proven useful. Short term fluoroquinolones and cephalosporins are the most studied antibiotics, and they are recommended by guidelines in clinical situations that depend on the severity of liver disease and resistance profile.

Lee YY, Tee HP, Mahadeva S. Role of prophylactic antibiotics in cirrhotic patients with variceal bleeding. *World J Gastroenterol* 2014; 20(7): 1790-1796 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1790.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1790>

INTRODUCTION

Upper gastrointestinal (GI) variceal bleeding is associated with significant mortality in cirrhosis. The prevalence of variceal bleed is known to occur in 20%-50% of patients with cirrhosis, with rebleeding as a significant cause of death^[1,2]. For the past 30 years, the mortality has improved markedly from intensive use of endoscopic

therapies, vasoactive drugs and antibiotics^[3,4]. However, rebleeding and failure to control bleeding remain a significant problem and factors that affect rebleeding are not well-established. Guidelines of major GI societies adopt the use of antibiotics in acute variceal bleeding due to its efficacy in the reduction of bacteremia and spontaneous bacterial peritonitis. The recommendations on the choice of antibiotics are however based on a limited number of studies and mostly with small sample sizes. Furthermore, the clinical effectiveness of antibiotics in preventing rebleeding and/or mortality is not firmly established^[5]. There are also issues with antibiotic resistance and emergence of hospital-acquired infections.

The current review aims to revisit issues surrounding infection and antibiotic usage in cirrhotics with variceal bleeding. PubMed/Medline was searched for English language scientific publications for human studies from 1980 to the present. MeSH terms including “antibiotic(s)”, “infection(s)”, “variceal”, “cirrhotic”, “hemorrhage or haemorrhage”, “bleeding” were searched with operators “AND” and “OR”. A total of 72 articles were returned from the search and these were further filtered and classified for the current review with additional articles taken from references in the above papers, if deemed necessary. Guidelines of societies including American Association of Study of Liver Diseases (AASLD), American College of Gastroenterology (ACG), World Gastroenterology Organisation (WGO), British Society of Gastroenterology (BSG) and the Baveno V consensus conference were also reviewed.

ROLE OF BACTERIAL INFECTIONS IN REBLEEDING AND MORTALITY

Bacterial infections are frequent in cirrhotic patients with liver failure. The reported in-hospital incidence is approximately 40% with mortality being related to a higher Child-Pugh score^[6]. Likewise, infections are common in cirrhotic patients with variceal bleeding, with the risk of death higher in those with infection diagnosed within 48 h. Bleichner *et al*^[7], retrospectively found 22% of bacterial infection occurring within 48 h of admission which was similar to a prospective study by Bernard *et al*^[8]. In the same study by Bernard *et al*^[8], early rebleeding, defined as recurrence of bleeding within 7 d after admission, was present in 43.5% of patients with bacterial infection compared to 9.8% in those without infection. Using stricter criteria including initial failure to control bleeding and early rebleeding or death within 5 d from admission, Goulis *et al*^[9] reported 47% of failures in the control of cirrhotic bleeding. Besides bacterial infections or prior antibiotic use, active bleeding during endoscopy and a higher Child-Pugh score were associated with failure to control bleeding in the multivariable analysis performed in the Goulis's study^[9]. These studies suggest that bacterial infection is responsible for early rebleeding (within 7 d) and is associated with failure to control bleeding but its role in mortality is not clear because of small sample

sizes of reported studies and there is an influence of severity of liver disease on death.

Which group of cirrhotic patients is at a higher risk for infection during bleeding? Patients with hepatocellular carcinoma (HCC) and variceal bleeding tend to have a greater rebleeding rate^[10] and a study from Taiwan suggests that this was due to a higher infection rate in these subjects^[11]. However, studies from the West, with patients mostly having alcoholic cirrhosis as the etiology, indicate that the severity of liver disease is a predictor of infection^[8,9].

BLEEDING FIRST OR INFECTION FIRST?

It remains debatable whether variceal bleeding results in bacterial infections or vice versa. The invasive nature of endoscopic procedures^[12] or other instrumentations that include urethral catheters and transjugular intrahepatic portosystemic shunts (TIPS) may cause a breakdown in natural defenses. Moreover, there is an increased risk for aspiration pneumonia as a result of hematemesis, during endoscopy and placement of balloon tamponade^[12]. The increased bacterial translocation^[13] and deficiency of complements^[14] in bleeding cirrhotics and hypovolemia may also predispose to infection. On the other hand, bacterial infections, through the release of endotoxins and the failure of cirrhotics to remove them, can result in a generalized intravascular activation of mediators (endothelins) that damage the vessels, increase the portal pressure as well as hematologic or hemostatic impairment^[15,16].

If infection is the cause of bleeding, then antibiotic administration as soon as possible is a logical approach, and those who are cirrhotics already on antibiotic prophylaxis may have a reduced chance for bleeding. If bleeding is the cause of infection, then likewise, antibiotics should be administered as soon as possible, even prior to endoscopic therapy. Retrospective studies have shown that infection occurred in 13% of patients within 24 h and increasing to 22% within 48 h, reiterating the importance of starting antibiotics early^[7]. Indeed, experts and guidelines recommend administration of antibiotics prior to endoscopy but again evidence are lacking on whether this approach does reduce rebleeding and improves mortality. There is a window of opportunity of 8 h for administering antibiotics following endoscopy if this is initially missed^[17]. A retrospective study suggested that antibiotics administered up to 8 h following endoscopy were associated with improved survival at 28 d and a trend in reducing 28-d rebleeding rate^[17].

WHICH ORGANISMS ARE INVOLVED IN CIRRHOTIC BLEEDING?

The most commonly isolated organisms during bleeding are aerobic gram negative bacilli of enteric origin which can include *Escherichia coli*, *Klebsiella* spp, *Enterococcus* and *Pseudomonas* spp^[11,18]. Bacteremia, spontaneous bacterial

peritonitis (SBP) and urinary tract infections were clinical infections most commonly reported in association with the above mentioned organisms. There are a number of case reports of less common organisms being isolated and which can be associated with more severe bleeding complications. These organisms include *Oerskovia xanthineolytica*^[19], methicillin-resistant *Staphylococcus aureus*^[19] and *Vibrio vulnificus*^[20]. Infections can also be introduced during endoscopic ligation therapy, which is occasionally severe, as in a reported case of pyogenic meningitis^[21]. Bacteremia is also more common following cyanoacrylate therapy for bleeding gastric varices but not elective cyanoacrylate injection for non-bleeding gastric varices^[22]. Organisms identified from blood cultures and needle-tip cultures performed in the former group were from the oral and GI tract^[22].

Helicobacter pylori (*H. pylori*) infection is also thought to have a role in cirrhotic GI bleeding. A recent study from Japan suggests a protective role for *H. pylori* in variceal bleeding through the induction of gastric mucosal atrophy and concomitant hypoacidity^[23]. The use of broad spectrum antibiotics and for long duration in cirrhotics may result in the emergence of health-care associated infections especially *Clostridium difficile* (*C. difficile*), which was recently shown to result in a higher mortality in this group of patients^[24]. Although the study by Brown *et al*^[17] did not find any significant difference in the incidence of *C. difficile* infection, the prevalent use of metronidazole in this retrospective study casted doubt on the validity of their result^[25].

CHOICE OF ANTIBIOTICS: WHAT IS THE EVIDENCE?

The use of antibiotics in cirrhotic bleeding arises from its success in the prevention of SBP and spontaneous bacteremia. This was first shown in a study by Rimola and colleagues in 1985^[26]. In this study, 140 cirrhotic patients were randomized into 2 groups - one with oral, non-absorbable antibiotics (gentamicin + vancomycin + nystatin or neomycin + colistin + nystatin) for 48 h and the other without antibiotics. The incidence of infection was observed to be significantly lower in the group receiving antibiotics compared to the group without (16.1% *vs* 34.7%)^[26].

Fluoroquinolones, namely norfloxacin and ciprofloxacin, seem to be obvious choices for prophylaxis since this group of antibiotics is active against the majority of enterobacteria and aerobic gram negative bacilli. Norfloxacin, given orally or through a nasogastric tube, 400 mg twice daily for 7 d immediately after emergency endoscopy, was shown to reduce infection, with a rate of only 10% in 60 patients compared to 37.2% in 59 controls^[27]. Ciprofloxacin has the advantage of being well-tolerated, has low hepatic toxicity and also less bacterial resistance even after 6 mo of treatment. In a case-control study from Taiwan, 120 cirrhotic patients with upper GI bleeding who had received ciprofloxacin 500 mg twice

daily after endoscopy for 7 d were found to have a lower incidence of proven bacterial infection, but not mortality, compared to 60 patients who had placebo (10% *vs* 45%)^[18]. Patients with Child-Pugh Class C and those with hepatocellular carcinoma are particularly prone to infection and ciprofloxacin in these patients were found to be especially useful. In a study by Pauwels *et al*^[28], 30 patients with advanced cirrhosis were found to have a higher rate of infection compared to 55 patients with Child-Pugh Class A-B (52.9% *vs* 18.2%). In the same study, a selected group of high risk patients were administered amoxicillin-clavulanic 1 g/200 mg *iv* q8h followed by ciprofloxacin 200 mg *po* q12h for 3 d after cessation of bleeding, and a significant reduction in infection was observed, compared to those who did not receive this regime (13.3% *vs* 52.9%)^[28].

In addition to reducing the incidence of infection, prophylactic quinolones given during cirrhotic bleeding have been shown to reduce early rebleeding and requirements for blood transfusion. This was shown in another study from Taiwan that randomized cirrhotic patients with bleeding into two groups - one group with 59 patients given prophylactic antibiotics (ofloxacin 200 mg *iv* q12h for 2 d followed by oral ofloxacin 200 mg q12h for 5 d) and the other group with 61 patients that only received antibiotics when infection became evident (on-demand group)^[11]. Again, survival was not shown to be different between the two groups despite the beneficial effect of prophylaxis on rebleeding rate.

Several earlier studies explored the administration of intravenous antibiotics prior and immediately after endoscopic sclerotherapy, but no efficacy could be demonstrated. Rolando *et al*^[29] commenced imipenem-cilastatin 500 mg *iv* at sedation and a further 500 mg 6 h after endoscopic sclerotherapy in 107 sessions and this was compared to dextrose-saline in 88 sessions. There was no significant difference in the infection rate (1.1% *vs* 5.6%), and the authors concluded that a short antibiotic regime does not affect the risk of early bacteremia following endoscopic sclerotherapy. Likewise, Selby *et al*^[30] could not demonstrate the clinical efficacy of prophylactic antibiotics (cefotaxime 1 g *iv*) given in 19 patients before sclerotherapy, as clinical infection attributable to sclerotherapy did not develop despite a reduction in bacteremia.

The choice of oral quinolones as the best antibiotic for preventing infection in cirrhotic bleeding has been questioned in recent studies. There are increasing reports of quinolone-resistant flora^[31] especially *Escherichia coli*^[32] and other infections which were gram-positive possibly related to invasive procedures^[33] performed in these patients. This led to a study from Barcelona that randomized 124 patients with advanced cirrhosis into 2 groups - one group with 63 patients given oral norfloxacin 400 mg q12h for 7 d and the other group given *iv* ceftriaxone 1 g once daily for 7 d^[34]. Ceftriaxone was chosen by the investigators for 2 reasons; one being that most quinolone-resistant bacteria are susceptible to third-generation cephalosporins and the other fact that the *iv* route is

more accessible during active GI bleeding. This study demonstrated that norfloxacin was associated with a higher probability of SBP and spontaneous bacteremia as compared to ceftriaxone, but hospital mortality was not different between the two groups. The changing microbiology of infection (susceptibility to both gram negative and positive organisms) and the delay in onset of action from an oral route of norfloxacin, might explain the failure of oral quinolones. Most importantly, *iv* ceftriaxone is more efficacious in the setting of severe liver failure since non-enterococcal streptococci and quinolone-resistant gram negative organisms were more common in this group of patients. A study from Poland, however, did not find any difference in early or late survival rate whether antibiotics were administered orally (norfloxacin) or *iv* (ampicillin/sulbactam), but this study suffered from a small sample size^[35].

Other cephalosporins that have been recently studied include *iv* cefotaxime (third generation) and *iv* cefazolin (first generation). In a prospective randomized trial from South Korea, *iv* cefotaxime 2 g q8h for 7 d was administered to 62 cirrhotics with GI bleeding and this was compared to 58 patients given on-demand quinolones^[36]. The prophylactic group was shown to have a lower infection and early rebleeding rate within 6 wk. However, it was not known whether *iv* cefotaxime was similarly effective as *iv* ceftriaxone in cirrhotics with more severe liver disease. A study from Taiwan recently studied *iv* cefazolin, a first generation cephalosporin, which has been shown to be effective in reducing infection in bleeding cirrhotics and is also cheaper than *iv* ceftriaxone^[37]. The study found that *iv* cefazolin is as similarly effective as *iv* ceftriaxone for patients with Child-Pugh Class A (group A; 51 patients) but not Class B and C (group B; 51 patients)^[38]. However, the small sample size and retrospective design did not allow a firm conclusion on the role of *iv* cefazolin.

Similar to esophageal varices, bleeding gastric varices is associated with bacteremia and antibiotic prophylaxis is recommended. In a study from Taiwan, 32% of patients with gastric varices developed bacteremia, and the risk was higher in emergency gastric varices obliteration^[39]. More patients injected with cyanoacrylate had positive blood cultures than the control group (15/47 *vs* 1/47). Most episodes of bacteremia were found to be transient and organisms cultured were identical to those cultured from endoscope accessory channel. In another study from the same group in Taiwan, routine antibiotic prophylaxis was given to all 20 subjects who received simultaneous injection of gastric varices and banding of esophageal varices^[40]. Even though the infection rate did not increase, the rebleeding rate did not differ either. In a long-term follow-up of four years of 31 patients receiving cyanoacrylate for gastric variceal bleeding, only two subjects developed pyrexia within 24 h of injection that settled with prophylactic antibiotic and had a low rebleeding rate of 16%^[41]. These studies indicate that while bacteremia is common after cyanoacrylate therapy for bleeding gastric varices, it is transient and easily treated

with prophylactic antibiotic.

Aside from their anti-microbial actions, prokinetic effects of antibiotics such as erythromycin have also been found to be beneficial in cirrhotic bleeding by improving visibility during endoscopy. In a randomized double-blind placebo-controlled study involving 102 patients, *iv* erythromycin lactobionate significantly improved visualization of the stomach and shortened the duration of endoscopy following a cirrhotic GI bleed^[42].

WHAT THE GUIDELINES SAY?

All practice guidelines and consensus statements agree that antibiotic prophylaxis is an integral part of medical therapy in cirrhotics with acute GI bleeding. The British Society of Gastroenterology (BSG) guideline in 2000 suggested that the choice of antibiotic and its dose should be decided by the unit where patients were being treated. However, it recognized that fluoroquinolones (ciprofloxacin) had the best evidence at that time^[43]. AASLD and ACG practice guideline in 2007 recommended the use of short term prophylactic antibiotics in cirrhotics and GI bleeding with or without ascites^[44,45]. Oral Norfloxacin 400 mg q12h for 7 d was the suggested schedule, with oral or *iv* ciprofloxacin the alternative. Although *iv* ceftriaxone was mentioned as being more effective than norfloxacin in advanced cirrhosis, the AASLD/ACG practice committee felt that the prevalence of quinolone-resistant organisms in individual studies would have affected the results. WGO practice guideline in 2008 gave similar recommendations but with *iv* ceftriaxone being recommended in advanced cirrhosis^[46]. The Baveno V consensus workshop in 2010 recommended oral quinolones for most patients and *iv* ceftriaxone in advanced cirrhotics only in hospital settings with a high prevalence of quinolone-resistant bacterial infections and in patients on previous quinolone prophylaxis^[47].

EFFICACY OF COMBINATION THERAPY THAT INCLUDES ANTIBIOTIC IN ACUTE VARICEAL BLEEDING

While the evidence suggests an integral role of antibiotics, it is not used in isolation. Antibiotics in combination with somatostatin and endoscopic ligation were shown in a study from Barcelona to be effective in reducing mortality in all stages of cirrhosis, even in the high risk groups (Child-Pugh Class B with active bleeding and Child-Pugh Class C)^[48]. Patients with Child-Pugh Class C and a baseline creatinine of ≥ 1.0 mg/dL are at an especially high risk, and an early use of covered TIPS in such patients may be beneficial^[49]. In a study from Italy, the 5-d mortality rate was related to Child-Pugh Class C, white cell count $> 10 \times 10^9$ /L and the presence of portal vein thrombosis^[4]. In this Italian study with a 28.1% hepatocellular carcinoma rate, treatment with a combination of vasoactive drugs, band ligation and antibiotics was found

Table 1 Summary of studies on antibiotics used in cirrhotics with acute variceal bleeding

Ref.	Year	Sample size, with antibiotics vs control group (total)	Type of antibiotic (s) vs control group	Infection rate in those with antibiotics vs control group
Rimola <i>et al</i> ^[26]	1985	68 vs 72 (140)	Gentamicin + vancomycin + nystatin	16.2% vs 34.7%
Soriano <i>et al</i> ^[27]	1992	60 vs 59 (119)	Or neomycin + colistin + nystatin vs without antibiotic	10% vs 37.2%
Rolando <i>et al</i> ^[29]	1993	107 vs 88 sessions	Norfloxacin 400 mg po q12h vs no antibiotic	1.1% vs 5.61%
Selby <i>et al</i> ^[30]	1994	19 vs 20 (39)	Imipenem-cilastatin 500 mg iv at sedation, further 500 mg 6 h after sclerotherapy vs dextrose-saline	5.3% vs 31.6%
Pauwels <i>et al</i> ^[28]	1996	34 vs 30 (64)	Cefotaxime 1 g iv before sclerotherapy vs no antibiotic	13.3% vs 52.9%
			Amoxicillin-clavulanic 1 g/200 mg iv q8h followed by ciprofloxacin 200 mg po q12h for 3 d after cessation of bleeding vs no antibiotics (high risk group)	
Hsieh <i>et al</i> ^[18]	1998	120 vs 60 (180)	Ciprofloxacin 500 mg iv q12h vs without antibiotic	10% vs 45%
Hou <i>et al</i> ^[11]	2004	59 vs 61 (120)	Ofloxacin 200 mg iv q12h 2 d followed by ofloxacin 200 mg po q12h 5 d vs without antibiotic	3.4% vs 26.2%
Fernández <i>et al</i> ^[34]	2006	61 vs 63 (124)	Ceftriaxone 1 g iv od 7 d vs norfloxacin 400 mg po q12h 7 d	2% vs 12%
Jun <i>et al</i> ^[36]	2006	62 vs 58 (120)	Cefotaxime 2 g iv q8h 7 d vs on-demand quinolone	3.2% vs 15.5%
Wu <i>et al</i> ^[38]	2013	Child-Pugh A: 51; Child-Pugh B + C: 51 (102)	Cefazolin 1 g iv q8h 2-7 d vs ceftriaxone 1 g q12h 2-7 d	6.9% vs 9.11% (Child-Pugh A) 22.2% vs 12.5% (Child-Pugh B + C)

¹P value not significant (> 0.05).

to be effective in controlling bleeding.

CONCLUSION

Bacterial infection is frequent in cirrhotic patients who present with upper GI bleeding. It is associated with early rebleeding and possibly mortality, especially in those patients with severe liver disease and HCC. To date, it is uncertain whether infection or bleeding is the primary event. Antibiotics given in combination with other standard therapy (including vasoactive agents and endoscopic therapy) and initiated for short term (7 d) before endoscopy and possibly up to 8 h following endoscopy are associated with a reduction in infection rate and a lower early rebleeding rate, but not improvement in survival. A summary of reported studies on antibiotic use in cirrhotics with acute variceal bleeding is given in Table 1. Oral quinolones, given for a short term (7 d) are useful in mild liver disease and in settings where quinolone-resistance is less of a problem. Intravenous ceftriaxone (or other third-generation cephalosporin) is likely useful in the setting of advanced liver disease and where quinolone-resistance is a concern.

REFERENCES

- Graham DY, Smith JL. The course of patients after variceal hemorrhage. *Gastroenterology* 1981; **80**: 800-809 [PMID: 6970703]
- Augustin S, Muntaner L, Altamirano JT, González A, Saperas E, Dot J, Abu-Suboh M, Armengol JR, Malagelada JR, Esteban R, Guardia J, Genescà J. Predicting early mortality after acute variceal hemorrhage based on classification and regression tree analysis. *Clin Gastroenterol Hepatol* 2009; **7**: 1347-1354 [PMID: 19699816 DOI: 10.1016/j.cgh.2009.08.011]
- McCormick PA, O'Keefe C. Improving prognosis following a first variceal haemorrhage over four decades. *Gut* 2001; **49**: 682-685 [PMID: 11600472 DOI: 10.1136/gut.49.5.682]
- Amitrano L, Guardascione MA, Manguso F, Bennato R, Bove A, DeNucci C, Lombardi G, Martino R, Menchise A, Orsini L, Picascia S, Riccio E. The effectiveness of current acute variceal bleed treatments in unselected cirrhotic patients: refining short-term prognosis and risk factors. *Am J Gastroenterol* 2012; **107**: 1872-1878 [PMID: 23007003 DOI: 10.1038/ajg.2012.313]
- Chavez-Tapia NC, Barrientos-Gutierrez T, Tellez-Avila F, Soares-Weiser K, Mendez-Sanchez N, Gluud C, Uribe M. Meta-analysis: antibiotic prophylaxis for cirrhotic patients with upper gastrointestinal bleeding - an updated Cochrane review. *Aliment Pharmacol Ther* 2011; **34**: 509-518 [PMID: 21707680 DOI: 10.1111/j.1365-2036.2011.04746.x]
- Caly WR, Strauss E. A prospective study of bacterial infections in patients with cirrhosis. *J Hepatol* 1993; **18**: 353-358 [PMID: 8228129 DOI: 10.1016/S0168-8278(05)80280-6]
- Bleichner G, Boulanger R, Squara P, Sollet JP, Parent A. Frequency of infections in cirrhotic patients presenting with acute gastrointestinal haemorrhage. *Br J Surg* 1986; **73**: 724-726 [PMID: 3489499 DOI: 10.1002/bjs.1800730916]
- Bernard B, Cadranet JF, Valla D, Escolano S, Jarlier V, Opolon P. Prognostic significance of bacterial infection in bleeding cirrhotic patients: a prospective study. *Gastroenterology* 1995; **108**: 1828-1834 [PMID: 7768389 DOI: 10.1016/0016-5085(95)90146-9]
- Goulis J, Armonis A, Patch D, Sabin C, Greenslade L, Burroughs AK. Bacterial infection is independently associated with failure to control bleeding in cirrhotic patients with gastrointestinal hemorrhage. *Hepatology* 1998; **27**: 1207-1212 [PMID: 9581672 DOI: 10.1002/hep.510270504]
- Chen WC, Hou MC, Lin HC, Lee FY, Yeh YY, Chang FY, Lee SD. Feasibility and potential benefit of maintenance endoscopic variceal ligation in patients with unresectable hepatocellular carcinoma and acute esophageal variceal hemorrhage: a controlled trial. *Gastrointest Endosc* 2001; **54**: 18-23 [PMID: 11427836 DOI: 10.1067/mge.2001.115731]
- Hou MC, Lin HC, Liu TT, Kuo BI, Lee FY, Chang FY, Lee SD. Antibiotic prophylaxis after endoscopic therapy prevents rebleeding in acute variceal hemorrhage: a randomized trial. *Hepatology* 2004; **39**: 746-753 [PMID: 14999693 DOI: 10.1002/hep.20126]
- Blaise M, Pateron D, Trinchet JC, Levacher S, Beaugrand M, Pourriat JL. Systemic antibiotic therapy prevents bacterial infection in cirrhotic patients with gastrointestinal hemorrhage. *Hepatology* 1994; **20**: 34-38 [PMID: 8020902 DOI: 10.1002/hep.1840200107]

- 13 **Rimola A**, Soto R, Bory F, Arroyo V, Piera C, Rodes J. Reticuloendothelial system phagocytic activity in cirrhosis and its relation to bacterial infections and prognosis. *Hepatology* 1984; **4**: 53-58 [PMID: 6693068 DOI: 10.1002/hep.1840040109]
- 14 **Homann C**, Varming K, Høgåsen K, Mollnes TE, Graudal N, Thomsen AC, Garred P. Acquired C3 deficiency in patients with alcoholic cirrhosis predisposes to infection and increased mortality. *Gut* 1997; **40**: 544-549 [PMID: 9176087]
- 15 **Ziegler EJ**, McCutchan JA, Fierer J, Glauser MP, Sadoff JC, Douglas H, Braude AI. Treatment of gram-negative bacteremia and shock with human antiserum to a mutant *Escherichia coli*. *N Engl J Med* 1982; **307**: 1225-1230 [PMID: 6752708 DOI: 10.1056/NEJM19821113072001]
- 16 **Hewett JA**, Roth RA. Hepatic and extrahepatic pathobiology of bacterial lipopolysaccharides. *Pharmacol Rev* 1993; **45**: 382-411 [PMID: 8127918]
- 17 **Brown MR**, Jones G, Nash KL, Wright M, Guha IN. Antibiotic prophylaxis in variceal hemorrhage: timing, effectiveness and *Clostridium difficile* rates. *World J Gastroenterol* 2010; **16**: 5317-5323 [PMID: 21072894 DOI: 10.3748/wjg.v16.i42.5317]
- 18 **Hsieh WJ**, Lin HC, Hwang SJ, Hou MC, Lee FY, Chang FY, Lee SD. The effect of ciprofloxacin in the prevention of bacterial infection in patients with cirrhosis after upper gastrointestinal bleeding. *Am J Gastroenterol* 1998; **93**: 962-966 [PMID: 9647029 DOI: 10.1111/j.1572-0241.1998.00288.x]
- 19 **Truant AL**, Satishchandran V, Eisenstaedt R, Richman P, McNeil MM. Oerskovia xanthineolytica and methicillin-resistant *Staphylococcus aureus* in a patient with cirrhosis and variceal hemorrhage. *Eur J Clin Microbiol Infect Dis* 1992; **11**: 950-951 [PMID: 1486895 DOI: 10.1007/BF01962383]
- 20 **Wongpaitoon V**, Sathapatayavongs B, Prachaktam R, Bunyaratvej S, Kurathong S. Spontaneous *Vibrio vulnificus* peritonitis and primary sepsis in two patients with alcoholic cirrhosis. *Am J Gastroenterol* 1985; **80**: 706-708 [PMID: 3898820]
- 21 **Nagamine N**, Kaneko Y, Kumakura Y, Ogawa Y, Ido K, Kimura K. Occurrence of pyogenic meningitis during the course of endoscopic variceal ligation therapy. *Gastrointest Endosc* 1999; **49**: 110-113 [PMID: 9869735 DOI: 10.1016/S0016-5107(99)70457-8]
- 22 **Rerknimitr R**, Chanyaswad J, Kongkam P, Kullavanijaya P. Risk of bacteremia in bleeding and nonbleeding gastric varices after endoscopic injection of cyanoacrylate. *Endoscopy* 2008; **40**: 644-649 [PMID: 18561097 DOI: 10.1055/s-2008-1077294]
- 23 **Sakamoto Y**, Oho K, Toyonaga A, Kumamoto M, Haruta T, Inoue H, Emori K, Tsuruta O, Sata M. Effect of *Helicobacter pylori* infection on esophagogastric variceal bleeding in patients with liver cirrhosis and portal hypertension. *J Gastroenterol Hepatol* 2013; **28**: 1444-1449 [PMID: 23577833 DOI: 10.1111/jgh.12221]
- 24 **Bajaj JS**, Ananthakrishnan AN, Hafeezullah M, Zadvornova Y, Dye A, McGinley EL, Saeian K, Heuman D, Sanyal AJ, Hoffmann RG. *Clostridium difficile* is associated with poor outcomes in patients with cirrhosis: A national and tertiary center perspective. *Am J Gastroenterol* 2010; **105**: 106-113 [PMID: 19844204 DOI: 10.1038/ajg.2009.615]
- 25 **Okano N**, Iwata K. Prophylactic antibiotics for variceal hemorrhage: *Clostridium difficile* infection still can be a risk. *World J Gastroenterol* 2011; **17**: 2356 [PMID: 21633604 DOI: 10.3748/wjg.v17.i18.2356]
- 26 **Rimola A**, Bory F, Teres J, Perez-Ayuso RM, Arroyo V, Rodes J. Oral, nonabsorbable antibiotics prevent infection in cirrhotics with gastrointestinal hemorrhage. *Hepatology* 1985; **5**: 463-467 [PMID: 3873389 DOI: 10.1002/hep.1840050320]
- 27 **Soriano G**, Guarner C, Tomás A, Villanueva C, Torras X, González D, Sainz S, Anguera A, Cussó X, Balanzó J. Norfloxacin prevents bacterial infection in cirrhotics with gastrointestinal hemorrhage. *Gastroenterology* 1992; **103**: 1267-1272 [PMID: 1397884]
- 28 **Pauwels A**, Mostefa-Kara N, Debenes B, Degoutte E, Lévy VG. Systemic antibiotic prophylaxis after gastrointestinal hemorrhage in cirrhotic patients with a high risk of infection. *Hepatology* 1996; **24**: 802-806 [PMID: 8855179 DOI: 10.1002/hep.510240408]
- 29 **Rolando N**, Gimson A, Philpott-Howard J, Sahathevan M, Casewell M, Fagan E, Westaby D, Williams R. Infectious sequelae after endoscopic sclerotherapy of oesophageal varices: role of antibiotic prophylaxis. *J Hepatol* 1993; **18**: 290-294 [PMID: 8228122 DOI: 10.1016/S0168-8278(05)80272-7]
- 30 **Selby WS**, Norton ID, Pokorny CS, Benn RA. Bacteremia and bacterascites after endoscopic sclerotherapy for bleeding esophageal varices and prevention by intravenous cefotaxime: a randomized trial. *Gastrointest Endosc* 1994; **40**: 680-684 [PMID: 7859964]
- 31 **Dupeyron C**, Mangeney N, Sedrati L, Campillo B, Fouet P, Leluan G. Rapid emergence of quinolone resistance in cirrhotic patients treated with norfloxacin to prevent spontaneous bacterial peritonitis. *Antimicrob Agents Chemother* 1994; **38**: 340-344 [PMID: 8192461 DOI: 10.1128/AAC.38.2.340]
- 32 **Ortiz J**, Vila MC, Soriano G, Miñana J, Gana J, Mirelis B, Novella MT, Coll S, Sábat M, Andreu M, Prats G, Solà R, Guarner C. Infections caused by *Escherichia coli* resistant to norfloxacin in hospitalized cirrhotic patients. *Hepatology* 1999; **29**: 1064-1069 [PMID: 10094947 DOI: 10.1002/hep.510290406]
- 33 **Fernández J**, Navasa M, Gómez J, Colmenero J, Vila J, Arroyo V, Rodés J. Bacterial infections in cirrhosis: epidemiological changes with invasive procedures and norfloxacin prophylaxis. *Hepatology* 2002; **35**: 140-148 [PMID: 11786970]
- 34 **Fernández J**, Ruiz del Arbol L, Gómez C, Durandez R, Serradilla R, Guarner C, Planas R, Arroyo V, Navasa M. Norfloxacin vs ceftriaxone in the prophylaxis of infections in patients with advanced cirrhosis and hemorrhage. *Gastroenterology* 2006; **131**: 1049-1056; quiz 1285 [PMID: 17030175 DOI: 10.1053/j.gastro.2006.07.010]
- 35 **Lata J**, Juránková J, Husová L, Senkyřík M, Díte P, Dastych M, Příbramská V, Kroupa R. Variceal bleeding in portal hypertension: bacterial infection and comparison of efficacy of intravenous and per-oral application of antibiotics—a randomized trial. *Eur J Gastroenterol Hepatol* 2005; **17**: 1105-1110 [PMID: 16148557 DOI: 10.1097/00042737-200510000-00015]
- 36 **Jun CH**, Park CH, Lee WS, Joo YE, Kim HS, Choi SK, Rew JS, Kim SJ, Kim YD. Antibiotic prophylaxis using third generation cephalosporins can reduce the risk of early rebleeding in the first acute gastroesophageal variceal hemorrhage: a prospective randomized study. *J Korean Med Sci* 2006; **21**: 883-890 [PMID: 17043424 DOI: 10.3346/jkms.2006.21.5.883]
- 37 **Xu HW**, Wang JH, Tsai MS, Wu KL, Chiou SS, Changchien CS, Hu TH, Lu SN, Chuah SK. The effects of cefazolin on cirrhotic patients with acute variceal hemorrhage after endoscopic interventions. *Surg Endosc* 2011; **25**: 2911-2918 [PMID: 21424196 DOI: 10.1007/s00464-011-1642-0]
- 38 **Wu CK**, Wang JH, Lee CH, Wu KL, Tai WC, Lu SN, Hu TH, Chuah SK. The outcome of prophylactic intravenous cefazolin and ceftriaxone in cirrhotic patients at different clinical stages of disease after endoscopic interventions for acute variceal hemorrhage. *PLoS One* 2013; **8**: e61666 [PMID: 23630607]
- 39 **Chen WC**, Hou MC, Lin HC, Yu KW, Lee FY, Chang FY, Lee SD. Bacteremia after endoscopic injection of N-butyl-2-cyanoacrylate for gastric variceal bleeding. *Gastrointest Endosc* 2001; **54**: 214-218 [PMID: 11474393 DOI: 10.1067/mge.2001.116566]
- 40 **Chang CJ**, Hou MC, Lin HC, Lee HS, Liao WC, Su CW, Lee SD. The safety and probable therapeutic effect of routine use of antibiotics and simultaneously treating bleeding gastric varices by using endoscopic cyanoacrylate injection and concomitant esophageal varices with banding ligation: a pilot study. *Gastrointest Endosc* 2010; **71**: 1141-1149 [PMID: 20362285 DOI: 10.1016/j.gie.2009.12.010]

- 41 **Rajoriya N**, Forrest EH, Gray J, Stuart RC, Carter RC, McKay CJ, Gaya DR, Morris AJ, Stanley AJ. Long-term follow-up of endoscopic Histoacryl glue injection for the management of gastric variceal bleeding. *QJM* 2011; **104**: 41-47 [PMID: 20871126 DOI: 10.1093/qjmed/hcq161]
- 42 **Altraif I**, Handoo FA, Aljumah A, Alalwan A, Dafalla M, Saeed AM, Alkhormi A, Albekairy AK, Tamim H. Effect of erythromycin before endoscopy in patients presenting with variceal bleeding: a prospective, randomized, double-blind, placebo-controlled trial. *Gastrointest Endosc* 2011; **73**: 245-250 [PMID: 21145052 DOI: 10.1016/j.gie.2010.09.043]
- 43 **Jalan R**, Hayes PC. UK guidelines on the management of variceal haemorrhage in cirrhotic patients. British Society of Gastroenterology. *Gut* 2000; **46** Suppl 3-4: III1-III15 [PMID: 10862604]
- 44 **Garcia-Tsao G**, Sanyal AJ, Grace ND, Carey W. Prevention and management of gastroesophageal varices and variceal hemorrhage in cirrhosis. *Hepatology* 2007; **46**: 922-938 [PMID: 17879356 DOI: 10.1002/hep.21907]
- 45 **Garcia-Tsao G**, Sanyal AJ, Grace ND, Carey WD. Prevention and management of gastroesophageal varices and variceal hemorrhage in cirrhosis. *Am J Gastroenterol* 2007; **102**: 2086-2102 [PMID: 17727436 DOI: 10.1111/j.1572-0241.2007.01481.x]
- 46 **Dite P**, Labrecque D, Fried M, Gangl A, Khan AG, Bjorkman D, Eliakim R, Bektaeva R, Sarin SK, Fedail S, Krabshuis JH, Le Mair A. Esophageal varices. Available from: URL: [http://gastroindia.net/wp-content/themes/gastro/images/practice_guidelines_pdf/esophageal varices.pdf](http://gastroindia.net/wp-content/themes/gastro/images/practice_guidelines_pdf/esophageal%20varices.pdf)
- 47 **de Franchis R**. Revising consensus in portal hypertension: report of the Baveno V consensus workshop on methodology of diagnosis and therapy in portal hypertension. *J Hepatol* 2010; **53**: 762-768 [PMID: 20638742 DOI: 10.1016/j.jhep.2010.06.004]
- 48 **Augustin S**, Altamirano J, González A, Dot J, Abu-Suboh M, Armengol JR, Azpiroz F, Esteban R, Guardia J, Genescà J. Effectiveness of combined pharmacologic and ligation therapy in high-risk patients with acute esophageal variceal bleeding. *Am J Gastroenterol* 2011; **106**: 1787-1795 [PMID: 21625271 DOI: 10.1038/ajg.2011.173]
- 49 **García-Pagán JC**, Caca K, Bureau C, Laleman W, Appenrodt B, Luca A, Abraldes JG, Nevens F, Vinel JP, Mössner J, Bosch J. Early use of TIPS in patients with cirrhosis and variceal bleeding. *N Engl J Med* 2010; **362**: 2370-2379 [PMID: 20573925]

P- Reviewers: Brian BB, Hori T, Takagi H **S- Editor:** Zhai HH
L- Editor: A **E- Editor:** Zhang DN



Increased susceptibility to *Trichuris muris* infection and exacerbation of colitis in Mdr1a^{-/-} mice

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Received: August 27, 2013 Revised: October 17, 2013

Accepted: November 18, 2013

Published online: February 21, 2014

Abstract

AIM: To investigate the influence of *Trichuris muris* (*T. muris*) infection in a mouse model of genetic susceptibility to inflammatory bowel disease, Mdr1a^{-/-}.

METHODS: Mdr1a^{-/-} mice were housed under specific pathogen free conditions to slow the development of colitis and compared to congenic FVB controls. Mice were infected with approximately 200 embryonated ova from *T. muris* and assessed for worm burden and histological and functional markers of gut inflammation on day 19 post infection.

RESULTS: Mdr1a^{-/-} mice exhibited a marked increase in susceptibility to *T. muris* infection with a 10-fold increase in colonic worm count by day 19 *pi* compared to FVB controls. Prior to infection, Mdr1a^{-/-} exhibited

low-level mucosal inflammation with evidence of an enhanced Th1 environment. *T. muris* infection accelerated the progression of colitis in Mdr1a^{-/-} as evidenced by marked increases in several indicators including histological damage score, mucosal CD4⁺ T-cell and DC infiltration and dramatically increased production of pro-inflammatory cytokines.

CONCLUSION: These data provide further evidence of the complex interaction between *T. muris* and an inflammatory bowel disease (IBD)-susceptible host which may have relevance to the application of helminth therapy in the treatment of human IBD.

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Key words: Helminth; Colitis; Inflammatory bowel disease; P-glycoprotein; *Mdr1a*

Core tip: This study investigates the interaction between the helminth parasite *Trichuris muris* (*T. muris*) and Mdr1a^{-/-} mice, a genetic model of inflammatory bowel disease linked to deficiency of a key transporter protein in the gut barrier. The main findings are that (1) Mdr1a mice exhibit dramatically increased susceptibility to worm infection compared to congenic controls and (2) challenge with *T. muris* induces severe pathological changes consistent with a marked exacerbation of colitis in this model with preliminary evidence pointing to worm persistence as a driver of this effect. These findings will be of interest in the emerging field of helminth therapy in inflammatory bowel disease (IBD) providing further evidence of the complexity of worm interaction with an IBD-susceptible host.

Bhardwaj EK, Else KJ, Rogan MT, Warhurst G. Increased susceptibility to *Trichuris muris* infection and exacerbation of colitis in Mdr1a^{-/-} mice. *World J Gastroenterol* 2014; 20(7): 1797-1806
Available from: URL: <http://www.wjgnet.com/1007-9327/full/>

INTRODUCTION

The outcome of infection with the gastrointestinal nematode worm *Trichuris*, which occurs in a range of mammalian species, is genetically determined and involves subversion of host Th1 and Th2 immune responses to allow the worm to survive and colonise the gut^[1,2]. This has led to the suggestion that helminths may have the potential to reduce dysregulated gut inflammation in human diseases such as Crohn's disease^[3]. Clinical trials using the pig worm *Trichuris suis* (*T. muris*) in patients with active Crohn's disease or ulcerative colitis have reported a significant improvement in disease symptoms in a majority of patients receiving this treatment although the numbers of subjects tested remains relatively small^[4,5]. There is currently a paucity of information on the mechanisms by which helminths regulate inflammation in man and, therefore, the possible impact of genetic variability on susceptibility and response to helminth challenge, including the potential for adverse effects remains uncertain^[6]. Studies in a range of murine models of colitis have shown variable responses to infection with helminth species such as *H. polygyrus*, *H. diminuta* and *T. spiralis* including amelioration and exacerbation of disease^[7]. To date, there is little information on the effects of *Trichuris* species in murine colitis with a single study showing severe inflammation in IL-10-/- mice infected with *T. muris*^[8], although the precise contribution of the infection to the inflammation that develops spontaneously in this model was unclear. The aim of the present study was to explore the impact of *T. muris* infection on the intestinal inflammation seen in the Mdr1a-/- mouse model of inflammatory bowel disease (IBD). These mice develop spontaneous colitis as a result of a primary defect in the gut epithelial barrier caused by the deletion of the *Mdr1a* gene which encodes P-glycoprotein, a xenobiotic transporter located in the apical membrane of epithelial cells^[9,10]. Recent studies have shown similarities between the transcriptomic changes occurring during early development of colitis in Mdr1a-/-^[11] and those associated with *T. muris* infection in susceptible mouse strains^[12] making this an interesting model in which to investigate interaction of the parasite with an IBD-susceptible host.

MATERIALS AND METHODS

Animals

Mdr1a-/- mice (KO) and the congenic background strain FVB (WT) were obtained from Taconic Farms (Germantown, New York) and bred and maintained under specific pathogen free condition in the Biological Services Facility of the University of Manchester. Mice had been backcrossed for 7 generations onto the FVB background and were periodically genotyped by poly-

merase chain reaction. Mice were housed individually in isolator cages with access to filtered water and standard chow and routinely monitored for outward signs of bowel disease (diarrhea, weight loss and rectal bleeding). Mice used in these studies were between 5 and 13 wk of age on infection with *T. muris*. A total of 43 animals (21 FVB and 22 Mdr1a-/-) were used in these studies. All animal studies conformed to current United Kingdom Home Office legislation.

Parasites

The maintenance of *T. muris* and the method used for infection were as described by Wakelin^[13]. Mice were infected with approximately 200 embryonated eggs by oral gavage at day 0. Worm burden was assessed at day 19 post infection (*pi*) by counting the number of worms present in the caecum and first 4 cm of large intestine as described previously^[14]. For a low dose, 20 egg infection, embryonated eggs were counted individually into Eppendorfs and dispensed to mice at day 0. *T. muris* excretory/secretory protein (*T. muris* E/S) was produced as previously described^[13].

Isolation of mesenteric lymph nodes

Mesenteric lymph nodes (MLNs) were removed on day 19 *pi* and placed in 10 mL complete RPMI-1640 (PAA) containing 10% foetal calf serum (FCS) (PAA, gold-inactivated), 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco) and 1% L-glutamine (Gibco) on ice. MLNC were washed three times and resuspended at 5×10^6 cells/mL in RPMI 1640 medium (Gibco) containing 10% fetal calf serum, antibiotics as above, 2 mmol/L L-glutamine (Gibco). The final cell suspension (1 mL) was placed in duplicate wells of a 48 well culture plate (Costar). Cell suspensions were stimulated with concanavalin A (ConA) 2.5 µg/mL or 50 µg/mL 4 h *T. muris* E/S and incubated at 37 °C in 5% CO₂. Con A stimulated mesenteric lymph node cell culture was recovered after 24 h. Similarly, after 48 h the *T. muris* E/S stimulated cell culture was harvested and stored at -20 °C for cytokine analysis.

Measurement of parasite specific antibody

Parasite specific IgG1 and IgG2a levels in mouse serum was determined by ELISA as described previously^[15]. Briefly, microplates were coated with adult *T. muris* excretory/secretory (E/S) antigen at 5 µg/mL (50 µL/well) in carbonate buffer pH 9.6. After washing with 0.05% Tween 20 in phosphate buffered saline (PBS-T), plates were blocked with PBS-T containing 2% bovine serum albumin. Serial dilutions of mouse serum from 1:20 to 1:2560 in PBS-T were added and plates were incubated for 1 h at 37 °C. After washing 50 µL/well of biotinylated rat anti-IgG1 (Serotec Ltd, Oxford, United Kingdom) or rat anti-IgG2a (BD Pharmingen, Oxford, United Kingdom) were added followed by streptavidin conjugated peroxidase and TMB detection at 405 nm.

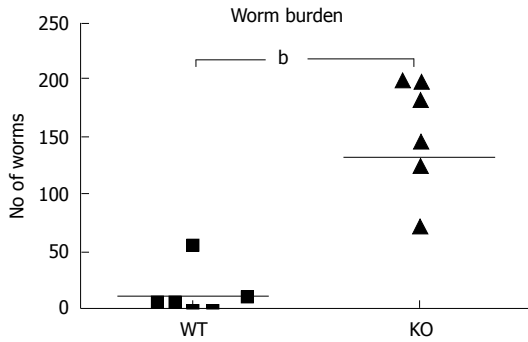


Figure 1 *Mdr1a*^{-/-} (KO) mice show increased susceptibility to *Trichuris muris* infection compared to FVB controls (WT). Data shows large intestinal worm burden measured on day 19 following infection with approximately 200 embryonated *Trichuris muris* eggs. Each data point represents a single mouse ($n = 6$ in each group); the horizontal bar denotes the mean for each group. ^b $P < 0.01$.

Cytokine analysis

The cytokines interleukin (IL)-5, IL-12p40 and interferon (IFN)- γ were quantified by an in-house sandwich ELISA using capture and detection antibodies from BD Pharmingen (Oxford, United Kingdom). Plates were washed with PBS-T and blocked with 10% fetal calf serum in phosphate buffered saline. Standard curves prepared with recombinant murine cytokine standards were used to quantify cytokines in experimental samples with OD at 405 nm on a MRXII microplate reader. IL-13 and tumor necrosis factor (TNF)- α were analysed using commercial ELISA kits obtained from R and D Systems (Abingdon, United Kingdom) according to the manufacturer's instructions.

Colonic epithelial cell isolation

Whole mouse colon was isolated, split longitudinally and washed in RPMI 1640 medium (PAA Laboratories, Yeovil, United Kingdom) supplemented with 100 U/mL penicillin and 100 μ g/mL streptomycin. Colon was cut into small pieces (approximately 5 mm) and incubated in 10 mL Dulbecco's modified Eagle medium (Invitrogen, Paisley, United Kingdom) containing 20% FCS (PAA Laboratories, Yeovil, United Kingdom), 100 U/mL penicillin, 100 μ g/mL streptomycin and 1 mg/mL Dispase (Sigma-Aldrich) with gentle rotation at 37 °C for 90 min. Epithelial cells were released by gentle aspiration with a pipette. Following brief centrifugation (750 g; 5 min), the epithelial cell pellet was washed in RPMI 1640 medium and re-pelleted. One milliliter of TRIzol reagent (Invitrogen, Paisley, United Kingdom) was added and RNA isolated according to the manufacturer's instructions.

Gene expression analysis

Colonocyte RNA was reverse transcribed using a commercial first-strand cDNA synthesis kit (Amersham Biosciences, Little Chalfont, United Kingdom) using random hexamers. Relative quantification of gene expression was performed using assays designed by ProbeFinder software (www.universalprobelibrary.com); this gives gene-specific primer pairs combined with the appropriate

hydrolysis probes [5-fluorescein (FAM)-labeled; 3-dark quencher dye] from the Universal Probe Library (UPL; Roche Diagnostics, Lewes, United Kingdom). Assays were performed using a Lightcycler480 (Roche Diagnostics) in a 20 μ L volume with 200 nmol/L of each primer, 100 nmol/L of the UPL probe and approximately 0.3 μ g of cDNA. Gene expression was quantified relative to β -actin.

Histology

Colon from *mdr1a*^{-/-} or FVB controls was assessed histologically for evidence of inflammation and tissue injury using a previously described grading system^[11]. Three main grading criteria with 4 different subgrades of severity (0-3) in each were applied. The resulting combined score (maximum 9) indicates disease severity with 0-normal, 1-3-mild changes, 3-6-moderate changes, 7-9-severe changes.

Immunohistochemistry

Samples of large intestine near to the caecum were obtained from naïve or *T. muris* infected (day 19) *mdr1a*^{-/-} and FVB mice were snap-frozen and stored in liquid nitrogen until analysis. Cryostat frozen sections (6 μ m) were fixed in 4% paraformaldehyde (for CD4⁺ staining) or acetone (for dendritic cells, DC). For CD4⁺, sections were incubated with 5 μ g/mL biotinylated CD4 antibody (Clone H129.19; BD Pharmingen), followed by Vectastain ABC kit (Vector Laboratories, Peterborough, United Kingdom) and colour development with 3,3'-diaminobenzidine. Sections were counterstained with haematoxylin QS. For DC, sections were rehydrated in PBS and incubated with biotinylated anti-CD11c (BD Biosciences) and anti-cytokeratin (Sigma-Aldrich). Following incubation with Vectastain ABC kit (Vector Laboratories, Peterborough, United Kingdom), slides were treated with tyramide amplification reagents (PerkinElmer) and washed and counterstained with the nuclear stain 4',6'-diamidino-2-phenylindole.

Statistical analysis

All data are presented as mean \pm SD with n indicating the number of mice used. Statistical comparisons were performed by either t test or ANOVA with Dunnett's post test for multiple comparisons using GraphPad Prism 3.02 software.

RESULTS

Mdr1a^{-/-} mice exhibit enhanced susceptibility to *T. muris* infection

Following infection of *Mdr1a*^{-/-} mice or the congenic background strain FVB with approximately 200 embryonated *T. muris* eggs on day 0, the worm burden in the large intestine was analysed in both groups on day 19 *pi* (Figure 1). Previous evidence suggests that resistant mouse strains generally expel *T. muris* by day 19 *pi* while susceptible strains will retain worms in the large intestine^[16]. The worm count in FVB control mice was relatively low (11

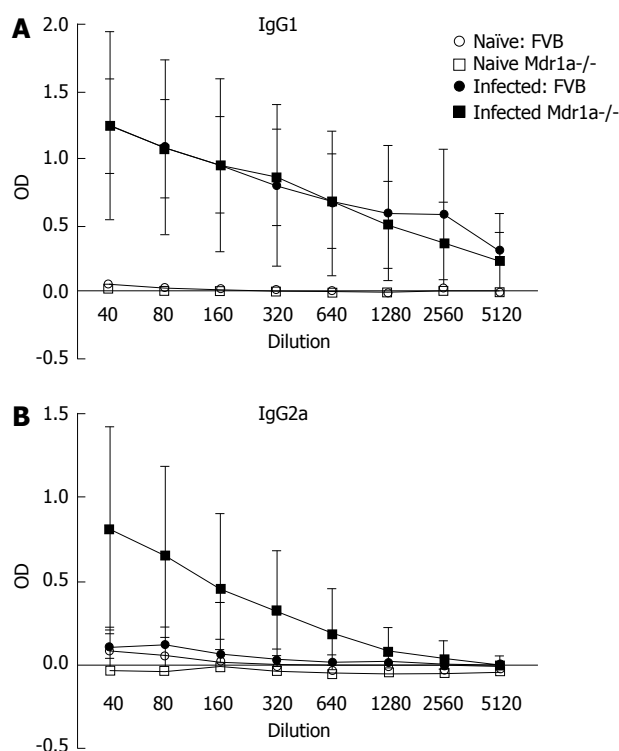


Figure 2 Parasite-specific IgG1 and IgG2a antibody responses in the serum of naïve and *Trichuris muris* infected FVB (WT) and *mdr1a*^{-/-} (KO) mice. Serum was taken from mice at day 19 post infection. Data shows mean \pm SD for $n = 5-7$ animals in each group. OD: Optical density.

± 7) consistent with this strain having a resistant phenotype. In marked contrast, mice lacking the *Mdr1a* gene showed a 10-fold higher worm count (134 ± 28 , $P < 0.01$) indicative of worm retention and a significantly increased susceptibility to *T. muris* infection (Figure 1). Infection with *T. muris* is associated with a strong anti-parasite antibody response which is suggested as a useful indicator of the Th1/Th2 balance and, therefore the resistance or susceptibility to parasite infection^[17]. Figure 2 shows the parasite-specific IgG1 and IgG2a antibody response in the serum of naïve and *T. muris* infected FVB control and *mdr1a*^{-/-} mice on day 19 *pi*. Sera from infected FVB and *mdr1a*^{-/-} mice contained parasite-specific IgG1 at equivalent levels. However, consistent with the increased susceptibility to *T. muris* infection seen in *Mdr1a*^{-/-}, levels of anti-*Trichuris* IgG2a were only increased in the *Mdr1a*^{-/-} mouse.

The increased retention of worms in *Mdr1a*^{-/-} compared to FVB control could be the result of an enhanced Th1 environment. Production of the Th1 cytokine, IFN γ by MLN from naïve animals was higher in *Mdr1a*^{-/-} than FVB (562 ± 561 pg/mL *vs* 149 ± 108 pg/mL, $n = 5$; see Figure 5) but this did not reach statistical significance ($P > 0.05$). However, analysis of colonocytes isolated from these mice showed significant up-regulation of genes that are known to be IFN γ -dependent. Expression of Indoleamine 2, 3 dioxygenase (IDO), a tryptophan catabolizing enzyme linked to susceptibility to *T. muris* infection^[18] and interferon gamma inducible protein-10, (IP-10;

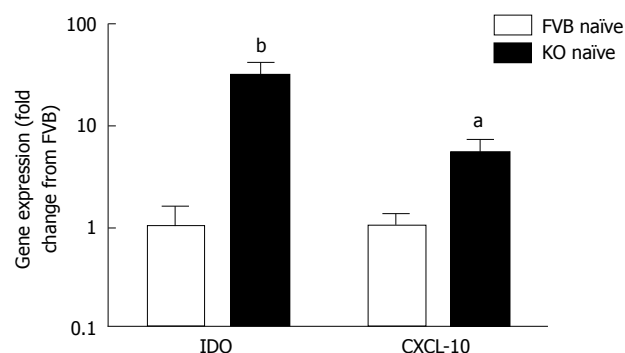


Figure 3 Expression of interferon γ -dependent genes IDO and CXCL-10 in colonocytes from naïve *Mdr1a*^{-/-} and FVB mice. mRNA expression was measured relative to β -actin in each case. Data is expressed as fold change in expression in *Mdr1a*^{-/-} colonocytes relative to FVB controls and is shown as mean \pm SD for $n = 4$ preparations for each group. ^a $P < 0.05$, ^b $P < 0.01$ vs FVB groups.

CXCL-10) were markedly higher (30- and 7-fold respectively) in naïve *Mdr1a*^{-/-} mice compared to FVB controls (Figure 3). These data provide evidence that *Mdr1a*^{-/-} mice have a pre-existing Th1 environment prior to infection with *T. muris*.

T. muris infection increases the severity of colitis in *Mdr1a*^{-/-} mice

We next investigated the impact of *T. muris* infection on colitis development in *Mdr1a*^{-/-} mice. *T. muris* infection in FVB mice produced no significant clinical or histological evidence of colitis (Figure 4); both naïve and infected animals had normal colonic mucosal morphology with the exception of one mouse with a minor inflammatory infiltrate on day 19 post infection (Figure 4B). In contrast, there was a major difference in colitis indicators in age-matched *Mdr1a*^{-/-}. Naïve *Mdr1a*^{-/-} showed a modest disease score (1.5) indicative of an existing low-level mucosal inflammation and consistent with the spontaneous, age-dependent development of colitis in this model (Figure 4C). However, infection of *Mdr1a*^{-/-} with *T. muris*, was associated with a dramatic increase in disease score (3.6) with the colon showing loss of architecture, a severe mixed inflammatory infiltrate and evidence of crypt abscess (Figure 4D and E). The pro-inflammatory effect of *T. muris* infection in *Mdr1a*^{-/-} was also evident in the cytokine response of MLN isolated at day 19 *pi* (Figure 5). Production of the Th1 cytokine IFN γ in FVB controls was not significantly increased by *T. muris* infection (Figure 5A) consistent with the ability of this mouse strain to expel *T. muris*. However, infection of *Mdr1a*^{-/-} mice led to a dramatic increase in IFN γ production with levels approximately 7 fold higher than naïve animals ($P < 0.01$). A second Th1-associated cytokine, IL-12p40 and the pro-inflammatory cytokine TNF α showed a similar pattern with marked increases in infected *Mdr1a*^{-/-} but not infected FVB mice. (Figure 5B and C). A different pattern was observed for Th2 cytokines (Figure 5D and E). IL-13 and IL-5 were elevated in both FVB and *Mdr1a*^{-/-} following *T. muris* infection (Figure 5D and E) and while

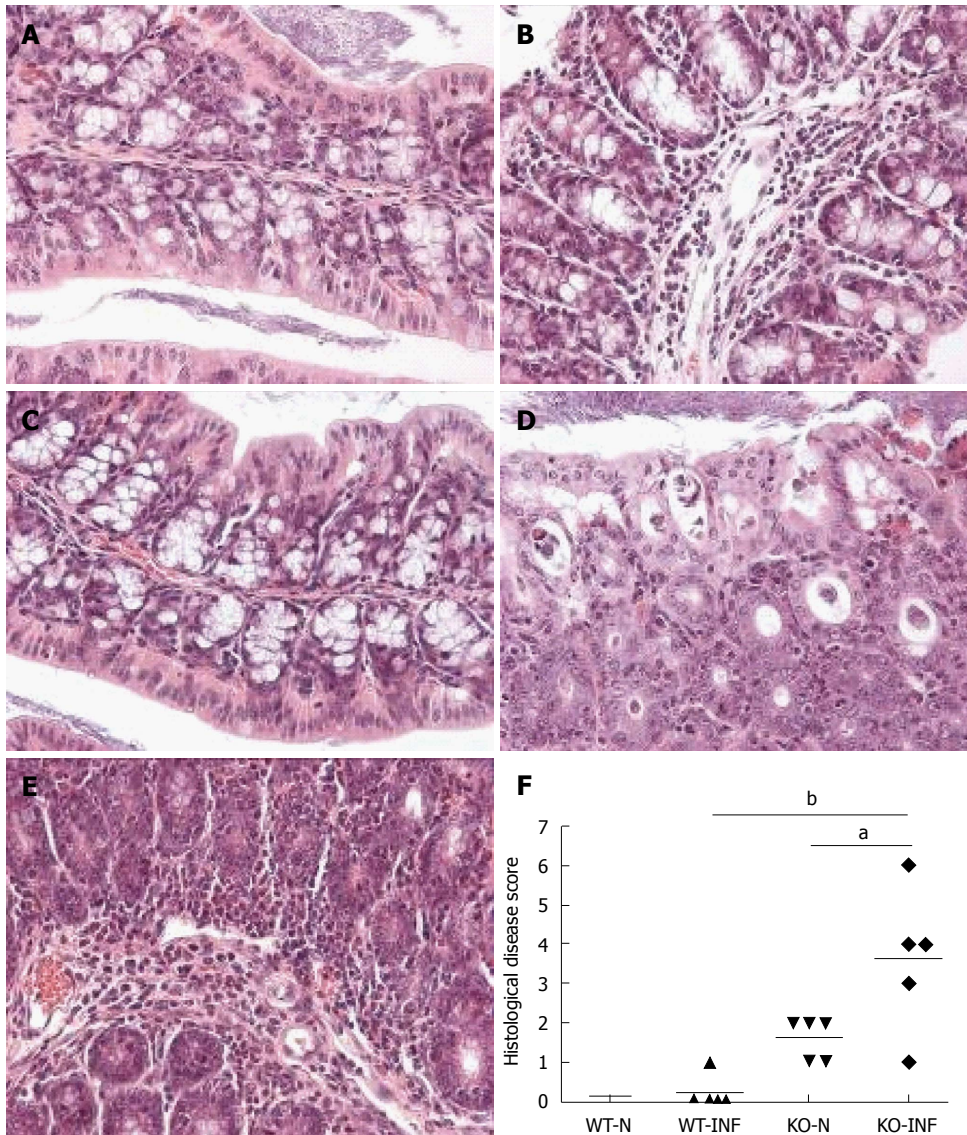


Figure 4 Histological analysis of proximal colon from FVB (WT) and *mdr1a*^{-/-} (PGP-KO) mice. A-E: H and E stained sections from proximal colon of WT-N (A), WT-INF (B), KO-N (C) and KO-INF (D-E). Tissues were isolated on day 19 post-infection in *Trichuris muris* (*T. muris*) infected mice; F: Histological disease scores for colon from naïve (WT-N; KO-N) and *T. muris* infected (WT-INF; KO-INF) mice based on the grading system described by Collett *et al*^[11], 2008. Bar shows mean values for each group **P* < 0.05, ***P* < 0.01, *n* = 5 for each group.

the increase was somewhat greater in *Mdr1a*^{-/-}, this was significant only for IL-5 (*Mdr1a*^{-/-}: 124.3 ± 78.7 pg/mL *vs* FVB: 48.3 ± 29.7 pg/mL, *P* < 0.05). Taken together, these data shows that whilst both FVB and *Mdr1a*^{-/-} mount a Th2 response *pi* a significantly heightened Th1/pro-inflammatory response is only seen in the *Mdr1a*^{-/-} mouse. Consistent with the significantly greater histological disease score seen in Figure 4, infected *Mdr1a*^{-/-} mice exhibited a significantly greater intestinal CD4⁺ T cell infiltrate compared to both infected wild type mice and uninfected *Mdr1a*^{-/-} mice (Figure 6). Likewise dendritic cells appeared more numerous in the gut tissue from infected *Mdr1a*^{-/-} mice compared to uninfected mice and infected wild type mice, indicative of a more inflamed environment (Figure 7). One possibility to explain these findings is that the worsened pathology in infected *Mdr1a*^{-/-} mice may simply be a reflection of the inflammatory response

associated with greater exposure to worms rather an increase in severity of the underlying colitis present in this model. To investigate this possibility, *mdr1a*^{-/-} and FVB mice were infected with a low dose *T. muris* infection. Low level infections established from 20 eggs are not expelled from any mouse strain^[19] thus ensuring more equivalent antigen exposure between mouse strains with different susceptibility to *T. muris* infection. In this situation, both FVB and *Mdr1a*^{-/-} strains harboured worms (4 ± 3 and 10 ± 3 ; *n* = 5 respectively) at day 19 post infection. This is in marked contrast to high dose infection where only *Mdr1a*^{-/-} mice harboured worms at this time point (Figure 1). Importantly, despite the similarity in worm burden, gut pathology in *Mdr1a*^{-/-} was still significantly worse as evidenced by the high disease score (mean score: infected *Mdr1a*^{-/-} 4.1; infected FVB 1.4) consistent with *T. muris* accelerating the disease process in these animals.

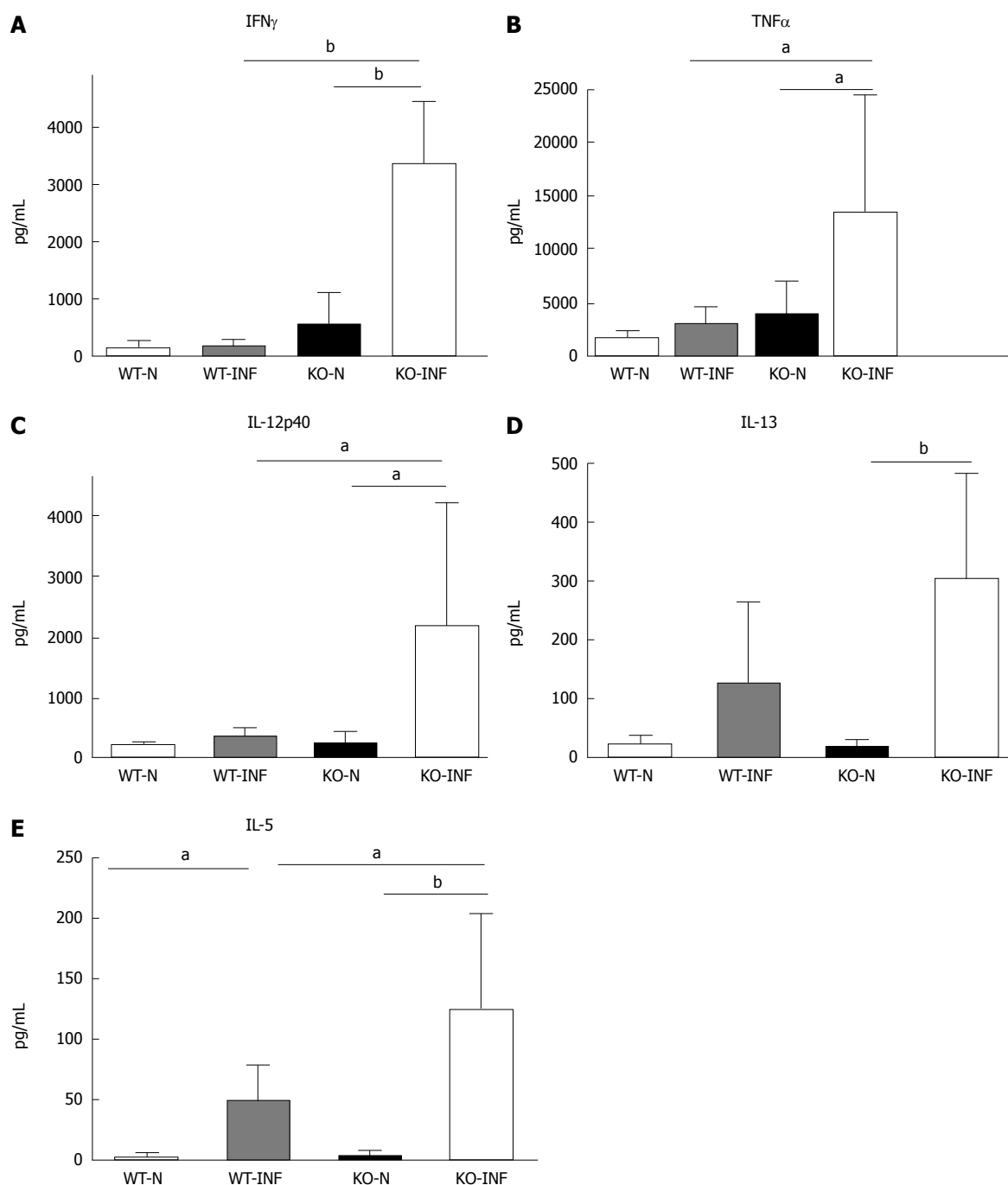


Figure 5 Cytokine production by mesenteric lymph nodes from naïve and *Trichuris muris* infected FVB (WT) and *mdr1a*^{-/-} (PGP-KO) mice. Levels of Th1 [interferon (IFN) γ , A; interleukin (IL)-12p40, C] and Th2 (IL-13, D; IL-5, E) and the pro-inflammatory cytokine [tumor necrosis factor (TNF) α , B] were measured in mesenteric lymph nodes (MLN) following *in vitro* stimulation with *Trichuris muris* (*T. muris*) E/S antigen. MLN were isolated on day 19 post infection in *T. muris* infected groups. Data is shown as mean + SD for *n* = 5 animals in each group ^a*P* < 0.05; ^b*P* < 0.01.

DISCUSSION

This study provides further evidence of the complex interaction between *T. muris* and IBD which may have relevance to the proposed use of helminth therapy in the treatment of IBD. Mice with a genetic predisposition to IBD, mediated by deletion of an epithelial transporter (p-glycoprotein) that forms a key part of the gut barrier, were shown to have a markedly increased susceptibility to infection with *T. muris* compared to the congenic

background strain that was resistant to infection. The most likely driver of increased susceptibility to infection in *Mdr1a*^{-/-} would seem to be an enhanced Th1 environment. A recent study by Collett *et al.*^[11] showed upregulation in expression of a range of IFN γ -dependent genes in *Mdr1a*^{-/-} colon including IDO, T-cell specific GTPase and MHC Class II subtypes that precedes the development of active colonic inflammation. This is consistent with the present observation that expression of IDO and CXCL-10 is elevated in colonocytes from *Mdr1a*^{-/-} mice

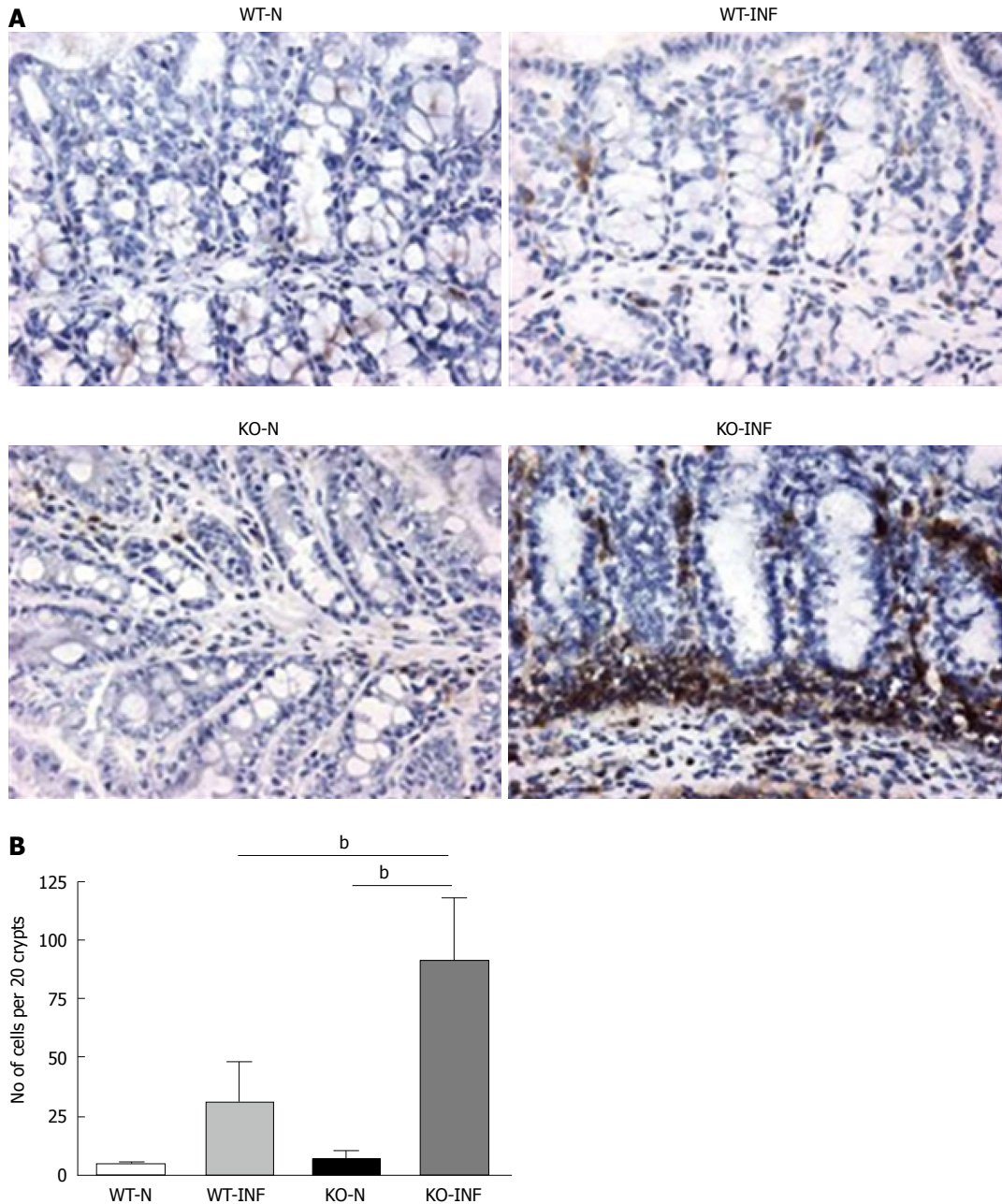


Figure 6 CD4⁺ T-cell infiltration associated with *Trichuris muris* infection in the proximal colon of FVB (WT) and *mdr1a*^{-/-} (PGP-KO) mice. A: Immunohistochemical staining for CD4⁺ T cells in naïve (WT-N; KO-N) and *Trichuris muris* (*T. muris*) infected (WT-INF; KO-INF) WT and PGP-KO mice; B: Mean data (\pm SD) from 5 animals in each group showing CD4⁺ T cells present per 20 crypts units. Tissues were isolated on day 19 post-infection in *T. muris* infected mice, ^b $P < 0.01$.

exhibiting little or no gut pathology. Similar increases in expression of IFN γ -dependent genes are found in *T. muris*-susceptible mouse strains^[12] while neutralisation of IFN γ effectively reversed susceptibility to infection by promoting worm expulsion^[20-22].

The *Mdr1a*^{-/-} strain is characterised by spontaneous and relatively slow development of colitis^[9,11] and the animals used in the present study were at an early stage with evidence of modest inflammatory changes. The finding that challenge with *T. muris* rapidly induced severe pathological changes consistent with an advanced stage of colitis in these animals was somewhat unexpected in light of evidence that infection with different parasitic helminths

can prevent or ameliorate colonic inflammation^[23].

Nevertheless, while a majority of studies report beneficial effects, there have been reports of enhanced disease following helminth infection. Infection with *Hy-menolepis diminuta* resulted in a significant exacerbation of oxalazone-induced colitis in mice^[24,25] despite evidence that the parasite prevents DNBS-induced colitis^[26]. Similarly, *Heligmosomoides polygyrus bakeri* enhances gut inflammation induced by enteric infection with *Citrobacter rodentium*^[27] but has protective effects in a number of other colitis models^[28,29]. Surprisingly, there is little information on *T. muris* in mouse models despite the fact that the porcine strain of this parasite (*T. suis*) has been used

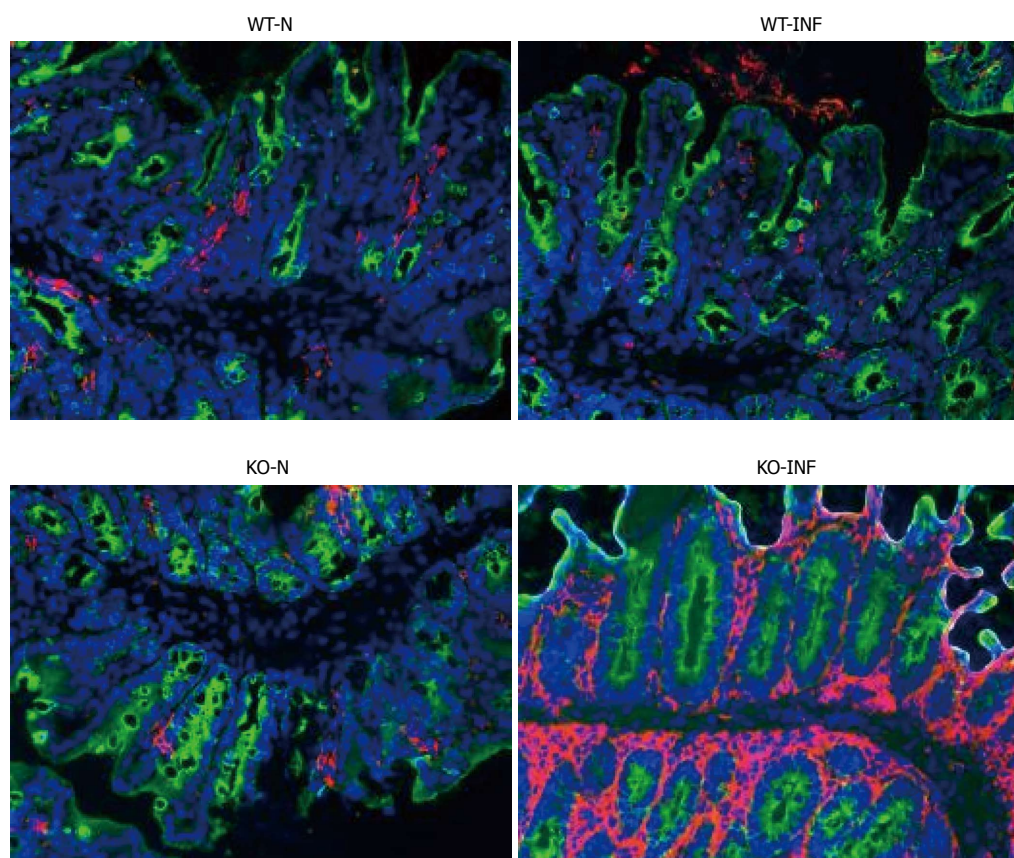


Figure 7 Dendritic cell infiltration associated with *Trichuris muris* infection in the proximal colon of FVB (WT) and *mdr1a*^{-/-} (PGP-KO) mice. Fluorescence staining (pink) for dendritic cells in frozen sections of proximal colon from naïve (WT-N; KO-N) and *Trichuris muris* (*T. muris*) infected (WT-INF; KO-INF) mice. Tissues were removed on day 19 post infection in *T. muris* infected mice. Original magnification $\times 20$. Images are representative of tissues from 5 mice in each group.

in the majority of human trials of helminth therapy^[30]. Wilson *et al.*^[8] showed development of a lethal colitis in IL-10^{-/-} mice infected with *T. muris* although the extent to which the parasite enhanced the colitis that develops spontaneously in these mice is unclear. A recent study showing close transcriptional similarities between human colitis and the response to *T. muris* infection, including changes in gene expression that could exacerbate inflammation, has urged caution in the therapeutic use of helminths^[6]. The present study adds some support to this view suggesting interdependence between genetic predisposition to colitis and susceptibility to *T. muris* infection, most likely linked to the prevailing immune environment that determines the degree to which worms persist and their overall impact on mucosal inflammation. This could at best compromise the efficacy of worm therapy but, at worst, potentially result in exacerbation of the disease as demonstrated here. For example, the prevailing immune profile will clearly depend on the type of IBD present; Crohn's disease is characteristically a Th1 driven, IFN γ -dominated inflammation while ulcerative colitis is primarily Th2-mediated with little evidence of an increased IFN γ response^[31].

Key questions remain regarding the precise mechanism by which *T. muris* exacerbates colitis in Mdr1a^{-/-}. These relate to whether the effects of *T. muris* infection vary according to the stage of the disease (*e.g.*, early devel-

opment or established colitis) and whether the exacerbation of colitis depends on worm persistence, number or both. The involvement of the IL-17/IL-23 pathway in the responses observed is also worthy of further investigation given recent evidence of its role in both IBD pathogenesis and host response to helminth infection^[32,33].

The relevance of these findings to the use of worm therapy in the treatment of IBD in man is difficult to determine at this stage. The pigworm *T. suis* may not be able to establish for any length of time in the human colon due to species incompatibility and the relatively few clinical trials conducted so far have not reported significant safety issues^[7,34]. However, the potential for pathological effects in man continues to be raised^[35] and information on the level and persistence of worms following human administration and whether this varies across individuals is necessary but not currently available. The present study in Mdr1a^{-/-} is relevant in that it shows that a predisposition to *T. muris* infection is associated with increased inflammation rather than amelioration. In this respect, the preliminary observation that low dose challenge with *T. muris*, which produces a persistent infection but with low worm burden, produced a similar enhancement of colitis to that seen with high dose in Mdr1a is intriguing. Further studies are clearly required but this could indicate that persistence of worms rather than worm burden *per se* may be a key factor in determin-

ing whether effects on host inflammation are beneficial or detrimental.

In conclusion, these data, taken together with similar studies in man and other animal model of IBD support the view that the impact of helminths on IBD in a given situation may be difficult to predict because the balance between disease amelioration and exacerbation will depend on a range of factors including species compatibility, the prevailing immune environment and host genetic susceptibility.

COMMENTS

Background

Preliminary clinical trials suggest that helminths such as *Trichuris* spp. may have therapeutic potential in the treatment inflammatory bowel disease (IBD) in man. The precise mechanisms are unclear although this may be related to subversion of host Th1 and Th2 responses which allows the worm to survive and colonise the gut.

Research frontiers

The success and acceptance of helminths as a treatment for IBD in man will depend on the ability to target therapy to individuals where the risk/benefit is clearly defined. Currently, there is a lack of information on the impact of host genetic variability on susceptibility and response to infection with *Trichuris* and the potential for adverse effects. In this study, the authors investigate the interaction of *Trichuris* with mice that are predisposed to IBD due to lack of the epithelial transporter gene *Mdr1a*.

Innovations and breakthroughs

A novel finding was that *Mdr1a*^{-/-} mice have a much greater susceptibility to *Trichuris* infection than congenic controls resulting in higher numbers of worms that persist for longer. This was associated with a marked exacerbation of gut inflammation rather than amelioration of disease as might have been expected. Preliminary evidence using worm infection at a lower doses points to worm persistence rather than worm number as being the main driver of increased disease severity, although further studies are needed to confirm this observation.

Applications

These findings will be of interest in the emerging field of helminth therapy in IBD providing further evidence of the complexity of worm interaction with an IBD-susceptible host. This study in mice highlights the unpredictability of the effects of helminth infection in hosts that have a genetic susceptibility to inflammatory bowel disease. The relevance of the current findings to the therapeutic use of *Trichuris* in human inflammatory bowel disease is unclear, however they indicate the need for further studies to determine how genetic factors influence the level and persistence of *Trichuris* infection and its impact on gut inflammation in man.

Terminology

IBD is characterized by dysregulation of mucosal immunity associated with an inappropriate response to the gut microbiota and has a significant genetic component. *Trichuris* is an intestinal helminth parasite that infects a range of mammalian species, modulating the host immune system to survive and colonise the intestine. The *Mdr1a* gene encodes a common xenobiotic transporter, P-glycoprotein which is an important component of the gut epithelial barrier and appears to be important in regulating interactions with the gut flora. *Mdr1a*^{-/-} mice lack P-glycoprotein in the intestinal epithelium and spontaneously develop inflammatory bowel disease.

Peer review

The investigation has profound therapeutical implication highlighting the new approaches to treatment of autoimmune diseases such as Crohn's disease. Exploring the effect of helminth therapy in this disease by following the cytokine expression of Th1 and Th2 immune responses as well as CD4⁺ and dendritic cells they showed the complexity of processes in worm infection especially in IBD-susceptible host.

REFERENCES

- 1 Cliffe LJ, Grecis RK. The *Trichuris muris* system: a paradigm of resistance and susceptibility to intestinal nematode

infection. *Adv Parasitol* 2004; **57**: 255-307 [PMID: 15504540 DOI: 10.1016/S0065-308X(04)57004-5]

- 2 Else KJ. Have gastrointestinal nematodes outwitted the immune system? *Parasite Immunol* 2005; **27**: 407-415 [PMID: 16179034 DOI: 10.1111/j.1365-3024.2005.00788.x]
- 3 Wang LJ, Cao Y, Shi HN. Helminth infections and intestinal inflammation. *World J Gastroenterol* 2008; **14**: 5125-5132 [PMID: 18777588 DOI: 10.3748/wjg.14.5125]
- 4 Summers RW, Elliott DE, Urban JF, Thompson R, Weinstock JV. *Trichuris suis* therapy in Crohn's disease. *Gut* 2005; **54**: 87-90 [PMID: 15591509 DOI: 10.1136/gut.2004.041749]
- 5 Summers RW, Elliott DE, Urban JF, Thompson RA, Weinstock JV. *Trichuris suis* therapy for active ulcerative colitis: a randomized controlled trial. *Gastroenterology* 2005; **128**: 825-832 [PMID: 15825065 DOI: 10.1053/j.gastro.2005.01.005]
- 6 Levison SE, McLaughlin JT, Zeef LA, Fisher P, Grecis RK, Pennock JL. Colonic transcriptional profiling in resistance and susceptibility to trichuriasis: phenotyping a chronic colitis and lessons for iatrogenic helminthosis. *Inflamm Bowel Dis* 2010; **16**: 2065-2079 [PMID: 20687192 DOI: 10.1002/ibd.21326]
- 7 Weinstock JV, Elliott DE. Translatability of helminth therapy in inflammatory bowel diseases. *Int J Parasitol* 2013; **43**: 245-251 [PMID: 23178819 DOI: 10.1016/j.ijpara.2012.10.016]
- 8 Wilson MS, Ramalingam TR, Rivollier A, Shenderov K, Mentink-Kane MM, Madala SK, Cheever AW, Artis D, Kelsall BL, Wynn TA. Colitis and intestinal inflammation in IL10^{-/-} mice results from IL-13Rα2-mediated attenuation of IL-13 activity. *Gastroenterology* 2011; **140**: 254-264 [PMID: 20951137 DOI: 10.1053/j.gastro.2010.09.047]
- 9 Panwala CM, Jones JC, Viney JL. A novel model of inflammatory bowel disease: mice deficient for the multiple drug resistance gene, *mdr1a*, spontaneously develop colitis. *J Immunol* 1998; **161**: 5733-5744 [PMID: 9820555]
- 10 Maggio-Price L, Shows D, Waggle K, Burich A, Zeng W, Escobar S, Morrissey P, Viney JL. *Helicobacter bilis* infection accelerates and H. hepaticus infection delays the development of colitis in multiple drug resistance-deficient (*mdr1a*^{-/-}) mice. *Am J Pathol* 2002; **160**: 739-751 [PMID: 11839595 DOI: 10.1016/S0002-9440(10)64894-8]
- 11 Collett A, Higgs NB, Gironella M, Zeef LA, Hayes A, Salmo E, Haboubi N, Iovanna JL, Carlson GL, Warhurst G. Early molecular and functional changes in colonic epithelium that precede increased gut permeability during colitis development in *mdr1a*^{-/-} mice. *Inflamm Bowel Dis* 2008; **14**: 620-631 [PMID: 18275070 DOI: 10.1002/ibd.20375]
- 12 Datta R, deSchoolmeester ML, Hedeler C, Paton NW, Brass AM, Else KJ. Identification of novel genes in intestinal tissue that are regulated after infection with an intestinal nematode parasite. *Infect Immun* 2005; **73**: 4025-4033 [PMID: 15972490 DOI: 10.1128/IAI.73.7.4025-4033.2005]
- 13 Wakelin D. Acquired immunity to *Trichuris muris* in the albino laboratory mouse. *Parasitology* 1967; **57**: 515-524 [PMID: 6048569 DOI: 10.1017/S0031182000072395]
- 14 Else KJ, Wakelin D, Wassom DL, Hauda KM. The influence of genes mapping within the major histocompatibility complex on resistance to *Trichuris muris* infections in mice. *Parasitology* 1990; **101** Pt 1: 61-67 [PMID: 2235076 DOI: 10.1017/S0031182000079762]
- 15 Else KJ, Entwistle GM, Grecis RK. Correlations between worm burden and markers of Th1 and Th2 cell subset induction in an inbred strain of mouse infected with *Trichuris muris*. *Parasite Immunol* 1993; **15**: 595-600 [PMID: 7877836]
- 16 Else K, Wakelin D. The effects of H-2 and non-H-2 genes on the expulsion of the nematode *Trichuris muris* from inbred and congenic mice. *Parasitology* 1988; **96** (Pt 3): 543-550 [PMID: 3136419 DOI: 10.1017/S0031182000080173]
- 17 Blackwell NM, Else KJ. A comparison of local and peripheral parasite-specific antibody production in different strains of mice infected with *Trichuris muris*. *Parasite Immunol* 2002; **24**: 203-211 [PMID: 12010485 DOI: 10.1046/

- j.1365-3024.2002.00452.x]
- 18 **Bell LV**, Else KJ. Regulation of colonic epithelial cell turnover by IDO contributes to the innate susceptibility of SCID mice to *Trichuris muris* infection. *Parasite Immunol* 2011; **33**: 244-249 [PMID: 21392042 DOI: 10.1111/j.1365-3024.2010.01272.x]
- 19 **Bancroft AJ**, Else KJ, Grecis RK. Low-level infection with *Trichuris muris* significantly affects the polarization of the CD4 response. *Eur J Immunol* 1994; **24**: 3113-3118 [PMID: 7805740 DOI: 10.1002/eji.1830241230]
- 20 **Else KJ**, Finkelman FD, Maliszewski CR, Grecis RK. Cytokine-mediated regulation of chronic intestinal helminth infection. *J Exp Med* 1994; **179**: 347-351 [PMID: 8270879 DOI: 10.1084/jem.179.1.347]
- 21 **Hepworth MR**, Grecis RK. Disruption of Th2 immunity results in a gender-specific expansion of IL-13 producing accessory NK cells during helminth infection. *J Immunol* 2009; **183**: 3906-3914 [PMID: 19692641 DOI: 10.4049/jimmunol.0900577]
- 22 **Taylor BC**, Zaph C, Troy AE, Du Y, Guild KJ, Comeau MR, Artis D. TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. *J Exp Med* 2009; **206**: 655-667 [PMID: 19273626 DOI: 10.1084/jem.20081499]
- 23 **Elliott DE**, Summers RW, Weinstock JV. Helminths and the modulation of mucosal inflammation. *Curr Opin Gastroenterol* 2005; **21**: 51-58 [PMID: 15687885]
- 24 **Hunter MM**, Wang A, McKay DM. Helminth infection enhances disease in a murine TH2 model of colitis. *Gastroenterology* 2007; **132**: 1320-1330 [PMID: 17408663 DOI: 10.1053/j.gastro.2007.01.038]
- 25 **Wang A**, Fernando M, Leung G, Phan V, Smyth D, McKay DM. Exacerbation of oxazolone colitis by infection with the helminth *Hymenolepis diminuta*: involvement of IL-5 and eosinophils. *Am J Pathol* 2010; **177**: 2850-2859 [PMID: 21037078 DOI: 10.2353/ajpath.2010.100537]
- 26 **Hunter MM**, Wang A, Hirota CL, McKay DM. Neutralizing anti-IL-10 antibody blocks the protective effect of tapeworm infection in a murine model of chemically induced colitis. *J Immunol* 2005; **174**: 7368-7375 [PMID: 15905584]
- 27 **Chen CC**, Louie S, McCormick B, Walker WA, Shi HN. Concurrent infection with an intestinal helminth parasite impairs host resistance to enteric *Citrobacter rodentium* and enhances *Citrobacter*-induced colitis in mice. *Infect Immun* 2005; **73**: 5468-5481 [PMID: 16113263 DOI: 10.1128/IAI.73.9.5468-5481.2005]
- 28 **Elliott DE**, Setiawan T, Metwali A, Blum A, Urban JF, Weinstock JV. Heligmosomoides polygyrus inhibits established colitis in IL-10-deficient mice. *Eur J Immunol* 2004; **34**: 2690-2698 [PMID: 15368285 DOI: 10.1002/eji.200324833]
- 29 **Blum AM**, Hang L, Setiawan T, Urban JP, Stoyanoff KM, Leung J, Weinstock JV. Heligmosomoides polygyrus bakeri induces tolerogenic dendritic cells that block colitis and prevent antigen-specific gut T cell responses. *J Immunol* 2012; **189**: 2512-2520 [PMID: 22844110 DOI: 10.4049/jimmunol.1102892]
- 30 **Elliott DE**, Weinstock JV. Where are we on worms? *Curr Opin Gastroenterol* 2012; **28**: 551-556 [PMID: 23079675 DOI: 10.1097/MOG.0b013e3283572f73]
- 31 **Strober W**, Fuss IJ. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology* 2011; **140**: 1756-1767 [PMID: 21530742 DOI: 10.1053/j.gastro.2011.02.016]
- 32 **Geremia A**, Jewell DP. The IL-23/IL-17 pathway in inflammatory bowel disease. *Expert Rev Gastroenterol Hepatol* 2012; **6**: 223-237 [PMID: 22375527 DOI: 10.1586/egh.11.107]
- 33 **Fasnacht N**, Greweling MC, Bollati-Fogolin M, Schippers A, Müller W. T-cell-specific deletion of gp130 renders the highly susceptible IL-10-deficient mouse resistant to intestinal nematode infection. *Eur J Immunol* 2009; **39**: 2173-2183 [PMID: 19593768 DOI: 10.1002/eji.200838710]
- 34 **Sandborn WJ**, Elliott DE, Weinstock J, Summers RW, Landry-Wheeler A, Silver N, Harnett MD, Hanauer SB. Randomised clinical trial: the safety and tolerability of *Trichuris suis* ova in patients with Crohn's disease. *Aliment Pharmacol Ther* 2013; **38**: 255-263 [PMID: 23730956 DOI: 10.1111/apt.12366]
- 35 **Tilp C**, Kapur V, Loging W, Erb KJ. Prerequisites for the pharmaceutical industry to develop and commercialise helminths and helminth-derived product therapy. *Int J Parasitol* 2013; **43**: 319-325 [PMID: 23291462 DOI: 10.1016/j.ijpara.2012.12.003]

P- Reviewers: Iijima H, Liu ZJ, Maric I **S- Editor:** Cui XM
L- Editor: A **E- Editor:** Wang CH



Diet high in fructose leads to an overexpression of lipocalin-2 in rat fatty liver

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Received: August 15, 2013 Revised: September 13, 2013

Accepted: October 14, 2013

Published online: February 21, 2014

Abstract

AIM: To explore lipocalin-2 (LCN-2) expression and its possible role and mechanism(s) of production in rat models of diet-inducible fatty liver.

METHODS: Fatty liver was triggered in male Sprague-Dawley rats fed either with liquid Lieber-DeCarli (LDC) or LDC + 70% cal fructose (L-HFr) diet for 4 or 8 wk. Chow-nourished animals served as controls. Hepatic expression of LCN-2 and other metabolic and inflammatory mediators was assessed by quantitative reverse transcription polymerase chain reaction and Western blotting. Serum LCN-2, fasting leptin, and lipid profile were evaluated *via* Enzyme-Linked Immunosorbent Assay, Radioimmunoassay, and colorimetric assays, respectively. The localization of LCN-2 in the liver was detected by using immunofluorescence staining. Furthermore, HE stain was used to evaluate hepatic fat

degeneration and inflammation.

RESULTS: Both LDC-fed and L-HFr-fed rat histologically featured fatty liver. In the liver, mRNA transcriptions of *Mcp-1*, *a2-m*, *Il-8* and *Glut5* were increased in the L-HFr group at both time points ($P < 0.001$), while the transcription of *Tlr4*, *Inos*, and *Tnf- α* was significantly up-regulated at week 4. Interestingly, hepatic *Lcn-2* expression was 90-fold at week 4 and 507-fold at week 8 higher in L-HFr-subjected rats *vs* control ($P < 0.001$). In contrast to HDL-cholesterol, systemic levels of LCN-2, fasting leptin and triglycerides were elevated in the L-HFr regimen ($P < 0.001$). Moreover, protein expression of hepatic LCN-2, CD14, phospho-MAPK, caspase-9, cytochrome *c* and 4-hydroxynonenal was increased in the L-HFr group. Conversely, the hepatic expression of PGC-1 α (a mitochondrial-biogenic protein) was reduced in the L-HFr category at week 8. The localization of LCN-2 in the liver was predominantly restricted to MPO⁺ granulocytes.

CONCLUSION: Fructose diet up-regulates hepatic LCN-2 expression, which correlates with the increased indicators of oxidative stress and mitochondrial dysfunction. The LCN-2 may be involved in liver protection.

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Key words: Non-alcoholic fatty liver disease; Inflammation; Endotoxins; Lipopolysaccharide; Oxidative stress; Mitochondrial dysfunction; Metabolic syndrome

Core tip: Both Lieber-DeCarli (LDC) and LDC + 70% cal fructose (L-HFr) models featured fatty liver. Fructose-enriched regimen induced metabolic syndrome in the corresponding rats. Lipocalin-2 (LCN-2) was strikingly increased in the liver and serum of the L-HFr group. In this group, the increase of LCN-2 synthesis was associated with inflammation at week 4, whereas the peak

value of LCN-2 at week 8 was mainly accompanied by impairment of the mitochondrial function. Nevertheless, an interaction coexists between both processes. The indicators of stress conditions and apoptosis were elevated at both time points. Evidently, the expression of LCN-2 was correlated to inflammatory and metabolic processes.

Alwahsh SM, Xu M, Seyhan HA, Ahmad S, Mihm S, Ramadori G, Schultze FC. Diet high in fructose leads to an overexpression of lipocalin-2 in rat fatty liver. *World J Gastroenterol* 2014; 20(7): 1807-1821 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1807.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1807>

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is presently the most prominent form of chronic liver diseases affecting people at different ages^[1]. This entity is also considered as the hepatic manifestation of metabolic disorders including insulin resistance (IR) and dyslipidemia^[2]. The prevalence of NAFLD is increasing among the population of Western countries^[3]. However, the pathogenesis of NAFLD remains poorly understood, and therapeutic options are quite limited.

There is strong evidence that the diet may affect the development of NAFLD^[4]. Fructose is a monosaccharide which is commonly used as a sweetener, *e.g.*, high fructose corn syrups. Industrially, it is frequently found in soft drinks and pre-packaged foods^[5]. A correlation is observed between dietary fructose intake and the prevalence of metabolic syndrome and fatty liver^[6].

Unlike glucose, which is widely used by tissues throughout the body, fructose is primarily metabolized in the liver, and it facilitates oxidative damage and lipid peroxidation^[7]; a process in which unsaturated lipids become oxidatively degraded to a variety of products at sites of inflammation. One of the end products of lipid peroxidation is 4-hydroxynonenal (4-HNE) which exhibits a chemotactic activity toward neutrophils^[8]. In mice, a chronic moderate fructose intake was shown to be associated with an increased translocation of lipopolysaccharide (LPS, endotoxin) from the gut into the portal vein, as a result of bacterial overgrowth and increased intestinal permeability, additionally. This may cause a further activation of hepatic Kupffer cells and formation of reactive oxygen species (ROS) in the liver and an induction of hepatic TNF- α expression *via* nuclear transcription factor κ B (NF- κ B)^[9].

The lipocalin family in general plays the role of transporters with various functions, *e.g.*, regulation of immune responses^[10], and in binding of small lipophilic substances (*e.g.*, arachidonic acid, iron, and lipids)^[11]. Lipocalin-2 (LCN-2) is a 25-kDa secretory glycoprotein initially identified in human neutrophils^[12] and cells that are exposed to microorganisms, and it is abundantly pres-

ent in the circulation^[13]. It was demonstrated that the liver is the main source of serum LCN-2^[14]. The latter plays a key role in implementing the acute-phase response^[15], and in the regulation of apoptosis^[16].

Interestingly, LCN-2 has been characterized as a critical regulator of energy metabolism, glucose and lipid homeostasis, and IR in LCN-2-deficient mice^[17]. It was reported that LCN-2 suppression could attenuate obesity-induced IR^[18]. In human beings, elevated serum LCN-2 concentration was also observed among diabetic patients, and this increase could be reversed by the insulin-sensitizing drug rosiglitazone^[19]. As most *in vivo* models involved in LCN-2 studies used genetically modified mice like *Lcn-2*, *ob/ob*, and *db/db*^[18-21], there are few studies regarding to LCN-2 in rat fatty liver models especially when caused by fructose.

Based on the current background, we hypothesized that dietary fructose-caused oxidative stress and gut-derived endotoxin could trigger hepatic LCN-2 expression in nutritionally induced (non-genetically modified) rat fatty liver models. We also assumed that LCN-2 may have hepatoprotective effects. Thus, we comparatively investigated LCN-2 expression in two rat fatty liver models under effects of different diets, to explore the mechanism(s) of LCN-2 induction and its relationship with inflammatory response and metabolism.

MATERIALS AND METHODS

Materials

Fructose and skim milk were purchased from Appli-Chem, Darmstadt, Germany; the chow and Lieber-De-Carli (LDC) diets, ssniff Spezialdiäten GmbH, Germany; Qiagen RNeasy Mini Kit, Qiagen GmbH, Germany; Moloney murine leukemia virus reverse transcriptase (M-MLV RT), Promega, Germany; SYBRGreen master mix, TaqMan and stepOne software, AB, Applied Biosystems, Germany; Complete Protease Inhibitor Cocktail Tablets, Roche, Germany; Hyoid-ECL nitrocellulose membranes, Habersham Biosciences, Buckinghamshire, United Kingdom. ECL chemiluminescent solutions A and B were from GE Healthcare, United States; Lipocalin-2 Enzyme-Linked Immunosorbent Assay (ELISA), Bioport Diagnostica Kit 046, Gentofte, Denmark; microtome, Microm HM325, Thermo Scientific, Germany; and 4,6-diamidino-2-phenylindole (DAPI) were from Molecular Probes Europe BV, Germany; Fluoromount-G, 0100-01, Southern Biotech. The Netherlands; Rabbit serum, Dako, Glostrup, Denmark; Ponceau S, SERVA Electrophoresis GmbH, Heidelberg, Germany; leptin, Radioimmunoassay (RIA) kit, was from Millipore, United States.

Experimental animals

Healthy male Sprague-Dawley rats (160-180 g) were used. All animals were purchased from Charles River, Sulzfeld, Germany. On arrival at facility, the rats were placed immediately into their respective experimental conditions. For house acclimatization, the rats were provided with

Table 1 List of primers that were used in this study

Name	Forward 5'-3'	Reverse 5'-3'
<i>Lcn-2</i>	GGAATATTCACAGCTACCCCTC	TTGTTATCCTTGAGGCCCAG
<i>Il-8</i>	GTGTCCCCAAGTAATGGAGAA	CGCCTACCATCTTTAAACTGC
<i>a2-m</i>	CTGTCACTCATCCGTGTGTC	ATCTCCTTCTTCGTGTCCTG
<i>Glut5</i>	TGCAGAGCAACGATGGAGAAA	ACAGCAGCGTCAGGGTGAAG
<i>Tnf-α</i>	AAATGGGCTCCCTCTCATCAGTTC	TCTGCTGGTGGTTTGCTACGAC
<i>Mcp-1</i>	CTCACCTGCTGCTACTCATTCACT	TTCTTATTGGGGTCAGCAC
<i>Lep-r</i>	GTTCTGGCCATCAATTCCAT	GCCCTCTGGTGTGCTTGTAT
<i>Actb</i>	ACCACCATGTACCCAGGCATT	CCACACAGAGTACTTGCGCTCA
<i>Ubc</i>	CACCAAGAAGGTCAAACAGGAA	AAGACACCTCCCATCAAACC

standard chow diet and tap water for 4 d *ad libitum*. To facilitate measures of food intake and to promote minimal sedentary movement patterns; the rats were maintained individually in conventional cages in a 12:12 h light-dark cycle and hygienically controlled room. Ethically, all animals received humane care within the provision of the German Law on the Protection of Animals and the institutional guidelines. All animal experiments were approved by the ethics review board and supervised by the local ethics commission.

Induction of fatty liver using different diets

Animals were randomly allocated ($n = 4$ per group/time point) as follows: oval chow pellets [control (Co)] group, modified liquid LDC group which is also called high-fat diet^[22], and LDC + high (70% cal) fructose (L-HFr) group. The animals were allowed access to a pre-weighed amount of food for 4 or 8 wk. Amounts of consumed food were recorded daily, while animals' body weights (BW) were measured weekly throughout the study. The animals were deprived of any food 10 h before being euthanized.

Collection of organs and blood samples

The animals were weighed and euthanized using sodium pentobarbital (Narcoren®, Merial GmbH, Hallbergmoos, Germany) (0.2 mL per 100 g BW *ip*). Under deep anesthesia, blood was withdrawn from *inferior vena cava* in plain (serum) and heparinized (plasma) tubes. Subsequently, livers were excised, weighed, and quickly dipped in physiological saline. Then, three portions of different liver lobes of each animal were fixed in 4:1% neutral-buffered formalin: glutaraldehyde for paraffin embedding. Alternatively, liver pieces were snap-frozen in liquid nitrogen and stored at -80 °C until use. Relative liver weights (RLW) were expressed as a percentage of the ratio of absolute liver weight (g), divided by the total BW (g) at the time of sacrifice multiplied by 100.

RNA isolation, quantitative reverse transcription polymerase chain reaction, and polymerase chain reaction analyses

Liver tissues RNA were isolated by Qiagen RNeasy Mini Kit. Extracted RNA concentrations were spectrophotometrically measured at $\lambda = 260$ nm, and RNA's purity was

controlled by the ratio of optical density (OD) OD₂₆₀/OD₂₈₀ nm and its integrity by 1.2% agarose gel electrophoresis.

DNase-treated total cellular RNA (1 µg) was denatured at 65 °C for 10 min in a total volume of 10 µL with RNase inhibitor. Thereafter, a master mix consisting of 100 nmol/L of dNTPs, 50 pmol/L of primer oligo(dT)₁₅, 200 units of M-MLV RT, 1× RT buffer, and 2.5 mL of 0.1 mol/L dithiothreitol was added to the denatured RNA samples and incubated for reverse transcription at 40 °C for 1 h, and 72 °C for 10 min. Complementary DNA (cDNA) was ready to use after addition of 120 µL deionized H₂O.

Primers (Table 1) that had been checked for potential hairpin formation and potential self-annealing were synthesized by Invitrogen. A 1 µL cDNA of each sample was added to 9 µL mixture of targeted primer-pair and Fast Platinum SYBR® Green Universal master mix. Predesigned TaqMan assay was used for *Inos* and *Tlr4* analysis, and the protocol was set according to the manufacturer. Ubiquitin C (*Ubc*) and β -actin (*Actb*) were designed as endogenous references (housekeeping genes). The amplification of a total 10 µL/well was performed in duplicate through two-step cycling (95 °C-60 °C) for 40 cycles in a stepone plus quantitative real time reverse transcription polymerase chain reaction (RT-PCR) detection system, following the instructions of the supplier. The comparative C_t-method was used to determine the amount of target gene, normalized to the housekeeping genes and relative to a calibrator ($2^{-\Delta\Delta C_t}$)^[23]. The purity of the RT-PCR products was verified by melting curves. For conventional PCR, 10 ng cDNA was used for *Lcn-2* and *Actb* study, and then the products were run in 1.1% agarose gel and photographed.

Protein extraction and measurement

Liver samples were prepared from the studied rats, and they were homogenized individually in ice-cold lysis buffer [containing 150 mmol/L sodium chloride, 10% (v/v) glycerol, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, 1% (v/v) Nonidet P-40, and 20 mmol/L Tris/HCl buffer, pH 7.5], a fresh Complete Protease Inhibitor Cocktail Tablets, phosphatase inhibitors, and 100 µg/mL of phenylmethanesulfonyl fluoride were added. Hepatic and serum proteins were quantified by Bradford method^[24].

Table 2 List of antibodies used in this study

Name	Clone	Species	Company	Dilution	Use
HNE	PC	Rabbit	Abcam	1:100	WB
LCN-2	MC	Mouse	Novus biological	1:250	WB
	PC	Goat	R&D	1:100	IF
GRP-78	PC	Rabbit	Novus Biocompare	1:800	WB
CD14	PC	Rabbit	Antibodies-online	1:700	WB
Casp 9	PC	Rabbit	Chemicon international	1:1200	WB
Cyt c	MC	Mouse	Millipore	1:600	WB
PGC-1 α		Goat	Abcam	1:200	WB
I κ B1 α	MC	Rabbit	Abcam	1:10000	WB
β -actin	MC	Mouse	Sigma Aldrich	1:5000	WB
MPO	PC	Rabbit	Dako, A0398	1:100	IF
ED1	MC	Mouse	AbD Serotec	1:100	IF
Anti-rabbit-HRP	PC	Swine	Dako	1:1500	WB
Anti-mouse-HRP	PC	Rabbit	Dako	1:1500	WB
AlexaFluor-555-conjugated anti-goat IgG		Donkey	Invitrogen	1:500	IF
AlexaFluor-488-conjugated anti-mouse IgG		Donkey	Invitrogen	1:500	IF
AlexaFluor-488-conjugated anti-rabbit IgG		Donkey	Invitrogen	1:500	IF

WB: Western blotting; MC: Monoclonal; PC: Polyclonal; IF: Immunofluorescence stain.

Western blot analysis for hepatic proteins

As much as 50 μ g of whole lysate was separated by (4%-12%) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then electroblotted onto Hybond-ECL nitrocellulose membranes, and equal loading was monitored in Ponceau S stain. Subsequently, non-specific binding sites on the membranes were blocked at room temperature for 1.5 h in 5% (w/v) skim milk and 0.1% (v/v) Tween-20 in PBS (PBS-T), and incubated with the first antibodies overnight on agitated plates at 4 °C. Subsequently, the membranes were washed 3 times for 5 min with PBS-T and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. Immunodetection was performed according to the ECL chemiluminescent solutions A and B Western blotting protocol. Antibodies that were used in the present study are listed in Table 2. Immunoreaction signals were viewed with enhanced chemiluminescence using a film processor machine. All Western blot experiments were performed in triplicate. The uniformity of protein loading in each lane was assessed by determining the signal of β -actin as a loading control.

Biochemical studies

After an overnight (10 h) fasting, the collected rat heparinized blood was centrifuged at 3500 *g* for 15 min at 4 °C. The concentrations of glucose, uric acid, total cholesterol, TG, high-density lipoprotein-containing cholesterol (HDL-C), LDL-C as well as the activity of ALT and AST were determined in harvested plasma by utilizing the automated systems of the central laboratory of the Institute of Clinical Chemistry in University Medical Center Goettingen. Serum leptin levels were evaluated by RIA.

Evaluation of serum LCN-2 by Sandwich ELISA and Western blotting

Hemolysis-free sera were stored at -80 °C. ELISA was

conducted to assess the levels of LCN-2 in the serum specimens according to the supplier's instructions. LCN-2 concentrations were expressed in ng/mL. The amount of LCN-2 was further validated by Western immunoblotting. Rat sera (75 μ g protein) samples were denatured at 95 °C for 5 min in reducing 3 \times Laemmli's loading buffer and subjected to standard Western-blot with an affinity-purified mouse monoclonal antibody raised against rat LCN-2 protein.

Histopathology

Paraffin-embedded liver slices of 5 μ m thickness were cut transversely into serial sections by using a microtome. Sections were then deparaffinized in xylene, rehydrated through an ascending ethanol series and stained automatically with haematoxylin-eosin (HE). After mounting with xylene-based media; a pathologist had carried out the histological examination by using a light microscope (Olympus BX43, Hamburg, Germany) with internal digital camera (Olympus DP21) having no information about the previous treatments of the rats.

Double immunofluorescent analysis

Immunofluorescence was used to detect the localization of the target antigens within liver tissue. Liver cryostatic sections (5 μ m) were air-dried and fixed in methanol/acetone for 9:1 min at -20 °C. After blocking of non-specific binding with serum (according to the species of the secondary antibody) for 1 h at room temperature; the sections were incubated with a solution of primary antibody anti-LCN-2 combined with either ED1 (CD68) or myeloperoxidase (MPO) overnight at 4 °C. In parallel, negative controls immunostainings were performed by replacing the primary antibodies with only PBS^[25]. Alexa flour-conjugated secondary antibodies were used to detect the primary antibodies (Table 2). Cells' Nuclei of the stained sections were marked by DAPI and the slides

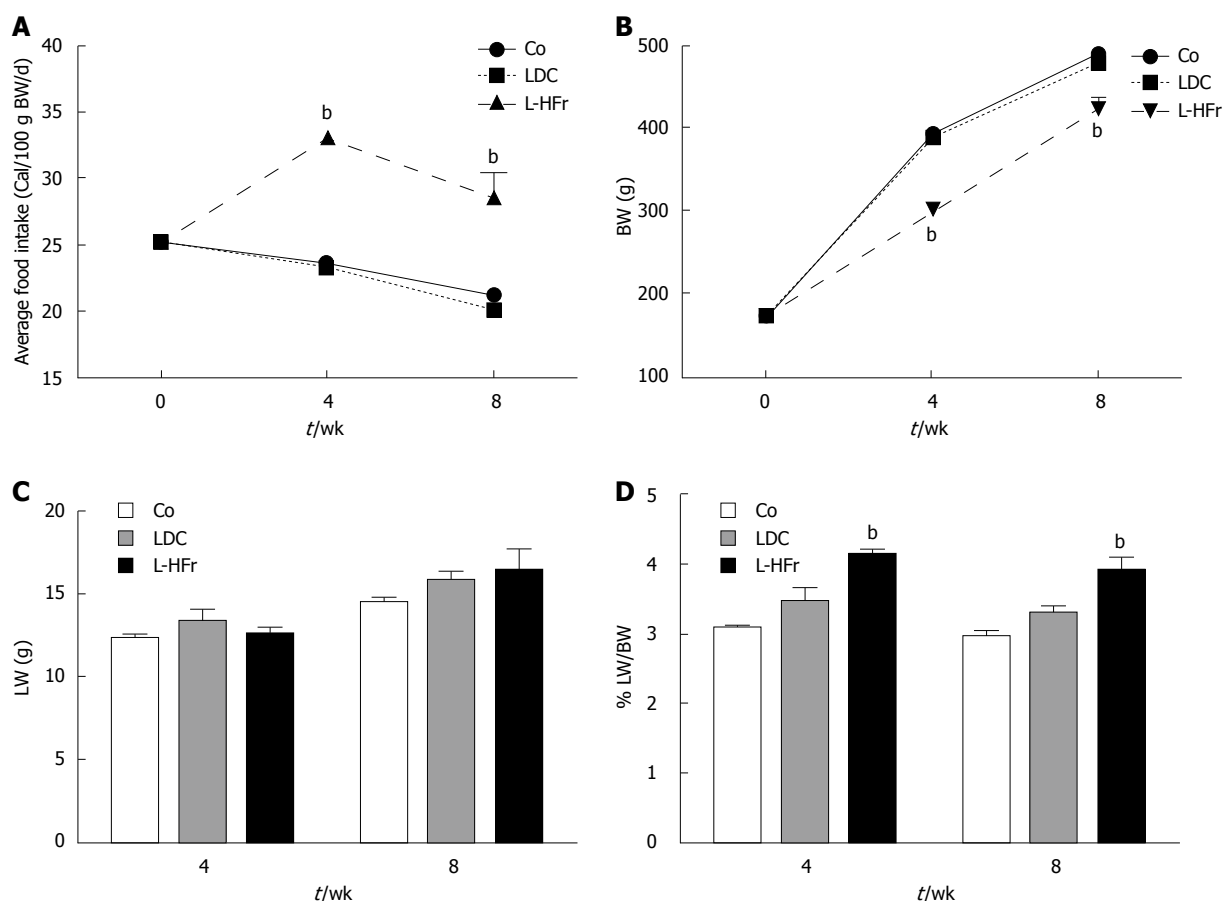


Figure 1 Phenotypes of the animals and daily food intake. Linear graph shows the average of animals' caloric intake (A) at weeks 0, 4 and 8 of the treatment (food intake was expressed as cal/100 g BW/d) and animals' body weight (BW; B). Bar blots show liver weight (LW; C) and relative liver weight (RLW; D). Data are presented as mean \pm SEM of 4 rats per group in correspondence to time points. Indicates statistically significant difference ^b $P < 0.01$ via one-way ANOVA. Standard chow pellets (Co), a fresh-prepared Lieber-DeCarli (LDC) liquid diet, and LDC combined with 70% cal fructose (L-HFr).

were covered with Fluoromount-G and observed using an epifluorescence microscope (Axiovert 200M, Zeiss, Jena, Germany).

Statistical analysis

Data were analyzed by using GraphPad Prism 5 software (San Diego, CA, United States) and described as mean \pm SEM. Statistical significance was calculated by one-way analysis of variance (ANOVA) (Dunnett's post-hoc test) to examine the statistical significance amongst experimental groups *vs* control. The null hypothesis was rejected when $P < 0.05$.

RESULTS

L-HFr fed rats exhibited increases of food intake

Fatty liver was induced either with LDC diet or fructose-enriched diet (L-HFr) for 4 or 8 wk. Chow diet served as control. The average of basal food intake (week 0) was 25.2 ± 0.5 cal/100 g BW/d for all animals. The food intake of controls was slightly declined at week 4 (23.5 ± 0.1) and week 8 (21.2 ± 0.1). There was no noticeable difference in food intake between Co and LDC groups. Instead, a significant increase of food intake was observed in the L-HFr group at week 4 (33.5 ± 0.7 cal/100 g BW/d)

and week 8 (29.0 ± 0.2 cal/100 g BW/d) (Figure 1A).

Modulations of body and liver weights

The phenotypes of the animals were characterized to observe the effect of the diets on the BW gain and liver weight (LW). The BW of the controls was 160-180 g at week 0, and then it increased to 393 ± 5 g at week 4 and 489 ± 7 g at week 8. The BW of chow and LDC-nourished rats were comparable over the study.

Despite markedly higher total caloric intake in high fructose-challenged rats, the absolute BW was significantly diminished at week 4 (300 ± 7 g; $P < 0.001$) and week 8 (420 ± 16 g; $P < 0.001$). This implies that the BW of L-HFr treated rats was reduced 30% and 15% relative to the corresponding control rats (Figure 1B). However, these animals behaviorally remained active and did not show any sign of distress.

In general, the LW of both treated animal groups was moderately higher than littermate controls (Figure 1C). LW of Co group was 12.3 ± 3 g at week 4 and 14.5 ± 0.3 g at week 8, while in LDC group LW was 13.1 ± 0.7 g at week 4 and 15.7 ± 0.5 g at week 8. In addition, the LW of L-HFr regimen was 12.6 ± 0.4 g and 16.4 ± 1.0 g at weeks 4 and 8, respectively.

Consequently, RLW was high in both experimental

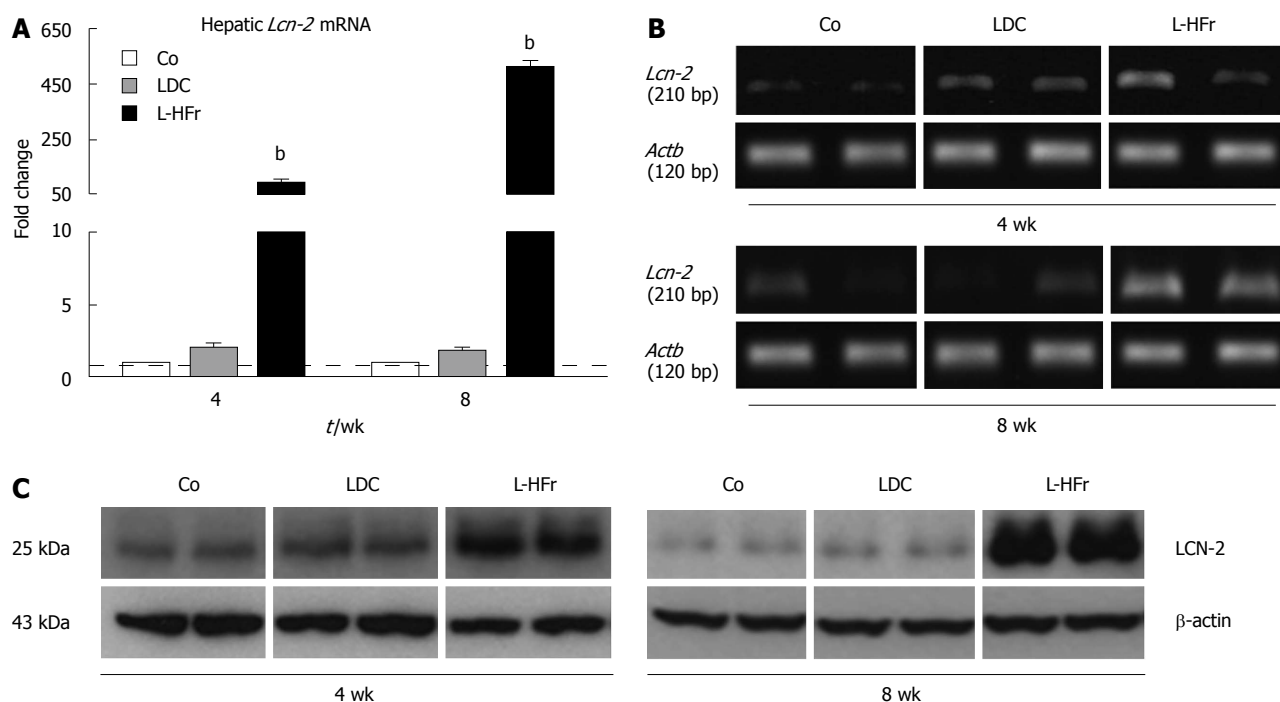


Figure 2 Changes in lipocalin-2 mRNA and protein expression in rats livers. A: Bar plots show relative specific mRNA levels in hepatic samples. Each bar represents as mean \pm SEM of 4 rats/group at weeks 4 and 8. Data were normalized by ubiquitin C. β -actin (*Actb*) was used as an additional reference housekeeping gene, yielding comparable results; B: Photographs of end-point polymerase chain reaction products run on 1.1% agarose gel electrophoresis for hepatic *Lcn-2* and *Actb*; C: Immunoblots of hepatic LCN-2 (two representative animals per group). ^b $P < 0.01$. Co: Chow pellets; LDC: Lieber-DeCarli liquid diet; L-HFr: LDC combined with 70% fructose.

groups *vs* controls. Animals got LDC diet had slightly higher RLW ($3.5\% \pm 0.2\%$, and $3.3 \pm 0.1\%$) compared with that received the standard chow ($3.1\% \pm 0.1\%$, $3.0\% \pm 0.1\%$) at week 4 and 8, respectively. On the other hand, the ratio of liver-to-body weight of L-HFr fed rats was the highest ($4.5\% \pm 0.1\%$ at week 4, and $4.0\% \pm 0.4\%$ at week 8) during the observation period ($P < 0.001$) (Figure 1D).

Changes of hepatic mRNA expression

Expression of hepatic *Lcn-2* mRNA: To elucidate whether both animal models of non-alcoholic induced fatty liver will similarly express LCN-2 in the liver, qRT-PCR and conventional PCR were used. Interestingly, the expression of *Lcn-2* was not significantly changed in the livers of LDC group at weeks 4 and 8 compared with the corresponding Co group. Alternatively, a significant increase of hepatic *Lcn-2* expression was detectable in the L-HFr category at week 4 (90 ± 11 fold; $P < 0.001$) and week 8 (507 ± 28 fold; $P < 0.001$) (Figure 2A). The extent of *Lcn-2* mRNA in the L-HFr group was the highest among the other studied genes. Furthermore, a qualitative evaluation for *Lcn-2* mRNA by conventional (end-point) PCR further showed similar changes in gene expression (Figure 2B).

Changes of inflammation-related mRNA expression in liver samples: The following inflammation-related genes were studied at weeks 4 and 8: *IL-8*; a potent activator of neutrophils, *Mcp-1*; recruits monocytes and granulocytes, *$\alpha 2-m$* , a positive acute-phase protein in human and rat, *Tnf- α* ; a pro-inflammatory cytokine, *Inos*;

involved in innate immunity and oxidative conditions, and *Thr4*; a pathogen-recognition receptor. The regulation of these genes did not significantly differ between LDC and control groups at both time points. Conversely, in the L-HFr group the mRNA levels of *IL-8* (30-fold), *Mcp-1* (4.9-fold), *$\alpha 2-m$* (9.4-fold), *Tnf- α* (4.5-fold), *Inos* (5.6-fold), and *Thr4* (7.1-fold) were significantly amplified at week 4 (Figure 3A-F). Noticeably, we also found an up-regulation of *IL-8* (13-fold), *Mcp-1* (3.6-fold), *$\alpha 2-m$* (5.7-fold) in the L-HFr at week 8 (Figure 3A-C). At this time point, however, there was no significant change in *Tnf- α* , *Inos*, and *Thr4* in the same group (Figure 3D-F).

Modulations of *Glut5* and *Lep-r* mRNA in liver samples:

In LDC group, levels of *Glut5* (the major fructose transporter) and *Lep-r* (leptin receptor) mRNA expression remained almost at the level of controls. In contrast, *Glut5* mRNA was considerably enhanced at week 4 (7-fold) and week 8 (6-fold) in the L-HFr-challenged rats compared to standard Co diet (Figure 3G). Concomitantly, hepatic *Lep-r* specific mRNA transcripts were substantially raised 9.2-fold (week 4) and 6-fold (week 8) in L-HFr-fed animals (Figure 3H).

Analyses of hepatic protein expression

Expression of hepatic LCN-2 protein: Western blotting were used to detect the amount of specific LCN-2 protein in total liver homogenates. The expression of LCN-2 protein was almost similar in LDC and control animals. Along with increased *Lcn-2* mRNA levels in the L-HFr group, the expression of LCN-2 was obviously

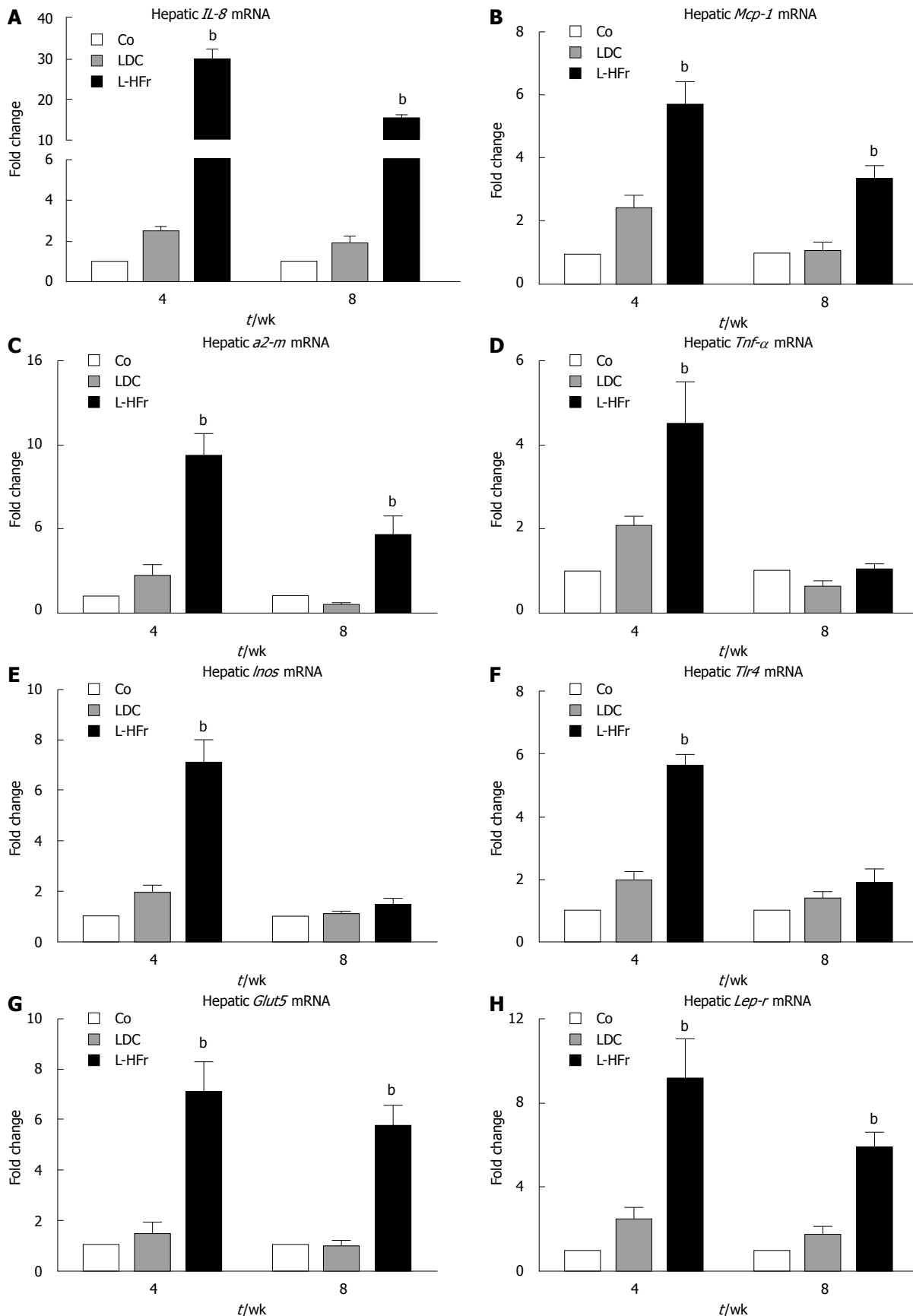


Figure 3 Alterations in hepatic mRNA levels of inflammation- and metabolism-related genes. A-C: Bar plots show relative specific mRNA levels of inflammation-related mediators in hepatic specimens that were up-regulated in the L-HFr group at both time points; D-F: Only increased at 4th week; G-H: Selected metabolism-related genes; Each bar represents mean \pm SEM of 4 rats/group at weeks 4 and 8; Data were normalized by *Ubc* (ubiquitin C), and *Actb* (β -actin) was additionally used as a reference housekeeping gene. ^b $P < 0.001$. Co: Chow pellets; LDC: Lieber-DeCarli liquid diet; L-HFr: LDC combined with 70% fructose.

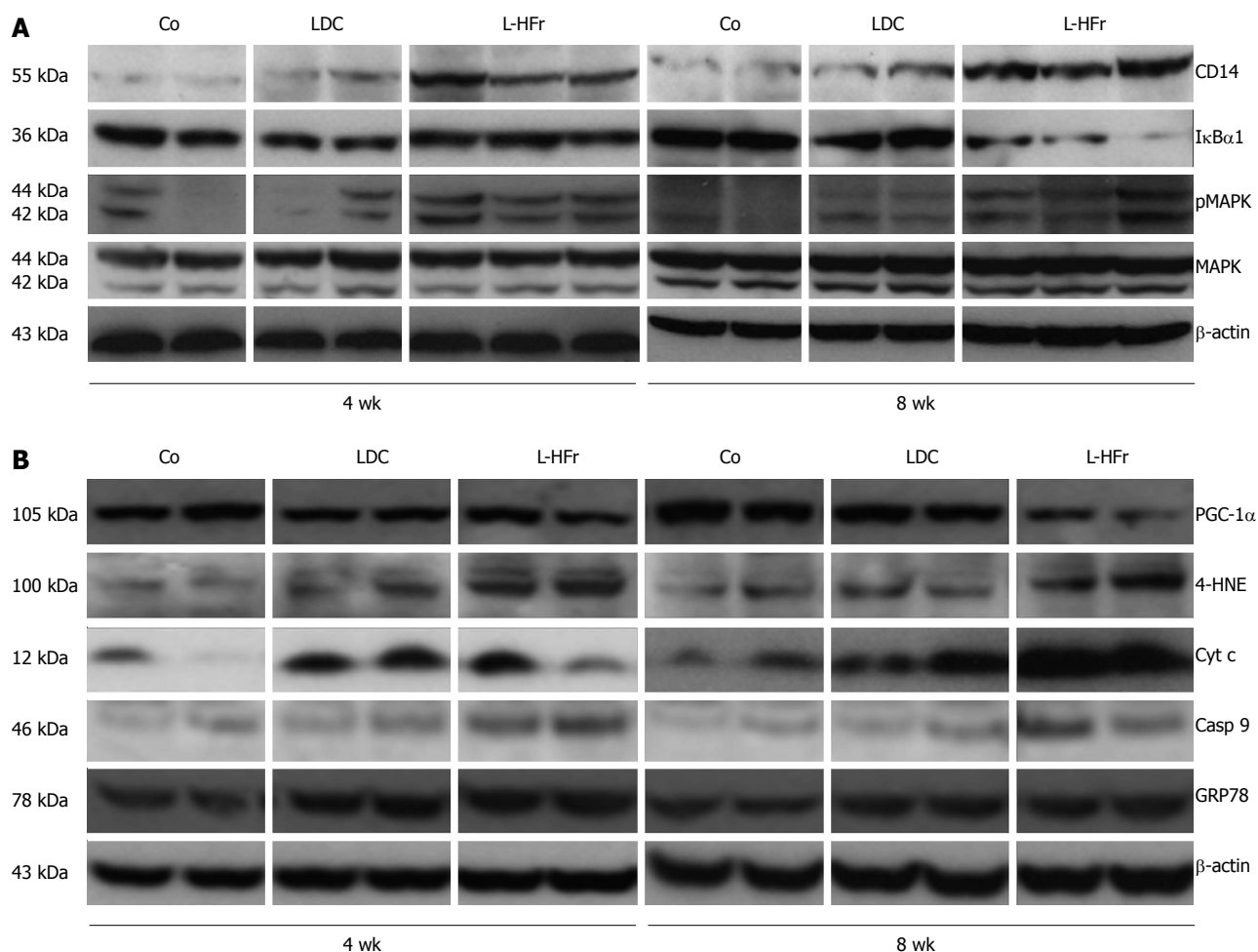


Figure 4 Changes in protein expression in rat liver. A: Representative immunoblots for inflammation-related proteins; B: mitochondrial functional impairment, apoptosis, and oxidative stress; Co: Chow diet, LDC: Lieber-DeCarli liquid diet; L-HFr: LDC + 70% fructose.

up-regulated in the L-HFr group (Figure 2C). Prominently, the maximal increase of LCN-2 was at week 8.

Variations of inflammation-related proteins in rats' livers:

Concurrently, fructose-enriched diets evidently stimulated the protein levels of CD14 (a multifunctional protein, involved in TLR4 signal transduction and apoptosis) and MAPK (is activated by phosphorylation in response to stress and coordinates pro-apoptotic functions). Markedly, hepatic IκBα1 protein expression was attenuated in the L-HFr group at week 8 (Figure 4A).

Metabolism and oxidative stress related indicators:

To study whether chronic dietary fructose could impair mitochondrial and (or) endoplasmic reticulum functions, Western blotting studies were performed on hepatic casp 9; the initial caspase in the mitochondrial apoptotic cascade in response of ROS, GRP78; a molecular chaperone correlated with endoplasmic reticulum stress, it regulates the unfolded protein response, and 4-HNE; a specific end product of lipid peroxidation. In contrast to the Co or LDC diet, the L-HFr induced casp 9, GRP78, and 4-HNE at both time points (Figure 4B). However,

Cyt *c* protein, which required for mitochondrial function and apoptosis, was maximally increased at week 8. Additionally, it's known that PGC-1α plays a crucial role in mitochondrial biogenesis^[26]. In the current study, our data showed that the L-HFr diet diminished the hepatic PGC-1α protein expression obviously at week 8 (Figure 4B). The latest result was inversely correlated to the patterns of liver LCN-2 protein.

Elevation of systemic LCN-2 levels in fructose-treated animals

At week 8, the concentration of serum LCN-2 tended to be higher than the concentration at week 4 in all groups (Figure 5). The LDC group showed a mild increase in serum LCN-2 when compared with the Co group. Oppositely, the concentration of LCN-2 in bloodstream revealed by ELISA was obviously augmented in fructose-targeted animals (Figure 5A). At week 4, L-HFr-given rats evidenced a (2-fold, $P < 0.05$) enhancement in the level of serum LCN-2, and more than 2.2-fold ($P < 0.05$) increase at week 8 in the LCN-2 magnitudes. The increase of circulatory LCN-2 protein was further confirmed by Western blotting (Figure 5B).

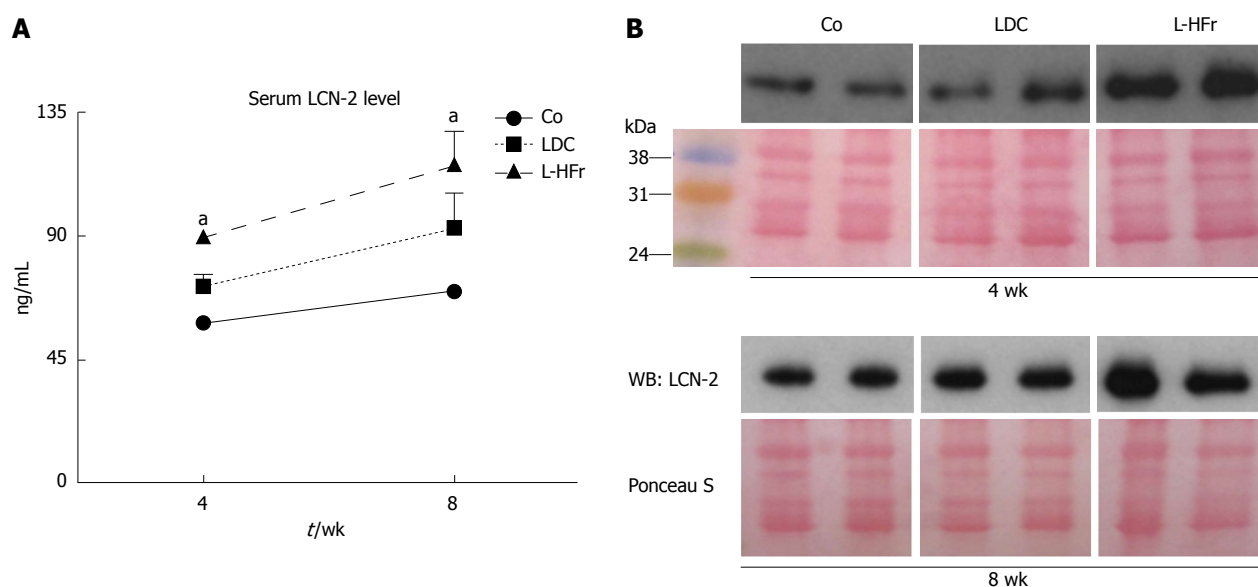


Figure 5 Up-regulation of a systemic lipocalin-2 mainly in animal model fed with Lieber-DeCarli + 70% cal fructose diet. A: Serum LCN-2 data; Values on y-axis were expressed in ng/mL; B: Representative autoradiographies from Western blotting determination and the corresponding Ponceau stained membrane of two animals per group are shown. ^a $P < 0.05$ Significance difference vs Co. Co: Chow diet, LDC: Lieber-DeCarli liquid diet; L-HFr: LDC + 70% fructose.

Table 3 Data of plasma biochemistry of Sprague-Dawley rats within 8 wk of feeding

Parameter	Time point/ Group (wk)	Co	LDC	L-HFr
Glucose (mg/dL)	4	152 ± 12	182 ± 5	243 ± 8 ^b
	8	210 ± 7	244 ± 5	292 ± 7 ^b
TG (mg/dL)	4	26 ± 5	74 ± 8 ^a	78 ± 13 ^a
	8	44 ± 13	62 ± 15	127 ± 21 ^a
Cholesterol (mg/dL)	4	55 ± 2	58 ± 4	58 ± 3
	8	47 ± 4	52 ± 4	53 ± 5
HDL-C (mg/dL)	4	49 ± 1	43 ± 2	46 ± 1
	8	44 ± 1	45 ± 3	32 ± 2 ^d
Uric acid (mg/dL)	4	0.6 ± 0.0	0.9 ± 0.2	0.5 ± 0.0
	8	1.1 ± 0.0	0.5 ± 0.1	2.2 ± 0.3 ^b
ALT (U/L)	4	41 ± 8	43 ± 3	69 ± 7 ^a
	8	55 ± 8	48 ± 4	80 ± 8 ^a
AST (U/L)	4	67 ± 5	74 ± 2	136 ± 8 ^b
	8	90 ± 7	117 ± 13	166 ± 10 ^b
Leptin (ng/mL)	4	1.8 ± 0.2	3.0 ± 0.3	5.0 ± 0.9 ^b
	8	3.7 ± 0.9	5.7 ± 2.1	12.1 ± 1.4 ^b

All animals were overnight fasted just before sacrificing. ^a $P < 0.05$, ^b $P < 0.01$ compared to controls. Data are expressed as mean ± SEM of 4 rats per group. Co: Chow (control); LDC: Lieber-DeCarli liquid diet; L-HFr: LDC + 70% cal fructose; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TG: Triglycerides; LDL-C: Low density lipoprotein cholesterol.

Biochemical parameters in rat blood samples

LDC diet had significantly increased fasting TGs in the plasma compared with littermate controls at week 4. Animal's administration with L-HFr had increased levels of fasting blood glucose and TGs compared to chow (Table 3). Consistently, the concentration of total plasma cholesterol was moderately higher in the L-HFr group compared with age-matched control. Furthermore, the plasma level of HDL-C was diminished in L-HFr fed rats after 8 wk ($P < 0.001$) (good cholesterol) (Table 3). LDL cholesterol levels weren't changed among the groups

(data not shown). In addition, the intake of L-HFr regimen was associated with a 2-fold increase in plasma uric acid concentration at week 8 ($P < 0.01$). Moreover, we found that the concentration of fasting serum leptin was elevated 2.8-fold and 3.3-fold in the L-HFr *vs* chow fed rats at weeks 4 and 8, respectively ($P < 0.01$). The difference in liver transaminases activities between the control and LDC groups was mild. Oppositely, the serum ALT and AST activities were significantly increased in L-HFr group ($P < 0.05$) at both studied time points (Table 3).

Histological manifestations of liver tissues

Control (chow-fed) rats showed normal hepatic architecture, and there was no sign of steatosis. The livers of rats fed liquid LDC diet showed micro- or mediovacuolar steatosis commonly observed in periportal areas at weeks 4 and 8, respectively. Instead, livers of the rats which were treated with L-HFr diet were distinguishable from those rats fed with chow or LDC (Figure 6A). The rats of L-HFr group had extremely macro-steatotic livers and moderate inflammation (grade 2) noticed in zone I, and II towards the central vein which was surrounded with few hepatocytes displaying micro-steatosis, along with a loss of structural integrity and the vascular architecture at both time points. The percentage of fat-loaded hepatocytes was histologically evaluated by a pathologist (Figure 6B).

Immunohistochemical detection of LCN-2, MPO and ED1 in rat liver

Immunofluorescence staining proved the tissue specific LCN-2 localization. The positivity of LCN-2 in the liver was restricted to MPO⁺ cells (Figure 7, left-panel). Only a few LCN-2⁺ cells were observed in the normal (healthy) liver tissues. LDC-treated animals presented similar view

as seen in control, but some LCN-2 signals were observed in MPO⁺ cells in periportal area mainly at week 8 of the study. Interestingly, the highest LCN-2 positivity was detected evidently in the MPO⁺ granulocytes in the livers of L-HFr groups. We also examined whether there is an overlapping between anti-MPO and anti-ED1 by double-immunostainings. The positivity of each marker was entirely separated (data not shown), indicating that MPO is a neutrophil-restricted marker.

DISCUSSION

A micro- or mediovacuolar steatosis was histologically revealed in the livers of LDC group and a macrovesicular steatosis in L-HFr treated rats. We demonstrated that LCN-2 protein was strikingly increased in the liver and serum of L-HFr group. In this group, the increase of LCN-2 synthesis was associated with inflammation at week 4, whereas the peak value of LCN-2 at week 8 was mainly accompanied by a decrease in the mitochondrial function. The indicators of stress conditions and apoptosis were elevated at both time points. Evidently, an interaction existed between the inflammatory and metabolic processes in this study.

By feeding with L-HFr regimen, it was able to induce metabolic syndrome in the corresponding rats. Indeed, we found an elevation of fasting plasma glucose, high TGs, and low HDL-C levels in this group. These findings are recognized as the core components of diabetic dyslipidemia^[27]. Furthermore, it is known that fructose phosphorylation in the liver consumes ATP, consequently the accumulated ADP serves as substrate for uric acid formation^[28]. In fact, the levels of fasting blood uric acid and leptin were significantly elevated in L-HFr-treated rats. The increased fasting leptin level was due to dietary fructose promoted leptin resistance^[29,30], in which the leptin's action was suppressed and it did not induce satiation (fullness) compared to chow or LDC diet. This could be the explanation of high food intake that was seen in the L-HFr group.

After intestinal uptake, fructose is typically extracted from the blood stream by hepatocytes, which is independent of insulin exertion and phosphofructokinase regulation step. Consequently, the up taken fructose is metabolized to glucose, fatty acids or lactate^[31]. We observed a significant up-regulation of hepatic *Glut5* (fructose transporter) gene expression in L-HFr nourished rats, which was correlated with the accumulated fat in the liver. It was stated that high fructose intake was associated with increased plasma TGs, most probably caused by an up-regulation of hepatic *de novo* lipogenesis and TGs secretion, and a decreased clearance of VLDL-TG^[32]. At that point, we found that L-HFr nourished rats promoted the onset of massive hepatic steatosis mainly in zone I and II.

The likelihood that changes in the concentration of hepatic fructose and its metabolites after intake could potentiate lipid peroxidation, alterations of endoplasmic reticulum and mitochondrial functions, and apoptosis was

assessed by 4-HNE adducts, GRP78, casp 9, and Cyt *c*. We found augmentations of those proteins expressions in L-HFr fed rats, suggesting that these rats compared with the LDC or chow-fed rats, were facing higher oxidative stress conditions. Additionally, 4-HNE chemotactically recruits neutrophil granulocytes into the stressed liver. It was regarded that PGC-1 α expression in liver is normally increased during fasting in response to glucagon^[33], and it plays a central role in the regulation of cellular energy metabolism^[34] and mitochondrial function^[35]. Interestingly, the protein expression of PGC-1 α was declined predominantly at week 8 in livers from L-HFr group, providing further evidence for a decreased mitochondrial function. Collectively, these results indicated that chronic fructose-enriched diet altered, at least partially, mitochondrial and endoplasmic reticulum functions and induced cellular apoptosis and stress conditions. These changes were related to a noticeable increase of hepatic LCN-2⁺ neutrophil granulocytes in the L-HFr group (Figure 8).

LCN-2 has emerged as a potential link among obesity, inflammation, and obesity-associated metabolic dysfunction such as IR^[19,36]. It was emphasized that fructose consumption is also correlated with elevated LPS level in liver, which can trigger the release of pro-inflammatory cytokines when bound to CD14/toll-like receptors (TLR4) which are presented on immune cells^[37]. Recognition of LPS by the CD14/TLR4/MD-2 complex activates intracellular signaling pathways involving mitogen-activated protein kinase (MAPK), resulting in the production of proinflammatory cytokines and chemokines^[38]. Even though we hadn't measured the endotoxin levels in portal blood, we found up-regulated levels of hepatic TLR4, ED1⁺ cells and p/ERK1/2 in L-HFr regimen obviously at week 4. However, the multi-functional CD14 was also elevated in livers of the L-HFr group at week 8. It was shown that CD14, in addition to its important role in inflammation and innate immunity, promotes cell survival and antagonizes programmed cell death "apoptosis"^[39]. Overall, these findings, indirectly, suggested that endotoxins influx from bowel *via* portal vein might also be involved in the modulation of liver inflammation in L-HFr-treated rats in a TLR4-dependent manner^[7].

Actually, there are controversial data coexisting regarding the role of LCN-2 in recruiting the inflammatory cells to the site of injury and exacerbating inflammation, and in its role as an anti-inflammatory and protective protein. It was shown that LCN-2 elicits its adverse effects at least partly by stimulating TNF- α , which may in turn magnify the local inflammation and cause impaired energy homeostasis^[40]. Furthermore, Fujino *et al*^[41] reported the pro-inflammatory NF- κ B has been shown to transactivate LCN-2 expression through binding with a consensus motif in its promoter region. On the other hand, the addition of LCN-2 protein to the culture media of adipocytes and macrophages leads to the suppression of TNF α - and LPS-induced cytokines/chemokines production, indicating an anti-inflammatory function^[42].

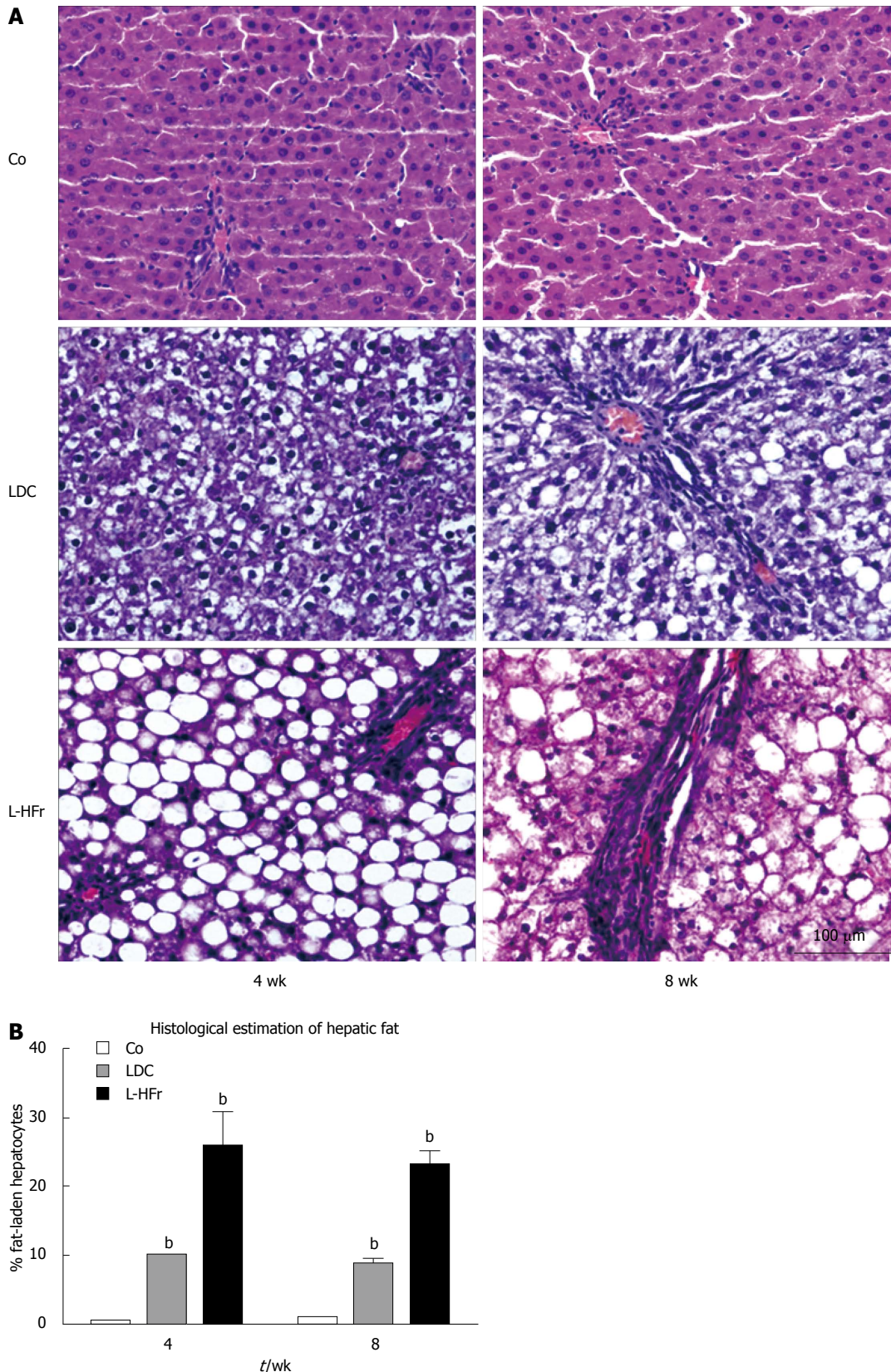


Figure 6 Microphotograph of representative livers of rats on different dietary regimens for 4 or 8 wk. A: The left panel (4-wk) and right panel (8-wk) of HE stained livers show: Co: control group exhibiting normal arrangement of hepatocytes around the sinusoids; LDC: Lieber-DeCarli nourished group represented principally micro- and randomly macro-steatosis at 4 and 8 wk respectively; L-HFr: 70% cal fructose supplemented LDC group showing almost hepatocytes around the portal triads with macrovesicular steatosis shows clear, rounded, and well-defined fat droplets (often single) within the cytoplasm and the nuclei are peripherally displaced by the fat droplets clearly at 4 wk more than 8 wk of experiment time, accompanied with loss of structural integrity and vessel architecture. Scale bar = 100 μ m; B: Percentage of hepatocytes with macro fat vesicles was microscopically evaluated by a pathologist in HE stained liver slices. ^b $P < 0.01$ vs Co.

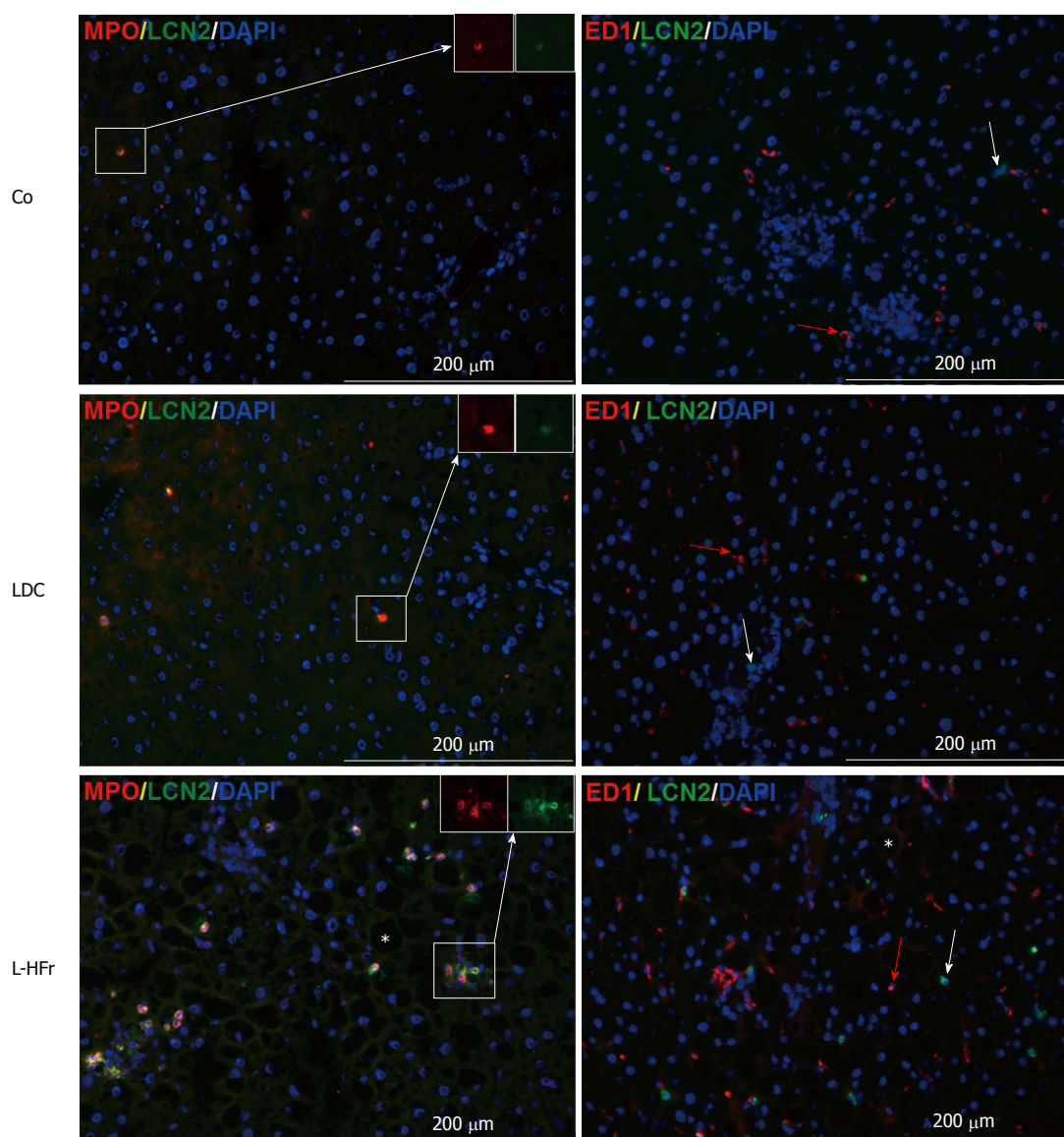


Figure 7 Representative photomicrographs show immunolocalization of key markers of Neutrophil granulocytes infiltration. Myeloperoxidase (MPO) double-stained with LCN-2 (left-panel), and tissue macrophages (ED1) plus LCN-2 (right-panel) at week 4; A co-localization of LCN-2 and MPO can be detected in liver sections. The black cavities (vesicles) that were marked by Star in L-HFr micrographs resulted from washing of fat from hepatocytes during fixation step. Red arrows in right panel show ED1⁺ cells. Scale bar = 200 µm. Co: Chow diet, LDC: Lieber-DeCarli liquid diet; L-HFr: LDC + 70% fructose.

Most strikingly, LCN-2 appears to protect against TNF α -induced IR in adipocytes.

Increased production of LCN-2 in obesity may be a protective mechanism against inflammation and IR^[17]. Guo *et al*^[17] found that in the absence of LCN-2, the expression of proinflammatory cytokines such as MCP-1 and TNF- α were up-regulated in adipose tissue, while expression levels of anti-inflammatory markers declined, suggesting an increased inflammatory status in *Lcn-2*^{-/-} mice. These results further suggest that *Lcn-2*^{-/-} mice are more susceptible to high fat diet-induced pro-inflammatory response than wild-type mice. Currently, we observed a reduction in *Tlr4*, *Inos*, and *Tnf- α* in the livers of L-HFr fed rats at 8th week accompanied by the most dramatic increase in LCN-2 level, supporting the point that LCN-2 acts more likely as an anti-inflammatory protein.

However, further studies are required to elucidate whether LCN-2 could act as a hepatoprotective protein (LPS-neutralizing and anti-inflammatory protein) especially in fructose-induced fatty liver.

Conclusion

In this comparative study, we verified that the increase of LCN-2 in liver and blood circulation was considerably provoked by fructose-enriched diet. The increase of hepatic LCN-2 was correlated with elevated indicators of apoptosis, mitochondrial dysfunction, and lipid peroxidation in the L-HFr group. LPS influx into the liver may contribute to LCN-2 induction. This study suggests that LCN-2 could serve as an indicator to distinguish between simple steatosis and non-alcoholic steatohepatitis. Moreover, it may have contra-regulatory hepatoprotective

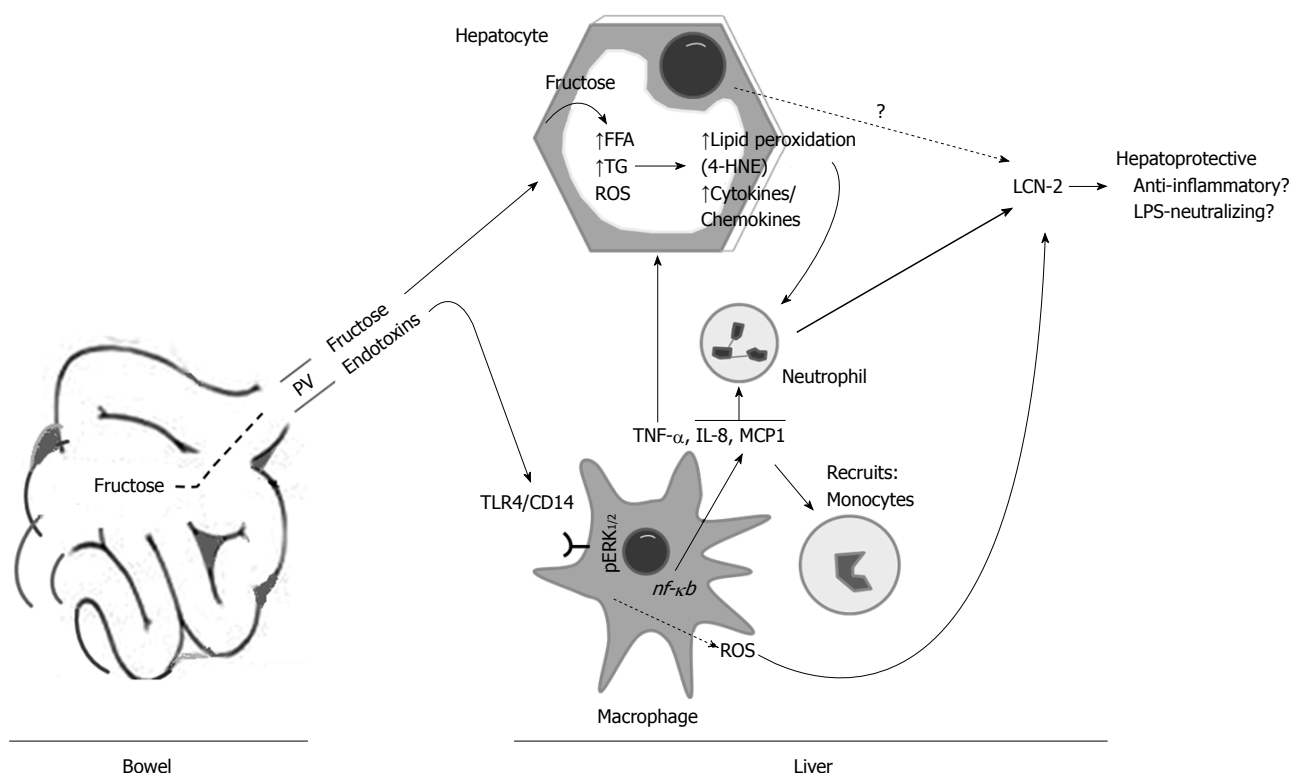


Figure 8 Hypothetical model of the mechanism whereby lipocalin-2 was produced in high fructose-induced rat fatty liver. Ingested fructose is delivered into the liver via portal vein. Once in liver, hepatocytes transformed fructose into FFAs and TGs. In addition, the chronic fructose consumption facilitates the influx of endotoxins LPS into the liver. Endotoxins stimulate Kupffer cells which, in turn, release ROS and cytokines. This results in recruitment of more neutrophil granulocytes and monocytes to the liver. As a result of fat accumulation in hepatocytes, lipid peroxidation end products (e.g., 4-HNE), iNOS and other cytokines/chemokines will be produced, which could recruit neutrophil granulocytes to the liver. LCN-2 could act as hepatoprotective protein. PV: Portal vein; LPS: Lipopolysaccharide; LCN-2: Lipocalin-2.

tive effects.

ACKNOWLEDGMENTS

The authors appreciate Prof. Dr. med. Tilman Sauerbruch, Dept. of Gastroenterology and Endocrinology, UMG, and Prof. Dr. Uwe Groß, Institute for Medical Microbiology, Faculty of Medicine, Georg-August-University, Göttingen, Germany for the critical advising and reviewing the study. This research did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sector. We also acknowledge support by the German Research Foundation and the Open Access Publication Funds of the Göttingen University.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) is a disease of increasing prevalence worldwide, which may develop into endstage liver disease. It was reported that fructose intake is related to NAFLD. Fructose can result in numerous metabolic changes including oxidative stress. The expression of hepatic lipocalin-2 (LCN-2, a secretory glycoprotein) is influenced by metabolic and inflammatory processes.

Research frontiers

The pathogenesis of NAFLD is still obscure. A few studies examined the expression of LCN-2 in rat fatty liver model especially that provoked by fructose. In contrast, most of them focused on (genetically-altered) mice e.g., *Lcn-2⁺* and

db/db models treated with high-fat diet. The authors explored LCN-2 expression in a novel rat fatty liver model induced by high (70% cal) fructose (L-HFr) vs high-fat and chow diets. In addition, the authors addressed the possible role of LCN-2 and its mechanism of production.

Innovations and breakthroughs

The role of fructose in the pathogenesis of NAFLD is not new. The health concerns raised pertain to the amounts of sugar in the current diet, primarily as beverages. Most meta-analyses have shown that the risk of obesity, diabetes, cardiovascular disease, and metabolic syndrome is directly related to consumption of beverages sweetened with sugar and/or high-fructose corn syrup. In support of these findings the authors found L-HFr diet induced more severe liver damage compared to LDC high fat and control diets. High fructose supplementation in a model of NAFLD worsens the liver pathology and that was associated with an increase in LCN-2, among several other mediators. The expression of LCN-2 correlated with the increased indicators of oxidative stress and mitochondrial dysfunction. Actually, there are controversial data coexisting regarding the role of LCN-2 in inflammation. The authors conclude that LCN-2 may be involved in liver protection.

Applications

The study speculates LCN-2 could function as a hepatoprotective protein and can be used as a biomarker to differentiate between simple steatosis (fatty liver) and NASH.

Terminology

NAFLD encompasses a wide spectrum of liver damage, ranging from simple steatosis into liver cancer. The pathogenesis of NAFLD is multifactorial. Fructose is a monosaccharide which is commonly used as a sweetener in food industry; it is used as a food additive and supplementary diet. LCN-2 is a 25-kDa secretory glycoprotein; it is also a positive acute phase protein. LCN-2 has been characterized as a critical regulator of energy and lipid homeostasis.

Peer review

This work is well-planned, executed and presented. The study is also quite interesting, since it refers to what the authors often find in common patients

due to the increased sugar intake in industrialized countries. The current data show clear differences between the two rat models of diet-inducible fatty liver and the possible mechanisms were discussed. It has novel findings and may help understanding the mechanism of the fatty liver disease based on fructose-enriched diet. This study could provide a future strategy for therapeutic intervention in the treatment of patients.

REFERENCES

- 1 **Wieckowska A**, McCullough AJ, Feldstein AE. Noninvasive diagnosis and monitoring of nonalcoholic steatohepatitis: present and future. *Hepatology* 2007; **46**: 582-589 [PMID: 17661414 DOI: 10.1002/hep.21768]
- 2 **Vanni E**, Bugianesi E, Kotronen A, De Minicis S, Yki-Järvinen H, Svegliati-Baroni G. From the metabolic syndrome to NAFLD or vice versa? *Dig Liver Dis* 2010; **42**: 320-330 [PMID: 20207596 DOI: 10.1016/j.dld.2010.01.016]
- 3 **Younossi ZM**. Review article: current management of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. *Aliment Pharmacol Ther* 2008; **28**: 2-12 [PMID: 18410557 DOI: 10.1111/j.1365-2036.2008.03710.x]
- 4 **Lê KA**, Bortolotti M. Role of dietary carbohydrates and macronutrients in the pathogenesis of nonalcoholic fatty liver disease. *Curr Opin Clin Nutr Metab Care* 2008; **11**: 477-482 [PMID: 18542010 DOI: 10.1097/MCO.0b013e328302f3ec]
- 5 **Akar F**, Uludağ O, Aydın A, Aytekin YA, Elbeg S, Tuzcu M, Sahin K. High-fructose corn syrup causes vascular dysfunction associated with metabolic disturbance in rats: protective effect of resveratrol. *Food Chem Toxicol* 2012; **50**: 2135-2141 [PMID: 22465803 DOI: 10.1016/j.fct.2012.03.061]
- 6 **Bantle JP**. Dietary fructose and metabolic syndrome and diabetes. *J Nutr* 2009; **139**: 1263S-1268S [PMID: 19403723 DOI: 10.3945/jn.108.098020]
- 7 **Anurag P**, Anuradha CV. Metformin improves lipid metabolism and attenuates lipid peroxidation in high fructose-fed rats. *Diabetes Obes Metab* 2002; **4**: 36-42 [PMID: 11874440]
- 8 **Quinn MT**, Linner JG, Siemsen D, Dratz EA, Buescher ES, Jesaitis AJ. Immunocytochemical detection of lipid peroxidation in phagosomes of human neutrophils: correlation with expression of flavocytochrome b. *J Leukoc Biol* 1995; **57**: 415-421 [PMID: 7884312]
- 9 **Spruss A**, Kanuri G, Wagnerberger S, Haub S, Bischoff SC, Berghelm I. Toll-like receptor 4 is involved in the development of fructose-induced hepatic steatosis in mice. *Hepatology* 2009; **50**: 1094-1104 [PMID: 19637282 DOI: 10.1002/hep.23122]
- 10 **Yang J**, Goetz D, Li JY, Wang W, Mori K, Setlik D, Du T, Erdjument-Bromage H, Tempst P, Strong R, Barasch J. An iron delivery pathway mediated by a lipocalin. *Mol Cell* 2002; **10**: 1045-1056 [PMID: 12453413]
- 11 **Flower DR**. The lipocalin protein family: structure and function. *Biochem J* 1996; **318** (Pt 1): 1-14 [PMID: 8761444]
- 12 **Chen X**, Cushman SW, Pannell LK, Hess S. Quantitative proteomic analysis of the secretory proteins from rat adipose cells using a 2D liquid chromatography-MS/MS approach. *J Proteome Res* 2005; **4**: 570-577 [PMID: 15822936 DOI: 10.1021/pr049772a]
- 13 **Cowland JB**, Borregaard N. Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans. *Genomics* 1997; **45**: 17-23 [PMID: 9339356 DOI: 10.1006/geno.1997.4896]
- 14 **Sultan S**, Pascucci M, Ahmad S, Malik IA, Bianchi A, Ramadori P, Ahmad G, Ramadori G. LIPOCALIN-2 is a major acute-phase protein in a rat and mouse model of sterile abscess. *Shock* 2012; **37**: 191-196 [PMID: 22249220 DOI: 10.1097/SHK.0b013e31823918c2]
- 15 **Liu Q**, Nilsen-Hamilton M. Identification of a new acute phase protein. *J Biol Chem* 1995; **270**: 22565-22570 [PMID: 7545679 DOI: 10.1074/jbc.270.38.22565]
- 16 **Devireddy LR**, Teodoro JG, Richard FA, Green MR. Induction of apoptosis by a secreted lipocalin that is transcriptionally regulated by IL-3 deprivation. *Science* 2001; **293**: 829-834 [PMID: 11486081 DOI: 10.1126/science.1061075]
- 17 **Guo H**, Jin D, Zhang Y, Wright W, Bazuine M, Brockman DA, Bernlohr DA, Chen X. Lipocalin-2 deficiency impairs thermogenesis and potentiates diet-induced insulin resistance in mice. *Diabetes* 2010; **59**: 1376-1385 [PMID: 20332347]
- 18 **Law IK**, Xu A, Lam KS, Berger T, Mak TW, Vanhoutte PM, Liu JT, Sweeney G, Zhou M, Yang B, Wang Y. Lipocalin-2 deficiency attenuates insulin resistance associated with aging and obesity. *Diabetes* 2010; **59**: 872-882 [PMID: 20068130]
- 19 **Wang Y**, Lam KS, Kraegen EW, Sweeney G, Zhang J, Tso AW, Chow WS, Wat NM, Xu JY, Hoo RL, Xu A. Lipocalin-2 is an inflammatory marker closely associated with obesity, insulin resistance, and hyperglycemia in humans. *Clin Chem* 2007; **53**: 34-41 [PMID: 17040956 DOI: 10.1373/clinchem.2006.075614]
- 20 **Jun LS**, Siddall CP, Rosen ED. A minor role for lipocalin 2 in high-fat diet-induced glucose intolerance. *Am J Physiol Endocrinol Metab* 2011; **301**: E825-E835 [PMID: 21771968 DOI: 10.1152/ajpendo.00147.2011]
- 21 **Jin D**, Guo H, Bu SY, Zhang Y, Hannaford J, Mashek DG, Chen X. Lipocalin 2 is a selective modulator of peroxisome proliferator-activated receptor-gamma activation and function in lipid homeostasis and energy expenditure. *FASEB J* 2011; **25**: 754-764 [PMID: 20974668 DOI: 10.1096/fj.10-165175]
- 22 **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]
- 23 **Malik IA**, Moriconi F, Sheikh N, Naz N, Khan S, Dudas J, Mansuroglu T, Hess CF, Rave-Fränk M, Christiansen H, Ramadori G. Single-dose gamma-irradiation induces up-regulation of chemokine gene expression and recruitment of granulocytes into the portal area but not into other regions of rat hepatic tissue. *Am J Pathol* 2010; **176**: 1801-1815 [PMID: 20185578 DOI: 10.2353/ajpath.2010.090505]
- 24 **Bradford MM**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248-254 [PMID: 942051 DOI: 10.1016/0003-2697(76)90527-3]
- 25 **Wójcik M**, Ramadori P, Blaschke M, Sultan S, Khan S, Malik IA, Naz N, Martius G, Ramadori G, Schultze FC. Immunodetection of cyclooxygenase-2 (COX-2) is restricted to tissue macrophages in normal rat liver and to recruited mononuclear phagocytes in liver injury and cholangiocarcinoma. *Histochem Cell Biol* 2012; **137**: 217-233 [PMID: 22131058 DOI: 10.1007/s00418-011-0889-9]
- 26 **Zhou L**, Yu X, Meng Q, Li H, Niu C, Jiang Y, Cai Y, Li M, Li Q, An C, Shu L, Chen A, Su H, Tang Y, Yin S, Raschke S, Eckardt K, Eckel J, Yang Z. Resistin reduces mitochondria and induces hepatic steatosis in mice by the protein kinase C/protein kinase G/p65/PPAR gamma coactivator 1 alpha pathway. *Hepatology* 2013; **57**: 1384-1393 [PMID: 23174781 DOI: 10.1002/hep.26167]
- 27 **Adiels M**, Olofsson SO, Taskinen MR, Borén J. Diabetic dyslipidemia. *Curr Opin Lipidol* 2006; **17**: 238-246 [PMID: 16680028 DOI: 10.1097/01.mol.0000226115.97436.c0]
- 28 **Choi HK**, Curhan G. Soft drinks, fructose consumption, and the risk of gout in men: prospective cohort study. *BMJ* 2008; **336**: 309-312 [PMID: 18244959 DOI: 10.1136/bmj.39449.819271.BE]
- 29 **Shapiro A**, Mu W, Roncal C, Cheng KY, Johnson RJ, Scarpace PJ. Fructose-induced leptin resistance exacerbates weight gain in response to subsequent high-fat feeding. *Am J Physiol Regul Integr Comp Physiol* 2008; **295**: R1370-R1375 [PMID: 18703413 DOI: 10.1152/ajpregu.00195.2008]
- 30 **Alwahsh S**, Xu M, Ramadori G, Schultze F. Lipocalin-2 is a biomarker in rat fatty liver induced by fructose-enriched diet. *Z Gastroenterol* 2013; **51**: P301 [DOI: 10.1055/s-0032-1331996]

- 31 **Tappy L**, Lê KA. Metabolic effects of fructose and the worldwide increase in obesity. *Physiol Rev* 2010; **90**: 23-46 [PMID: 20086073 DOI: 10.1152/physrev.00019.2009]
- 32 **Johnson RJ**, Segal MS, Sautin Y, Nakagawa T, Feig DI, Kang DH, Gersch MS, Benner S, Sánchez-Lozada LG. Potential role of sugar (fructose) in the epidemic of hypertension, obesity and the metabolic syndrome, diabetes, kidney disease, and cardiovascular disease. *Am J Clin Nutr* 2007; **86**: 899-906 [PMID: 17921363]
- 33 **Herzig S**, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P, Spiegelman B, Montminy M. CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature* 2001; **413**: 179-183 [PMID: 11557984 DOI: 10.1038/35093131]
- 34 **Liang H**, Ward WF. PGC-1 α : a key regulator of energy metabolism. *Adv Physiol Educ* 2006; **30**: 145-151 [PMID: 17108241 DOI: 10.1152/advan.00052.2006]
- 35 **Holmström MH**, Iglesias-Gutierrez E, Zierath JR, Garcia-Roves PM. Tissue-specific control of mitochondrial respiration in obesity-related insulin resistance and diabetes. *Am J Physiol Endocrinol Metab* 2012; **302**: E731-E739 [PMID: 22252943 DOI: 10.1152/ajpendo.00159.2011]
- 36 **Alwahsh SM**, Xu M, Ramadori G, Mihm S, Schultze FC. Fructose-enriched diet induced overexpression of lipocalin-2 in rat fatty liver. *J Clin Exp Hepat* 2013; **3**: S31-S32 [DOI: 10.1016/j.jceh.2013.03.065]
- 37 **Baker RG**, Hayden MS, Ghosh S. NF- κ B, inflammation, and metabolic disease. *Cell Metab* 2011; **13**: 11-22 [PMID: 21195345 DOI: 10.1016/j.cmet.2010.12.008]
- 38 **Lin SM**, Frevert CW, Kajikawa O, Wurfel MM, Ballman K, Mongovin S, Wong VA, Selk A, Martin TR. Differential regulation of membrane CD14 expression and endotoxin-tolerance in alveolar macrophages. *Am J Respir Cell Mol Biol* 2004; **31**: 162-170 [PMID: 15059784 DOI: 10.1165/rcmb.2003-0307OC]
- 39 **Heidenreich S**. Monocyte CD14: a multifunctional receptor engaged in apoptosis from both sides. *J Leukoc Biol* 1999; **65**: 737-743 [PMID: 10380893]
- 40 **Pedersen BK**, Steensberg A, Fischer C, Keller C, Ostrowski K, Schjerling P. Exercise and cytokines with particular focus on muscle-derived IL-6. *Exerc Immunol Rev* 2001; **7**: 18-31 [PMID: 11579746]
- 41 **Fujino RS**, Tanaka K, Morimatsu M, Tamura K, Kogo H, Hara T. Spermatogonial cell-mediated activation of an I κ B α -independent nuclear factor- κ B pathway in Sertoli cells induces transcription of the lipocalin-2 gene. *Mol Endocrinol* 2006; **20**: 904-915 [PMID: 16322095 DOI: 10.1210/me.2005-0423]
- 42 **Zhang J**, Wu Y, Zhang Y, Leroith D, Bernlohr DA, Chen X. The role of lipocalin 2 in the regulation of inflammation in adipocytes and macrophages. *Mol Endocrinol* 2008; **22**: 1416-1426 [PMID: 18292240 DOI: 10.1210/me.2007-0420]

P- Reviewers: Gong ZJ, Sazci A **S- Editor:** Qi Y **L- Editor:** A
E- Editor: Ma S



Sophocarpine attenuates liver fibrosis by inhibiting the TLR4 signaling pathway in rats

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Supported by The National Natural Science Foundation of China, Nos. 30971343, 81270486, 81000167 and 81370009

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Received: September 7, 2013 Revised: November 13, 2013

Accepted: November 28, 2013

Published online: February 21, 2014

Abstract

AIM: To explore the effect of sophocarpine on experimental liver fibrosis and the potential mechanism involved.

METHODS: Sophocarpine was injected intraperitoneally in two distinct rat hepatic fibrosis models induced either by dimethylnitrosamine or bile duct ligation. Masson's trichrome staining, Sirius red staining and hepatic hydroxyproline level were used for collagen determination. Primary hepatic stellate cells (HSCs) were isolated and treated with different concentrations of sophocarpine. Real-time reverse transcription-polymerase chain reaction was used to detect the mRNA levels of fibrotic markers and cytokines. The expression of pathway proteins was measured by Western blot. The Cell Counting

Kit-8 test was used to detect the proliferation rate of activated HSCs treated with a gradient concentration of sophocarpine.

RESULTS: Sophocarpine decreased serum levels of aminotransferases and total bilirubin in rats under chronic insult. Moreover, administration of sophocarpine suppressed extracellular matrix deposition and prevented the development of hepatic fibrosis. Furthermore, sophocarpine inhibited the expression of α -smooth muscle actin (SMA), interleukin (IL)-6, transforming growth factor- β 1 (TGF- β 1), Toll-like receptor 4 (TLR4), and extracellular-related kinase (ERK) in rats. Sophocarpine also down-regulated the mRNA expression of α -SMA, collagen I, collagen III, TGF- β 1, IL-6, tumor necrosis factor- α and monocyte chemoattractant protein-1, and decreased protein levels of TLR4, p-ERK, p-JNK, p-P38 and p-IKK *in vitro* after Lipopolysaccharide induction. In addition, sophocarpine inhibited the proliferation of HSCs accompanied by a decrease in the expression of Cyclin D1. The protein level of proliferating cell nuclear antigen was decreased in activated HSCs following a gradient concentration of sophocarpine.

CONCLUSION: Sophocarpine can alleviate liver fibrosis mainly by inhibiting the TLR4 pathway. Sophocarpine may be a potential chemotherapeutic agent for chronic liver diseases.

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Key words: Liver fibrosis; Sophocarpine; Toll-like receptor 4; Hepatic stellate cells; Cytokines

Core tip: Sophocarpine significantly ameliorated liver function and hepatic fibrosis in both the dimethylnitrosamine and bile duct ligation models. In addition, sophocarpine inhibited the activation and proliferation of hepatic stellate cells *in vitro*, which contributed to

the anti-fibrotic effect of sophocarpine *in vivo*. Toll-like receptor 4 signaling was blocked by sophocarpine *in vivo* and *in vitro*, accompanied by a reduction in pro-inflammatory and fibrotic cytokines, as well a decrease in the expression of Cyclin D1 and proliferating cell nuclear antigen.

Qian H, Shi J, Fan TT, Lv J, Chen SW, Song CY, Zheng ZW, Xie WF, Chen YX. Sophocarpine attenuates liver fibrosis by inhibiting the TLR4 signaling pathway in rats. *World J Gastroenterol* 2014; 20(7): 1822-1832 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1822.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1822>

INTRODUCTION

Liver fibrosis is a wound-healing response to chronic liver injury and is characterized by the accumulation of extracellular matrix (ECM), which depends on the balance between ECM synthesis and degradation. Following liver injury, hepatic stellate cells (HSCs) are the predominant ECM-producing cell type in the liver^[1,2]. The activation and proliferation of HSCs, characterized by the morphological transition to myofibroblast-like cells, have been well established as the central events in the pathogenesis of hepatic fibrosis^[3]. Previous studies have suggested that inhibition of the activation, proliferation and migration of HSCs may be an attractive anti-fibrotic therapy^[4].

In cirrhotic rats and patients, intestinal bacterial overgrowth can occur, including Gram-negative and -positive bacteria^[5]. Lipopolysaccharide (LPS), a major cellular component of Gram-negative bacteria, aggravates liver cirrhosis with increased permeability of the intestinal mucosal barrier. LPS-induced HSC activation has been proved to be an important mechanism in liver injury^[6]. Quiescent murine HSCs responsive to LPS can express Toll-like receptor 4 (TLR4) similar to *in vivo*-activated HSCs, even at the low LPS dose of 1 ng/mL^[7]. In addition, activated murine HSCs express TLR4 and respond to LPS with up-regulation of extracellular-related kinase (ERK) phosphorylation, interleukin (IL)-6 and transforming growth factor- β 1 (TGF- β 1)^[7,8]. TLR4-mutant mice displayed a profound reduction in hepatic fibrogenesis in three different experimental models of biliary or toxic fibrosis^[7]. These results confirm the critical role of TLR4 signaling in regulating HSC activation which affects the risk of hepatic fibrosis progression.

Sophocarpine is a matrine-type quinolizidine alkaloid widespread in the genus *Sophora*. Basic and clinical studies have shown that sophocarpine possesses a variety of pharmacological effects, such as anti-inflammatory, immuno-regulatory, anti-virus and anti-tumor^[9-11]. Moreover, our previous research proved that sophocarpine alleviated the progression of non-alcohol steatohepatitis through the down-regulation of inflammatory cytokines *in vivo*^[12]. More importantly, sophocarpine has minor toxic side effects and significant potential for clinical application.

In this study, we demonstrated that sophocarpine ameliorated liver fibrosis by inhibiting the activation and proliferation of HSCs in rats. In addition, negative regulation of the TLR4 signaling pathway contributed to the effects of sophocarpine which decreased the expression of profibrotic and inflammatory cytokines such as tumor necrosis factor (TNF)- α , TGF- β 1 and IL-6, and reduced Cyclin D1 and proliferating cell nuclear antigen (PCNA).

MATERIALS AND METHODS

Sophocarpine

Sophocarpine (High Performance Liquid Chromatography purity > 98%) was obtained from Winherb Medical Science Co., Ltd (Shanghai, China). The sophocarpine used was endotoxin-free, as determined by the limulus lysate assay, with a minimum detectable level of 8 pg/L.

Animal fibrosis models and sophocarpine administration *in vivo*

Male Sprague-Dawley rats (190 \pm 15 g) were housed in cages with stainless-steel wire tops under standard animal laboratory conditions in the specific pathogen-free-grade animal room of the Experimental Animal Center of the Second Military Medical University. The rats had free access to standard rat chow and water. This study was approved by the Ethics Committee of the Second Military Medical University, Shanghai, China. Two distinct models of hepatic fibrosis were induced in rats using dimethylnitrosamine (DMN) injection (10 mg/kg, three injections per week for 4 wk) or bile duct ligation (BDL) as described previously^[13]. For the BDL model, seven rats served as controls and underwent sham surgery. Three days after surgery, 24 BDL rats were randomly divided into two groups and treated as follows: the model group (n = 12, Ringer's solution) and sophocarpine treatment group (n = 12, 20 mg/kg sophocarpine dissolved in Ringer's solution). For the DMN model, seven rats were included as controls. Fourteen days after DMN injection, the 24 DMN rats were divided as described above (12 in the model group and 12 in the sophocarpine treatment group). Sophocarpine and placebo solution were injected intraperitoneally once a day. The animals were sacrificed 3 wk after BDL or 4 wk after DMN administration.

Serum biochemical analysis

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TB) were determined in all experimental rats using the appropriate kits from Sigma-Aldrich.

Histological examination and immunohistological staining

All paraffin-embedded liver samples were stained with hematoxylin and eosin for histopathological examination. Masson's trichrome staining and Sirius red staining were used for collagen determination. For the semiquantitative analysis, connective tissues stained with Sirius red were

Table 1 Primers used for detection of gene transcription

Gene	Forward	Reverse
α -SMA	5'-ACTGGGACGACATGGAAAAG-3'	5'-CATCTCCAGAGTCCAGCACA-3'
Pro-collagen I	5'-AGGCATAAAGGGTCATCGTG-3'	5'-ACCGTTGAGTCCATCTTTC-3'
Collagen III	5'-GTCCACGAGGTGACAAAAGGT-3'	5'-CATCTTTTCCAGGAGGTCCA-3'
TNF α	5'-AGATGTGGAACCTGGCAGAGG-3'	5'-CCCATTGGGAACCTCTCCT-3'
IL-6	5'-CCGGAGAGGAGACTTCACAG-3'	5'-ACAGTGCATCATCGTGTTC-3'
TGF β 1	5'-ATACGCTGAGTGGCTGTCT-3'	5'-TGGGACTGATCCCATTTGATT-3'
MCP-1	5'-ATGCAGTTAATGCCCCACTC-3'	5'-TTCCTTATGGGGTCAGCAC-3'
TLR4	5'-TGCTCAGACATGGCAGTTTC-3'	5'-TCAAGGCTTTTCCATCCAAC-3'
Myd88	5'-GAGATCCGCGAGTTTGAGAC-3'	5'-CTGTTTCTGCTGGTTGCGTA-3'
TRAF6	5'-AGGGTACAATACGCCTCACG-3'	5'-GCGGGTAGAGACTTCACAGC-3'
ERK1	5'-TCCAAGGGCTACACCAAATC-3'	5'-AGGTAGTTTCGGGCCTTCAT-3'
JNK1	5'-GCCACAAAATCCTCTTTCCA-3'	5'-CACATCGGGGAACAGTTTCT-3'
Cyclin D1	5'-GCGTACCCTGACACCAATCT-3'	5'-GGCTCCAGAGACAAGAAACG-3'
β -actin	5'-GCCAACACAGTGTGTCTGG-3'	5'-TGATCCACATCTGCTGGAAGG-3'

measured on an image analyzer (Image-Pro Plus, Media Cybernetics) by a technician blinded to the samples. The intensity of collagen deposition or protein expression was calculated as the percentage of the positive area in the corresponding field of liver tissue. Immunohistochemical examinations were carried out to determine the expression of α -smooth muscle actin (SMA) (BM0002, Boster, Wuhan, China), TGF- β 1 (sc-146, Santa Cruz), IL-6 (ab6672, Abcam), TLR4 (ab13556, Abcam) and ERK1/2 (4695, Cell Signaling).

Measurement of hepatic hydroxyproline content

Total hepatic hydroxyproline levels in all experimental rats were determined in the hydrolysates of liver samples as previously described^[13]. One hundred mg of wet liver samples were subjected to acid hydrolysis to determine the amount of hydroxyproline according to the protocol outlined in the Hydroxyproline Testing Kit (A030-2, Jincheng, Nanjing, China).

Cell culture and treatment

Primary HSCs were freshly isolated as previously described^[14]. The primary HSCs were cultured in plastic cell culture dishes. Forty-eight hours later, sophocarpine was added at the concentrations of 25, 50 or 200 mg/mL for 48 or 72 h, and the cells were then treated with LPS (Sigma, 2 ng/mL) for 30 min (Western blotting) or 12 h [Real-time reverse transcription-polymerase chain reaction (RT-PCR)]. Activated HSCs were derived from the primary HSCs which were cultured for approximately 14 d and passaged for 2-3 passages, the cells were then treated with sophocarpine (100-1000 μ g/mL) for 0-5 d. These cells were cultured in Dulbecco's modified medium containing 10% fetal bovine serum.

RT-PCR

Total RNA was extracted from the cells and liver tissues (3 animals representative of each group) with Trizol reagent (Invitrogen, Carlsbad, CA, United States), and cDNA was synthesized according to the manufacturer's instructions (TAKARA, Japan). Transcription levels were detected by

real-time RT-PCR with a SYBR Green PCR Kit (Applied Biosystems, Foster City, CA, United States). Primer sequences are listed in Table 1.

Western blot analysis

Western blot analyses of α -SMA (ab5694, Abcam), Collagen I (ab6308, Abcam), TLR4 (ab13556, Abcam), PCNA (ab29, Abcam), GAPDH (BSAP0063, Bioworld), p-JNK (4668, Cell Signaling), JNK (9258, Cell Signaling), p-ERK1/2 (4370, Cell Signaling), ERK1/2 (4695, Cell Signaling), p-P38 (4511, Cell Signaling), P38 (8690, Cell Signaling), p-IKK α / β (2697, Cell Signaling) and IKK α / β (sc-7607, Santa Cruz) were performed according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, United States).

Measurement of HSC proliferation

Activated HSCs were plated in triplicate wells on a 96-well plate at 3×10^3 cells/well and cultured for 24 h. These cells were then treated with sophocarpine at the concentrations of 0, 100, 200, 400, 600 and 1000 μ g/mL. The number of metabolically active and viable cells was detected colorimetrically at 450 nm using the Cell Counting Kit-8 (DOJINDO, Tokyo, Japan) assays.

Statistical analysis

Results are presented as the mean of three independent experiments (mean \pm SD). The two-sided independent Student's *t* test was performed to analyze the differences in gene expression levels, hydroxyproline content and histology data. *P* < 0.05 was considered statistically significant.

RESULTS

Sophocarpine ameliorates liver function in fibrotic rats

To assess the effect of sophocarpine on liver function, serum aminotransferases and total bilirubin were determined. Liver function tests showed that sophocarpine significantly down-regulated the concentrations of aminotransferases and total bilirubin in both the DMN and BDL models (Table 2, Figure 1A and B). TB concentra-

Table 2 Effect of sophocarpine on the improvement in serum alanine aminotransferase, aspartate aminotransferase and total bilirubin levels in both models of liver fibrosis

Group	ALT (U/L)	AST (U/L)	TB (mmol/L)
Normal	29.29 ± 1.76	123.67 ± 29.06	1.29 ± 0.28
DMN model	68.27 ± 3.43	176.9 ± 8.99	8.09 ± 1.35
DMN + sophocarpine	48.58 ± 4.52 ¹	108.42 ± 15.46 ¹	3.00 ± 0.48 ¹
Sham operation	28.83 ± 2.22	121.00 ± 36.72	2.00 ± 0.63
BDL model	150.50 ± 23.6	959.50 ± 255	139.67 ± 16.23
BDL + sophocarpine	91.86 ± 6.71 ¹	464.14 ± 182.6 ¹	123.86 ± 9.69

¹Compared with the model group, $P < 0.05$. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TB: Total bilirubin; DMN: Dimethylnitrosamine; BDL: Bile duct ligation.

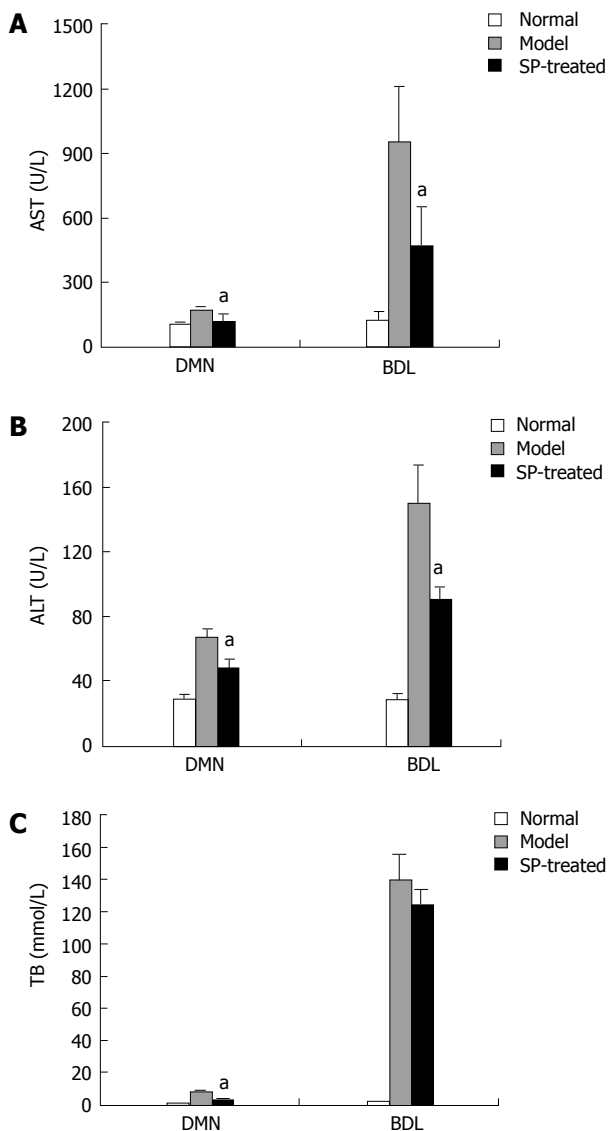


Figure 1 Sophocarpine ameliorates liver function in fibrotic rats. Serum was collected from each group of rats. AST (A), ALT (B) and TB (C) levels were determined to assess liver function in the sophocarpine-treated group compared to each model group ($P < 0.05$, by two-tailed Student's *t* test). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TB: Total bilirubin; DMN: Dimethylnitrosamine; BDL: Bile duct ligation.

tion in sophocarpine-treated rats showed a decrease by approximately 70% compared with the DMN rat model,

however, there was no significant decrease in the sophocarpine-treated BDL group (Table 2, Figure 1C).

Sophocarpine attenuates hepatic fibrosis induced by DMN or BDL in rats

We next examined the potential effect of sophocarpine on the two distinct fibrotic models. Liver sections stained with Masson's trichrome stain showed that periportal fibrosis with fibrous septa extended to adjacent portal tracts and the terminal hepatic venue in the DMN model; in the BDL model, livers showed extensive bile duct proliferation, a detrimental collapse of liver parenchyma and overt ECM deposition around the reactive bile ductile (Figure 2A). Following administration of sophocarpine, the ECM area (Masson's staining) was reduced by 55.6% and 39.3% ($P < 0.05$) in DMN and BDL rats, respectively (Figure 2B). The contents of hydroxyproline in the sophocarpine-treated rats were lower than those in the model rats (261.17 ± 20.45 $\mu\text{g/g}$ in the sophocarpine-treated DMN group *vs* 361.17 ± 20.55 $\mu\text{g/g}$ in the DMN model group, $P < 0.05$; 178 ± 15.89 $\mu\text{g/g}$ in the sophocarpine-treated BDL group *vs* 259.33 ± 23.18 $\mu\text{g/g}$ in the BDL model group, $P < 0.05$) (Figure 2C). The mRNA expression of α -SMA, TGF- β 1 and alpha 1 type I procollagen detected by RT-PCR was reduced significantly in the sophocarpine-treated groups in both the DMN and BDL models ($P < 0.05$) (Figure 2D and E).

Expression of pro-fibrotic cytokines and TLR4 signaling pathway-related proteins is suppressed in sophocarpine-treated rats

We subsequently determined the effects of sophocarpine on the expression of pro-fibrotic cytokines. Immunohistochemical examination revealed that α -SMA, IL-6 and TGF- β 1 protein expression was significantly elevated in both the DMN and BDL models, and was suppressed following sophocarpine administration (Figure 3). Furthermore, from the observed effects of sophocarpine on HSCs *in vitro*, the expression of TLR4 signaling pathway-related proteins, such as TLR4 and ERK1/2, was also increased in the DMN and BDL models. Sophocarpine down-regulated these proteins *in vivo* as shown by immunohistochemistry (Figure 3). These results indicated that sophocarpine blocked the TLR4 signaling pathway which aggravated the progression of liver fibrosis and reduced pro-fibrotic cytokine expression.

Sophocarpine inhibits the activation of hepatic stellate cells

RT-PCR was performed to determine the gene expression of inflammatory and fibrotic markers in HSCs treated with a gradient concentration of sophocarpine at 48 and 72 h to observe the effect of sophocarpine during activation of HSCs. In contrast to the freshly isolated and self-activated HSCs, dose gradient sophocarpine-treated cells showed significantly lower expression of α -SMA, collagen I, collagen III, TGF- β 1 and inflammatory cytokines, including IL-6, TNF- α and monocyte chemoattractant protein-1 (MCP-1) (Figure 4A). Furthermore,

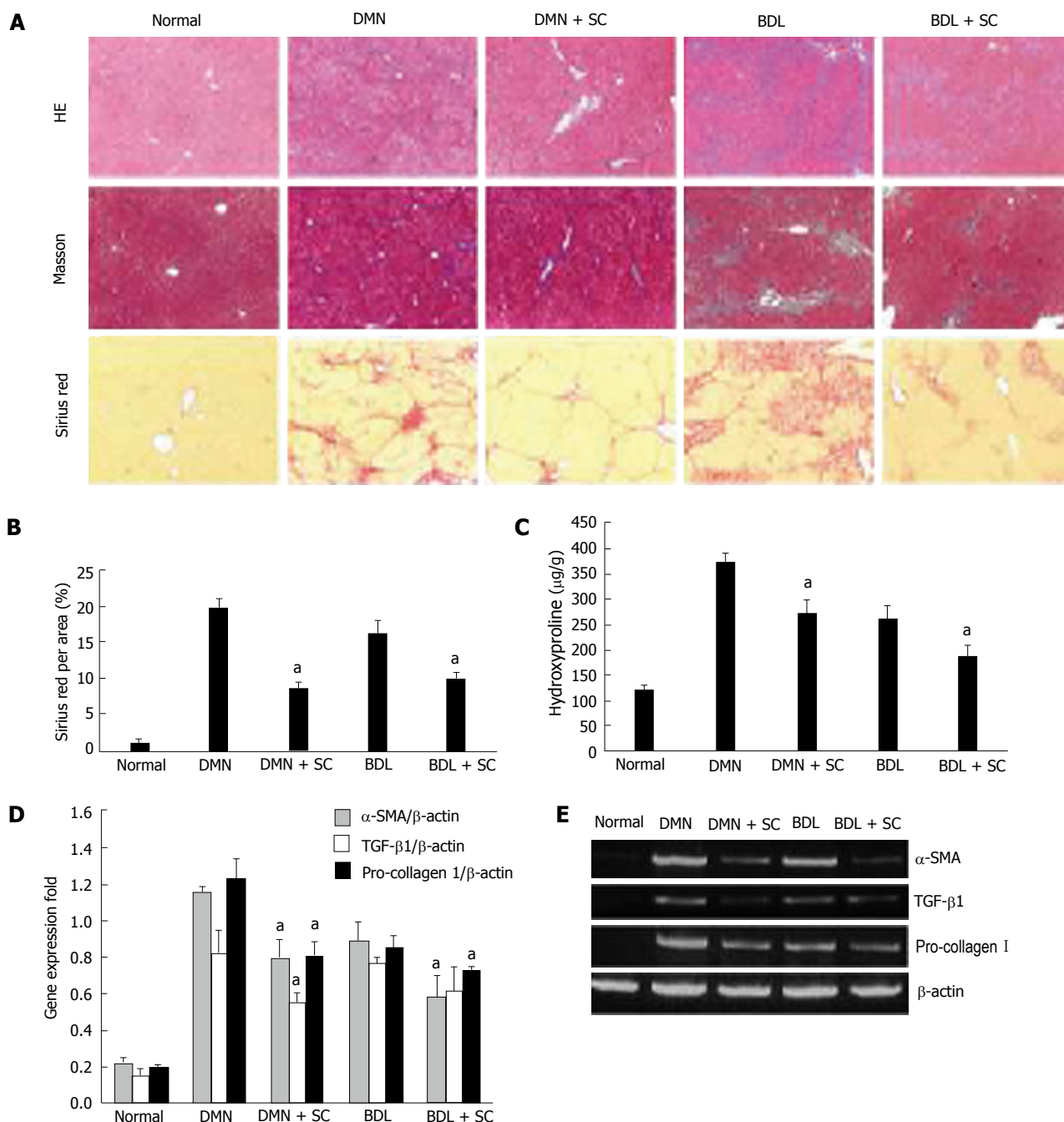


Figure 2 Sophocarpine attenuates hepatic fibrosis induced by dimethylnitrosamine or bile duct ligation in rats. DMN and BDL were used to construct two types of hepatic fibrosis models to evaluate the therapeutic effect of sophocarpine. A: Liver fibrosis in each group was assessed by HE ($\times 40$), Masson's trichrome ($\times 40$) and Sirius red staining ($\times 40$); B: The percentage of Sirius-red in fibrotic livers was quantified using an image analysis system ($^aP < 0.05$); C: The amount of hydroxyproline in fibrotic livers was detected in the sophocarpine-treated group compared with each model group ($^aP < 0.05$); D, E: Real-time RT-PCR was employed to examine the expression of α -SMA, TGF- β and pro-collagen I in fibrotic livers following sophocarpine administration compared with the control models ($^aP < 0.05$ by two-tailed Student's *t* test). DMN: Dimethylnitrosamine; BDL: Bile duct ligation; TGF- β : Transforming growth factor- β ; RT-PCR: Reverse transcription-polymerase chain reaction; SMA: Smooth muscle actin.

the protein expression of α -SMA and collagen I was depressed by sophocarpine in HSCs (Figure 4B). We also measured the above-mentioned gene expression at different culture times. The mRNA expression of α -SMA, collagen III, IL-6, TNF- α and MCP-1 decreased by 38.6%, 43.7%, 59.5%, 45.2% and 55.6% at 72 h compared to 48 h, respectively (Figure 4C). These results confirmed that sophocarpine had a dose- and time-dependent inhibitory effect on the activation of HSCs.

Sophocarpine blocks the LPS-induced TLR4 signaling pathway affecting the activation of HSCs

We then detected mRNA expression of TLR4 pathway-related genes induced by LPS using RT-PCR at 72 h. The gene expression of TLR4 and Myd88 showed a significant dose-dependent decrease in sophocarpine-treated HSCs (Figure 4D). Subsequently, we found that the protein expression of TLR4, p-ERK1, p-JNK1, p-P38 and p-IKK decreased markedly in sophocarpine-treated

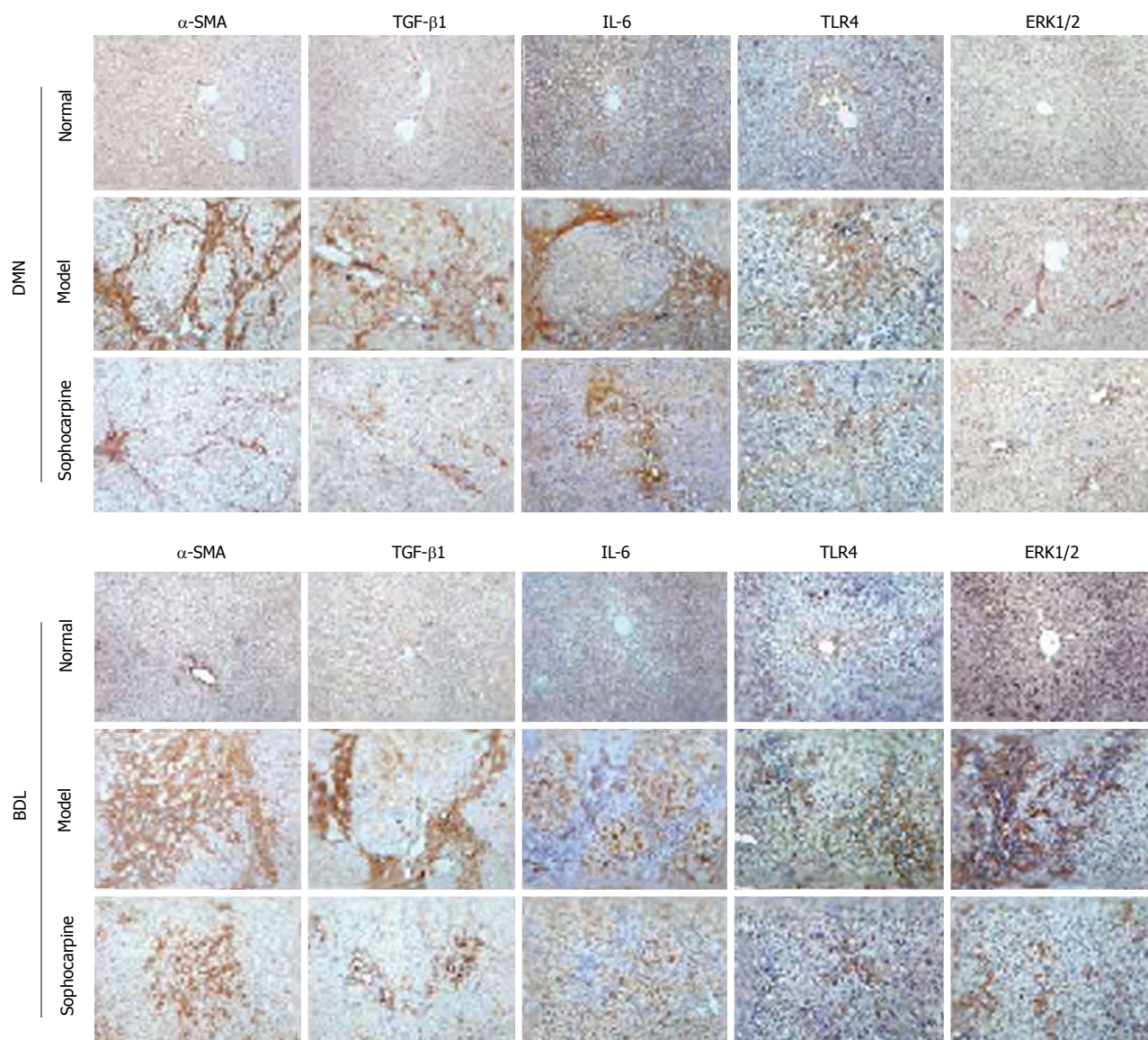


Figure 3 Expression of pro-fibrotic cytokines and toll-like receptor 4 signaling pathway related-proteins is suppressed in sophocarpine-treated rats. Immunohistochemical analysis of the protein expression of α -SMA, TGF- β 1, IL-6, TLR4 and ERK1/2 in the liver tissue of each group was performed as described in Materials and Methods. The results show the protein expression of α -SMA ($\times 200$), TGF- β 1 ($\times 200$), IL-6 ($\times 200$), TLR4 ($\times 200$) and ERK1/2 ($\times 200$) in the fibrotic livers of each group. TLR4: Toll-like receptor 4; IL-6: Interleukin-6; TGF- β : Transforming growth factor- β ; SMA: Smooth muscle actin.

HSCs compared with control cells induced by LPS at 72 h. Thus, the Myd88-dependent TLR4 signaling pathway was blocked by sophocarpine which inhibited the activation of HSCs (Figure 4E).

Sophocarpine suppresses the proliferation of HSCs

To determine the effects of sophocarpine on inhibition of HSC proliferation, CCK-8 assays were performed. The results of CCK-8 measurement showed that sophocarpine at doses ≥ 200 μ L/mL significantly suppressed the proliferation of HSCs. These inhibitory effects were dose- and time-dependent (Figure 5A). We also detected mRNA expression of Cyclin D1 in self-activated HSCs treated with sophocarpine and in control cells, and sophocarpine significantly down-regulated the expression of Cyclin D1 at 72 h (Figure 5B). The inhibitory effect of sophocarpine on the proliferation of HSCs was confirmed by decreased

protein expression of PCNA in HSCs (Figure 5C).

DISCUSSION

Liver fibrosis occurs as a wound-healing scar response following acute and chronic inflammation, including viral hepatitis, alcohol consumption, autoimmune and metabolic liver diseases^[15]. Liver inflammation results in the activation of HSCs through various inflammatory or fibrogenic mediators including TNF- α , IL-1 β , IL-6 and TGF- β ^[16-21]. In recent studies, sophocarpine, a monomer used in traditional Chinese medicine, inhibited the production of inflammatory cytokines IL-6 and TNF- α in murine macrophages^[11]. In addition, sophocarpine prevented cachexia-related symptoms induced by adenocarcinoma and alleviated non-alcoholic steatohepatitis in rats^[11,12]. Based on the validated effects of sophocarpine

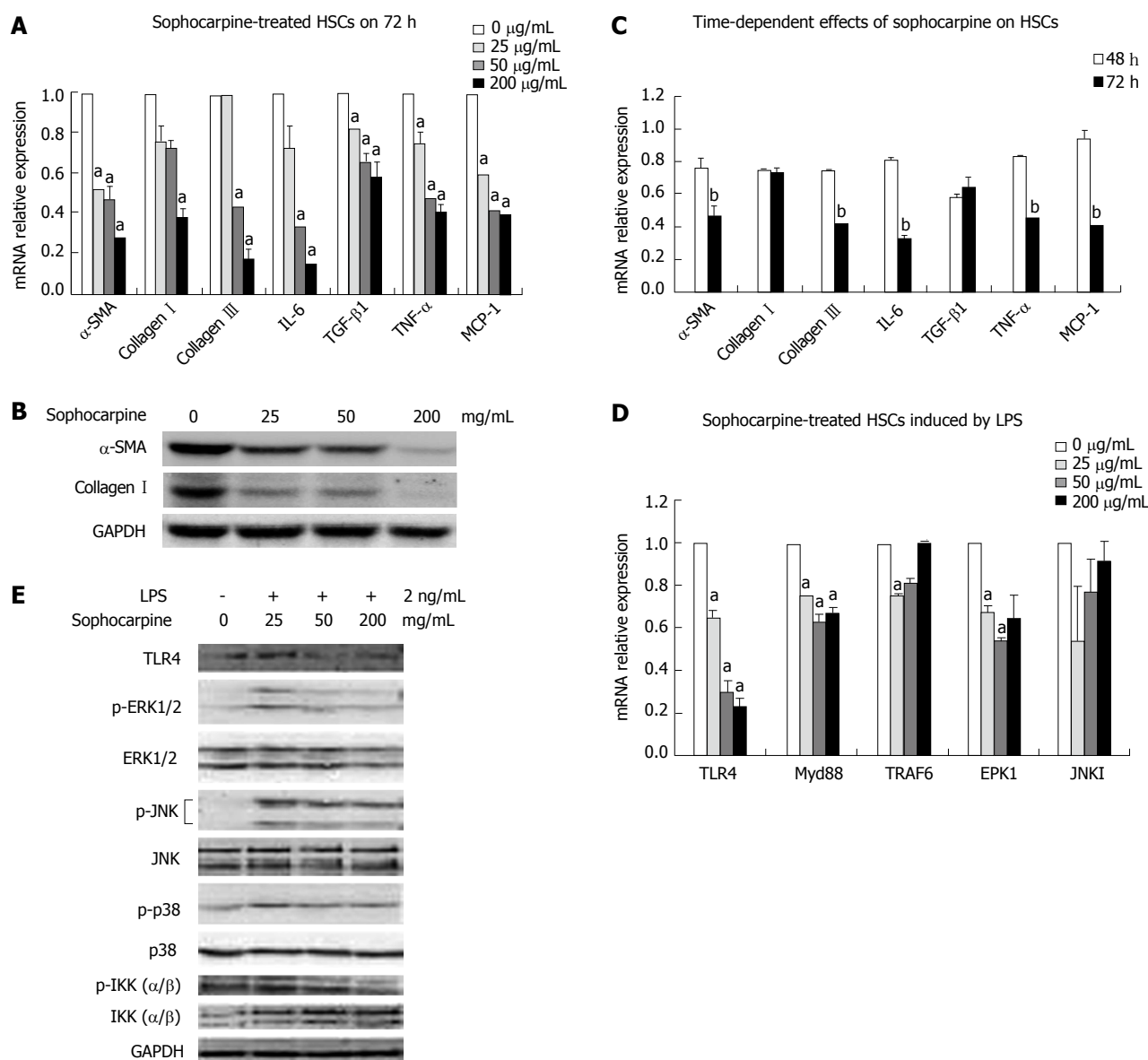


Figure 4 Sophocarpine inhibits the activation of hepatic stellate cells by blocking the lipopolysaccharide-induced toll-like receptor 4 signaling pathway. Primary HSCs were isolated and plated in 6-well plates (1×10^6 cells/well). Forty-eight hours later, the HSCs were treated with a gradient concentration of sophocarpine for 48 or 72 h. A: Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed to analyze the mRNA levels of α -SMA, collagen I, collagen III, IL-6, TGF- β 1, TNF- α and MCP-1 in HSCs treated with a gradient concentration of sophocarpine for 72 h (compared to 0 µg/mL, $^aP < 0.05$); B: Immunoblots of α -SMA, collagen I and GAPDH were detected by Western blot from HSCs treated with a gradient concentration of sophocarpine for 72 h; C: The mRNA expression of the above genes was detected in HSCs treated with sophocarpine (50 µg/mL) at 72 h compared to that at 48 h, $^bP < 0.05$; D, E: Gradient concentration sophocarpine-treated HSCs were incubated with LPS (2 ng/mL), and real-time RT-PCR (compared to 0 µg/mL, $^aP < 0.05$, D) and Western blot analysis (E) were employed to detect the expression of TLR4 pathway-related genes at the gene and protein levels (P value by two-tailed Student's t test). LPS: Lipopolysaccharide; HSCs: Hepatic stellate cells; TGF- β : Transforming growth factor- β ; IL-6: Interleukin-6; SMA: Smooth muscle actin; TNF: Tumor necrosis factor; MCP: Monocyte chemoattractant protein.

on inflammation regulation, we hypothesized that sophocarpine may have a restorative effect on liver fibrosis.

In this study, we used sophocarpine to treat hepatic fibrosis induced by two mechanistically different fibrosis models: DMN administration and BDL. Based on the histopathological and immunohistochemical results, hepatocellular injury and HSC activation improved following sophocarpine administration in both models. More importantly, it was shown for the first time that sophocarpine administration attenuated ECM deposition and hydroxyproline content in liver fibrosis induced by DMN and BDL, indicating that sophocarpine suppressed

hepatic fibrosis in these rat models. Furthermore, we demonstrated that reduced production of inflammatory cytokines, such as IL-6 and TNF- α , contributed to the anti-fibrotic effect of sophocarpine. This was accompanied by alleviation of liver fibrosis and a reduction in pro-fibrotic cytokines such as TGF- β 1 *in vivo*^[22,23]. The expression of α -SMA which is considered a marker of myofibroblasts^[24] decreased significantly in fibrotic rats treated with sophocarpine. Based on these findings, we hypothesize that sophocarpine inhibited the activation and proliferation of HSCs which mediate the central pathological effects in the progression of liver fibrosis.

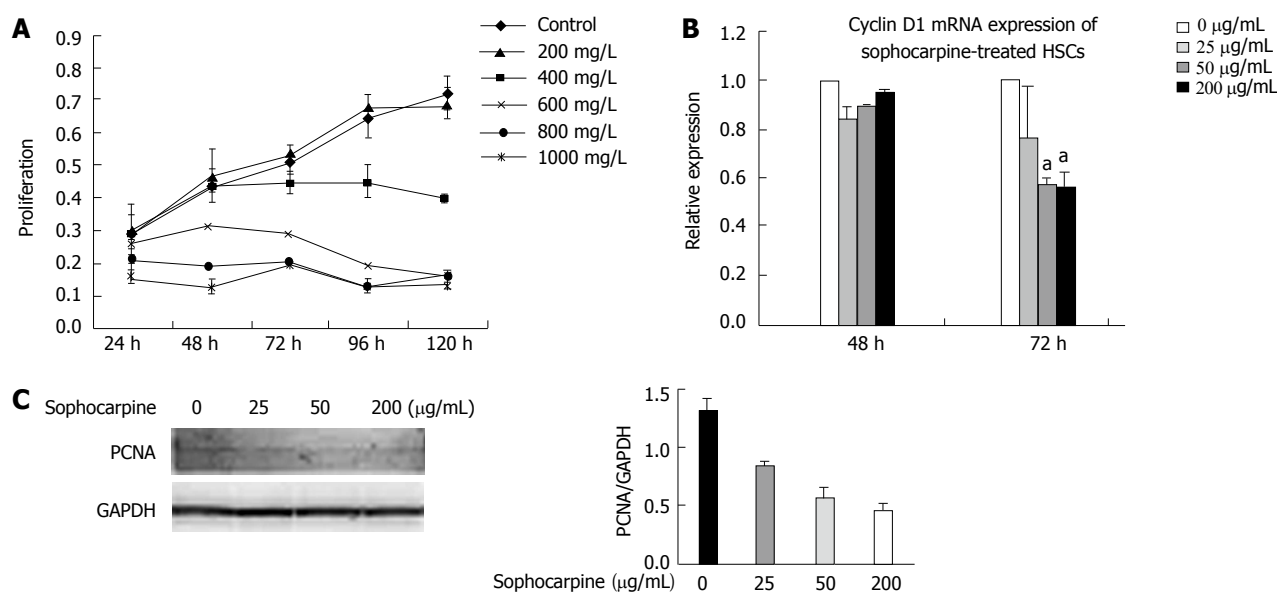


Figure 5 Sophocarpine suppresses the proliferation of hepatic stellate cells. A: Activated HSCs were treated with a gradient concentration of sophocarpine and the proliferation of HSCs was assessed using the CCK-8 kit; B: Real-time polymerase chain reaction was performed to examine the expression of Cyclin D1 in HSCs after treatment with a gradient concentration of sophocarpine ($P < 0.05$); C: Western blot was employed to detect PCNA expressed in HSCs after treatment with sophocarpine. HSCs: Hepatic stellate cells; PCNA: Proliferating cell nuclear antigen.

During hepatic fibrosis, HSCs undergo activation and conversion to myofibroblast-like cells which secrete collagens and aggravate the deposition of ECM. Reversal of these processes is critical in the treatment of liver fibrosis. In the present study, sophocarpine decreased the expression of α -SMA, collagen I and III *in vitro*, which indicated that sophocarpine inhibited the activation and conversion of HSCs. Moreover, sophocarpine reduced the expression of TGF- β 1 which is a major pro-fibrogenic molecule. Furthermore, sophocarpine attenuated the hepatic inflammation reaction and reduced the expression of IL-6, MCP-1 and TNF- α during the *in vivo* activation of HSCs. Sophocarpine also inhibited the proliferation of HSCs with a reduction in Cyclin D1 which participates in cell cycle regulation. These results demonstrated that the inhibition of activation and proliferation of HSCs was the major cytological mechanism involved in the alleviation of liver fibrosis by sophocarpine.

Toll-like receptors (TLRs) play an important role in the regulation of inflammation, even under sterile conditions, such as injury and wound healing. The healthy liver contains lower mRNA levels of TLRs and signaling molecules such as MD-2 and MyD88 than other organs, suggesting that the low expression of TLR signaling molecules may contribute to the high tolerance of the liver to TLR ligands from the intestinal microbiota to which the liver is constantly exposed^[25-28]. Numerous studies have demonstrated that LPS is elevated in experimental models of hepatic fibrosis^[7,29,30] and in cirrhotic patients^[31-33]. Cirrhotic patients have markedly elevated endotoxin levels compared with healthy subjects^[33]. In view of the critical role of the intestinal microbiota in hepatic fibrogenesis, the LPS-induced TLR4 signaling pathway contributes significantly to the progression of liver cirrhosis^[34]. Activated human HSCs expressed TLR4 and its

coreceptors MD-2 and CD-14 *in vitro*^[35]. LPS treatment can induce strong activation of the nuclear factor kappa B (NF- κ B) and JNK/AP-1 pathways as well as the secretion of pro-inflammatory cytokines in activated HSCs^[36]. Activated murine HSCs expressed TLR4 and responded to LPS with an up-regulation of extracellular-related kinase (ERK) phosphorylation and IL-6, TGF- β 1 and MCP-1 secretion^[7,8].

As IL-6, TNF- α , TGF- β 1 and MCP-1, whose expression decreased after sophocarpine administration, are all regulated by the LPS-induced TLR4 signal pathway, we suspected that sophocarpine may affect the TLR4 signal pathway and inhibit liver fibrosis. We found that sophocarpine reduced the expression of TLR4 and Myd88, but not TLR2 or TLR9 which can also mediate the progression of liver fibrosis^[37,38]. During TLR4 signaling, the MyD88-dependent pathway mediates the up-regulation of inflammatory cytokines through activation of NF- κ B and mitogen-activated protein kinases (MAPK)^[39]. The MAPK signal pathway involves ERK, JNK, p38 and their phosphorylation in the pathogenesis of liver fibrosis^[40-42]. SiRNAs or selective inhibitors targeting these molecules reduced their expression or activity, and alleviated liver fibrosis *in vivo*^[13,43]. We found that the phosphorylation of ERK, JNK, p38 and IKK was significantly down-regulated after sophocarpine administration, which indicated that sophocarpine suppressed the MyD88-dependent TLR4 pathway and inhibited the activation of HSCs. The ERK/AP-1 pathway also induces c-Myc and Cyclin D1 expression which facilitates the proliferation of HSCs^[14,44,45]. Sophocarpine inhibited the phosphorylation of ERK, and then down-regulated the expression of Cyclin D1, which may contribute to the inhibitory effect of sophocarpine on the proliferation of HSCs.

In our study, sophocarpine exhibited potent control

of liver inflammation, which mainly contributed to the inhibition of hepatic fibrosis and HSC activation. For decades many researchers have investigated many stimuli, with the exception of inflammatory cytokines, that can drive the activation of HSCs including hepatocellular necrosis due to oxidative stress and apoptosis^[46-48]. The TLR4 and complements also play important roles in oxidative stress and hepatotoxicity, especially in the initiation of alcoholic steatohepatitis and fibrosis^[49,50]. It is likely that sophocarpine has an impact on suppressing oxidative stress and subsequently protecting hepatocytes from necrosis or apoptosis, which merits investigation. Moreover, as a monomer derived from matrine, although sophocarpine blocked the TLR4 pathway which was confirmed by our investigation, the direct target molecules of sophocarpine in the LPS-induced TLR4 pathway are unknown and require further study.

In summary, our investigation provides strong evidence for a suppressive effect of sophocarpine on hepatic fibrosis through inhibition of the activation and proliferation of HSCs. Moreover, sophocarpine exhibited potent blockage of the TLR4 signaling pathway and subsequently decreased the expression of pro-inflammatory and fibrotic cytokines. Based on the present study, sophocarpine may emerge as a novel option for the clinical therapy of chronic liver diseases.

COMMENTS

Background

The activation and proliferation of hepatic stellate cells (HSCs) are central events in the pathogenesis of hepatic fibrosis. However, there is no efficient treatment for chronic liver diseases in clinical practice.

Research frontiers

Previous studies have suggested that inhibition of the activation, proliferation and migration of HSCs may be an attractive anti-fibrotic therapy. Inflammatory cells and the inflammatory response are involved in driving the activation of HSCs through various inflammatory or fibrogenic mediators and pathways. Lipopolysaccharide toll-like receptor 4 (LPS-TLR4) signaling plays a critical role in regulating HSC activation and affects the risk of hepatic fibrosis progression.

Innovations and breakthroughs

Sophocarpine is a matrine-type quinolizidine alkaloid and exhibits a variety of pharmacological effects including anti-inflammatory, immuno-regulatory, anti-virus and anti-tumor. In this study, the authors demonstrated that sophocarpine administration ameliorated liver fibrosis by inhibiting the activation and proliferation of HSCs in rats. Moreover, blockage of the TLR4 signaling pathway contributed to the effects of sophocarpine by inhibiting the expression of fibrotic cytokines.

Applications

Due to the inhibitory effect of sophocarpine on hepatic fibrosis, it is likely that sophocarpine may have potential application in the clinical treatment of chronic liver diseases.

Terminology

HSCs are pericytes found in the perisinusoidal space (a small area between the sinusoids and hepatocytes) of the liver also known as the space of Disse. The stellate cell is the major cell type involved in liver fibrosis, which is the formation of scar tissue in response to liver damage.

Peer review

This study suggests that sophocarpine can alleviate liver fibrosis mainly through inhibiting the TLR4 signaling pathway. Sophocarpine might be present as a potential agent for chronic liver diseases.

REFERENCES

- Friedman SL. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem* 2000; **275**: 2247-2250 [PMID: 10644669 DOI: 10.1074/jbc.275.4.2247]
- Yang C, Zeisberg M, Mosterman B, Sudhakar A, Yerramalla U, Holthaus K, Xu L, Eng F, Afdhal N, Kalluri R. Liver fibrosis: insights into migration of hepatic stellate cells in response to extracellular matrix and growth factors. *Gastroenterology* 2003; **124**: 147-159 [PMID: 12512039 DOI: 10.1053/gast.2003.50012]
- Sato M, Suzuki S, Senoo H. Hepatic stellate cells: unique characteristics in cell biology and phenotype. *Cell Struct Funct* 2003; **28**: 105-112 [PMID: 12808230 DOI: 10.1247/csf.28.105]
- Wynn TA. Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008; **214**: 199-210 [PMID: 18161745 DOI: 10.1002/path.2277]
- Nolan JP. The role of endotoxin in liver injury. *Gastroenterology* 1975; **69**: 1346-1356 [PMID: 1104401]
- Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest* 2005; **115**: 209-218 [PMID: 15690074 DOI: 10.1172/JCI24282]
- Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, Schwabe RF. TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med* 2007; **13**: 1324-1332 [PMID: 17952090 DOI: 10.1038/nm1663]
- Brun P, Castagliuolo I, Pinzani M, Palù G, Martines D. Exposure to bacterial cell wall products triggers an inflammatory phenotype in hepatic stellate cells. *Am J Physiol Gastrointest Liver Physiol* 2005; **289**: G571-G578 [PMID: 15860640 DOI: 10.1152/ajpgi.00537.2004]
- Lin Z, Huang CF, Liu XS, Jiang J. In vitro anti-tumour activities of quinolizidine alkaloids derived from *Sophora flavescens* Ait. *Basic Clin Pharmacol Toxicol* 2011; **108**: 304-309 [PMID: 21159130 DOI: 10.1111/j.1742-7843.2010.00653.x]
- Gao Y, Li G, Li C, Zhu X, Li M, Fu C, Li B. Anti-nociceptive and anti-inflammatory activity of sophocarpine. *J Ethnopharmacol* 2009; **125**: 324-329 [PMID: 19607897 DOI: 10.1016/j.jep.2009.06.036]
- Zhang Y, Wang S, Li Y, Xiao Z, Hu Z, Zhang J. Sophocarpine and matrine inhibit the production of TNF-alpha and IL-6 in murine macrophages and prevent cachexia-related symptoms induced by colon26 adenocarcinoma in mice. *Int Immunopharmacol* 2008; **8**: 1767-1772 [PMID: 18775799 DOI: 10.1016/j.intimp.2008.08.008]
- Song CY, Zeng X, Chen SW, Hu PF, Zheng ZW, Ning BF, Shi J, Xie WF, Chen YX. Sophocarpine alleviates non-alcoholic steatohepatitis in rats. *J Gastroenterol Hepatol* 2011; **26**: 765-774 [PMID: 21054517 DOI: 10.1111/j.1440-1746.2010.06561.x]
- Zhong W, Shen WF, Ning BF, Hu PF, Lin Y, Yue HY, Yin C, Hou JL, Chen YX, Zhang JP, Zhang X, Xie WF. Inhibition of extracellular signal-regulated kinase 1 by adenovirus mediated small interfering RNA attenuates hepatic fibrosis in rats. *Hepatology* 2009; **50**: 1524-1536 [PMID: 19787807 DOI: 10.1002/hep.23189]
- Marra F, Efsen E, Romanelli RG, Caligiuri A, Pastacaldi S, Batignani G, Bonacchi A, Caporale R, Laffi G, Pinzani M, Gentilini P. Ligands of peroxisome proliferator-activated receptor gamma modulate profibrogenic and proinflammatory actions in hepatic stellate cells. *Gastroenterology* 2000; **119**: 466-478 [PMID: 10930382 DOI: 10.1053/gast.2000.9365]
- Friedman SL. Seminars in medicine of the Beth Israel Hospital, Boston. The cellular basis of hepatic fibrosis. Mechanisms and treatment strategies. *N Engl J Med* 1993; **328**: 1828-1835 [PMID: 8502273 DOI: 10.1056/NEJM199306243282508]
- Byl B, Roucloux I, Crusiaux A, Dupont E, Devière J. Tumor necrosis factor alpha and interleukin 6 plasma levels in infected cirrhotic patients. *Gastroenterology* 1993; **104**: 1492-1497 [PMID: 8482461]
- Spirli C, Nathanson MH, Fiorotto R, Duner E, Denson LA, Sanz JM, Di Virgilio F, Okolicsanyi L, Casagrande F, Strazzabosco M. Proinflammatory cytokines inhibit secretion in rat bile duct epithelium. *Gastroenterology* 2001; **121**: 156-169

- [PMID: 11438505 DOI: 10.1053/gast.2001.25516]
- 18 **Albillos A**, Muñoz L, Nieto M, Ubeda M, de-la-Hera A, Alvarez-Mon M. Systemic effects of TNF- α secreted by circulating monocytes and fatigue in cirrhosis. *Hepatology* 2006; **43**: 1399; author reply 1399-1400 [PMID: 16729299 DOI: 10.1002/hep.21205]
 - 19 **Tiggelman AM**, Boers W, Linthorst C, Sala M, Chamuleau RA. Collagen synthesis by human liver (myo)fibroblasts in culture: evidence for a regulatory role of IL-1 β , IL-4, TGF β and IFN γ . *J Hepatol* 1995; **23**: 307-317 [PMID: 8550995]
 - 20 **Napoli J**, Bishop GA, McCaughan GW. Increased intrahepatic messenger RNA expression of interleukins 2, 6, and 8 in human cirrhosis. *Gastroenterology* 1994; **107**: 789-798 [PMID: 8076766 DOI: 10.1016/0016-5085(94)90128-7]
 - 21 **Tilg H**, Wilmer A, Vogel W, Herold M, Nölchen B, Judmaier G, Huber C. Serum levels of cytokines in chronic liver diseases. *Gastroenterology* 1992; **103**: 264-274 [PMID: 1612333]
 - 22 **Koff RS**. Transforming growth factors in human chronic hepatitis and cirrhosis: correlations with fibrogenesis and hepatic regeneration. *Gastroenterology* 1991; **101**: 1445-1446 [PMID: 1936817]
 - 23 **Milani S**, Herbst H, Schuppan D, Stein H, Surrenti C. Transforming growth factors β 1 and β 2 are differentially expressed in fibrotic liver disease. *Am J Pathol* 1991; **139**: 1221-1229 [PMID: 1750499]
 - 24 **Cassiman D**, Libbrecht L, Desmet V, Denef C, Roskams T. Hepatic stellate cell/myofibroblast subpopulations in fibrotic human and rat livers. *J Hepatol* 2002; **36**: 200-209 [PMID: 11830331 DOI: 10.1016/S0168-8278(01)00260-4]
 - 25 **De Creus A**, Abe M, Lau AH, Hackstein H, Raimondi G, Thomson AW. Low TLR4 expression by liver dendritic cells correlates with reduced capacity to activate allogeneic T cells in response to endotoxin. *J Immunol* 2005; **174**: 2037-2045 [PMID: 15699133]
 - 26 **Lichtman SN**, Wang J, Lemasters JJ. LPS receptor CD14 participates in release of TNF- α in RAW 264.7 and peritoneal cells but not in kupffer cells. *Am J Physiol* 1998; **275**: G39-G46 [PMID: 9655682]
 - 27 **Zarembek KA**, Godowski PJ. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J Immunol* 2002; **168**: 554-561 [PMID: 11777946]
 - 28 **Mencin A**, Kluwe J, Schwabe RF. Toll-like receptors as targets in chronic liver diseases. *Gut* 2009; **58**: 704-720 [PMID: 19359436 DOI: 10.1136/gut.2008.156307]
 - 29 **Nolan JP**, Leibowitz AI. Endotoxin and the liver. III. Modification of acute carbon tetrachloride injury by polymyxin b—an antiendotoxin. *Gastroenterology* 1978; **75**: 445-449 [PMID: 210083]
 - 30 **Grinko I**, Geerts A, Wisse E. Experimental biliary fibrosis correlates with increased numbers of fat-storing and Kupffer cells, and portal endotoxemia. *J Hepatol* 1995; **23**: 449-458 [PMID: 8655963 DOI: 10.1016/0168-8278(95)80204-5]
 - 31 **Scaffidi P**, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 2002; **418**: 191-195 [PMID: 12110890 DOI: 10.1038/nature00858]
 - 32 **Chan CC**, Hwang SJ, Lee FY, Wang SS, Chang FY, Li CP, Chu CJ, Lu RH, Lee SD. Prognostic value of plasma endotoxin levels in patients with cirrhosis. *Scand J Gastroenterol* 1997; **32**: 942-946 [PMID: 9299675 DOI: 10.3109/00365529709011206]
 - 33 **Lin RS**, Lee FY, Lee SD, Tsai YT, Lin HC, Lu RH, Hsu WC, Huang CC, Wang SS, Lo KJ. Endotoxemia in patients with chronic liver diseases: relationship to severity of liver diseases, presence of esophageal varices, and hyperdynamic circulation. *J Hepatol* 1995; **22**: 165-172 [PMID: 7790704 DOI: 10.1016/0168-8278(95)80424-2]
 - 34 **Aoyama T**, Paik YH, Seki E. Toll-like receptor signaling and liver fibrosis. *Gastroenterol Res Pract* 2010; **2010**: pii:192543 [PMID: 20706677 DOI: 10.1155/2010/192543]
 - 35 **Scott MJ**, Billiar TR. β 2-integrin-induced p38 MAPK activation is a key mediator in the CD14/TLR4/MD2-dependent uptake of lipopolysaccharide by hepatocytes. *J Biol Chem* 2008; **283**: 29433-29446 [PMID: 18701460 DOI: 10.1074/jbc.M803905200]
 - 36 **Paik YH**, Schwabe RF, Bataller R, Russo MP, Jobin C, Brenner DA. Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. *Hepatology* 2003; **37**: 1043-1055 [PMID: 12717385 DOI: 10.1053/jhep.2003.50182]
 - 37 **Seki E**, Brenner DA. Toll-like receptors and adaptor molecules in liver disease: update. *Hepatology* 2008; **48**: 322-335 [PMID: 18506843 DOI: 10.1002/hep.22306]
 - 38 **Gäbele E**, Mühlbauer M, Dorn C, Weiss TS, Froh M, Schnabl B, Wiest R, Schölmerich J, Obermeier F, Hellerbrand C. Role of TLR9 in hepatic stellate cells and experimental liver fibrosis. *Biochem Biophys Res Commun* 2008; **376**: 271-276 [PMID: 18760996 DOI: 10.1016/j.bbrc.2008.08.096]
 - 39 **Akira S**, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004; **4**: 499-511 [PMID: 15229469 DOI: 10.1038/nri1391]
 - 40 **Davis BH**, Chen A, Beno DW. Raf and mitogen-activated protein kinase regulate stellate cell collagen gene expression. *J Biol Chem* 1996; **271**: 11039-11042 [PMID: 8626642 DOI: 10.1074/jbc.271.19.11039]
 - 41 **Marra F**, Arrighi MC, Fazi M, Caligiuri A, Pinzani M, Romanelli RG, Efsen E, Laffi G, Gentilini P. Extracellular signal-regulated kinase activation differentially regulates platelet-derived growth factor's actions in hepatic stellate cells, and is induced by in vivo liver injury in the rat. *Hepatology* 1999; **30**: 951-958 [PMID: 10498647 DOI: 10.1002/hep.510300406]
 - 42 **Svegliati-Baroni G**, Ridolfi F, Caradonna Z, Alvaro D, Marziani M, Saccomanno S, Candelaesi C, Trozzi L, Macarri G, Benedetti A, Folli F. Regulation of ERK/JNK/p70S6K in two rat models of liver injury and fibrosis. *J Hepatol* 2003; **39**: 528-537 [PMID: 12971962 DOI: 10.1016/S0168-8278(03)00291-5]
 - 43 **Kluwe J**, Pradere JP, Gwak GY, Mencin A, De Minicis S, Osterreicher CH, Colmenero J, Bataller R, Schwabe RF. Modulation of hepatic fibrosis by c-Jun-N-terminal kinase inhibition. *Gastroenterology* 2010; **138**: 347-359 [PMID: 19782079 DOI: 10.1053/gastro.2009.09.015]
 - 44 **Wang Y**, Gao J, Zhang D, Zhang J, Ma J, Jiang H. New insights into the antifibrotic effects of sorafenib on hepatic stellate cells and liver fibrosis. *J Hepatol* 2010; **53**: 132-144 [PMID: 20447716 DOI: 10.1016/j.jhep.2010.02.027]
 - 45 **Liu Y**, Wang Z, Kwong SQ, Lui EL, Friedman SL, Li FR, Lam RW, Zhang GC, Zhang H, Ye T. Inhibition of PDGF, TGF- β , and Abl signaling and reduction of liver fibrosis by the small molecule Bcr-Abl tyrosine kinase antagonist Nilotinib. *J Hepatol* 2011; **55**: 612-625 [PMID: 21251937 DOI: 10.1016/j.jhep.2010.11.035]
 - 46 **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]
 - 47 **Bellafante E**, Murzilli S, Salvatore L, Latorre D, Villani G, Moschetta A. Hepatic-specific activation of peroxisome proliferator-activated receptor γ coactivator-1 β protects against steatohepatitis. *Hepatology* 2013; **57**: 1343-1356 [PMID: 23299802 DOI: 10.1002/hep.26222]
 - 48 **Robert K**, Nehmé J, Bourdon E, Pivert G, Friguet B, Delcayre C, Delabar JM, Janel N. Cystathionine β synthase deficiency promotes oxidative stress, fibrosis, and steatosis in mice liver. *Gastroenterology* 2005; **128**: 1405-1415 [PMID: 15887121]

DOI: 10.1053/j.gastro.2005.02.034]

- 49 **Suliman HB**, Welty-Wolf KE, Carraway MS, Schwartz DA, Hollingsworth JW, Piantadosi CA. Toll-like receptor 4 mediates mitochondrial DNA damage and biogenic responses after heat-inactivated *E. coli*. *FASEB J* 2005; **19**: 1531-1533

[PMID: 15994412 DOI: 10.1096/fj.04-3500]

- 50 **Gao B**, Seki E, Brenner DA, Friedman S, Cohen JL, Nagy L, Szabo G, Zakhari S. Innate immunity in alcoholic liver disease. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**: G516-G525 [PMID: 21252049 DOI: 10.1152/ajpgi.00537.2010]

P- Reviewers: Apte MV, Abdel-Raheem IT, Trapero-Marugan M
S- Editor: Cui XM **L- Editor:** Wang TQ **E- Editor:** Wu HL



Colonic manifestations of *PTEN* hamartoma tumor syndrome: Case series and systematic review

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Received: July 28, 2013 Revised: September 12, 2013

Accepted: September 29, 2013

Published online: February 21, 2014

Abstract

AIM: To investigate our clinical experience with the colonic manifestations of phosphatase and tensin homolog on chromosome ten (*PTEN*) hamartoma tumor syndrome (PHTS) and to perform a systematic literature review regarding the same.

METHODS: This study was approved by the appropriate institutional review board prior to initiation. A clinical genetics database was searched for patients with PHTS or a component syndrome that received gastrointestinal endoscopy or pathology interpretation at our center. These patient's records were retrospectively reviewed for clinical characteristics (including family history and genetic testing), endoscopy results and pathology findings. We also performed a systematic review of the literature for case series of PHTS or component

syndromes that reported gastrointestinal manifestations and investigations published after consensus diagnostic criteria were established in 1996. These results were compiled and reported.

RESULTS: Eight patients from our institution met initial inclusion criteria. Of these, 5 patients underwent 4.2 colonoscopies at mean age 45.8 ± 10.8 years. All were found to have colon polyps during their clinical course and polyp histology included adenoma, hyperplastic, ganglioneuroma and juvenile. No malignant lesions were identified. Two had multiple histologic types. One patient underwent colectomy due to innumerable polyps and concern for future malignant potential. Systematic literature review of PHTS patients undergoing endoscopy revealed 107 patients receiving colonoscopy at mean age 37.4 years. Colon polyps were noted in 92.5% and multiple colon polyp histologies were reported in 53.6%. Common polyp histologies included hyperplastic (43.6%), adenoma (40.4%), hamartoma (38.3%), ganglioneuroma (33%) and inflammatory (24.5%) polyps. Twelve (11.2%) patients had colorectal cancer at mean age 46.7 years (range 35-62). Clinical outcomes secondary to colon polyposis and malignancy were not commonly reported.

CONCLUSION: PHTS has a high prevalence of colon polyposis with multiple histologic types. It should be considered a mixed polyposis syndrome. Systematic review found an increased prevalence of colorectal cancer and we recommend initiating colonoscopy for colorectal cancer surveillance at age 35 years.

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Key words: Adenoma; Bannayan-Riley-Ruvalcaba syndrome; Colon polyps; Colorectal cancer; Cowden syndrome; Endoscopy; Ganglioneuroma; Hamartoma; Hyperplastic; Phosphatase and tensin homolog on chromosome ten

Core tip: Phosphatase and tensin homolog on chromosome ten (*PTEN*) hamartoma tumor syndrome has a high rate of colonic polyposis. In contrast with prior dogma, the majority of patients will have mixed polyp histologies including adenoma, hamartoma and hyperplastic. Thus, multiple polyp types should spur investigation for this syndrome with a thorough clinical exam and possibly genetic testing. There is likely an increased risk of colorectal cancer at a young age and surveillance colonoscopy is recommended. We recommend starting at age 35 years.

Stanich PP, Pilarski R, Rock J, Frankel WL, El-Dika S, Meyer MM. Colonic manifestations of the *PTEN* hamartoma tumor syndrome: Case series and systematic review. *World J Gastroenterol* 2014; 20(7): 1833-1838 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1833.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1833>

INTRODUCTION

The phosphatase and tensin homolog on chromosome ten (*PTEN*) gene acts as a tumor suppressor through regulation of cell growth^[1]. The *PTEN* hamartoma tumor syndrome (PHTS) incorporates several rare diseases that occur secondary to germline mutations within the *PTEN* gene. The component syndromes include Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome (BRRS), which many now consider a single entity with age-related phenotypic presentations^[2-5]. Lhermitte-Duclos disease (also known as dysplastic gangliocytoma of the cerebellum) and autism spectrum disorders with macrocephaly are variably included^[6,7]. The defining characteristic of PHTS is the development of hamartomas in multiple organ systems and an increased risk of malignancy, including breast, thyroid, endometrial, kidney and possibly melanoma^[8].

Cowden syndrome is thought to be the most common of the component syndromes, with an estimated prevalence of 1 in 200000-250000^[9]. A landmark study by Nelen *et al*^[10] in 1996 both localized the causative gene and described the first iteration of the International Cowden Consortium (ICC) diagnostic criteria, which included hamartomatous intestinal polyps as a minor criterion. The diagnostic criteria has since been revised, but there have been only minor changes and gastrointestinal (GI) hamartomas are still considered a minor criterion^[11].

Although Cowden syndrome and BRRS were known to have intestinal polyps as a manifestation, they were long thought to not infer an increased risk of malignancy due to the presumed hamartomatous nature of the polyps^[12]. However, this was based on case series compiled prior to modern flexible endoscopy and routine polypectomy. Recent work has begun to change this perception, with a higher rate of intestinal polyposis and an increased risk of colorectal cancer reported^[13,14]. This has led to a

change in the clinical guidelines by the National Comprehensive Cancer Network and the recommendation of endoscopic colorectal cancer surveillance^[15]. Unfortunately, beyond the risk of colorectal cancer minimal data are available regarding the clinical outcomes resulting from the intestinal polyposis, although one case series suggests colectomy may be more common than expected^[16].

The aim of the current study was to investigate our institution's experience with the gastrointestinal manifestations of PHTS and perform a systematic review of the modern literature with a similar focus.

MATERIALS AND METHODS

Case series

The appropriate institutional review board approved this study. An existing clinical genetics database was searched for patients that had been diagnosed with PHTS or a component syndrome including Cowden syndrome, BRRS or Lhermitte-Duclos disease and had a medical record number established at our institution. Forty-three patients were identified and their charts were retrospectively reviewed. Patients were evaluated further if they underwent an endoscopic evaluation or had intestinal tissue pathologic interpretation at our institution. Records were initially accessed on November 2, 2012 and any data collected after that point was censored. Exclusion criteria included insufficient documentation to confirm the diagnosis of PHTS or component syndrome. Eight patients met study criteria and were further characterized. Demographic data, including age at the time of initial procedure, manifestations of PHTS or component syndrome, family history, genetic testing results, endoscopic reports, surgical reports and pathology reports were collected. Estimation of colon polyp number and location were obtained directly from reports. Left-sided polyp location was considered when polyps were limited to distal to the splenic flexure. Pancolonic polyp location included polyps from both proximal and distal to the splenic flexure. Age is reported as mean \pm SD (range) in the text and mean in tables, number of procedures is reported as mean (range) in text and mean in tables. Other data are presented as proportions.

Systematic review

A systematic review of the medical literature for relevant case series of PHTS or component syndromes and GI manifestations that were published after the ICC diagnostic criteria were established was also performed^[10]. Inclusion and exclusion criteria were developed a priori. Inclusion criteria included the presentation of 3 or more patients with PHTS or a component syndrome published in a peer-reviewed journal with detailed information available regarding the endoscopic and GI pathologic investigations performed. Exclusion criteria included publication prior to 1996 and the presentation of 2 or fewer patients to limit ascertainment and publication bias. The literature search was independently performed by two authors (Stan-

Table 1 Clinical characteristics of the *PTEN* hamartoma tumor syndrome cohort receiving colonoscopy

	<i>n</i> (%)
Patients with colonoscopy	5
Female sex	5 (100)
Phenotype	
Cowden syndrome	5 (100)
BRRS	0 (0)
<i>PTEN</i> mutation	4 (80)
Family history of PHTS	2 (40)
ICC diagnostic criteria ^[10]	
Pathognomonic	
Acral keratoses	1 (20)
Papillomatous lesions	0 (0)
Trichilemmomas	0 (0)
Major criteria	
Breast cancer	4 (80)
Dysplastic gangliocytoma of the cerebellum	0 (0)
Endometrial cancer	0 (0)
Macrocephaly	4 (80)
Thyroid cancer	1 (20)
Minor criteria	
Benign thyroid lesions	4 (80)
Fibroma	0 (0)
Fibrocystic breast disease	2 (40)
Genitourinary tumors	1 (20)
Hamartomatous intestinal polyps	1 (20)
Lipoma	3 (60)
Mental retardation	0 (0)

BRRS: Bannayan-riley-ruvalcaba syndrome; ICC: International cowden consortium; PHTS: *PTEN* hamartoma tumor syndrome.

ich PP and Pilarski R). Medline (<http://www.ncbi.nlm.nih.gov/pubmed/>), Scopus and the Cochrane library were searched using the strategy of: (*PTEN* OR Cowden* OR Bannayan-Riley-Ruvalcaba syndrome) AND (gastrointestinal OR polyp) with a limit of publication in 1996 or later. The reference sections of identified manuscripts were also searched for relevant reports. This was initially performed on January 22, 2013 and publications after this were censored. Five manuscripts were identified that met criteria^[14,16-19]. Data abstraction from the selected manuscripts was independently performed by two authors (Stanich PP and Meyer MM). Age and number of procedures were reported as means only given limitations of source material. In the tables with data acquired from the systematic literature review, information that was unavailable was demarcated “not reported” and excluded from the reported proportions.

RESULTS

Case series

Eight patients from our institution met inclusion criteria for the study and all were women. Six patients (75%) were consistent with a Cowden syndrome phenotype and 2 patients (25%) had a BRRS phenotype. All 8 patients underwent *PTEN* mutation testing and 7 (87.5%) were found to have deleterious mutations (3 deleterious nonsense mutations, 2 deleterious missense mutations and 2 with deleterious splice-site mutations). The patient without a

detected mutation met ICC diagnostic criteria for Cowden syndrome with macrocephaly, breast cancer, thyroid cancer, fibrocystic breast disease and lipomas. Three patients were from a single family and the others were unrelated.

Five patients (4 with *PTEN* mutation) from this cohort underwent colonoscopy at our institution. All were female and unrelated, with 4 (80%) Caucasian and 1 (20%) African-American. Further details regarding their PHTS clinical manifestations are included in Table 1. They underwent a mean 4.2 (range 1-10) colonoscopies, with the age at first procedure 45.8 ± 10.8 (range 33.8-63.7). Indications for initial colonoscopy were GI bleeding in 3 and to follow or confirm outside findings in 2.

All patients were noted to have polyps during their clinical course. Four patients had polyps on initial colonoscopy and 1 patient did not develop polyps until the third procedure at age 49.5 years (12 years after first colonoscopy). Among the patients with colon polyps on initial exam, 3 patients were reported to have “multiple” polyps without a numerical estimate and 1 patient had a single polyp identified.

Polypectomy was performed in 4 patients (1 patient with multiple polyps had no specimens removed due to anticoagulation and was then lost to follow-up). Histology included tubular and tubulovillous adenomas, hyperplastic, ganglioneuroma and a juvenile polyp (the ganglioneuroma and juvenile polyp occurred in the same patient). No malignant lesions were identified. Further details are reported in Table 2. One patient received a colectomy due to innumerable ganglioneuromatous colon polyps (estimated to be 200 on gross pathology examination) and concern for future malignant potential by providers, 3 patients underwent polypectomies during serial colonoscopies with complete clearance of polyps from colon and 1 patient was lost to follow-up.

Systematic review

Colon findings from the systematic literature review are reported in Table 2. Colonoscopy was performed in 107 patients at mean age 37.4. Ninety-nine (92.5%) had polyps and 64% of patients were estimated to have 50 or more polyps when the number of polyps were reported. They were most often pancolonial (71.4%) when location was described. There was a wide array of histologies, with PHTS patients having hyperplastic (43.6%), adenoma (40.4%), hamartoma (38.3%), ganglioneuroma (33%) and inflammatory (24.5%) polyps commonly reported. Many patients had more than a single histology, with 31% having 2 types and 22.6% having 3 or more types. Colorectal cancer was found in 12 patients (11.2% of total cohort, 12.8% of patients with pathology reviewed) with mean age 46.7 (range 35-62) years. Findings were similar when limited to patients with confirmed *PTEN* mutations.

DISCUSSION

Intestinal hamartomatous polyposis has been described as a feature of Cowden syndrome, Bannayan-Riley-Ruvalca-

Table 2 Colon findings from the *PTEN* hamartoma tumor syndrome cohort and systematic review

Author	OSU cohort	Levi <i>et al.</i> ^[19]	Coriat <i>et al.</i> ^[18]	Stanich <i>et al.</i> ^[16]	Heald <i>et al.</i> ^[14]	Kim <i>et al.</i> ^[17]	Total, all patients	Percentage	Total, + <i>PTEN</i> mutation	Percentage
<i>n</i> , with colonoscopy	5	10	10	10	67	5	107		88	
<i>PTEN</i> mutation	4	10	NR	5	67	2	88	82%	88	100%
Age (yr), mean	45.8	31.7	37	48	36.4	34	37.4		36.9	
# of colonoscopies, mean	4.2	2.4	3.1	2	NR	NR	2.7		2.9	
Patients with colon polyps	5	8	10	9	62	5	99	92.5%	81	92%
> 50 polyps	1	NR	8	7	NR	NR	16	64%	5	55.6%
Pancolonic location	3	7	7	8	NR	NR	25	71.4%	14	73.7%
Left-sided location	2	1	NR	1	NR	NR	4	16%	3	15.8%
<i>n</i> , with colonic pathology	4	8	10	11	56	5	94		75	
Adenocarcinoma	0	1	0	2	9	0	12	12.8%	12	16%
Adenoma	2	3	10	6	16	1	38	40.4%	24	32%
Ganglioneuroma	1	3	5	6	16	0	31	33%	23	30.7%
Hamartoma	0	5	6	7	18	0	36	38.3%	27	36%
Hyperplastic	2	8	0	4	27	0	41	43.6%	39	52%
Inflammatory	0	0	0	7	11	5	23	24.5%	18	24%
Juvenile	1	2	0	2	0	0	5	5.3%	5	6.7%
Lymphoid hyperplasia/polyp	0	0	0	0	4	1	5	5.3%	5	6.7%
Sessile serrated polyp	0	0	0	0	2	0	2	2.1%	2	2.7%
1 polyp type	2	1	NR	2	29	3	37	44%	32	42.7%
2 polyp types	2	4	NR	2	16	2	26	31%	25	33.3%
≥ 3 polyp types	0	3	NR	7	9	0	19	22.6%	16	21.3%

NR: Not reported.

ba syndrome and the composite PHTS since the earliest reports of the disease, but the prevalence of findings and the pathologic diversity was underestimated due to lack of routine endoscopy and polypectomy. Current research is changing the perception of the GI polyposis prevalence, pathology and most importantly the risk of malignancy.

Our cohort included 5 patients with PHTS that underwent colonoscopy. We noted a 100% prevalence of colonic polyps, which corresponds with the high prevalence of colonic polyposis that has been reported recently. Although polypectomy was not performed on all patients, we found a higher incidence of adenomatous and hyperplastic polyps and relatively few of the classically associated hamartomatous polyps, which were only reported in a single patient. This patient was noted to have diffuse colonic ganglioneuromatous polyps with an isolated juvenile polyp found on gross pathology specimen after colectomy.

Our systematic review of case series published after the establishment of Cowden syndrome diagnostic criteria and the widespread use of endoscopy also supports the high rate of endoscopic findings and polyposis in PHTS. Colonic polyps were found in 92.5% of patients, with similar rates when only *PTEN* mutation positive patients were considered. In addition, a variety of polyp histologies were reported, with hyperplastic, adenomatous, hamartomatous, ganglioneuromatous and inflammatory polyps all being common in the colon. Importantly, the majority of patients with colon polyp pathology interpretation were found to have 2 or more histologic types.

Given the prevalence of multiple polyp histologies—including hyperplastic polyps, adenomas and hamartomas—we suggest that PHTS be reclassified as a mixed pol-

yposis syndrome rather than a hamartomatous polyposis syndrome. If hamartomatous polyps were relied upon to suggest PHTS as a possible diagnosis, many patients in our series would have gone undetected. In further support of this nomenclature shift, Sweet *et al.*^[20] have previously investigated patients with unexplained polyposis syndromes consisting of hyperplastic and adenomatous polyps without hamartomas and detected *PTEN* mutations in 2 (9%) patients from a cohort of 23^[20]. Thus, we believe the current classification is misleading and investigation for PHTS should not depend on the presence of hamartomatous GI polyps.

There has been a paradigm shift regarding the risk of colorectal cancer in PHTS. Recent work has shown an increased risk of colorectal cancer in this population, with a 9%-18% prevalence of colorectal cancer and standardized incidence rate of 10.3 (95%CI: 5.6-17.4) reported^[8,13,14,21]. There are also reports of young onset colorectal cancer in this population, with Kersseboom *et al.*^[22] reporting diagnoses at 28 and 39 years of age. This has resulted in the National Comprehensive Cancer Network changing their recommendations to include considering colonoscopy starting at age 35 years, then every 5-10 years or more frequently if patient is symptomatic or polyps found^[15].

Based on our systematic review, we found a 11.2% prevalence of colorectal cancer in PHTS patients undergoing GI work-up and 13.6% in *PTEN* mutation positive patients. The mean age of colorectal cancer diagnosis was 46.7, with the earliest found at age 35. Based on this, it is reasonable to start colonic surveillance at either age 35 or 10 years younger than the earliest colorectal cancer diagnosis in a first-degree relative, whichever is sooner.

We then recommend proceeding with further colonic surveillance based on the findings, with colonoscopy every 1-2 years if multiple polyps and/or adenomatous polyps are present or every 3-5 years if either sparse, non-adenomatous polyps or no polyps are present. The use of additional techniques such as narrow-band imaging^[23], probe-based confocal laser endomicroscopy^[24] or other emerging technologies to help discern polyp histology in real time and direct polypectomy efforts towards higher risk lesions should be considered if the technology and expertise is available.

Unfortunately, minimal data on the clinical outcomes resulting from the GI polyposis in this population are available. The mean number of colonoscopies per patient based on the systematic review was 2.7, although the time course these took place over is unknown. In our cohort, 1 (20%) that received a colonoscopy underwent colectomy due to concern for future malignant potential. In the Mayo Clinic cohort, 5 (38%) patients underwent colectomy due to dysplastic colon lesions^[16]. The other case series do not report clinical outcomes. When initiating colonic surveillance, it is important to discuss possible outcomes with patients including the need for repeated procedures and the possibility of colectomy if colorectal cancer, high risk lesions or multiple polyps are present. Further investigation is needed in this area to clarify the optimal surveillance protocol and to allow for adequate counseling and treatment of PHTS patients with GI polyposis.

Several areas of caution should be noted when interpreting this data. Due to the method of subject accrual being based on review of outside records in the Heald *et al.*^[14] study, we cannot definitively rule out the possibility that some patients from other cohorts may be included in their cohort. Ascertainment bias should also be taken into account, as PHTS patients without GI manifestations may be less likely to undergo endoscopic evaluation and may be under-represented. Thus, neither our data nor that in the published literature can be used to determine the true prevalence of GI abnormalities in PHTS. Future work in this area, aided by the new recommendations favoring colorectal cancer surveillance, should help clarify this and confirm the current data.

In summary, PHTS has a high prevalence of colonic polyposis. The classically associated hamartomatous polyps cannot be relied on to suggest the diagnosis, and patients with multiple polyp histologies need to be examined further for PHTS. An increased risk of colon cancer is now reported and surveillance with colonoscopy is indicated. We recommend starting at age 35 or 10 years younger than the earliest colorectal cancer diagnosis in a first-degree relative with future surveillance intervals based on the results.

COMMENTS

Background

The phosphatase and tensin homolog on chromosome ten (*PTEN*) gene is a tumor suppressor that regulates cell growth. Germline mutations of this gene leads to Cowden syndrome and other disorders that are now collectively known

as the *PTEN* hamartoma tumor syndrome. Gastrointestinal polyposis is a known manifestation of the disorder since the initial reports, but were thought to be benign hamartomas without malignant potential. Recent case series have indicated mixed polyp histology and an increased colon cancer risk.

Research frontiers

Continued investigation into the malignant potential and clinical outcomes of colonic polyposis in the *PTEN* hamartoma tumor syndrome, including cost effectiveness of colon cancer screening and surveillance.

Innovations and breakthroughs

The authors report findings from our *PTEN* hamartoma tumor syndrome patients undergoing colonoscopy which show all with colon polyps and 40% with multiple polyp histologies. They detail the clinical outcomes of these polyps, including a patient that received colectomy. They also performed a systematic review of the modern literature focusing on gastrointestinal manifestations; this revealed 92.5% with colon polyps and a majority of patients with multiple polyp histologies. The systematic review also supports an increased risk of colon cancer.

Applications

PTEN hamartoma tumor syndrome should be considered a mixed polyposis syndrome and this diagnosis should be considered in patients with multiple polyp histologies. The authors' findings also support an increased risk of colon cancer and we recommend colonoscopy for colorectal cancer surveillance beginning at either age 35 or 10 years younger than the earliest colorectal cancer diagnosis in a first-degree relative, whichever is sooner. Future research should include clinical outcomes.

Peer review

In this paper, the authors analysed and reviewed patient data from their institute and literature to identify the connection between gastrointestinal abnormalities (in particular colon lesions/cancer) and *PTEN* hamartoma tumor syndrome (PHTS) to make recommendation for future clinical examination of patients. The study was well organized and clearly set up. The authors reported that PHTS had a high prevalence of colonic polyposis. It will be interesting to know the connection of the high prevalence of colonic polyposis in PHTS patients to the risk of gastrointestinal (colon in particular) cancers.

REFERENCES

- 1 **Stambolic V**, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 1998; **95**: 29-39 [PMID: 9778245]
- 2 **Nelen MR**, van Staveren WC, Peeters EA, Hassel MB, Gorlin RJ, Hamm H, Lindboe CF, Fryns JP, Sijmons RH, Woods DG, Mariman EC, Padberg GW, Kremer H. Germline mutations in the PTEN/MMAC1 gene in patients with Cowden disease. *Hum Mol Genet* 1997; **6**: 1383-1387 [PMID: 9259288]
- 3 **Liaw D**, Marsh DJ, Li J, Dahia PL, Wang SI, Zheng Z, Bose S, Call KM, Tsou HC, Peacocke M, Eng C, Parsons R. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat Genet* 1997; **16**: 64-67 [PMID: 9140396 DOI: 10.1038/ng0597-64]
- 4 **Marsh DJ**, Dahia PL, Zheng Z, Liaw D, Parsons R, Gorlin RJ, Eng C. Germline mutations in PTEN are present in Bannayan-Zonana syndrome. *Nat Genet* 1997; **16**: 333-334 [PMID: 9241266 DOI: 10.1038/ng0897-333]
- 5 **Marsh DJ**, Kum JB, Lunetta KL, Bennett MJ, Gorlin RJ, Ahmed SF, Bodurtha J, Crowe C, Curtis MA, Dasouki M, Dunn T, Feit H, Geraghty MT, Graham JM, Hodgson SV, Hunter A, Korf BR, Manchester D, Miesfeldt S, Murday VA, Nathanson KL, Parisi M, Pober B, Romano C, Eng C. PTEN mutation spectrum and genotype-phenotype correlations in Bannayan-Riley-Ruvalcaba syndrome suggest a single entity with Cowden syndrome. *Hum Mol Genet* 1999; **8**: 1461-1472 [PMID: 10400993]
- 6 **Zhou XP**, Marsh DJ, Morrison CD, Chaudhury AR, Maxwell M, Reifemberger G, Eng C. Germline inactivation of PTEN and dysregulation of the phosphoinositide-3-kinase/Akt pathway cause human Lhermitte-Duclos disease in adults. *Am J Hum Genet* 2003; **73**: 1191-1198 [PMID: 14566704 DOI: 10.1086/37500]

- 10.1086/379382]
- 7 **Butler MG**, Dasouki MJ, Zhou XP, Talebizadeh Z, Brown M, Takahashi TN, Miles JH, Wang CH, Stratton R, Pilarski R, Eng C. Subset of individuals with autism spectrum disorders and extreme macrocephaly associated with germline PTEN tumour suppressor gene mutations. *J Med Genet* 2005; **42**: 318-321 [PMID: 15805158 DOI: 10.1136/jmg.2004.024646]
- 8 **Tan MH**, Mester JL, Ngeow J, Rybicki LA, Orloff MS, Eng C. Lifetime cancer risks in individuals with germline PTEN mutations. *Clin Cancer Res* 2012; **18**: 400-407 [PMID: 22252256 DOI: 10.1158/1078-0432.CCR-11-2283]
- 9 **Nelen MR**, Kremer H, Konings IB, Schoute F, van Essen AJ, Koch R, Woods CG, Fryns JP, Hamel B, Hoefsloot LH, Peeters EA, Padberg GW. Novel PTEN mutations in patients with Cowden disease: absence of clear genotype-phenotype correlations. *Eur J Hum Genet* 1999; **7**: 267-273 [PMID: 10234502 DOI: 10.1038/sj.ejhg.5200289]
- 10 **Nelen MR**, Padberg GW, Peeters EA, Lin AY, van den Helm B, Frants RR, Coulon V, Goldstein AM, van Reen MM, Easton DF, Eeles RA, Hodgson S, Mulvihill JJ, Murday VA, Tucker MA, Mariman EC, Starink TM, Ponder BA, Ropers HH, Kremer H, Longy M, Eng C. Localization of the gene for Cowden disease to chromosome 10q22-23. *Nat Genet* 1996; **13**: 114-116 [PMID: 8673088 DOI: 10.1038/ng0596-114]
- 11 **Pilarski R**, Eng C. Will the real Cowden syndrome please stand up (again)? Expanding mutational and clinical spectra of the PTEN hamartoma tumour syndrome. *J Med Genet* 2004; **41**: 323-326 [PMID: 15121767]
- 12 **Starink TM**, van der Veen JP, Arwert F, de Waal LP, de Lange GG, Gille JJ, Eriksson AW. The Cowden syndrome: a clinical and genetic study in 21 patients. *Clin Genet* 1986; **29**: 222-233 [PMID: 3698331]
- 13 **Riegert-Johnson DL**, Gleeson FC, Roberts M, Tholen K, Youngborg L, Bullock M, Boardman LA. Cancer and Lhermitte-Duclos disease are common in Cowden syndrome patients. *Hered Cancer Clin Pract* 2010; **8**: 6 [PMID: 20565722 DOI: 10.1186/1897-4287-8-6]
- 14 **Heald B**, Mester J, Rybicki L, Orloff MS, Burke CA, Eng C. Frequent gastrointestinal polyps and colorectal adenocarcinomas in a prospective series of PTEN mutation carriers. *Gastroenterology* 2010; **139**: 1927-1933 [PMID: 20600018 DOI: 10.1053/j.gastro.2010.06.061]
- 15 **National Comprehensive Cancer Network**. Genetic/Familial High Risk Assessment: Breast and Ovarian. NCCN Clinical Practice Guidelines in Oncology. Version 1.2013 ed. United States: National Comprehensive Cancer Network, 2013
- 16 **Stanich PP**, Owens VL, Sweetser S, Khambatta S, Smyrk TC, Richardson RL, Goetz MP, Patnaik MM. Colonic polyposis and neoplasia in Cowden syndrome. *Mayo Clin Proc* 2011; **86**: 489-492 [PMID: 21628613 DOI: 10.4065/mcp.2010.0816]
- 17 **Kim DK**, Myung SJ, Yang SK, Hong SS, Kim KJ, Byeon JS, Lee GH, Kim JH, Min YI, Lee SM, Jeong JY, Song K, Jung SA. Analysis of PTEN gene mutations in Korean patients with Cowden syndrome and polyposis syndrome. *Dis Colon Rectum* 2005; **48**: 1714-1722 [PMID: 16007494 DOI: 10.1007/s10350-005-0130-9]
- 18 **Coriat R**, Mozer M, Caux F, Chryssostalis A, Terris B, Grandjouan S, Benamouzig R, Martin A, Chaussade S. Endoscopic findings in Cowden syndrome. *Endoscopy* 2011; **43**: 723-726 [PMID: 21437855 DOI: 10.1055/s-0030-1256342]
- 19 **Levi Z**, Baris HN, Kedar I, Niv Y, Geller A, Gal E, Gingold R, Morgenstern S, Baruch Y, Leach BH, Bronner MP, Eng C. Upper and Lower Gastrointestinal Findings in PTEN Mutation-Positive Cowden Syndrome Patients Participating in an Active Surveillance Program. *Clin Transl Gastroenterol* 2011; **2**: e5 [PMID: 23238744 DOI: 10.1038/ctg.2011.4]
- 20 **Sweet K**, Willis J, Zhou XP, Gallione C, Sawada T, Alhopuro P, Khoo SK, Patocs A, Martin C, Bridgeman S, Heinz J, Pilarski R, Lehtonen R, Prior TW, Frebourg T, Teh BT, Marchuk DA, Aaltonen LA, Eng C. Molecular classification of patients with unexplained hamartomatous and hyperplastic polyposis. *JAMA* 2005; **294**: 2465-2473 [PMID: 16287957 DOI: 10.1001/jama.294.19.2465]
- 21 **Nieuwenhuis MH**, Kets CM, Murphy-Ryan M, Colas C, Möller P, Hes FJ, Hodgson SV, Olderoode-Berends MJ, Aretz S, Heinimann K, Gomez Garcia EB, Douglas F, Spigelman A, Timshel S, Lindor NM, Vasen HF. Is colorectal surveillance indicated in patients with PTEN mutations? *Colorectal Dis* 2012; **14**: e562-e566 [PMID: 22672595 DOI: 10.1111/j.1463-1318.2012.03121.x]
- 22 **Kerseboom R**, Dubbink HJ, Corver WE, van Tilburg AJ, Poley JW, van Leerdam ME, Atmodimedjo PN, van de Laar IM, Collée JM, Dinjens WN, Morreau H, Wagner A. PTEN in colorectal cancer: a report on two Cowden syndrome patients. *Clin Genet* 2012; **81**: 555-562 [PMID: 21291452 DOI: 10.1111/j.1399-0004.2011.01639.x]
- 23 **Hewett DG**, Huffman ME, Rex DK. Leaving distal colorectal hyperplastic polyps in place can be achieved with high accuracy by using narrow-band imaging: an observational study. *Gastrointest Endosc* 2012; **76**: 374-380 [PMID: 22695207 DOI: 10.1016/j.gie.2012.04.446]
- 24 **Buchner AM**, Shahid MW, Heckman MG, Krishna M, Ghabril M, Hasan M, Crook JE, Gomez V, Raimondo M, Woodward T, Wolfsen HC, Wallace MB. Comparison of probe-based confocal laser endomicroscopy with virtual chromoendoscopy for classification of colon polyps. *Gastroenterology* 2010; **138**: 834-842 [PMID: 19909747 DOI: 10.1053/j.gastro.2009.10.053]

P- Reviewers: He H, Pichler M **S- Editor:** Qi Y
L- Editor: A **E- Editor:** Wang CH



Risk factors for bleeding after endoscopic submucosal dissection of colorectal neoplasms

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Received: August 21, 2013 Revised: November 14, 2013

Accepted: January 2, 2014

Published online: February 21, 2014

Abstract

AIM: To investigate the risk factors for delayed bleeding following endoscopic submucosal dissection (ESD) treatment for colorectal neoplasms.

METHODS: We retrospectively reviewed the medical records of 317 consecutive patients with 325 lesions who underwent ESD for superficial colorectal neoplasms at our hospital from January 2009 to June 2013. Delayed post-ESD bleeding was defined as bleeding that resulted in overt hematochezia 6 h to 30 d after ESD and the observation of bleeding spots as

confirmed by repeat colonoscopy or a required blood transfusion. We analyzed the relationship between risk factors for delayed bleeding following ESD and the following factors using univariate and multivariate analyses: age, gender, presence of comorbidities, use of antithrombotic drugs, use of intravenous heparin, resected specimen size, lesion size, lesion location, lesion morphology, lesion histology, the device used, procedure time, and the presence of significant bleeding during ESD.

RESULTS: Delayed post-ESD bleeding was found in 14 lesions from 14 patients (4.3% of all specimens, 4.4% patients). Patients with episodes of delayed post-ESD bleeding had a mean hemoglobin decrease of 2.35 g/dL. All episodes were treated successfully using endoscopic hemostatic clips. Emergency surgery was not required in any of the cases. Blood transfusion was needed in 1 patient (0.3%). Univariate analysis revealed that lesions located in the cecum ($P = 0.012$) and the presence of significant bleeding during ESD ($P = 0.024$) were significantly associated with delayed post-ESD bleeding. The risk of delayed bleeding was higher for larger lesion sizes, but this trend was not statistically significant. Multivariate analysis revealed that lesions located in the cecum (OR = 7.26, 95%CI: 1.99-26.55, $P = 0.003$) and the presence of significant bleeding during ESD (OR = 16.41, 95%CI: 2.60-103.68, $P = 0.003$) were independent risk factors for delayed post-ESD bleeding.

CONCLUSION: Location in the cecum and significant bleeding during ESD predispose patients to delayed post-procedural bleeding. Therefore, careful and additional management is recommended for these patients.

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Key words: Adverse event; Bleeding; Colorectal neo-

plasms; Endoscopic submucosal dissection; Hemorrhage

Core tip: Endoscopic submucosal dissection (ESD) has recently been accepted as an effective treatment for colorectal neoplasms, but the risk factors for bleeding following ESD have not been elucidated. We analyzed the relationship between delayed post-ESD bleeding and various factors related to ESD for colorectal neoplasms. The rate of delayed post-ESD bleeding was 4.3%, and univariate and multivariate analyses showed that the location of lesions in the cecum and the presence of significant bleeding during ESD were significantly associated with delayed post-ESD bleeding. Therefore, patients with these risk factors should be carefully managed with additional interventions if necessary.

Suzuki S, Chino A, Kishihara T, Uragami N, Tamegai Y, Suganuma T, Fujisaki J, Matsuura M, Itoi T, Gotoda T, Igarashi M, Moriyasu F. Risk factors for bleeding after endoscopic submucosal dissection of colorectal neoplasms. *World J Gastroenterol* 2014; 20(7): 1839-1845 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1839.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1839>

INTRODUCTION

Endoscopic resection (ER) is an effective and safe procedure for the treatment of superficial colorectal neoplasms because this procedure is minimally invasive and provides good clinical outcomes^[1-3]. Conventional endoscopic mucosal resection (EMR) is widely performed, but this procedure is often inadequate for *en bloc* resection in cases involving large tumors, tumors in difficult locations, or poor tumor elevation after submucosal injection, which results in a high rate of local recurrence^[4-6]. Endoscopic submucosal dissection (ESD) was initially developed for early gastric cancers, and it is a highly effective and safe treatment. Furthermore, ESD has a high *en bloc* resection rate, regardless of tumor size or location^[7,8]. ESD has recently been accepted as an effective endoscopic treatment for superficial colorectal neoplasms^[9,10].

ER is associated with a low but significant rate of serious adverse events, including delayed post-procedural bleeding. The rate and risk factors of post-procedural bleeding after conventional polypectomy and EMR have been examined previously^[11,12], but investigations into the risk factors for delayed bleeding following ESD treatment for superficial colorectal neoplasms are lacking.

Delayed post-ESD bleeding can result in serious adverse effects that can increase morbidity and hospital admissions and require additional expenditures for medical resources and procedures, such as endoscopy, rarely for angiography, or surgical interventions and/or blood transfusions. Therefore, an understanding of the risk factors for delayed bleeding following ESD for colorectal neoplasms is important to avoid this complication. The

present study determined the incidence of delayed bleeding following ESD and identified the clinical factors that are associated with this adverse event.

MATERIALS AND METHODS

Patients

A total of 319 patients (who had 327 colorectal neoplasms) underwent ESD at the Cancer Institute Hospital (Tokyo, Japan) between January 2009 and June 2013. The indications for ESD were defined according to the guidelines proposed by the Japanese Colorectal ESD Standardization Implementation Working Group (Table 1). Two patients were excluded because they had undergone emergency surgical repair for bowel perforation. Therefore, the medical records of 325 colorectal neoplasms in 317 patients were reviewed retrospectively.

ESD procedure and post-procedural treatment

Patients with multiple lesions received multiple ESD procedures on different days to treat each lesion separately. Antiplatelet and/or anticoagulant drugs were discontinued for 7 d before and after treatment if patients were considered low risk for thromboembolism. Intravenous heparin was administered to patients considered high risk for thromboembolism until 6 h before ESD, and then restarted on first post-procedural day if hemostasis was confirmed by stable vital signs and laboratory data.

Expert endoscopists, who were certified by the Japanese Society of Gastrointestinal Endoscopy, performed ESD. All patients were treated under sedation with midazolam and pethidine hydrochloride. Submucosal injections of 10% glycerol and 5% fructose in normal saline solution (Glycerol®; Chugai Pharmaceutical, Tokyo, Japan) and undiluted 0.4% sodium hyaluronate (MucoUp®; Johnson and Johnson, New Brunswick, NJ, United States) were administered to lift the mucosa. Circumferential incisions and all lesion dissections were performed using endoscopic knives. Carbon dioxide insufflation was used. Endoscopic hemostasis during ESD was achieved whenever active bleeding occurred using hemostatic forceps (FD-411QR; Olympus, Tokyo, Japan) and an electrosurgical unit (ERBE-ICC200 or ERBE-VIO300D; Erbe Elektromedizin, Tübingen, Germany) in the 80-W, soft coagulation mode. Hemostatic clips (EZ Clip; Olympus) were used when hemostasis could not be achieved using hemostatic forceps alone. Endoscopic hemostasis using hemostatic forceps was performed if bleeding occurred on the artificial ulcer after resection of the lesion.

Patients without adverse events began drinking water on the first postoperative day and eating soft food on the second postoperative day. All patients were discharged on postoperative days 4-5. Patients were informed to contact our hospital and visit an emergency department immediately if they experienced hematochezia. All patients had visited the outpatient department to confirm their final pathological results within 3 wk after discharge.

Table 1 Indications for colorectal endoscopic submucosal dissection

Large lesions (diameter > 20 mm) for which endoscopic treatment is indicated but <i>en bloc</i> resection by snare endoscopic mucosal dissection would be difficult
Laterally spreading tumor of the non-granular type, particularly the pseudo-depressed type
Lesions showing a type V1 pit pattern
Cancer with submucosal infiltration
Large depressed type tumor
Large lesions of the protruded type suspected to be carcinoma ¹
Mucosal lesions with fibrosis caused by prolapse due to biopsy or peristalsis of the lesions ²
Local residual early cancer after endoscopic resection
Sporadic localized tumors with chronic inflammation, such as ulcerative colitis

¹Including laterally spreading tumors of the granular type consisting of large nodules; ²Caused by biopsy or peristalsis of the lesion (prolapse).

Data analysis

Delayed post-ESD bleeding was defined as bleeding that resulted in overt hematochezia 6 h to 30 d after ESD and the observation of bleeding spots as confirmed by repeat colonoscopy or the requirement of a blood transfusion.

The risk factors for delayed post-ESD bleeding were assessed from data based on patient-, treatment-, and lesion-related variables. The patient-related factors included age, gender, presence of comorbidities (*e.g.*, hypertension, diabetes mellitus, hyperlipidemia, cardiovascular disease, liver cirrhosis, and chronic renal failure), use of antithrombotic drugs (including anticoagulants and/or antiplatelet drugs), and the use of intravenous heparin. The lesion-related factors were the size of the resected specimen and the size, location, and morphology of the lesions [protruded type (I s, I sp, I p), depressed type (II c), laterally spreading tumor granular type (LST-G), or laterally spreading tumor nongranular type (LST-NG)]. Histology was sub-classified as serrated lesion, adenoma, intra-mucosal adenocarcinoma, submucosal minutely invasive adenocarcinoma (SM invasion < 1000 μ m), or submucosal deeply invasive adenocarcinoma (SM invasion \geq 1000 μ m). The treatment-related factors were the device used [needle type (Hook/Flush/Dual/B knife), scissor type (SB knife Jr)], procedure time, and the presence of significant bleeding during ESD.

The procedure time was defined as the interval between submucosal injection and the completion of specimen resection. Significant bleeding during ESD was defined as active bleeding during the procedure that resulted in the use of endoscopic hemostatic clips because hemostasis could not be achieved using hemostatic forceps alone.

Ethics

This study was conducted in accordance with the Declaration of Helsinki, and the Institutional Review Board of our hospital approved the study protocol.

Statistical analysis

Univariate analysis was performed using Fisher's exact test or the χ^2 test for associations between the categorical variables and delayed post-ESD bleeding. The Mann-Whitney *U*-test was used for associations between the continuous variables and delayed post-ESD bleeding. Variables with a *P* value < 0.1 in univariate analysis were considered potential risk factors, and these variables were entered into multivariate logistic regression analysis. OR with 95% CIs quantified the extent of the association. A *P* < 0.05 was considered significant for all tests. All analyses were performed using the SPSS statistical software package (IBM SPSS statistic version 19.0; IBM, New York, United States).

RESULTS

Patient baseline and outcomes

The baseline characteristics of the patients are shown in Table 2. A total of 325 lesions from 317 patients were treated with ESD. Post-ESD bleeding was observed in 14 patients (4.3% of all specimens, 4.4% patients). The onset of bleeding occurred between postoperative days 1 and 7 (mean postoperative day, 2.5). Bleeding onset occurred by postoperative day 4 in all cases except for 1. Only 1 patient had more than one bleeding episode (on postoperative days 4 and 7), which required a repeat colonoscopy with endoscopic hemostasis. Patients with episodes of delayed post-ESD bleeding had a mean hemoglobin decrease of 2.35 g/dL (range, 0.8–5.9 g/dL). All patients with hematochezia received a repeat colonoscopy without bowel preparation. All hemorrhagic episodes were successfully treated using endoscopic hemostatic clips, and no cases required surgical intervention. Only 1 patient needed a blood transfusion (0.3% of all specimens, 0.3% of patients); this patient had overt hematochezia and a hemoglobin decrease of 5.9 g/dL. There were no treatment-related deaths.

Risk factor assessment

Univariate analysis for associations between the various clinical factors and the risk of delayed post-ESD bleeding are shown in Tables 3 and 4. Significant bleeding during the ESD procedure was associated with an increased risk of delayed post-ESD bleeding (*P* = 0.024). Lesions that showed delayed post-ESD bleeding significantly differed from lesions without bleeding in terms of location (*P* = 0.042). The presence of lesions in the cecum was significantly associated with an increased risk of delayed post-ESD bleeding (Table 4; *P* = 0.012). A larger lesion size tended to be associated with bleeding, but this association failed to reach statistical significance (*P* = 0.070).

The results of the multivariate logistic regression analysis are shown in Table 5. This analysis examined lesion size (mm), location in the cecum (yes *vs* no), and significant bleeding during ESD (yes *vs* no). Lesions located in the cecum (OR = 7.26, 95%CI: 1.99–26.55, *P* =

Table 2 Baseline characteristics of the 325 superficial colorectal neoplasms in 317 patients *n* (%)

Characteristic	<i>n</i>
Patient characteristics	
Number	317
Age (yr)	
Mean \pm SD	65.5 \pm 10.9
Median (range)	67 (29-86)
Gender	
Male	183 (57.7)
Female	134 (42.3)
Comorbidities	
Hypertension	103 (32.5)
Diabetes mellitus	31 (9.8)
Hyperlipidemia	54 (17.0)
Cardiovascular disease	16 (5.0)
Liver cirrhosis	0 (0)
Chronic renal failure	0 (0)
Use of antithrombotic drugs	26 (8.2)
Use of intravenous heparin	5 (1.6)
Lesion characteristics	
Number	325
Lesion size, mm	
Mean \pm SD	34.1 \pm 16.6
Median (range)	30 (7-115)
Location	
Cecum	23 (7.1)
Ascending colon	58 (17.8)
Transverse colon	51 (15.7)
Descending colon	12 (3.7)
Sigmoid colon	59 (18.2)
Rectum	122 (37.5)
Morphology	
Protruded	31 (9.5)
Depressed	4 (1.2)
LST-G	180 (55.4)
LST-NG	110 (33.8)
Histology and depth	
Serrated lesion	5 (1.5)
Adenoma	26 (8.0)
M	249 (76.6)
SM < 1000 μ m	27 (8.3)
SM \geq 1000 μ m	18 (5.5)
Resectability	
En bloc resection	284 (87.4)
Complete resection	282 (86.8)
Procedure time, min	
Mean \pm SD	101.0 \pm 80.2
Median (range)	80 (10-630)
Procedure-related adverse events	
Delayed bleeding	14 (4.3)
Patients needed transfusion	1 (0.3)
Death related to the procedure	0 (0)

M: Intra-mucosal adenocarcinoma; SM < 1000 μ m: Submucosal, minutely invasive adenocarcinoma; SM \geq 1000 μ m: Submucosal, deeply invasive adenocarcinoma.

0.003) and significant bleeding during ESD (OR = 16.41, 95%CI: 2.60-103.68, P = 0.003) were independent risk factors for delayed post-ESD bleeding in multivariate logistic regression analysis.

DISCUSSION

This is the first study to identify risk factors that are as-

Table 3 Univariate analysis for risk factors of delayed bleeding *n* (%)

Variable	Delayed bleeding	Non-bleeding	<i>P</i> value
Number of patients	14	303	
Number of lesions	14	311	
Patient-related factors			
Median age (yr) (range)	68 (29-79)	67 (31-86)	0.871
Gender (male/female)	7/6	175/124	> 0.999
Comorbidities			
Hypertension	5 (35.7)	103 (33.1)	> 0.999
Diabetes mellitus	1 (7.1)	34 (10.9)	> 0.999
Hyperlipidemia	2 (14.3)	52 (16.7)	> 0.999
Cardiovascular disease	1 (7.1)	17 (5.5)	0.557
Use of antithrombotic drugs	1 (7.1)	27 (8.7)	> 0.999
Use of intravenous heparin	0 (0)	6 (1.9)	> 0.999
Lesion related factors			
Mean size of tumor (mm) (range)	40.9 (20-70)	33.8 (7-115)	0.070
Mean size of specimen (mm) (range)	45.4 (20-75)	38.4 (8-120)	0.142
Location			0.042
Cecum	4 (28.6)	19 (6.1)	
Ascending colon	3 (21.4)	55 (17.7)	
Transverse colon	2 (14.3)	49 (15.8)	
Descending colon	0 (0)	12 (3.9)	
Sigmoid colon	1 (7.1)	58 (18.6)	
Rectum	4 (28.6)	118 (37.9)	
Morphology			0.897
Protruded	1 (7.1)	30 (9.6)	
Depressed	0 (0)	4 (1.3)	
LST-G	9 (64.3)	171 (55.0)	
LST-NG	4 (28.6)	106 (34.1)	
Histology and depth			0.312
Serrated lesion	0 (0)	5 (1.6)	
Adenoma	2 (14.3)	24 (7.7)	
M	8 (57.1)	241 (77.5)	
SM < 1000 μ m	3 (21.4)	24 (7.7)	
SM \geq 1000 μ m	1 (7.1)	17 (5.5)	
Treatment-related factors			
Device used			
Needle type ¹ /scissor type ²	12/2	280/31	0.642
Mean procedure time (min) (range)	90.4	101.5	0.965
	(20-180)	(10-630)	
Significant bleeding during ESD	2 (14.3)	4 (1.3)	0.024

¹Including Flush knife, Dual knife, Hook knife, and B knife; ²SB knife Jr. LST-G: Laterally spreading tumor granular type; LST-NG: Laterally spreading tumor nongranular type; M: Intra-mucosal adenocarcinoma; SM < 1000 μ m, submucosal, minutely invasive adenocarcinoma; SM \geq 1000 μ m, submucosal, deeply invasive adenocarcinoma; ESD: Endoscopic submucosal dissection.

sociated with delayed bleeding following ESD treatment of colorectal neoplasms. Delayed bleeding occurred in 4.3% of all lesions in this study (4.4% of all patients). This finding varies from the published studies on delayed post-ESD bleeding, which ranges between 0% and 12% for colorectal neoplasms^[13-17]. This wide variation is largely due to differences in study design, patient selection criteria, the operative technique used, and the definition of bleeding. Bleeding occurred within 4 postoperative days in all cases except for 1. Therefore, patients who undergo ESD should be observed carefully for at least 4 d after ESD.

We also discovered that lesions located in the cecum have an increased risk for the development of delayed

Table 4 Univariate analysis of specific locations in the colon as risk factors for delayed bleeding *n* (%)

Location of the lesions	Delayed bleeding	Non-bleeding	<i>P</i> value
Cecum	4 (28.6)	19 (6.1)	0.012
Ascending colon	3 (21.4)	55 (17.7)	0.722
Transverse colon	2 (14.3)	49 (15.8)	> 0.999
Descending colon	0 (0)	12 (3.9)	> 0.999
Sigmoid colon	1 (7.1)	58 (18.6)	0.479
Rectum	4 (28.6)	118 (37.9)	0.581

bleeding complications (7.3-fold higher) compared to lesions located in other parts of the colon. This result is supported by studies of post-EMR and post-polypectomy bleeding in which delayed bleeding occurred more often in lesions proximal to the hepatic flexure, including the cecum^[18-22]. This difference may be due to differences in the anatomical structure and physiology of the colon. For example, the submucosal vasculature is easily affected by thermal injury because the intestinal wall of the cecum is thin, and the cecum wall receives higher tension than the other parts of the colon^[21]. Buddingh *et al*^[18] suggested that fresh ileal fluids contain bile acids and digestive enzymes that have not been fully resorbed and/or have been inactivated in the colon prior to their transit into the cecum, and these factors underlie the high occurrence of delayed post-polypectomy bleeding in the cecum. These fluids and enzymes are postulated to remove post-polypectomy protective substances in the ulcer site, which damages blood vessels. A previous retrospective study reported that the prophylactic clipping of resection sites after endoscopic resection of large, flat, colorectal lesions was associated with a reduced incidence of delayed post-procedural bleeding^[22]. The prophylactic closure may reduce the exposure of cecal ulcer sites to bile acids and digestive enzymes. However, this proposed mechanism has not been fully elucidated.

We also found that significant bleeding during the ESD procedure was associated with an increased (16.4-fold) incidence of delayed bleeding compared to cases in which bleeding was easily stopped using only hemostatic forceps during the procedure. Kim *et al*^[23] also found that immediate post-polypectomy bleeding was significantly correlated with delayed bleeding. Higashiyama *et al*^[24] reported poor control of bleeding during ESD as an independent risk factor for delayed bleeding in their ESD study on gastric lesions. These authors suggested that the delayed bleeding might be caused by difficulties in the identification of the exposed blood vessel and the failure of coagulation with hemostatic forceps. This failure may be due to the adhesion of the coagula to the ulcer floor immediately after the resection and the occurrence of bleeding during the procedure. However, no prospective trial has been conducted to confirm these reports, and preventive coagulation treatment of the ulcer floor following ESD remains safe and appropriate in gastric ESD. Preventive coagulation treatment using hemostatic forceps for colorectal lesions is rarely performed

Table 5 Multivariate analysis of risk factors for delayed bleeding

Variable	OR (95%CI)	<i>P</i> value
Lesion size (per mm)	1.02 (0.99-1.05)	0.212
Location in the cecum (yes vs no)	7.26 (1.99-26.55)	0.003
Significant bleeding during ESD (yes vs no)	16.41 (2.60-103.68)	0.003

ESD: Endoscopic submucosal dissection.

after ESD because the muscularis propria of the colon is much thinner and more easily perforated. Accordingly, there may be an association between the lack of hemostasis and/or coagulation treatment and the occurrence of delayed bleeding in cases with bleeding during ESD, which our results suggest. Therefore, hemostasis and/or coagulation treatment should be performed on the bleeding spots during ESD to prevent delayed bleeding complications after the procedure.

The lesion location and bleeding during the ESD procedure were significantly associated with delayed post-ESD bleeding, but no association was found for lesion size. The resection of large lesions exposes larger areas of the submucosa and creates deeper wounds, which suggests that larger lesions are more likely to bleed. However, there is a lack of consensus on the association between lesion size and delayed post-procedural bleeding in the EMR and polypectomy literature. Some reports have shown a significant difference^[23,25], some studies have shown a trend without significance^[21] and some studies have not demonstrated an association^[19,26]. The resection area is larger in ESD procedures than EMR because of the absence of a size limitation for tumors suitable for *en bloc* ESD resection. The diameter of the largest lesion removed in the present study was 115 mm (mean, 34.1 mm), which is larger than typical EMR procedures. However, significant associations were not found between the size of the lesion and the rate of delayed bleeding in either the univariate or multivariate analyses.

This study has a number of limitations. The study was retrospective in nature. In addition, this study was a single-institution analysis, and future studies are required to replicate our findings at other sites. Finally, the number of subjects enrolled was relatively small. We aim to conduct a multicenter, prospective study with a larger number of subjects in the future.

In conclusion, we found that the rate of delayed post-procedural bleeding following ESD for colorectal neoplasms was 4.3%, and an increased risk of post-procedural bleeding was associated with the presence of lesions in the cecum and significant bleeding events during the ESD procedure. Therefore, additional post-ESD procedures are recommended in patients with these risk factors.

ACKNOWLEDGMENTS

We sincerely thank the staff of the endoscopy unit at the Cancer Institute Hospital of the Japanese Foundation for

Cancer Research for their valuable assistance in conducting this study.

COMMENTS

Background

Endoscopic submucosal dissection (ESD) was initially developed for early gastric cancers, and it is a highly effective and safe treatment. ESD has recently been accepted as an effective endoscopic treatment for superficial colorectal neoplasms. ESD is associated with a small but finite rate of serious adverse events, including delayed post-procedural bleeding. Delayed post-ESD bleeding can result in serious adverse effects that can lead to increased morbidity and require additional medical resources. However, the risk factors for delayed post-ESD bleeding following the treatment of colorectal neoplasms remain unknown.

Research frontiers

ESD for colorectal neoplasms is not a widely used procedure because of its technical difficulties and the high incidence of severe adverse events, including delayed post-procedural bleeding. ESD for colorectal neoplasms may be performed more safely to determine the incidence of delayed post-ESD bleeding and identify the clinical factors that are associated with this adverse event.

Innovations and breakthroughs

The rate of delayed post-ESD bleeding was 4.3%, and bleeding occurred within 4 postoperative days in most cases. The increased risk of post-ESD bleeding was significantly associated with the presence of lesions in the cecum and significant bleeding events during the ESD procedure.

Applications

The study results suggest that patients who undergo ESD should be observed carefully during the 4 d after ESD, and additional post-ESD procedures, such as prophylactic closure of the resection site or hemostasis treatment in the bleeding spots, should be performed in patients with these risk factors.

Terminology

Delayed post-ESD bleeding: Delayed post-ESD bleeding was defined as bleeding that resulted in overt hematochezia 6 h to 30 d after ESD and the observation of bleeding spots confirmed by repeat colonoscopy or the requirement of blood transfusion. Significant bleeding during ESD: Significant bleeding during ESD was defined as active bleeding during the procedure that resulted in the use of endoscopic hemostatic clips because hemostasis could not be achieved using hemostatic forceps alone.

Peer review

This is a good descriptive study of the risk factors of delayed bleeding after colorectal ESD. The results are interesting and suggest that location in the cecum and significant bleeding during ESD were the risk factors for delayed bleeding after ESD, but a larger lesion size was not significantly associated with delayed bleeding.

REFERENCES

- 1 Winawer SJ, Zauber AG, Ho MN, O'Brien MJ, Gottlieb LS, Sternberg SS, Wayne JD, Schapiro M, Bond JH, Panish JF. Prevention of colorectal cancer by colonoscopic polypectomy. The National Polyp Study Workgroup. *N Engl J Med* 1993; **329**: 1977-1981 [PMID: 8247072 DOI: 10.1056/NEJM199312303292701]
- 2 Kudo S. Endoscopic mucosal resection of flat and depressed types of early colorectal cancer. *Endoscopy* 1993; **25**: 455-461 [PMID: 8261988 DOI: 10.1055/s-2007-1010367]
- 3 Tanaka S, Haruma K, Oka S, Takahashi R, Kunihiro M, Kitadai Y, Yoshihara M, Shimamoto F, Chayama K. Clinicopathologic features and endoscopic treatment of superficially spreading colorectal neoplasms larger than 20 mm. *Gastrointest Endosc* 2001; **54**: 62-66 [PMID: 11427843 DOI: 10.1067/mge.2001.115729]
- 4 Saito Y, Fujii T, Kondo H, Mukai H, Yokota T, Kozu T, Saito D. Endoscopic treatment for laterally spreading tumors in the colon. *Endoscopy* 2001; **33**: 682-686 [PMID: 11490384 DOI: 10.1055/s-2001-16213]
- 5 Tamura S, Nakajo K, Yokoyama Y, Ohkawauchi K, Yamada T, Higashidani Y, Miyamoto T, Ueta H, Onishi S. Evaluation of endoscopic mucosal resection for laterally spreading rectal tumors. *Endoscopy* 2004; **36**: 306-312 [PMID: 15057679 DOI: 10.1055/s-2004-814204]
- 6 Terasaki M, Tanaka S, Oka S, Nakadoi K, Takata S, Kanao H, Yoshida S, Chayama K. Clinical outcomes of endoscopic submucosal dissection and endoscopic mucosal resection for laterally spreading tumors larger than 20 mm. *J Gastroenterol Hepatol* 2012; **27**: 734-740 [PMID: 22098630 DOI: 10.1111/j.1440-1746.2011.06977.x]
- 7 Gotoda T. Endoscopic resection of early gastric cancer. *Gastric Cancer* 2007; **10**: 1-11 [PMID: 17334711 DOI: 10.1007/s10120-006-0408-1]
- 8 Ono H, Kondo H, Gotoda T, Shirao K, Yamaguchi H, Saito D, Hosokawa K, Shimoda T, Yoshida S. Endoscopic mucosal resection for treatment of early gastric cancer. *Gut* 2001; **48**: 225-229 [PMID: 11156645 DOI: 10.1136/gut.48.2.225]
- 9 Uraoka T, Parra-Blanco A, Yahagi N. Colorectal endoscopic submucosal dissection in Japan and Western countries. *Dig Endosc* 2012; **24** Suppl 1: 80-83 [PMID: 22533758 DOI: 10.1111/j.1443-1661.2012.01279.x]
- 10 Lee EJ, Lee JB, Lee SH, Kim do S, Lee DH, Lee DS, Youk EG. Endoscopic submucosal dissection for colorectal tumors--1,000 colorectal ESD cases: one specialized institute's experiences. *Surg Endosc* 2013; **27**: 31-39 [PMID: 22729707 DOI: 10.1007/s00464-012-2403-4]
- 11 Sawhney MS, Salfiti N, Nelson DB, Lederle FA, Bond JH. Risk factors for severe delayed postpolypectomy bleeding. *Endoscopy* 2008; **40**: 115-119 [PMID: 18253906 DOI: 10.1055/s-2007-966959]
- 12 Watabe H, Yamaji Y, Okamoto M, Kondo S, Ohta M, Ikemoue T, Kato J, Togo G, Matsumura M, Yoshida H, Kawabe T, Omata M. Risk assessment for delayed hemorrhagic complication of colonic polypectomy: polyp-related factors and patient-related factors. *Gastrointest Endosc* 2006; **64**: 73-78 [PMID: 16813806 DOI: 10.1016/j.gie.2006.02.054]
- 13 Takeuchi Y, Uedo N, Ishihara R, Iishi H, Kizu T, Inoue T, Chatani R, Hanaoka N, Taniguchi T, Kawada N, Higashino K, Shimokawa T, Tatsuta M. Efficacy of an endo-knife with a water-jet function (Flushknife) for endoscopic submucosal dissection of superficial colorectal neoplasms. *Am J Gastroenterol* 2010; **105**: 314-322 [PMID: 19773749 DOI: 10.1038/ajg.2009.547]
- 14 Hurlstone DP, Atkinson R, Sanders DS, Thomson M, Cross SS, Brown S. Achieving R0 resection in the colorectum using endoscopic submucosal dissection. *Br J Surg* 2007; **94**: 1536-1542 [PMID: 17948864 DOI: 10.1002/bjs.5720]
- 15 Saito Y, Sakamoto T, Fukunaga S, Nakajima T, Kiriya S, Matsuda T. Endoscopic submucosal dissection (ESD) for colorectal tumors. *Dig Endosc* 2009; **21** Suppl 1: S7-12 [PMID: 19691740 DOI: 10.1111/j.1443-1661.2009.00870.x]
- 16 Toyonaga T, Man-i M, Fujita T, East JE, Nishino E, Ono W, Morita Y, Sanuki T, Yoshida M, Kutsumi H, Inokuchi H, Azuma T. Retrospective study of technical aspects and complications of endoscopic submucosal dissection for laterally spreading tumors of the colorectum. *Endoscopy* 2010; **42**: 714-722 [PMID: 20806155 DOI: 10.1055/s-0030-1255654]
- 17 Kuroki Y, Hoteya S, Mitani T, Yamashita S, Kikuchi D, Fujimoto A, Matsui A, Nakamura M, Nishida N, Iizuka T, Yahagi N. Endoscopic submucosal dissection for residual/locally recurrent lesions after endoscopic therapy for colorectal tumors. *J Gastroenterol Hepatol* 2010; **25**: 1747-1753 [PMID: 21039836 DOI: 10.1111/j.1440-1746.2010.06331.x]
- 18 Buddingh KT, Herngreen T, Haringsma J, van der Zwet WC, Vleggaar FP, Breumelhof R, Ter Borg F. Location in the right hemi-colon is an independent risk factor for delayed post-polypectomy hemorrhage: a multi-center case-control study. *Am J Gastroenterol* 2011; **106**: 1119-1124 [PMID: 21266961 DOI: 10.1038/ajg.2010.507]
- 19 Metz AJ, Bourke MJ, Moss A, Williams SJ, Swan MP, Byth K.

- Factors that predict bleeding following endoscopic mucosal resection of large colonic lesions. *Endoscopy* 2011; **43**: 506-511 [PMID: 21618150 DOI: 10.1055/s-0030-1256346]
- 20 **Rex DK**, Lewis BS, Waye JD. Colonoscopy and endoscopic therapy for delayed post-polypectomy hemorrhage. *Gastrointest Endosc* 1992; **38**: 127-129 [PMID: 1568607]
 - 21 **Sorbi D**, Norton I, Conio M, Balm R, Zinsmeister A, Gostout CJ. Postpolypectomy lower GI bleeding: descriptive analysis. *Gastrointest Endosc* 2000; **51**: 690-696 [PMID: 10840301 DOI: 10.1067/mge.2000.105773]
 - 22 **Liaquat H**, Rohn E, Rex DK. Prophylactic clip closure reduced the risk of delayed postpolypectomy hemorrhage: experience in 277 clipped large sessile or flat colorectal lesions and 247 control lesions. *Gastrointest Endosc* 2013; **77**: 401-407 [PMID: 23317580 DOI: 10.1016/j.gie.2012.10.024]
 - 23 **Kim HS**, Kim TI, Kim WH, Kim YH, Kim HJ, Yang SK, Myung SJ, Byeon JS, Lee MS, Chung IK, Jung SA, Jeon YT, Choi JH, Choi KY, Choi H, Han DS, Song JS. Risk factors for immediate postpolypectomy bleeding of the colon: a multi-center study. *Am J Gastroenterol* 2006; **101**: 1333-1341 [PMID: 16771958 DOI: 10.1111/j.1572-0241.2006.00638.x]
 - 24 **Higashiyama M**, Oka S, Tanaka S, Sanomura Y, Imagawa H, Shishido T, Yoshida S, Chayama K. Risk factors for bleeding after endoscopic submucosal dissection of gastric epithelial neoplasm. *Dig Endosc* 2011; **23**: 290-295 [PMID: 21951088 DOI: 10.1111/j.1443-1661.2011.01151.x]
 - 25 **Rosen L**, Bub DS, Reed JF, Nastase SA. Hemorrhage following colonoscopic polypectomy. *Dis Colon Rectum* 1993; **36**: 1126-1131 [PMID: 8253009 DOI: 10.1007/BF02052261]
 - 26 **Hui AJ**, Wong RM, Ching JY, Hung LC, Chung SC, Sung JJ. Risk of colonoscopic polypectomy bleeding with anticoagulants and antiplatelet agents: analysis of 1657 cases. *Gastrointest Endosc* 2004; **59**: 44-48 [PMID: 14722546 DOI: 10.1016/S0016-5107(03)02307-1]

P- Reviewer: Ishii N S- Editor: Wen LL

L- Editor: A E- Editor: Wang CH



Is the AIMS65 score useful in predicting outcomes in peptic ulcer bleeding?

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Supported by Catholic Research Coordinating Center of the Korea health 21 R and D Project, No. A070001, Ministry of Health and Welfare South Korea

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Received: September 2, 2013 Revised: November 3, 2013

Accepted: November 18, 2013

Published online: February 21, 2014

bleeding were analysed. The poor outcome group comprised 28 patients [male: 23 (82.1%) vs female: 5 (10.7%)] while the good outcome group included 121 patients [male: 93 (76.9%) vs female: 28 (23.1%)]. The mean age in each group was not significantly different. The mean serum albumin levels in the poor outcome group were slightly lower than those in the good outcome group ($P = 0.072$). For the prediction of poor outcome, the AIMS65 score had a sensitivity of 35.5% (95%CI: 27.0-44.8) and a specificity of 82.1% (95%CI: 63.1-93.9) at a score of 0. The AIMS65 score was insufficient for predicting outcomes in peptic ulcer bleeding (area under curve = 0.571; 95%CI: 0.49-0.65).

CONCLUSION: The AIMS65 score may therefore not be suitable for predicting clinical outcomes in peptic ulcer bleeding. Low albumin levels may be a risk factor associated with high mortality in peptic ulcer bleeding.

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Key words: Gastrointestinal haemorrhage; Peptic ulcer; Mortality; Morbidity; Prognosis

Abstract

AIM: To evaluate the applicability of AIMS65 scores in predicting outcomes of peptic ulcer bleeding.

METHODS: This was a retrospective study in a single center between January 2006 and December 2011. We enrolled 522 patients with upper gastrointestinal haemorrhage who visited the emergency room. High-risk patients were regarded as those who had re-bleeding within 30 d from the first endoscopy as well as those who died within 30 d of visiting the Emergency room. A total of 149 patients with peptic ulcer bleeding were analysed, and the AIMS65 score was used to retrospectively predict the high-risk patients.

RESULTS: A total of 149 patients with peptic ulcer

Core tip: AIMS65 score is a novel simple score for predicting outcomes for acute upper gastrointestinal bleeding (UGIB). However, this scoring system is based on analyses of data from a mixed patient population with both variceal and non-variceal UGIB. The present study focused on the effectiveness of the AIMS65 score in predicting outcomes of peptic ulcer bleeding. This retrospective single-centre study, which included 149 patients, revealed that the AIMS65 score may not be suitable for predicting outcomes in peptic ulcer bleeding. Further, low albumin levels may be a risk factor associated with high mortality in peptic ulcer bleeding.

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SW. Is the AIM65 score useful in predicting outcomes in peptic ulcer bleeding? *World J Gastroenterol* 2014; 20(7): 1846-1851 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1846.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1846>

INTRODUCTION

Acute upper gastrointestinal (GI) bleeding is a common emergency associated with high morbidity and medical expense. The yearly incidence of acute upper GI bleeding is 50-150 per 100000 of the population, with a mortality rate of 10%-14%^[1]. A major cause of acute upper gastrointestinal bleeding is peptic ulcer bleeding^[2]. Endoscopic treatment and acid suppression with proton-pump inhibitors are most important in the management of peptic ulcer bleeding and these treatments have been reduced mortality^[2-4]. Despite recent advances in endoscopic and pharmacological management, non-variceal upper gastrointestinal bleeding (NVUGIB) is still associated with considerable mortality and morbidity^[5]. The recently published International Consensus Recommendations on the management of patients with non-variceal upper GI bleeding recommend "early risk stratification", by using validated prognostic scales^[1]. Several prognostic indices are available, including the Rockall^[6] and Baylor^[7] scores; however, these include clinical and endoscopic components and are therefore unsuitable for pre-endoscopic triage. The Glasgow-Blatchford score^[8], which may be used for pre-endoscopic triage, compares favourably with the pre-endoscopic component of the Rockall score^[9,10]. However, it has not been adopted in routine clinical practice, because of its limitations: it is weighted and assigns points to elements in the patient's medical history, some of which lack a clear definition^[11]. Recently, AIMS65-a new simple risk score for acute upper gastrointestinal bleeding-has been developed and validated^[12-14]. The 5 parameters of AIMS65 are as follows: albumin levels, international normalized ratio (prothrombin time), altered mental status, systolic blood pressure, and age > 65 years. However, the role and utility of this for peptic ulcer bleeding has not yet been clarified since this scoring system was based on analysis of data from a mixed patient population, with acute upper GI bleeding that included both variceal and non-variceal UGIB. We considered whether this score would be useful in patients with peptic ulcer bleeding since the parameters evaluated in AIMS65, such as albumin and INR, appear to be associated with variceal bleeding. Therefore, in the present study, we aimed to evaluate the applicability of the AIMS65 score in predicting outcomes of peptic ulcer bleeding.

MATERIALS AND METHODS

Patients

This study was performed in St. Paul's Hospital, Catholic Medical Center, South Korea. This retrospective analysis included patients enrolled consecutively between January

2006 and December 2011. The study was reviewed and approved by the institutional review board. Patients were considered eligible for inclusion if they were over 18 years of age and had visited the emergency room (ER) for any upper GI bleeding symptoms, including melena, haematemesis and/or haematochezia. Of these, only patients who underwent endoscopy were included in the analysis. Exclusion criteria for the study were as follows: patients who did not undergo upper GI endoscopy; presence of variceal bleeding, bleeding ulcer from the anastomosis following gastrectomy, bleeding due to stomach cancer, obscure GI bleeding, Mallory-Weiss syndrome or angiodysplastic bleeding; and inability to follow up after 30 d from visiting the ER (determined from patient charts).

The variables examined included demographic factors (age and sex), vital signs (pulse, systolic blood pressure, diastolic blood pressure, temperature and respiratory rate), mental status, results of laboratory tests and underlying co-morbid conditions. Altered mental status was defined as physician-charted findings of "disoriented", "stupor", or "coma". Vital signs, mental status and laboratory test results on the day of admission, including routine chemistry and haematology, were recorded.

Definitions

High-risk patients were defined as those who suffered re-bleeding within 30 d of the first endoscopy along with those who died within 30 d from visiting the ER. Re-bleeding was characterized as fresh haematemesis and/or melena associated with the development of shock (pulse > 100 beats/min, systolic blood pressure < 100 mmHg), or a reduction in haemoglobin concentration greater than 2 g/dL over 24 h^[15]. Re-bleeding also included cases requiring repeat endoscopy, surgical intervention or any interventional radiology procedure. Patient charts and/or electronic patient records were used to evaluate 30-d mortality. All high-risk patients were included in the "poor outcome" group.

Regarding the AIMS65 score, the following 5 factors were included: serum albumin < 3.0 g/dL, INR > 1.5, altered mental status, systolic blood pressure ≤ 90 mmHg, and age > 65 years. Each risk factor carries 1 point. Mortality risk can be differentiated as low (AIMS65 0-1 risk factors) or high (AIMS65 2-5 risk factors)^[12]. We investigated whether the AIMS65 scores could predict patients with poor outcomes.

Statistical analyses

Categorical data are presented as mean ± SD. The χ^2 test or Fisher's exact test were applied to evaluate categorical variables. The *t*-test was used to evaluate continuous variables. Differences between good and poor outcomes were assessed using the χ^2 test and *t*-tests. Both univariate and multivariate analyses were performed [SAS system for Windows (release 9.2; SAS Institute, Cary, NC, United States)]. *P* < 0.05 was considered statistically significant. The score for the area under curve (AUC) was suggested through the receiver operating curve (ROC)

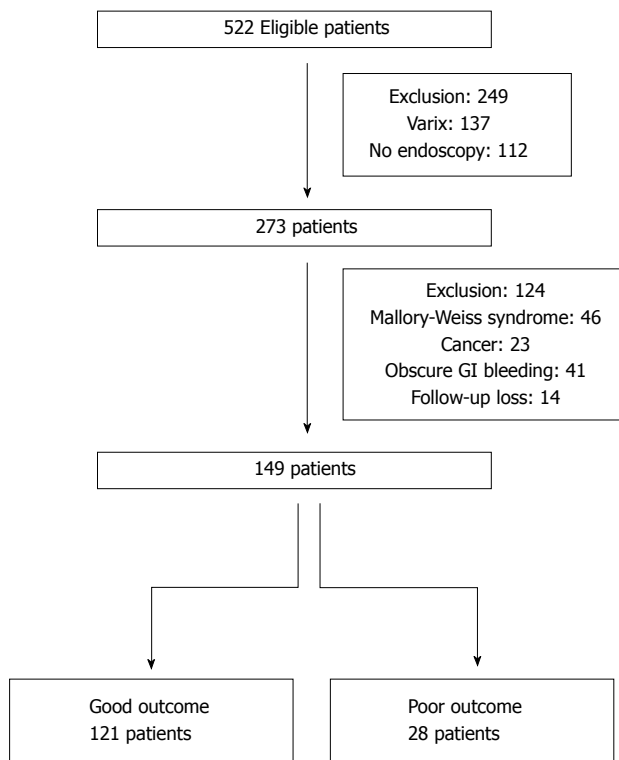


Figure 1 Study enrolment. GI: Gastrointestinal.

using the cut-off value of the AIMS65 score (MedCalc ver. 11.2.1.0).

RESULTS

We reviewed 522 patients over the age of 18 years who visited the ER of St. Paul's Hospital, Catholic Medical Center, South Korea, for complaints of upper GI bleeding, including haematemesis, melena, and/or haematochezia. Overall, we excluded 373 patients from the study for the following reasons: 112 patients did not undergo endoscopy, 206 patients were diagnosed with gastrointestinal bleeding from causes other than peptic ulcer (variceal bleeding, 137; Mallory-Weiss syndrome, 46; gastric cancer bleeding or bleeding ulcer during gastrectomy, 23; and other causes such as obscure GI bleeding or angiodysplastic bleeding, 41), and 14 patients were lost to follow-up. Thus, 149 patients with peptic ulcer bleeding were included in the final analysis (Figure 1).

The poor outcome group comprised 28 patients [male: 23 (82.1%) *vs* female: 5 (10.7%)] while the good outcome group included 121 patients [male: 93 (76.9%) *vs* female: 28 (23.1%)]. The mean age in each group was not significantly different (good outcome group *vs* poor outcome group; 66.4 ± 13.0 *vs* 62.9 ± 15.9 ; $P = 0.216$). The serum albumin level in the poor outcome group was slightly lower than that in the good outcome group; however, this difference was not statistically significant (Table 1). The poor outcome group included the following outcomes: repeat endoscopy ($n = 24$), operation ($n = 3$), and death ($n = 1$).

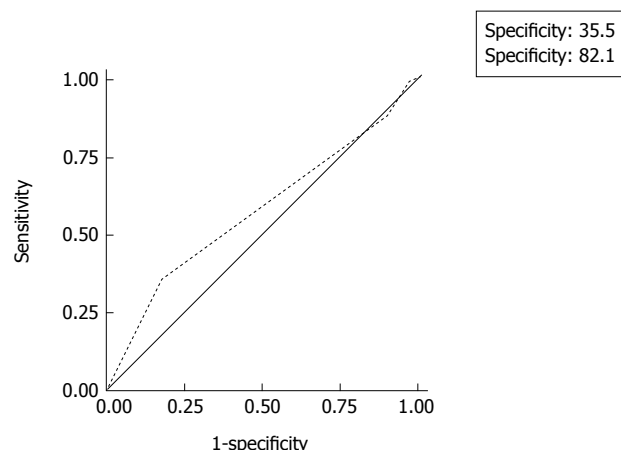


Figure 2 The receiver operating curve using the cut-off value of the AIMS65 score.

With regard to the AIMS65 score, 43 (35.5%) patients from the good outcome group and 5 (17.9%) patients from the poor outcome group scored 0 ($P = 0.071$). For the prediction of poor outcome, the AIMS65 score had a sensitivity of 35.5% (95%CI: 27.0-44.8) and a specificity of 82.1% (95%CI: 63.1-93.9) at a score of 0. Sensitivity and specificity were also suboptimal at higher decision thresholds (≤ 1 , ≤ 2 , and ≤ 3) (Table 2). The AIMS65 score was thus insufficient in predicting outcomes in peptic ulcer bleeding (AUC = 0.571; 95%CI: 0.49-0.65) (Figure 2).

DISCUSSION

Peptic ulcer bleeding is the most common cause of acute non-variceal upper GI bleeding with high mortality, especially in older patients^[16-18]. It is widely accepted that endoscopy should be performed as soon as possible, *i.e.*, within 24 h of presentation at the hospital, and it is recommended that validated prognostic scales are applied to such patients for optimal management^[1,19]. These strategies make it possible to identify high-risk lesions, such as active haemorrhage, non-bleeding visible vessels or non-bleeding adherent clots, and apply endoscopic therapy to these for improved prognosis.

The most consistently reported predictors of mortality and re-bleeding in NVUGIB have been age, number of co-morbid conditions and haemodynamic instability^[20-23]. Several prognostic scales have been developed; however, these are not often adopted in routine clinical practice because of their complexity. In comparison, the AIMS65 score, which accurately predicts in-hospital mortality and length of stay, is a very simple risk score predicting outcomes in patients with acute upper GI bleeding^[12]. Two recent reports confirmed the applicability of AIMS65 in acute upper GI bleeding patients, including bleeding of variceal and non-variceal origin^[12,13]. However, whether the AIMS65 score is applicable for predicting outcomes in patients of non-variceal GI bleeding remains uncertain, since 2 of the 5 risk factors

Table 1 Characteristics of good vs poor outcome *n* (%)

		Total (<i>n</i> = 149)	Good outcome (<i>n</i> = 121)	Poor outcome (<i>n</i> = 28)	<i>P</i> value
Diagnosis	Gastric ulcer	117 (78.5)	92 (76.0)	25 (89.3)	0.124
	Duodenal ulcer	32 (21.5)	29 (24.0)	3 (10.7)	
Sex	Male	116 (77.9)	93 (76.9)	23 (82.1)	0.544
	Female	33 (22.1)	28 (23.1)	5 (17.9)	
Age	mean ± SD	62.9 ± 15.9	62.1 ± 16.4	66.3 ± 13.0	0.216
	< 65 yr	72 (48.3)	62 (51.2)	10 (35.7)	0.139
	≥ 65 yr	77 (51.7)	59 (48.8)	18 (64.3)	
Systolic BP	mean ± SD	110.9 ± 22.6	109.8 ± 22.7	115.4 ± 22.4	0.243
	≤ 90	36 (24.2)	31 (25.6)	5 (17.9)	0.387
	> 90	113 (75.8)	90 (74.4)	23 (82.1)	
Albumin	mean ± SD	3.3 ± 0.6	3.4 ± 0.6	3.1 ± 0.6	0.072
	< 3.0	47 (31.5)	35 (28.9)	12 (42.9)	0.153
	≥ 3.0	102 (68.5)	86 (71.1)	16 (57.1)	
INR (PT)	mean ± SD	1.2 ± 0.8	1.2 ± 0.8	1.2 ± 0.2	0.537
	≤ 1.5	138 (92.6)	113 (93.4)	25 (89.3)	0.434 ¹
	> 1.5	11 (7.4)	8 (6.6)	3 (10.7)	
Mental status	alert	144 (96.6)	117 (96.7)	27 (96.4)	0.999
	drowsy, coma	5 (3.4)	4 (3.3)	1 (3.6)	
AIMS65 score	0	48 (32.2)	43 (35.5)	5 (17.9)	0.272 ¹
	1	49 (32.9)	37 (30.6)	12 (42.9)	
	2	34 (22.8)	26 (21.5)	8 (28.6)	
	3	15 (10.1)	13 (10.7)	2 (7.1)	
	4	3 (2.0)	2 (1.7)	1 (3.6)	
	< 2 (0–1)	97 (65.1)	80 (66.1)	17 (60.7)	0.589
	≥ 2 (2–5)	52 (34.9)	41 (33.9)	11 (39.3)	
	< 1	48 (32.2)	43 (35.5)	5 (17.9)	0.071
	≥ 1	101 (67.8)	78 (64.5)	23 (82.1)	

¹Mean ± SD tested by *t*-test, *n* (%) tested using the χ^2 test and Fisher's exact test. BP: Blood pressure; INR (PT): International normalized ratio (prothrombin time).

Table 2 Sensitivity, specificity, positive and negative predictive values, and area under the receiver operating curve using the AIMS65 score cut-off point

AIMS65 score cut-off point	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Area under the ROC curve
≤ 0	35.5%	82.1%	89.6	22.8	0.571
≤ 1	66.1%	39.3%	82.5	21.2	(SE: 0.054)
≤ 2	87.6%	10.7%	80.9	16.7	95%CI: 0.49–0.65)
≤ 3	98.4%	3.6%	81.5	33.3	

ROC: Receiver operating curve; SE: Standard error; CI: Confidence interval.

in AIMS65 scores are generally accepted as poor prognostic factors of liver cirrhosis, *i.e.* serum albumin < 3.0 g/dL and INR > 1.5. Therefore, the AIMS65 score might be useful for predicting outcomes in variceal GI bleeding but not in non-variceal GI bleeding. Our present results revealed a disappointing ROC value for the AIMS65 score, indicating that the AIMS65 score was not particularly useful for predicting poor outcomes in patients with peptic ulcer bleeding.

Interestingly, the mean serum albumin level in the poor outcomes group was slightly lower than that in the good outcomes group although this difference was not statistically significant (*P* = 0.072). This may have been caused by the inclusion of patients with co-morbidities other than liver cirrhosis in the poor outcomes group. On the other hand, low serum albumin levels may be a single prognostic factor predicting outcomes in patients with peptic ulcer bleeding. Two recent studies have demonstrated that serum albumin level ≤ 3 g/dL or <

2.6 g/dL are associated with the in-hospital mortality in patients with non-variceal GI bleeding^[24,25]. In terms of INR, systemic review has shown that the INR does not predict re-bleeding among NVUGIB patients^[26]. However, INR ≥ 1.5 has been shown to be independently associated with in-hospital mortality in upper GI bleeding in the UK^[27]. More research is needed to clarify whether the albumin level and INR can indeed predict outcomes in patients with non-variceal GI bleeding.

This study has certain limitations. First, this is a retrospective single-centre study. Second, we enrolled only patients who underwent endoscopy and excluded patients who refused endoscopy or were discharged by the emergency department. In addition, patients with bleeding due to stress ulcers in the ICU were excluded because this was considered to be related to other co-morbidities rather than peptic ulcer disease specifically. These exclusions may create a bias. Third, it is possible that the small sample size especially that for the poor outcome

group, could affect the results of this study. However, the current study is the first to examine the applicability of the AIMS65 score in patients with peptic ulcer bleeding taking re-bleeding into consideration, which was not evaluated previously in their study^[12]. Fourth, ethnic differences between Western population and Asian may have affected our results. Although the Blatchford score and Rockall score are useful for predict prognoses in Western populations, a recent study demonstrated that in Asians, only the Blatchford score was appropriate for predicting low-risk patients who do not need therapeutic endoscopy^[28].

In conclusion, the AIMS65 score may not be suitable for predicting outcomes in patients with peptic ulcer bleeding. A low albumin level may be a risk factor associated with high mortality in patients with peptic ulcer bleeding. However, further studies are necessary to validate the role of the AIMS65 score in variceal and non-variceal GI bleeding and its usefulness in identifying high-risk patients needing endoscopic therapy.

COMMENTS

Background

Acute upper gastrointestinal bleeding is a common emergency associated with high morbidity and medical expenses. Several prognostic indices are clinically available, including the Rockall score, Baylor score, and the Glasgow-Blatchford score. Recently, AIMS65 has been proposed as a new simple risk score.

Research frontiers

Acute upper gastrointestinal bleeding remains widely prevalent, since anti-platelet medications, such as aspirin, are currently commonly used. Despite recent advances in endoscopic and pharmacological management, non-variceal upper gastrointestinal bleeding (UGIB) continues to be associated with considerable mortality and morbidity. It is important to accurately select high-risk patients with UGIB since these patients need emergent endoscopy.

Innovations and breakthroughs

This study focused on the effectiveness of the AIMS65 score in predicting outcomes of peptic ulcer bleeding. The AIMS65 score is based on analysis of data from a mixed patient population with both variceal and non-variceal UGIB. The present study showed that the AIMS65 score appeared to be unsuitable for predicting outcomes in patients with peptic ulcer bleeding in an Asian population.

Applications

Low albumin levels may be a risk factor associated with high mortality in patients with peptic ulcer bleeding. Further studies are necessary to validate the role of the AIMS65 score in variceal and non-variceal gastrointestinal (GI) bleeding and its usefulness in identifying high-risk patients needing endoscopic therapy.

Peer review

This is an interesting manuscript studying whether a validated score for predicting outcomes in patients with upper GI bleeding is useful for evaluating patients with bleeding peptic ulcers. Although there are several limitations, the negative results merit publication. A large-scale multi-centric trial across Asia would provide the evidence for supporting these findings.

REFERENCES

- 1 Marshall JK, Collins SM, Gafni A. Prediction of resource utilization and case cost for acute nonvariceal upper gastrointestinal hemorrhage at a Canadian community hospital. *Am J Gastroenterol* 1999; **94**: 1841-1846 [PMID: 10406245 DOI: 10.1111/j.1572-0241.1999.01215.x]
- 2 Lau JY, Barkun A, Fan DM, Kuipers EJ, Yang YS, Chan FK. Challenges in the management of acute peptic ulcer bleeding. *Lancet* 2013; **381**: 2033-2043 [PMID: 23746903 DOI: 10.1016/s0140-6736(13)60596-6]

- 3 Sreedharan A, Martin J, Leontiadis GI, Dorward S, Howden CW, Forman D, Moayyedi P. Proton pump inhibitor treatment initiated prior to endoscopic diagnosis in upper gastrointestinal bleeding. *Cochrane Database Syst Rev* 2010; (7): CD005415 [PMID: 20614440 DOI: 10.1002/14651858.CD005415.pub3]
- 4 Barkun AN, Martel M, Toubouti Y, Rahme E, Bardou M. Endoscopic hemostasis in peptic ulcer bleeding for patients with high-risk lesions: a series of meta-analyses. *Gastrointest Endosc* 2009; **69**: 786-799 [PMID: 19152905 DOI: 10.1016/j.gie.2008.05.031]
- 5 Loffroy RF, Abualsaud BA, Lin MD, Rao PP. Recent advances in endovascular techniques for management of acute non-variceal upper gastrointestinal bleeding. *World J Gastrointest Surg* 2011; **3**: 89-100 [PMID: 21860697 DOI: 10.4240/wjgs.v3.i7.89]
- 6 Rockall TA, Logan RF, Devlin HB, Northfield TC. Risk assessment after acute upper gastrointestinal haemorrhage. *Gut* 1996; **38**: 316-321 [PMID: 8675081 DOI: 10.1136/gut.38.3.316]
- 7 Saeed ZA, Winchester CB, Michaelitz PA, Woods KL, Graham DY. A scoring system to predict rebleeding after endoscopic therapy of nonvariceal upper gastrointestinal hemorrhage, with a comparison of heat probe and ethanol injection. *Am J Gastroenterol* 1993; **88**: 1842-1849 [PMID: 8237930]
- 8 Blatchford O, Murray WR, Blatchford M. A risk score to predict need for treatment for upper-gastrointestinal haemorrhage. *Lancet* 2000; **356**: 1318-1321 [PMID: 11073021 DOI: 10.1016/s0140-6736(00)02816-6]
- 9 Ananthakrishnan AN, McGinley EL, Saeian K. Outcomes of weekend admissions for upper gastrointestinal hemorrhage: a nationwide analysis. *Clin Gastroenterol Hepatol* 2009; **7**: 296-302e1 [PMID: 19084483 DOI: 10.1016/j.cgh.2008.08.013]
- 10 Chen IC, Hung MS, Chiu TF, Chen JC, Hsiao CT. Risk scoring systems to predict need for clinical intervention for patients with nonvariceal upper gastrointestinal tract bleeding. *Am J Emerg Med* 2007; **25**: 774-779 [PMID: 17870480 DOI: 10.1016/j.ajem.2006.12.024]
- 11 Mungan Z. An observational European study on clinical outcomes associated with current management strategies for non-variceal upper gastrointestinal bleeding (ENERGIB-Turkey). *Turk J Gastroenterol* 2012; **23**: 463-477 [PMID: 23161291]
- 12 Saltzman JR, Tabak YP, Hyett BH, Sun X, Travis AC, Johannes RS. A simple risk score accurately predicts in-hospital mortality, length of stay, and cost in acute upper GI bleeding. *Gastrointest Endosc* 2011; **74**: 1215-1224 [PMID: 21907980 DOI: 10.1016/j.gie.2011.06.024]
- 13 Hyett BH, Abougergi MS, Charpentier JP, Kumar NL, Brozovic S, Claggett BL, Travis AC, Saltzman JR. The AIMS65 score compared with the Glasgow-Blatchford score in predicting outcomes in upper GI bleeding. *Gastrointest Endosc* 2013; **77**: 551-557 [PMID: 23357496 DOI: 10.1016/j.gie.2012.11.022]
- 14 Chandra S. AIMS65 score predicts short-term mortality but not the need for intervention in acute upper GI bleeding. *Gastrointest Endosc* 2013; **78**: 381-382 [PMID: 23867377 DOI: 10.1016/j.gie.2013.02.034]
- 15 Manta R, Galloro G, Mangiavillano B, Conigliaro R, Pasquale L, Arezzo A, Masci E, Bassotti G, Frazzoni M. Over-the-scope clip (OTSC) represents an effective endoscopic treatment for acute GI bleeding after failure of conventional techniques. *Surg Endosc* 2013; **27**: 3162-3164 [PMID: 23436101 DOI: 10.1007/s00464-013-2871-1]
- 16 Barkun AN, Bardou M, Kuipers EJ, Sung J, Hunt RH, Martel M, Sinclair P. International consensus recommendations on the management of patients with nonvariceal upper gastrointestinal bleeding. *Ann Intern Med* 2010; **152**: 101-113 [PMID: 20083829 DOI: 10.7326/0003-4819-152-2-201001190-00009]
- 17 El Ouali S, Barkun AN, Wyse J, Romagnuolo J, Sung JJ, Gralnek IM, Bardou M, Martel M. Is routine second-look endoscopy effective after endoscopic hemostasis in acute peptic ulcer bleeding? A meta-analysis. *Gastrointest Endosc* 2012;

- 76: 283-292 [PMID: 22695209 DOI: 10.1016/j.gie.2012.04.441]
- 18 **Gralnek IM**, Barkun AN, Bardou M. Management of acute bleeding from a peptic ulcer. *N Engl J Med* 2008; **359**: 928-937 [PMID: 18753649 DOI: 10.1056/NEJMra0706113]
 - 19 **Lim LG**, Ho KY, Chan YH, Teoh PL, Khor CJ, Lim LL, Rajnakova A, Ong TZ, Yeoh KG. Urgent endoscopy is associated with lower mortality in high-risk but not low-risk nonvariceal upper gastrointestinal bleeding. *Endoscopy* 2011; **43**: 300-306 [PMID: 21360421 DOI: 10.1055/s-0030-1256110]
 - 20 **Barkun A**, Bardou M, Marshall JK. Consensus recommendations for managing patients with nonvariceal upper gastrointestinal bleeding. *Ann Intern Med* 2003; **139**: 843-857 [PMID: 14623622 DOI: 10.7326/0003-4819-139-10-200311180-00012]
 - 21 **Lahiff C**, Shields W, Cretu I, Mahmud N, McKiernan S, Norris S, Silke B, Reynolds JV, O'Toole D. Upper gastrointestinal bleeding: predictors of risk in a mixed patient group including variceal and nonvariceal haemorrhage. *Eur J Gastroenterol Hepatol* 2012; **24**: 149-154 [PMID: 22113209 DOI: 10.1097/MEG.0b013e32834e37d6]
 - 22 **Lanas A**, Aabakken L, Fonseca J, Mungan ZA, Papatheodoridis GV, Piessevaux H, Cipolletta L, Nuevo J, Tafalla M. Clinical predictors of poor outcomes among patients with nonvariceal upper gastrointestinal bleeding in Europe. *Aliment Pharmacol Ther* 2011; **33**: 1225-1233 [PMID: 21480935 DOI: 10.1111/j.1365-2036.2011.04651.x]
 - 23 **Marmo R**, Koch M, Cipolletta L, Capurso L, Pera A, Bianco MA, Rocca R, Dezi A, Fasoli R, Brunati S, Lorenzini I, Germani U, Di Matteo G, Giorgio P, Imperiali G, Minoli G, Barberani F, Boschetto S, Martorano M, Gatto G, Amuso M, Pastorelli A, Torre ES, Triossi O, Buzzi A, Cestari R, Della Casa D, Proietti M, Tanzilli A, Aragona G, Giangregorio F, Allegretta L, Tronci S, Michetti P, Romagnoli P, Nucci A, Rogai F, Piubello W, Tebaldi M, Bonfante F, Casadei A, Cortini C, Chiozzini G, Girardi L, Leoci C, Bagnalasta G, Segato S, Chianese G, Salvagnini M, Rotondano G. Predictive factors of mortality from nonvariceal upper gastrointestinal hemorrhage: a multicenter study. *Am J Gastroenterol* 2008; **103**: 1639-1647; quiz 1648 [PMID: 18564127 DOI: 10.1111/j.1572-0241.2008.01865.x]
 - 24 **Weng SC**, Shu KH, Tarng DC, Tang YJ, Cheng CH, Chen CH, Yu TM, Chuang YW, Huang ST, Sheu WH, Wu MJ. In-hospital mortality risk estimation in patients with acute non-variceal upper gastrointestinal bleeding undergoing hemodialysis: a retrospective cohort study. *Ren Fail* 2013; **35**: 243-248 [PMID: 23336331 DOI: 10.3109/0886022x.2012.747140]
 - 25 **González-González JA**, Vázquez-Elizondo G, García-Compeán D, Gaytán-Torres JO, Flores-Rendón ÁR, Jáquez-Quintana JO, Garza-Galindo AA, Cárdenas-Sandoval MG, Maldonado-Garza HJ. Predictors of in-hospital mortality in patients with non-variceal upper gastrointestinal bleeding. *Rev Esp Enferm Dig* 2011; **103**: 196-203 [PMID: 21526873]
 - 26 **Shingina A**, Barkun AN, Razzaghi A, Martel M, Bardou M, Gralnek I. Systematic review: the presenting international normalised ratio (INR) as a predictor of outcome in patients with upper nonvariceal gastrointestinal bleeding. *Aliment Pharmacol Ther* 2011; **33**: 1010-1018 [PMID: 21385193 DOI: 10.1111/j.1365-2036.2011.04618.x]
 - 27 **Jairath V**, Kahan BC, Stanworth SJ, Logan RF, Hearnshaw SA, Travis SP, Palmer KR, Murphy MF. Prevalence, management, and outcomes of patients with coagulopathy after acute nonvariceal upper gastrointestinal bleeding in the United Kingdom. *Transfusion* 2013; **53**: 1069-1076 [PMID: 22897615 DOI: 10.1111/j.1537-2995.2012.03849.x]
 - 28 **Pang SH**, Ching JY, Lau JY, Sung JJ, Graham DY, Chan FK. Comparing the Blatchford and pre-endoscopic Rockall score in predicting the need for endoscopic therapy in patients with upper GI hemorrhage. *Gastrointest Endosc* 2010; **71**: 1134-1140 [PMID: 20598244 DOI: 10.1016/j.gie.2010.01.028]

P- Reviewers: Basoli A, Mayol J, Reddy DN
S- Editor: Ma YJ **L- Editor:** A **E- Editor:** Liu XM



Intravenous iron supplementation may be superior to observation in acute isovolemic anemia after gastrectomy for cancer

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Received: August 5, 2013 Revised: November 20, 2013

Accepted: December 12, 2013

Published online: February 21, 2014

RESULTS: The initial Hb level was higher in the iron group than in the observation group (7.3 ± 1.0 g/dL vs 8.4 ± 0.5 g/dL, $P < 0.001$). The slope of the changes in the Hb level was significantly higher in the iron group than in the observation group (0.648 ± 0.054 vs 0.349 ± 0.038 , $P < 0.001$). The Hb level 1 and 3 mo post-operatively increased from 10.7 ± 1.3 to 11.9 ± 1.3 g/dL in the iron group ($P = 0.033$) and from 10.1 ± 1.0 to 10.8 ± 1.4 g/dL in the observation group ($P < 0.001$). The postoperative hospital stay was significantly longer in the iron group than in the observation group (10.5 ± 6.8 d vs 7.6 ± 5.5 d, $P = 0.011$). There were no significant differences in the major and surgical complications between the groups (6.3% vs 13.3%, $P = 0.192$; 9.5% vs 3.3%, $P = 0.164$).

CONCLUSION: IV-iron supplementation may be an effective treatment for post-operative isovolemic post-gastrectomy anemia and may be a better alternative than observation.

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Key words: Intravenous iron; Observation; Gastric cancer; Acute isovolemic anemia; Gastrectomy

Core tip: Acute isovolemic anemia frequently occurs after major surgery. Intravenous iron supplementation was more effective in elevating the hemoglobin level than observation, and the complications were comparable to observation in 123 acute post-gastrectomy anemia patients.

Abstract

AIM: To determine whether the application of post-operative intravenous (IV)-iron for acute isovolemic anemia after gastrectomy for cancer may be effective.

METHODS: Among 2078 gastric cancer patients who underwent surgery between February 2007 and August 2009 at the National Cancer Center Korea, 368 patients developed post-operative anemia [hemoglobin-(Hb)-level < 9 g/dL] within the first postoperative week. Patients requiring transfusions were excluded. IV-iron was administered to 63 patients (iron group). Sixty patients were observed without treatment (observation group). The clinical outcomes of the groups were compared concerning clinicopathologic data, morbidity, and changes in Hb levels using Fisher's exact test, Student's *t*-test and the Z-test.

Yoon HM, Kim YW, Nam BH, Reim D, Eom BW, Park JY, Ryu KW. Intravenous iron supplementation may be superior to observation in acute isovolemic anemia after gastrectomy for cancer. *World J Gastroenterol* 2014; 20(7): 1852-1857 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1852.htm>

INTRODUCTION

Perioperative anemia occurs in 25%-75% of cancer patients, and the prevalence of anemia in the immediate post-operative period after major surgery is reported to be as high as 90%^[1]. Because acute blood loss normally leads to intraoperative hypovolemia, volume expanders (crystalloids or colloids) are usually used to stabilize volume status. Transfusion is often deferred until the amount of bleeding is considered excessive according to transfusion guidelines^[2,3]. Under such conditions, acute isovolemic anemia develops. Postoperative acute isovolemic anemia can affect the recovery and quality of life of patients by subtly slowing reaction time, deteriorating memory, increasing the heart rate and decreasing oxygen levels^[4,5].

Acute isovolemic anemia can frequently occur after gastrectomy for gastric cancer due to the invasiveness of the procedure. Oncologic gastrectomy with a D2 lymphadenectomy is considered the standard treatment for gastric cancer, and there is thus a high probability of blood loss with subsequent acute isovolemic anemia. Some surgeons balance acute isovolemic anemia with a red blood cell (RBC) transfusion. The postoperative transfusion rates for patients with colorectal and gastric cancers have been reported to be 10%-38% and 21%, respectively^[6,7]. Other surgeons prefer observation for postoperative anemia because spontaneous correction occurs within several months if oral intake is adequate. Moreover, RBC transfusion can result in potential complications and is associated with poor prognosis^[8-11]. Indeed, many patients do not recover from postoperative anemia.

Refractory iron deficiency anemia thus remains a clinical problem particularly in the postoperative setting. In recent years, intravenous (IV)-iron and red cell substitutes, such as erythropoietin stimulants and artificial oxygen carriers, have been developed and used under clinical investigation^[12]. An IV-iron sucrose infusion is considered safe and effective in patients on dialysis^[13] and before orthopedic surgery^[14]. However, treatment with IV-iron for acute isovolemic anemia after oncologic gastrectomy has not been investigated.

The purpose of this retrospective analysis was to determine whether the postoperative use of IV-iron for acute severe isovolemic post-gastrectomy anemia in patients not requiring urgent transfusion may be effective.

MATERIALS AND METHODS

Patients

This retrospective case-control study was approved by the Institutional Review Board of the National Cancer Center Korea (NCC NCS-10-388). The study protocol conformed to the ethical guidelines of the Declaration of Helsinki.

Between February 2007 and August 2009, 2,078

patients underwent surgery for gastric cancer at the National Cancer Center in Korea. Three hundred sixty-eight patients (17.7%) exhibited hemoglobin (Hb) levels < 9.0 g/dL during the postoperative period and were diagnosed with anemia. We excluded 245 patients from this analysis with documented substitutions of a RBC-unit pre-, intra- or post-operatively; preoperative iron treatments; unstable vital signs (hypotension or tachycardia); dyspnea; heart disease (angina and myocardial infarction); more than one treatment modality for postoperative anemia; and any other surgical procedure during the follow-up period. Thus, 123 patients who were either treated with an IV-infusion of iron (iron group) or who underwent clinical observation without treatment (observation group) were enrolled in this analysis.

Treatments

The target Hb level was considered 12.0 g/dL. Sixty-three patients were treated with IV-iron sucrose, and 60 patients were observed without treatment. The iron group received 10 mL of Fe³⁺ (200 mg) mixed with 100 mL of normal saline every other day and had a mean IV infusion of 13.7 ± 8.3 ampules of iron sucrose.

Complications

All postoperative adverse events requiring treatment or hospitalization were considered relevant complications. Surgical complications included ileus, anastomotic site leakage, anastomotic site stenosis, fluid collection and abscess formation. Non-surgical complications included pleural effusion, voiding difficulty, brain infarction and stress-induced cardiomyopathy. Postoperative clinically unapparent events not requiring hospitalization or treatment such as minor pleural effusion, atelectasis or abdominal fluid collections without signs of infection were omitted from the analysis as relevant complications.

Possible adverse events related to IV-iron administration, including nausea/vomiting, headache, anaphylactic reaction, dyspnea, abdominal pain and allergic reactions, such as urticaria and fever, were retrospectively reviewed.

Statistical analysis

The following parameters were recorded and analyzed using a Pearson χ^2 test or Fisher's exact test: patient age and gender, clinicopathologic data and morbidity. Student's *t* test was used to analyze the Hb levels before treatment and throughout the hospital stay after treatment. The *Z* test was performed to determine whether a significant difference existed between the groups with respect to the slopes of the changes in the Hb level during follow-up. A *P* value < 0.05 was considered statistically significant. The slopes of the two groups were estimated using a linear regression test.

RESULTS

Demographics

The mean patient ages were 63.2 ± 12.3 and 64.4 ± 10.2

Table 1 Demographic and clinicopathologic data *n* (%)

Variables	Iron group (<i>n</i> = 63)	Observation group (<i>n</i> = 60)	<i>P</i> value
Age (yr), mean ± SD	63.2 ± 12.3	64.4 ± 10.2	0.560 ¹
Gender (male/female)	37:26	30:30	0.331 ²
Operation type			0.878 ²
Subtotal gastrectomy	38	36	
Total gastrectomy	25	24	
Stage of cancer (AJCC 6 th)			0.036 ²
I and II	28 (44.4)	38 (63.3)	
III and IV	35 (55.6)	22 (36.7)	
Combined resection			0.610 ²
None	48 (76.2)	48 (75)	
Done	15 (23.8)	12 (25)	
Adjuvant chemotherapy			0.002 ²
No	39 (61.9)	52 (86.7)	
Yes	24 (38.1)	8 (13.3)	

¹Student's *t* test; ²Pearson χ^2 test. AJCC: American Joint Committee on Cancer.

years in the iron and observation groups, respectively ($P = 0.560$). The gender ratio was not significantly different between the groups ($P = 0.331$). There were no statistically significant differences between the groups with respect to the type of surgical procedure and combined resection ($P = 0.184$ and $P = 0.610$, respectively). The cancer stage was more advanced in the iron group than in the observation group ($P = 0.036$). The number of patients undergoing adjuvant chemotherapy in the iron group was significantly higher than that in the observation group ($P = 0.002$). Demographic data are presented in Table 1.

Hematologic laboratory data before treatment

The Hb and hematocrit level before treatment was lower in the iron group than in the observation group (7.3 ± 1.0 g/dL *vs* 8.4 ± 0.5 g/dL, $P < 0.001$; 23.8 ± 3.3 g/dL *vs* 26.4 ± 1.9 g/dL, $P < 0.001$). The mean corpuscular volume in the iron group before treatment did not differ from that of the observation group ($P = 0.13$), and the mean corpuscular hemoglobin concentration in the iron group was lower than that in the observation group ($P < 0.001$). Hyperchromic anemia was ruled out for all patients in the present analysis (Table 2).

Changes in the Hb level

The Hb level was higher in the iron group one month post-operatively than in the observation group (10.7 ± 1.3 and 10.1 ± 1.0 g/dL, respectively, $P = 0.033$). Three months post-operatively, the Hb level was higher in the iron group than in the observation group (11.9 ± 1.3 and 10.8 ± 1.4 g/dL, respectively, $P < 0.001$). Six months post-operatively, the Hb level was again higher in the iron group than in the observation group (11.5 ± 1.3 g/dL *vs* 10.7 ± 1.3 g/dL, $P = 0.003$). At 12 mo post-operatively, the Hb level was higher in the iron group than in the observation group (12.0 ± 1.4 and 11.1 ± 1.5 g/dL, respectively, $P = 0.003$) (Figure 1).

Table 2 Hematologic laboratory data and morbidity before treatment *n* (%)

	Iron group (<i>n</i> = 63)	Observation group (<i>n</i> = 60)	<i>P</i> value ¹
Hematologic laboratory data			
Hemoglobin (g/dL), mean ± SD	7.3 ± 1.0	8.4 ± 0.5	< 0.001
Hematocrit, mean ± SD	23.8 ± 3.3	26.4 ± 1.9	< 0.001
Mean MCV (fL), mean ± SD	85.1 ± 8.9	87.4 ± 7.8	0.130
Mean MCHC (g/dL), mean ± SD	31.0 ± 1.3	31.9 ± 1.4	< 0.001
Morbidity			
Surgical	4 (6.3)	8 (13.3)	0.192
Non-surgical	8 (12.7)	3 (5.0)	0.135
Major	6 (9.5)	2 (3.3)	0.164
Minor	16 (25.4)	10 (16.7)	0.236
Adverse events	0 (0.0)	NA	

¹Student's *t* test, Pearson χ^2 test. MCV: Mean corpuscular volume; MCHC: Mean corpuscular hemoglobin concentration; NA: Not applicable.

Increase in the Hb levels after treatment

The slopes ($\beta \pm SE$) for the changes in the Hb levels in the iron and observation groups were 0.628 ± 0.054 and 0.349 ± 0.038 according to a linear regression test ($P < 0.001$ and $P < 0.001$, respectively). The slope in the iron group was significantly steeper than that in the observation group, as determined by Z-test (Z-score 2.777, $P = 0.006$), indicating that the Hb level in the iron group increased more rapidly than in the observation group. No patient suffered from chronic anemia (Hb level < 9 g/dL one year after treatment) in the iron group compared with six patients (10.5%) in the observation group ($P = 0.012$) (Figure 2).

Post-operative courses and complications

The postoperative hospital stay was significantly longer in the iron group than in the observation group (10.5 ± 6.8 and 7.6 ± 5.5 d, respectively, $P = 0.011$).

Table 2 demonstrates no significant differences between the two groups with respect to surgical complications (6.3% in the iron group and 13.3% in the observation group; $P = 0.192$) and non-surgical complications (12.7% in the iron group and 5.0% in the observation group; $P = 0.135$). There were no significant differences between the groups with respect to major and minor complications ($P = 0.164$ and $P = 0.236$, respectively). There were no 30-d mortalities in either of the groups. There were no patients with adverse events related to IV-iron.

DISCUSSION

Recently, several clinical studies on IV-iron treatment in surgery have been reported^[14-16]. Theusinger *et al.*^[14] reported on the beneficial effects of preoperative IV-iron administration in orthopedic patients, and Van Wyck *et al.*^[15] reported the advantages of IV-iron for postpartum

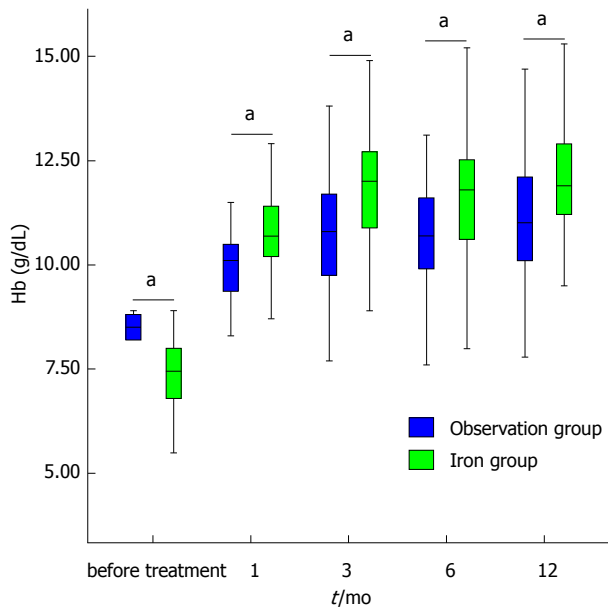


Figure 1 Changes in hemoglobin levels 1, 3, and 6 mo after treatment in the iron and observation groups. ^a $P < 0.05$ between iron and observation groups.

anemia. In contrast, a consensus statement by Beris *et al.*^[16] reported only moderate- to low-quality evidence for IV-iron application in surgical patients until further data from prospective randomized controlled trials become available. However, there are few studies on acute post-operative anemia in patients with gastric cancer. This retrospective analysis was designed to evaluate the efficacy of IV-iron for the treatment of post-operative anemia in patients with gastric cancer. The current analysis demonstrated that the Hb level in the iron group increased more rapidly than in the observation group and that there were no significant differences between the groups with respect to complications or any adverse events related to iron application. The results of the present analysis are consistent with those of earlier studies that reported that IV-iron sucrose treatment for patients with dialysis-associated anemia, pre-arthroplasty and pre-hysterectomy resulted in increased Hb levels^[13-15].

When intra-operative bleeding occurs, anesthesiologists do not use packed RBCs as long as the patient's vital signs are stable and the amount of bleeding is not considered excessive. As a consequence, postoperative acute isovolemic anemia has been largely neglected by surgeons. We found that one, three and six months postoperatively, the Hb level in the observation group increased spontaneously. However, the Hb level in the observation group was lower than that in the iron group at every timepoint and increased more slowly than did the levels in the iron group. These findings suggest that the active treatment of acute isovolemic anemia by the administration of IV-iron might improve postoperative recovery because impaired cognitive function and circulatory homeostasis might be restored earlier^[4,5].

The postoperative Hb levels in the iron group and

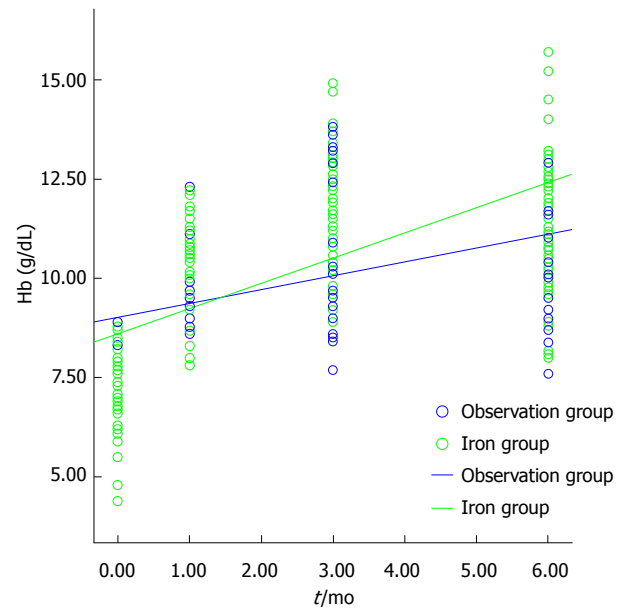


Figure 2 Estimation of slopes for changes in hemoglobin levels in the iron and observation groups by univariate linear regression test.

the observation group differed significantly before any treatment, which might imply a selection bias. Further, there were more advanced cancers in the iron group. It is possible that advanced gastric cancer influences iron metabolism and that preoperative Hb levels might thus have been lower. It is also conceivable that due to the larger number of advanced cancers, surgical invasiveness and thus blood loss was higher in the iron group. Another finding was that the number of patients undergoing adjuvant chemotherapy was significantly higher in the iron group. Interestingly, though chemotherapy may influence erythropoiesis, the Hb levels in the iron group recovered more quickly than they did in the observation group, which might suggest the beneficial effect of IV-iron administration.

The present analysis also reveals that IV-iron use was commonly effective irrespective of the preoperative iron deficiency. Although some studies for different surgical procedures reported that iron supplementation after surgery is unnecessary for recovery if the iron level before surgery is adequate, those prior studies were not focused on the specific clinical setting of acute isovolemic anemia^[17,18]. The pre- and immediate postoperative iron level should be checked to determine whether IV-iron supplementation for acute isovolemic anemia is equally effective for preoperative iron deficiency and normal iron storage in a future prospective study.

Although the Hb level after transfusion therapy might increase more rapidly than the level following iron treatment, transfusion alone does not replenish the iron stores, which could eventually be depleted for several months^[19]. Iron supplementation is necessary to restore the Hb level effectively. Furthermore, for post-gastrectomy patients, the diet is severely restricted during the early postoperative months, and iron absorption is reduced.

Furthermore, the clinical observation of acute post-operative isovolemic anemia without iron supplementation may be tolerable for some patients to return to a normal Hb level but may result in chronic anemia in other patients. The present analysis revealed that 10.5% of the patients in the observation group developed chronic anemia (Hb < 9 g/dL) by 12 mo post-operatively. On the contrary, no patient in the iron group developed chronic anemia.

Many physicians may be reluctant to use iron dextran as a transfusion alternative because iron dextran is known to cause life-threatening anaphylactic reactions in up to 0.6% of treated patients because of the high molecular weight^[20]. Furthermore, IV-iron is believed to produce oxidative stress, inflammation, endothelial dysfunction, and renal injuries^[21]. However, a recent study reported that the rates of adverse drug effects, life-threatening events, and allergic reactions for iron sucrose are extremely rare: 19.8 per million, 0.6 per million and 2.0 per million, respectively^[22]. In the current analysis with iron sucrose, there were no adverse events, such as severe anaphylactic reactions or mortality. The rate of adverse events of low molecular weight IV-iron was recently reported to be lower than that of high molecular weight IV-iron^[23].

The Hb levels became steady three months postoperatively irrespective of iron supplementation. As Liedman *et al.*^[24] demonstrated, energy intake decreases in the early postoperative period after gastrectomy due to the difficulty of food intake and remained constant or increased only after the first three months after surgery, a dietary factor that could possibly explain these findings. Most importantly the Hb level in the iron group was higher than in the observation group at 3, 6, 9 and 12 mo postoperatively and may support the idea that iron supplementation in the early postoperative period might be helpful in maintaining normal Hb levels.

The current retrospective analysis has several limitations. The data were retrospectively collected from a prospectively documented database. Furthermore, the clinical outcomes of earlier Hb level recovery were not evaluated, and the quality of life of the patients was not measured and compared. It is thus debatable whether earlier Hb recovery actually translates into measurable patient benefits. Further, no data were collected concerning the patients' ferritin, iron, and TIBC levels. Finally, IV-iron was used based on clinical experience. The authors are well aware of the limitations of this retrospective analysis, but the main intention was to demonstrate that IV-iron administration for acute isovolemic anemia may be more beneficial than clinical observation alone. Therefore, our institution is initiating a randomized controlled trial to clarify these uncertainties.

In conclusion, the current analysis demonstrates that IV-iron supplementation might be an effective treatment for postoperative isovolemic post-gastrectomy anemia and appears to be a better alternative than clinical observation. An additional prospective trial is needed to deter-

mine the proper indications of IV-iron in postoperative isovolemic anemia for gastric cancer patients.

COMMENTS

Background

Perioperative anemia occurs in 25%-75% of cancer patients, and the prevalence of anemia in the immediate postoperative period after major surgery is reported to be as high as 90%. Because acute blood loss normally leads to intraoperative hypovolemia, volume expanders (crystalloids or colloids) are usually used to stabilize volume status. Transfusion is often deferred until the amount of bleeding is considered excessive according to transfusion guidelines.

Research frontiers

The current analysis demonstrates that intravenous (IV)-iron supplementation might be an effective treatment for postoperative isovolemic post-gastrectomy anemia and appears to be a better alternative than clinical observation. An additional prospective trial is needed to determine the proper indications of IV-iron in postoperative isovolemic anemia for gastric cancer patients.

Innovations and breakthroughs

Acute isovolemic anemia frequently occurs after major surgery. IV-iron supplementation was more effective in elevating the hemoglobin level than observation, and the complications were comparable to observation in 123 acute post-gastrectomy anemia patients.

Peer review

This retrospective analysis was conducted to determine whether the postoperative use of IV-iron for acute severe isovolemic post-gastrectomy anemia in patients not requiring urgent transfusion may be effective. This study enrolled 63 patients with IV-iron sucrose treatment and 60 patients without treatment. Then the authors observed the Hb levels for a period of time. As a consequence, Hb level in the iron group increased more rapidly than in the observation group. In conclusion, IV-iron supplementation might be an effective treatment for postoperative isovolemic post-gastrectomy anemia and appears to be a better alternative than clinical observation.

REFERENCES

- 1 Shander A, Knight K, Thurer R, Adamson J, Spence R. Prevalence and outcomes of anemia in surgery: a systematic review of the literature. *Am J Med* 2004; **116** Suppl 7A: 58S-69S [PMID: 15050887 DOI: 10.1016/j.amjmed.2003.12.013]
- 2 Murphy MF, Wallington TB, Kelsey P, Boulton F, Bruce M, Cohen H, Duguid J, Knowles SM, Poole G, Williamson LM. Guidelines for the clinical use of red cell transfusions. *Br J Haematol* 2001; **113**: 24-31 [PMID: 11328275 DOI: 10.1046/j.1365-2141.2001.02701.x]
- 3 American Association of Blood Banks. Circular of information for the use of human blood and blood components. Available from: URL: http://www.aabb.org/Documents/About_Blood/Circulars_of_Information/coi0702.pdf Accessed January 2013
- 4 Weiskopf RB, Kramer JH, Viele M, Neumann M, Feiner JR, Watson JJ, Hopf HW, Toy P. Acute severe isovolemic anemia impairs cognitive function and memory in humans. *Anesthesiology* 2000; **92**: 1646-1652 [PMID: 10839915 DOI: 10.1097/0000542-200006000-00023]
- 5 Weiskopf RB, Feiner J, Hopf HW, Viele MK, Watson JJ, Kramer JH, Ho R, Toy P. Oxygen reverses deficits of cognitive function and memory and increased heart rate induced by acute severe isovolemic anemia. *Anesthesiology* 2002; **96**: 871-877 [PMID: 11964594 DOI: 10.1097/0000542-200204000-00014]
- 6 Gombotz H, Rehak PH, Shander A, Hofmann A. Blood use in elective surgery: the Austrian benchmark study. *Transfusion* 2007; **47**: 1468-1480 [PMID: 17655591 DOI: 10.1111/j.1537-2995.2007.01286.x]
- 7 Adachi Y, Mimori K, Mori M, Maehara Y, Sugimachi K. Morbidity after D2 and D3 gastrectomy for node-positive

- gastric carcinoma. *J Am Coll Surg* 1997; **184**: 240-244 [PMID: 9060918]
- 8 **Weber RS**, Jabbour N, Martin RC. Anemia and transfusions in patients undergoing surgery for cancer. *Ann Surg Oncol* 2008; **15**: 34-45 [PMID: 17943390 DOI: 10.1245/s10434-007-9502-9]
 - 9 **Eder AF**, Chambers LA. Noninfectious complications of blood transfusion. *Arch Pathol Lab Med* 2007; **131**: 708-718 [PMID: 17488156]
 - 10 **Wu HS**, Little AG. Perioperative blood transfusions and cancer recurrence. *J Clin Oncol* 1988; **6**: 1348-1354 [PMID: 3045268]
 - 11 **Hyung WJ**, Noh SH, Shin DW, Huh J, Huh BJ, Choi SH, Min JS. Adverse effects of perioperative transfusion on patients with stage III and IV gastric cancer. *Ann Surg Oncol* 2002; **9**: 5-12 [PMID: 11829431 DOI: 10.1245/aso.2002.9.1.5]
 - 12 **Goodnough LT**, Shander A, Brecher ME. Transfusion medicine: looking to the future. *Lancet* 2003; **361**: 161-169 [PMID: 12531595 DOI: 10.1016/S0140-6736(03)12195-2]
 - 13 **Charytan C**, Levin N, Al-Saloum M, Hafeez T, Gagnon S, Van Wyck DB. Efficacy and safety of iron sucrose for iron deficiency in patients with dialysis-associated anemia: North American clinical trial. *Am J Kidney Dis* 2001; **37**: 300-307 [PMID: 11157370 DOI: 10.1053/ajkd.2001.21293]
 - 14 **Theusinger OM**, Leyvraz PF, Schanz U, Seifert B, Spahn DR. Treatment of iron deficiency anemia in orthopedic surgery with intravenous iron: efficacy and limits: a prospective study. *Anesthesiology* 2007; **107**: 923-927 [PMID: 18043060 DOI: 10.1097/01.anes.0000291441.10704.82]
 - 15 **Van Wyck DB**, Martens MG, Seid MH, Baker JB, Mangione A. Intravenous ferric carboxymaltose compared with oral iron in the treatment of postpartum anemia: a randomized controlled trial. *Obstet Gynecol* 2007; **110**: 267-278 [PMID: 17666600 DOI: 10.1097/01.AOG.0000275286.03283.18]
 - 16 **Beris P**, Muñoz M, García-Erce JA, Thomas D, Maniatis A, Van der Linden P. Perioperative anaemia management: consensus statement on the role of intravenous iron. *Br J Anaesth* 2008; **100**: 599-604 [PMID: 18372258 DOI: 10.1093/bja/aen054]
 - 17 **Karkouti K**, McCluskey SA, Ghannam M, Salpeter MJ, Quirt I, Yau TM. Intravenous iron and recombinant erythropoietin for the treatment of postoperative anemia. *Can J Anaesth* 2006; **53**: 11-19 [PMID: 16371604 DOI: 10.1007/BF03021522]
 - 18 **van Iperen CE**, Kraaijenhagen RJ, Biesma DH, Beguin Y, Marx JJ, van de Wiel A. Iron metabolism and erythropoiesis after surgery. *Br J Surg* 1998; **85**: 41-45 [PMID: 9462381 DOI: 10.1046/j.1365-2168.1998.00571.x]
 - 19 **Koch CG**, Li L, Sessler DI, Figueroa P, Hoeltge GA, Mihajevic T, Blackstone EH. Duration of red-cell storage and complications after cardiac surgery. *N Engl J Med* 2008; **358**: 1229-1239 [PMID: 18354101 DOI: 10.1056/NEJMoa070403]
 - 20 **Hamstra RD**, Block MH, Schocket AL. Intravenous iron dextran in clinical medicine. *JAMA* 1980; **243**: 1726-1731 [PMID: 6154155 DOI: 10.1001/jama.1980.03300430028018]
 - 21 **Bishu K**, Agarwal R. Acute injury with intravenous iron and concerns regarding long-term safety. *Clin J Am Soc Nephrol* 2006; **1** Suppl 1: S19-S23 [PMID: 17699372 DOI: 10.2215/CJN.01420406]
 - 22 **Chertow GM**, Mason PD, Vaage-Nilsen O, Ahlmén J. Update on adverse drug events associated with parenteral iron. *Nephrol Dial Transplant* 2006; **21**: 378-382 [PMID: 16286429 DOI: 10.1093/ndt/gfi253]
 - 23 **Anker SD**, Comin Colet J, Filippatos G, Willenheimer R, Dickstein K, Drexler H, Lüscher TF, Bart B, Banasiak W, Niegowska J, Kirwan BA, Mori C, von Eisenhart Rothe B, Pocock SJ, Poole-Wilson PA, Ponikowski P. Ferric carboxymaltose in patients with heart failure and iron deficiency. *N Engl J Med* 2009; **361**: 2436-2448 [PMID: 19920054 DOI: 10.1056/NEJMoa0908355]
 - 24 **Liedman B**, Andersson H, Berglund B, Bosaeus I, Hugosson I, Olbe L, Lundell L. Food intake after gastrectomy for gastric carcinoma: the role of a gastric reservoir. *Br J Surg* 1996; **83**: 1138-1143 [PMID: 8869329 DOI: 10.1002/bjs.1800830835]

P- Reviewers: Aoyagi K, Baba H, Symeonidis NG, Wang SK

S- Editor: Gou SX **L- Editor:** A **E- Editor:** Ma S



Statins and the risk of colorectal cancer: An updated systematic review and meta-analysis of 40 studies

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Received: September 30, 2013 Revised: November 13, 2013

Accepted: January 6, 2014

Published online: February 21, 2014

timates with their 95%CI were calculated using fixed- and random-effects models. Then, we assessed the potential presence of publication bias and between-studies heterogeneity. To evaluate the results, we also performed a "leave-one-out" sensitivity analysis.

RESULTS: A total of 40 studies, involving more than eight million subjects, contributed to the analysis. They were grouped on the basis of study design and, consequently, three separate meta-analyses were conducted. A similar modest reduction in the risk of colorectal cancer with statin use was observed, which was not statistically significant among RCTs (RR = 0.89, 95%CI: 0.74-1.07; $n = 8$), but reached statistical significance among cohort studies (RR = 0.91, 95%CI: 0.83-1.00; $n = 13$) and case-control studies (RR = 0.92, 95%CI: 0.87-0.98; $n = 19$). While we did not find significant evidence of selective outcome reporting or publication bias, substantial heterogeneity was detected, mainly among the observational studies. The sensitivity analysis confirmed the stability of our results.

CONCLUSION: A modest reduction in risk of colorectal cancer among statin users cannot be disproved. Further targeted research is warranted.

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Abstract

AIM: To investigate the association between statin use and colorectal cancer risk, we conducted an updated meta-analysis of published studies.

METHODS: We performed a comprehensive search for studies published up to July 2013. Eligible studies for this meta-analysis were either randomized controlled trials (RCTs) or observational studies (case-control or cohort) evaluating any exposure to statins and the risk of colorectal cancer. Two reviewers selected studies based on predefined inclusion criteria, and abstracted the data. Pooled relative risk (RR) es-

Key words: 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors; Statins; Colorectal cancer; Systematic review; Meta-analysis; Cancer chemoprevention

Core tip: To investigate the association between statin use and colorectal cancer risk, we conducted a systematic review and meta-analysis of published studies. A total of 40 studies, involving more than eight million subjects, contributed to our analysis. A modest reduction in the risk of colorectal cancer with statin use was observed, which was not statistically significant among RCTs (RR = 0.89, 95%CI: 0.74-1.07; $n = 8$), but reached statistical significance among cohort stud-

ies (RR = 0.91, 95%CI: 0.83-1.00; n = 13) and case-control studies (RR = 0.92, 95%CI: 0.87-0.98; n = 19). Further targeted research is warranted.

Lytras T, Nikolopoulos G, Bonovas S. Statins and the risk of colorectal cancer: An updated systematic review and meta-analysis of 40 studies. *World J Gastroenterol* 2014; 20(7): 1858-1870 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1858.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1858>

INTRODUCTION

Statins are some of the most widely prescribed drugs worldwide^[1], as a result of their proven efficacy in the primary and secondary prevention of cardiovascular morbidity and mortality, in a variety of populations^[2-7]. Their main mechanism of action is the reduction of serum cholesterol, by means of competitive inhibition of hepatic 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is the rate-limiting enzyme in the mevalonate synthesis pathway^[8]; this reduces endogenous cholesterol biosynthesis, leading to decreased levels of low-density lipoprotein (LDL), a major risk factor for atherosclerosis^[9]. In addition, statins exert a variety of so-called “pleiotropic” effects on human physiology^[10]; these are thought to contribute to their principal cardiovascular benefit^[11,12], although this has not as yet been reflected in clinical trial data^[13].

Because of their pleiotropic effects, it has been suggested that statins might have an effect on cancer risk and play a role in cancer chemoprevention^[10,14-16]. Data from in vitro and animal model studies have been encouraging^[17], but the epidemiological evidence remains inconclusive^[18]. In addition, several meta-analyses of randomized controlled trials (RCTs) of statins for cardiovascular outcomes failed to find an association between statin use and overall cancer risk^[19-22]. Cancer is not a homogenous disease entity however, and the effects of statins might significantly differ according to anatomical site and molecular type. Thus, overall cancer risk is not a very sensitive outcome, and may mask important effects of statins at particular sites.

The relation between statins and colorectal cancer has been the focus of a growing body of both basic and epidemiological research^[23,24]. A fair amount of epidemiological studies have examined the effect of statins on colorectal cancer risk, with often inconsistent results ranging from very protective^[25] (47% risk reduction) to moderately harmful^[26] (7% risk increase). In 2007, we undertook a meta-analysis of published studies reporting on statin use and colorectal cancer risk^[27]; at that time we identified 18 studies (6 RCTs and 12 observational) and concluded that the evidence did not support a strong reduction in colorectal cancer risk by the use of statins in usual dosage, although a modest protective effect or an effect at higher doses could not be excluded. Since

then, many additional studies have been published, and therefore we sought to update our previous systematic review and meta-analysis to reflect the current totality of evidence on statins and colorectal cancer risk.

MATERIALS AND METHODS

Search strategy

We identified studies by a systematic literature search of MEDLINE electronic database (1966 through July 2013). We ran two queries, one aimed at RCTs and one aimed at observational studies (case-control or cohort). Query I was: [“Randomized Controlled Trial” (ptyp)] AND (“HMG-CoA reductase inhibitor” OR “HMG-CoA reductase inhibitors” OR “HMG-CoA reductase inhibitor” OR “HMG-CoA reductase inhibitors” OR statin OR statins OR atorvastatin OR cerivastatin OR fluvastatin OR lovastatin OR mevastatin OR pravastatin OR rivastatin OR rosuvastatin OR simvastatin). Query II was: (“HMG-CoA reductase inhibitor” OR “HMG-CoA reductase inhibitors” OR statin OR statins OR atorvastatin OR cerivastatin OR fluvastatin OR lovastatin OR mevastatin OR pravastatin OR rivastatin OR rosuvastatin OR simvastatin OR pitavastatin) AND (cancer OR cancers OR neoplasm OR neoplasms OR malignancy OR malignancies).

In addition, we browsed the reference lists of relevant narrative and systematic reviews, and asked a knowledgeable expert to identify any additional studies. We browsed the title and abstract of all identified studies to exclude any that were clearly irrelevant. The full text of the remaining articles was read to determine whether it contained information on the topic of interest.

Selection criteria

Eligible studies for this meta-analysis were either RCTs or observational studies (case-control or cohort) evaluating any exposure to statins and the risk of colorectal cancer. In order to be included in the meta-analysis, studies had to report an estimated measure of effect size (risk ratio, rate ratio, hazard ratio or odds ratio) and its associated CI, or had to provide enough data to calculate such an effect measure and CI. In cases of multiple publications from the same population, only data from the most recent report were included.

In particular, RCTs were considered eligible if they (1) evaluated a statin therapy compared with placebo or no treatment; (2) had no other intervention difference between the experimental and the control group; (3) enrolled at least 2000 participants; (4) had a minimum duration of 2 years; and (5) reported the incidence of colorectal cancer in both arms during the trial period. The fourth criterion was used because the effects of the intervention may require long-term exposure. In addition, as colorectal cancer is a rare disease, RCTs of short duration are unlikely to register any significant number of cases.

We did not assess the methodological quality of the

primary studies, since quality scoring in meta-analyses of observational studies is controversial, as it is for RCTs^[28,29]. Instead, we performed subgroup and sensitivity analyses, as is recommended.

Data extraction

Two reviewers (Lytras T, Bonovas S) abstracted the data independently. The following information was collected from each study: (1) citation data, first author's last name, year of publication, and country of the population studied; (2) study design; (3) number of subjects; (4) relative risk (RR) and 95%CI; (5) for RCTs, the number of events (colorectal cancer cases) in the statin and control groups; (6) definition of statin exposure; and (7) control for confounding factors by matching or adjustment, if applicable.

Risk ratios and 95%CI were calculated for each RCT by reconstructing contingency tables based on the number of subjects randomly assigned and the number of subjects with incident colorectal cancer (analysis in accordance with the intention-to-treat principle). In observational studies, we extracted the RR estimates that reflected the greatest degree of control for potential confounders. Differences in data extraction were resolved by consensus, referring back to the original article.

Statistical analysis

We included in this meta-analysis studies reporting different measures of relative risk: RCTs (risk ratio), cohort studies (rate ratio, hazard ratio), and case-control studies (odds ratio). In practice, these measures of effect yield very similar RR estimates, since the absolute risk of colorectal cancer is very low^[30].

Studies were grouped on the basis of study design, and three separate meta-analyses were conducted: one each for RCTs, cohort studies and case-control studies, using both fixed-effects and random-effects models. This was done to examine consistency of results across varying study designs with different potential biases. We also compared the summary RR estimates derived from the three separate meta-analyses with a test of interaction^[31].

Each meta-analysis was performed twice, assuming either a fixed-effects or a random-effects model. In the absence of heterogeneity, the fixed-effects and the random-effects models provide similar results. When heterogeneity is found, the random-effects model is considered to be more appropriate, though both models may be biased^[32].

For all statistical analyses we used the R software environment^[33], version 3.0.1, and the “meta” package for R^[34], version 2.3-0. For RCTs we used the function “metabin” to perform meta-analysis of binary outcome data, using as input the number of colorectal cases per group in each study; the Mantel-Haenszel method^[35] was used to calculate pooled estimates, and the DerSimonian-Laird method^[36] to estimate between-study variance in the random-effects model. For cohort and case-control studies we used the function “metagen”, inputting the

log RR and its standard error (SE) for each study; the inverse variance weighting method was used to calculate pooled estimates, and the DerSimonian-Laird method^[36] to estimate between-studies variance in the random-effects model. The standard error of the log RR was calculated from the upper (U) and lower (L) limits of the 95%CI published in each study, using the formula $SE = (\log U - \log L) / (2 \times 1.96)$.

Selective outcome reporting or publication bias was assessed using the Begg and Mazumdar adjusted rank correlation test^[37] and the Egger regression asymmetry test^[38]. To evaluate whether the results of the studies were homogeneous, we used the Cochran's *Q* test with a 0.10 level of significance. We also calculated the *I*² statistic^[39] that describes the percentage variation across studies that is due to heterogeneity rather than chance. Negative values of *I*² were put equal to zero, so that *I*² lies between 0% (*i.e.*, no observed heterogeneity) and 100%. We regarded an *I*² value less than 40% as indicative of “not important heterogeneity” and a value higher than 75% as indicative of “considerable heterogeneity”^[40].

All *P*-values are two-tailed. For all tests (except for heterogeneity), a probability level less than 0.05 was considered statistically significant. This work was performed according to the guidelines proposed by the Meta-analysis of Observational Studies in Epidemiology (MOOSE) group^[41], and the PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) statement^[42].

RESULTS

Search results

The results of our search process are presented on Figure 1. We identified and analyzed 40 independent studies that met the predefined inclusion criteria^[4,5,24,25,39-74]. Eight out of 40 were RCTs of statins for cardiovascular outcomes^[4,5,43-48], 13 were cohort studies^[49-61], and 19 were case-control studies^[25,26,62-78]. Sixteen studies had been included in the previous meta-analysis^[4,5,25,45-48,60,61,72-78] while 24 were newly published^[26,43,44,49-59,62-71], of which 2 RCTs^[43,44], 11 cohort studies^[49-59] and 11 case-control studies^[26,62-71].

In total, the 40 studies involved more than 8.2 million subjects. The number of colorectal cancer cases ranged from 32 to 245 in the RCTs, from 76 to 6637 in the cohort studies, and from 56 to 36736 in the case-control studies. Three out of the 40 studies had been published solely in abstract form^[73-75], and one was an academic dissertation^[62]. One study from Japan^[54] was a pooled analysis of two randomized trials and one cohort study, providing a single summary RR for statin use and colorectal cancer; we treated this as a single cohort study.

Seven out of eight RCTs were placebo-controlled^[4,5,43-45,47,48], while one RCT was a non-blinded trial comparing statin treatment with a usual care control group^[46]. All RCTs reported site-specific cancer outcomes, including colorectal cancer, as secondary endpoints. We were thus able to conduct a post hoc analysis of these trials and calculate risk

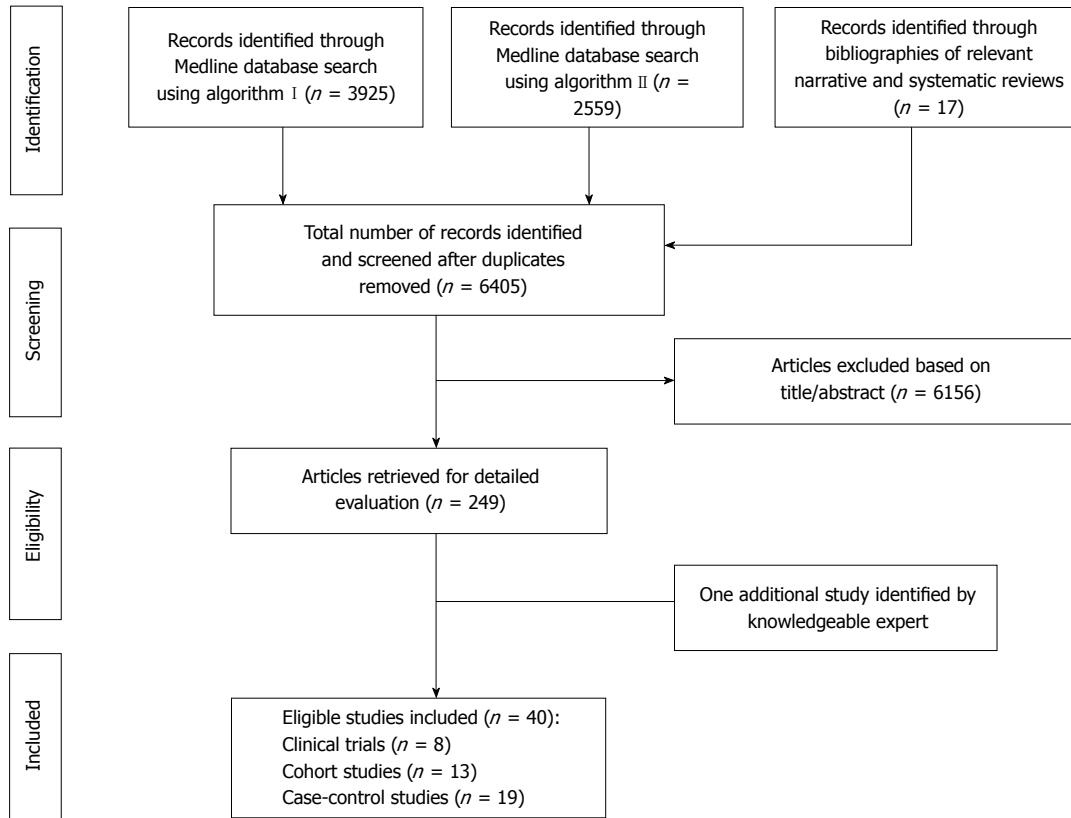


Figure 1 Summary of evidence search and selection.

Table 1 Randomized controlled trials included in the meta-analysis

Study	Agent	All subjects	Duration (yr)	Incident colorectal cancer cases		RR	95%CI
				Statin group	Control group		
JUPITER ^[43]	Rosuvastatin	16304	Median: 2.0	16 of 8154	28 of 8150	0.57	0.31-1.05
WOSCOPS ^[44]	Pravastatin	6577	Mean: 4.9	12 of 3291	20 of 3286	0.60	0.29-1.23
4S ^[47]	Simvastatin	4444	Median: 10.4	25 of 2221	32 of 2223	0.78	0.46-1.32
ALLHAT-LLT ^[46]	Pravastatin	10355	Mean: 4.8	46 ¹ of 5170	38 ¹ of 5185	1.21	0.79-1.86
HPS ^[45]	Simvastatin	20536	Mean: 5.0	114 of 10269	131 of 10267	0.87	0.68-1.12
LIPID ^[48]	Pravastatin	9014	Mean: 8.0	75 of 4512	71 of 4502	1.05	0.76-1.45
AFCAPS ^[5]	Lovastatin	6605	Mean: 5.2	25 ¹ of 3304	20 ¹ of 3301	1.25	0.70-2.24
CARE ^[4]	Pravastatin	4159	Mean: 4.8	12 of 2081	21 of 2078	0.57	0.28-1.16

¹Figures for colon cancer rather than colorectal cancer.

ratios for colorectal cancer in an intention-to-treat analysis. All observational studies^[24,25,47-76] evaluated exposure to statins and risk of colorectal cancer, and were controlled for various potential confounding factors by matching or adjustments. The publication dates of the studies included in the meta-analysis ranged between 1996 and 2013. Study designs, along with the RR estimates and 95%CI are shown in Table 1 for the RCTs, in Table 2 for the cohort studies, and in Table 3 for the case-control studies.

Meta-analysis of RCTs

Eight large RCTs contributed to the analysis^[4,5,43-48]. A total of 77994 individuals participated in these trials; 39002 in treatment groups and 38992 in control groups. The participants had a mean follow-up of approximately 5.0

years and a total experience of 390000 person-years. Five trials^[4,43-45,47] reported a lower risk of colorectal cancer in the treatment group, while the other three trials^[5,46,48] reported a higher risk (Table 1). None was statistically significant. The overall rate of colorectal cancer on all 8 RCTs was 0.83% in the statin group (325 incident cases) and 0.93% in the control group (361 incident cases). We found a modest but not statistically significant protective association (about 10% risk reduction) of statin use against colorectal cancer, both under the assumption of a fixed-effects model (RR = 0.90, 95%CI: 0.78-1.04), or a random-effects model (RR = 0.89, 95%CI: 0.74-1.07) (Table 4). Figure 2 shows the forest plot of the RR estimates and 95%CI from the individual trials and the pooled results. The Cochran's *Q* test had a *P*-value of

Table 2 Cohort studies included in the meta-analysis

Study	Study location	All subjects	CRC cases	RR	95%CI	Control for potential confounding factors ²
Clancy <i>et al</i> ^[50] , 2013	Italy	266109	2420	0.84	0.76-0.93	1-9
Leung <i>et al</i> ^[49] , 2013	Taiwan	34205	654	0.57	0.45-0.72 ¹	-
Simon <i>et al</i> ^[51] , 2012	United States	159219	2000	0.99	0.83-1.20	1, 4, 10-27
Jacobs <i>et al</i> ^[53] , 2011	United States	133255	1739	1.03	0.94-1.14 ¹	-
Lee <i>et al</i> ^[52] , 2011	United States	131922	1680	0.97	0.84-1.12	1, 6, 10, 12, 13, 28-34
Haukka <i>et al</i> ^[55] , 2010	Finland	944962	5016	1.04	0.98-1.10 ¹	-
Matsushita <i>et al</i> ^[54] , 2010	Japan	13724	76	1.22	0.77-1.94	-
Flick <i>et al</i> ^[57] , 2009	United States	69115	171	0.89	0.61-1.30	1, 4, 6, 10-13, 15, 18, 19, 28, 35-38
Singh <i>et al</i> ^[56] , 2009	Canada	413271	6637	1.13	1.02-1.25	1-4, 6, 7, 16, 39-42
Friedman <i>et al</i> ^[58] , 2008	United States	4243067	5684	0.88	0.82-0.96 ¹	-
Farwell <i>et al</i> ^[59] , 2008	United States	62842	687	0.65	0.55-0.78	1, 6, 11, 13, 19, 29, 43-53
Setoguchi <i>et al</i> ^[60] , 2007	United States	31723	249	0.96	0.70-1.31	1-9, 39, 43, 54-64
Friis <i>et al</i> ^[61] , 2005	Denmark	334754	3006	0.85	0.65-1.11	1, 2, 4, 16, 66

¹Calculated crude relative risk (RR); ²1: Age; 2: Sex; 3: Inflammatory bowel disease; 4: Use of nonsteroidal anti-inflammatory drugs; 5: Obesity; 6: Colonoscopy; 7: Comorbidity score; 8: Distinct generic medicines taken; 9: Prior hospitalizations; 10: Body mass index; 11: Smoking status; 12: Family History of colorectal cancer; 13: Alcohol use; 14: Education; 15: Physical activity level; 16: Hormone replacement therapy; 17: Ethnic group; 18: Colorectal polyps; 19: Cardiovascular disease; 20: Calcium intake; 21: Percent energy from fat; 22: Fruit and vegetable intake; 23: Calcium supplement use; 24: Selenium supplement use; 25: Current healthcare provider; 26: Last medical visit within one year; 27: Colon screening; 28: Red meat consumption; 29: Use of aspirin; 30: Calendar year; 31: Study; 32: Pack-years of smoking before age 30; 33: Height; 34: Total energy intake; 35: Hypercholesterolemia; 36: Multivitamin use; 37: Energy-adjusted fibre intake; 38: Folate intake; 39: Benign mammary dysplasia; 40: Coronary heart disease; 41: Resective colorectal surgery; 42: Socioeconomic status; 43: Diabetes; 44: Weight; 45: Thyroid disease; 46: Hypertension; 47: Renal failure; 48: Chest pain; 49: Mental illness; 50: Lung disease; 51: Gastrointestinal Disease; 52: Prostate disease; 53: Total cholesterol; 54: Race; 55: Arthritis; 56: Use of gastroprotective drugs; 57: Estrogen use; 58: Tobacco abuse; 59: Mammography; 60: Gynecologic examination; 61: Pap smear; 62: Fecal occult blood; 63: Number of physician visits; 64: Prior nursing home stay; 65: History of heart attack; 66: Use of cardiovascular drugs. CRC: Colorectal cancer.

Table 3 Case-control studies included in the meta-analysis

Study	Study location	All subjects	CRC cases	OR	95%CI	Control for potential confounding factors ²
Deshpande ^[62] , 2013	United States	73472	36736	0.96	0.89-1.05	1-4
Broughton <i>et al</i> ^[64] , 2012	United Kingdom	233	101	0.43	0.25-0.80	1, 2, 5-8
Lakha <i>et al</i> ^[63] , 2012	United Kingdom	603	309	0.33	0.15-0.69	1-3, 9-15
Cheng <i>et al</i> ^[65] , 2011	Taiwan	5780	1156	1.09	0.91-1.30	1-3, 5, 8, 9, 16-21
Vinogradova <i>et al</i> ^[26] , 2011	United Kingdom	60373	11749	1.07	1.00-1.15	1-3, 7, 9, 11, 12, 22-27
Robertson <i>et al</i> ^[66] , 2010	Denmark	109769	9979	0.87	0.80-0.96	1, 2, 5-7, 9, 10, 19
Hachem <i>et al</i> ^[68] , 2009	United States	30400	6080	0.91	0.86-0.96	1-4, 9, 16, 19, 20, 28, 29
Shadman <i>et al</i> ^[67] , 2009	United States	2044	669	1.17	0.74-1.85	1, 4, 11, 12, 30-33
Boudreau <i>et al</i> ^[70] , 2008	United States	1330	665	1.02	0.65-1.59	1, 2, 5, 9, 11, 12, 32
Yang <i>et al</i> ^[69] , 2008	United Kingdom	48724	4432	1.10	0.50-2.20	1, 2, 4, 6, 7, 9, 11, 12, 32, 34
Hoffmeister <i>et al</i> ^[71] , 2007	Germany	1154	540	0.69	0.45-1.06	1, 2, 4-7, 9, 11, 12, 30-32, 35-37
Coogan <i>et al</i> ^[72] , 2007	United States	3618	1809	0.92	0.78-1.09	1, 2, 4, 9, 10
Li <i>et al</i> ^[73] , 2006	United States	741	339 ¹	0.8	0.34-1.87	1, 2, 5, 6, 9, 11, 12, 30, 38
Poynter <i>et al</i> ^[25] , 2005	Israel	3968	1953	0.53	0.38-0.74	1, 2, 9, 30, 36, 39-41
Rubin <i>et al</i> ^[74] , 2005	United States	387240	18440	0.92	0.89-0.96	1, 2, 4, 9, 17, 42-45
Kaye <i>et al</i> ^[76] , 2004	United Kingdom	18088	329 ¹	1.0	0.6-1.7	1, 2, 11, 12, 42
Graaf <i>et al</i> ^[77] , 2004	Netherlands	20105	292 ¹	0.87	0.48-1.57	1, 2, 5, 9, 17, 18, 32, 34, 46-49
Khurana <i>et al</i> ^[75] , 2004	United States	534273	5339 ¹	0.94	0.89-1.00	1, 2, 6, 9, 12, 38, 50
Blais <i>et al</i> ^[78] , 2000	Canada	5962	56 ¹	0.83	0.37-1.89	1, 2, 14, 18, 34, 46

¹Figures for colon cancer rather than colorectal cancer; ²1: Age; 2: Sex; 3: Inflammatory bowel disease; 4: Colonoscopy; 5: Diabetes; 6: Alcohol use; 7: Use of aspirin; 8: Use of metformin; 9: Use of NSAIDs; 10: Precinct of residence; 11: Body mass index; 12: Smoking status; 13: Physical activity level; 14: History of neoplasia; 15: Family history of cancer; 16: Fecal occult blood; 17: Prior hospitalizations; 18: Other lipid-lowering therapy; 19: Cholecystectomy; 20: Liver disease; 21: Colorectal polyps; 22: History of heart attack; 23: Hypertension; 24: Coronary heart disease; 25: Socioeconomic status; 26: Rheumatoid arthritis; 27: Use of COX-2 inhibitors; 28: Diabetic nephropathy; 29: Use of sulfonylurea; 30: Family history of colorectal cancer; 31: Education; 32: Hormone replacement therapy; 33: Calendar year; 34: Duration of follow-up; 35: Red meat consumption; 36: Hypercholesterolemia; 37: History of rheumatic disease; 38: Race; 39: Ethnic group; 40: Sports participation; 41: Level of vegetable consumption; 42: Number of physician visits; 43: Use of glucocorticosteroids; 44: Use of immunomodulators; 45: Use of 5-aminosalicylic acids; 46: Comorbidity score; 47: Use of diuretics; 48: Use of ACE inhibitors; 49: Use of CCBs; 50: Obesity. CRC: Colorectal cancer.

0.23 and the corresponding I^2 statistic was 25%, both indicating little variability between studies that cannot be explained by chance. The P -values for the Begg's and the Egger's tests were $P = 0.22$ and $P = 0.31$, respectively,

both suggesting a low probability of selective outcome reporting or publication bias. Figure 3 shows the contribution of the randomized studies (represented by the blue points) in the overall funnel plot, while Figure 4

Table 4 Meta-analysis results

	No. of studies	Fixed-effects model		Random-effects model		Tests of homogeneity			Tests of publication bias	
		RR	95%CI	RR	95%CI	Q value (df)	P value	I ²	Begg's P value	Egger's P value
Randomized Controlled Trials	8	0.90	0.78-1.04	0.89	0.74-1.07	9.31 (7)	0.23	25%	0.22	0.31
Placebo-controlled RCTs	7	0.86	0.74-1.01	0.85	0.71-1.03	7.21 (6)	0.30	17%	0.18	0.24
RCTs of lipophilic statins	3	0.90	0.73-1.10	0.90	0.73-1.10	1.55 (2)	0.46	0%	0.60	0.70
RCTs of lipophobic statins	5	0.90	0.73-1.12	0.83	0.60-1.15	7.75 (4)	0.10	48%	0.62	0.05
Cohort Studies	13	0.96	0.93-0.99	0.91	0.83-1.00	70.85 (12)	< 0.001	83%	0.54	0.22
Case-control studies	19	0.93	0.91-0.96	0.92	0.87-0.98	50.31 (18)	< 0.001	64%	0.46	0.27
Published in full text form	16	0.94	0.91-0.98	0.90	0.83-0.99	49.12 (15)	< 0.001	69%	0.47	0.19
Observational studies	32	0.94	0.92-0.96	0.92	0.87-0.96	122.68 (31)	< 0.001	75%	0.36	0.16
All studies	40	0.94	0.92-0.96	0.91	0.87-0.96	132.3 (39)	< 0.001	71%	0.33	0.11

RCT: Randomized controlled trials.

Randomized controlled trials	RR	95%CI	Weights
JUPITER	0.57	0.31-1.05	7.8%
WOSCOPS	0.60	0.29-1.22	6.0%
4S	0.78	0.46-1.32	10.3%
ALLHAT-LLT	1.21	0.79-1.86	13.9%
HPS	0.87	0.68-1.12	27.0%
LIPID	1.05	0.76-1.45	20.4%
AFCAPS	1.25	0.70-2.24	8.5%
CARE	0.57	0.28-1.16	6.1%
Pooled effect estimate (n = 8)	0.89	0.74-1.07	100%
Cohort studies	RR	95%CI	Weights
Clancy <i>et al.</i> , 2013	0.84	0.76-0.93	9.9%
Leung <i>et al.</i> , 2013	0.57	0.45-0.72	6.5%
Simon <i>et al.</i> , 2012	0.99	0.82-1.19	7.8%
Jacobs <i>et al.</i> , 2011	1.03	0.94-1.13	10.0%
Lee <i>et al.</i> , 2011	0.97	0.84-1.12	8.9%
Haukka <i>et al.</i> , 2010	1.04	0.98-1.10	10.8%
Matsushita <i>et al.</i> , 2010	1.22	0.77-1.94	3.0%
Flick <i>et al.</i> , 2009	0.89	0.61-1.30	3.9%
Singh <i>et al.</i> , 2009	1.13	1.02-1.25	9.9%
Friedman <i>et al.</i> , 2008	0.88	0.81-0.95	10.4%
Farwell <i>et al.</i> , 2008	0.65	0.55-0.77	8.1%
Setoguchi <i>et al.</i> , 2007	0.96	0.70-1.31	4.9%
Friis <i>et al.</i> , 2005	0.85	0.65-1.11	5.8%
Pooled effect estimate (n = 13)	0.91	0.83-1.00	100%
Case-control studies	OR	95%CI	Weights
Deshpande, 2013	0.96	0.88-1.04	11.5%
Broughton <i>et al.</i> , 2012	0.43	0.24-0.77	0.9%
Lakka <i>et al.</i> , 2012	0.33	0.15-0.71	0.5%
Cheng <i>et al.</i> , 2011	1.09	0.91-1.30	6.1%
Vinogradova <i>et al.</i> , 2011	1.07	1.00-1.15	12.4%
Robertson <i>et al.</i> , 2010	0.87	0.79-0.95	10.9%
Hachem <i>et al.</i> , 2009	0.91	0.86-0.96	13.3%
Shadman <i>et al.</i> , 2009	1.17	0.74-1.85	1.4%
Boudreau <i>et al.</i> , 2008	1.02	0.65-1.60	1.5%
Yang <i>et al.</i> , 2008	1.10	0.52-2.31	0.6%
Hoffmeister <i>et al.</i> , 2007	0.69	0.45-1.06	1.6%
Coogan <i>et al.</i> , 2007	0.92	0.78-1.09	6.6%
Li <i>et al.</i> , 2006	0.80	0.34-1.88	0.4%
Poynter <i>et al.</i> , 2005	0.53	0.38-0.74	2.5%
Rubin <i>et al.</i> , 2005	0.92	0.89-0.96	14.2%
Kaye and Jick, 2004	1.00	0.59-1.68	1.1%
Graaf <i>et al.</i> , 2004	0.87	0.48-1.57	0.9%
Khurana <i>et al.</i> , 2004	0.94	0.89-1.00	13.1%
Blais <i>et al.</i> , 2000	0.83	0.37-1.88	0.5%
Pooled effect estimate (n = 19)	0.92	0.87-0.98	100%

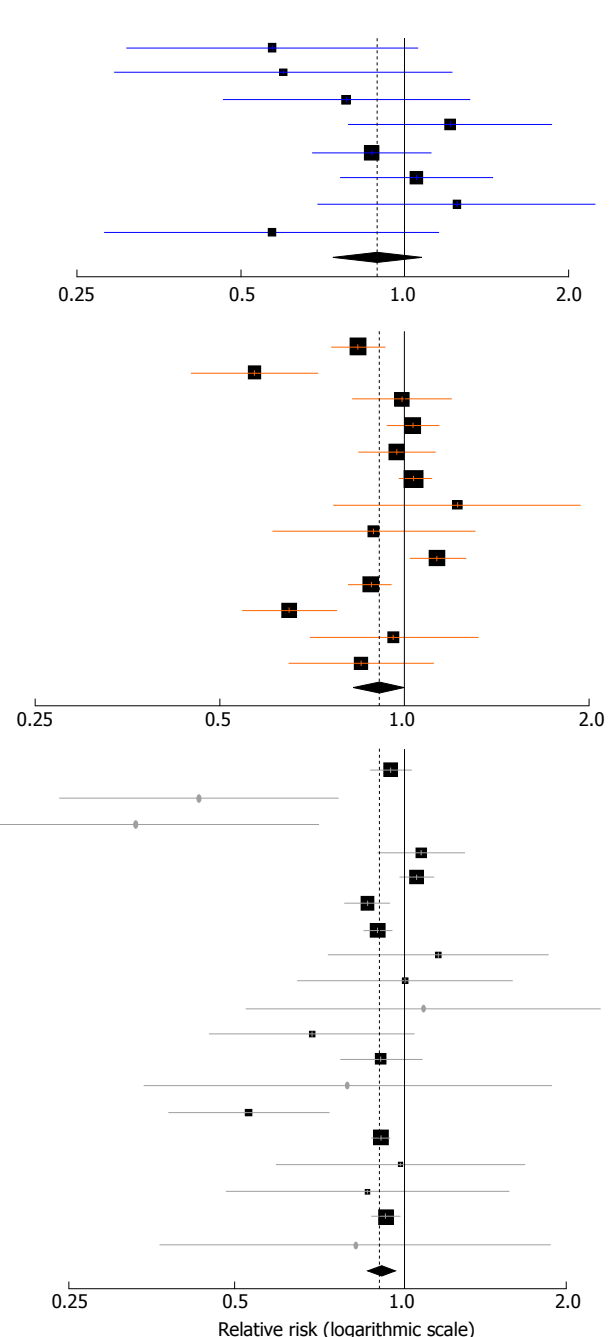


Figure 2 Forest plot: results from individual studies and meta-analyses. The RR and 95%CI for each study are displayed on a logarithmic scale. Pooled estimates are from a random-effects model.

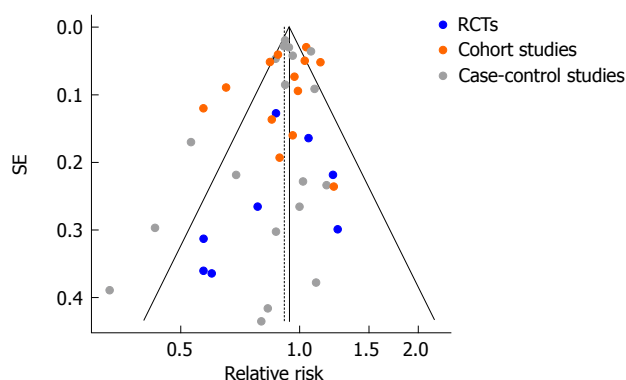


Figure 3 Funnel plot of observed relative risk against standard error (as a surrogate of study size) for all studies analyzed. RCT: Randomized controlled trials.

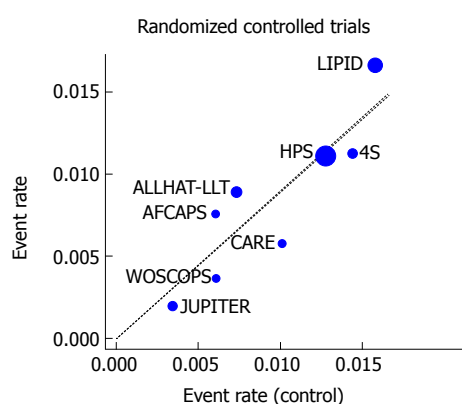


Figure 4 L'Abbé plot of the incidence of colorectal cancer in the experimental (statin) group, against the incidence in the control group, across the analyzed randomized controlled trials ($n = 8$).

shows the incidence of colorectal cancer in the statin group against the incidence in the control group, across the 8 RCTs (L'Abbé plot)^[79]. This plot demonstrates no substantial heterogeneity between studies, and no relation of statin effect to baseline risk of colorectal cancer.

When the analysis was restricted to trials that evaluated statin therapy compared with placebo^[4,3,43-45,47,48], the results did not substantially change (fixed-effects model: $RR = 0.86$, 95%CI: 0.74-1.01; random-effects model: $RR = 0.85$, 95%CI: 0.71-1.03; Cochran's $P = 0.30$ and $I^2 = 17\%$; Begg's $P = 0.18$ and Egger's $P = 0.24$). Similarly, after stratifying the data in two subgroups (lipophilic^[5,45,47] vs lipophobic statins^[4,43,44,46,48]), we did not find any statistically significant association between lipophilic or lipophobic statins and risk of colorectal cancer (Table 4). Last, to explore whether the results were dominated by a single study, we performed a "leave-one-out" sensitivity analysis, removing one study at a time (Figure 5A). This approach confirmed the stability of our results.

Meta-analysis of cohort studies

Thirteen cohort studies^[49-61] evaluating exposure to statins and colorectal cancer risk were included in the meta-analysis (Table 2, Figure 2). Approximately seven million

patients participated in these studies, with the occurrence of 30019 colorectal cancer cases. Four cohort studies reported not an overall, but two or more "correlated" subgroup effect-estimates^[49,53,55,58]; however, based on the available data, we were able to calculate study-specific crude RR estimates for these four studies for the purpose of our meta-analysis (Table 2).

Statin use was associated with a modest reduction in the risk of colorectal cancer, and this association reached statistical significance both under a fixed-effects model ($RR = 0.96$, 95%CI: 0.93-0.99) and under a random-effects model ($RR = 0.91$, 95%CI: 0.83-1.00) (Table 4, Figure 2). However, the Cochran's Q test had a P -value lower than 0.001 and the corresponding I^2 statistic was 83%, indicating substantial heterogeneity between studies. In the sensitivity analysis (Figure 5B), omitting any single study did not lower the I^2 further than 78%. The P -values for the Begg's and the Egger's tests were $P = 0.54$ and $P = 0.22$, respectively, suggesting a low probability of publication bias.

Meta-analysis of case-control studies

Nineteen case-control studies^[25,26,62-78] evaluated exposure to statins and colorectal cancer risk (Table 3). A total of 1.3 million patients participated in these studies, of which 100000 were colorectal cancer cases. Once again, statin use was associated with a similar modest reduction in the risk of colorectal cancer, which was statistically significant under both a fixed-effects model ($RR = 0.93$, 95%CI: 0.91-0.96) and a random-effects model ($RR = 0.92$, 95%CI: 0.87-0.98) (Table 4, Figure 2). We found substantial heterogeneity between studies; the Cochran's Q test had a P -value lower than 0.001 and the corresponding I^2 statistic was 64%. In the "leave-one-out" sensitivity analysis, we identified the study by Vinogradova *et al*^[26] as contributing most to the between-studies variability, but not to a crucial degree; excluding this study from the analysis lowered the I^2 to 50%. The P -values for the Begg's and the Egger's tests were $P = 0.46$ and $P = 0.27$, respectively, suggesting a low probability of publication bias.

When the analysis was limited to studies published in full-text, *i.e.*, excluding those published solely in abstract form^[73-75], the results did not appreciably change (Table 4). The association between statins and colorectal cancer risk remained statistically significant assuming either a fixed-effects model ($RR = 0.94$, 95%CI: 0.91-0.98), or a random-effects model ($RR = 0.90$, 95%CI: 0.83-0.99). The Cochran's Q test had a P -value lower than 0.001, and the corresponding I^2 was 69%. The P -values for the Begg's and the Egger's tests were $P = 0.47$ and $P = 0.19$, respectively, but the funnel plot was slightly asymmetric, indicating a small likelihood of publication bias. Thus, selective publication of smaller case-control studies with statistically significant results might have occurred to some extent. It should be noted, however, that the result of our meta-analysis of case-control studies was fairly robust in the "leave-one-out" sensitivity analysis (Figure

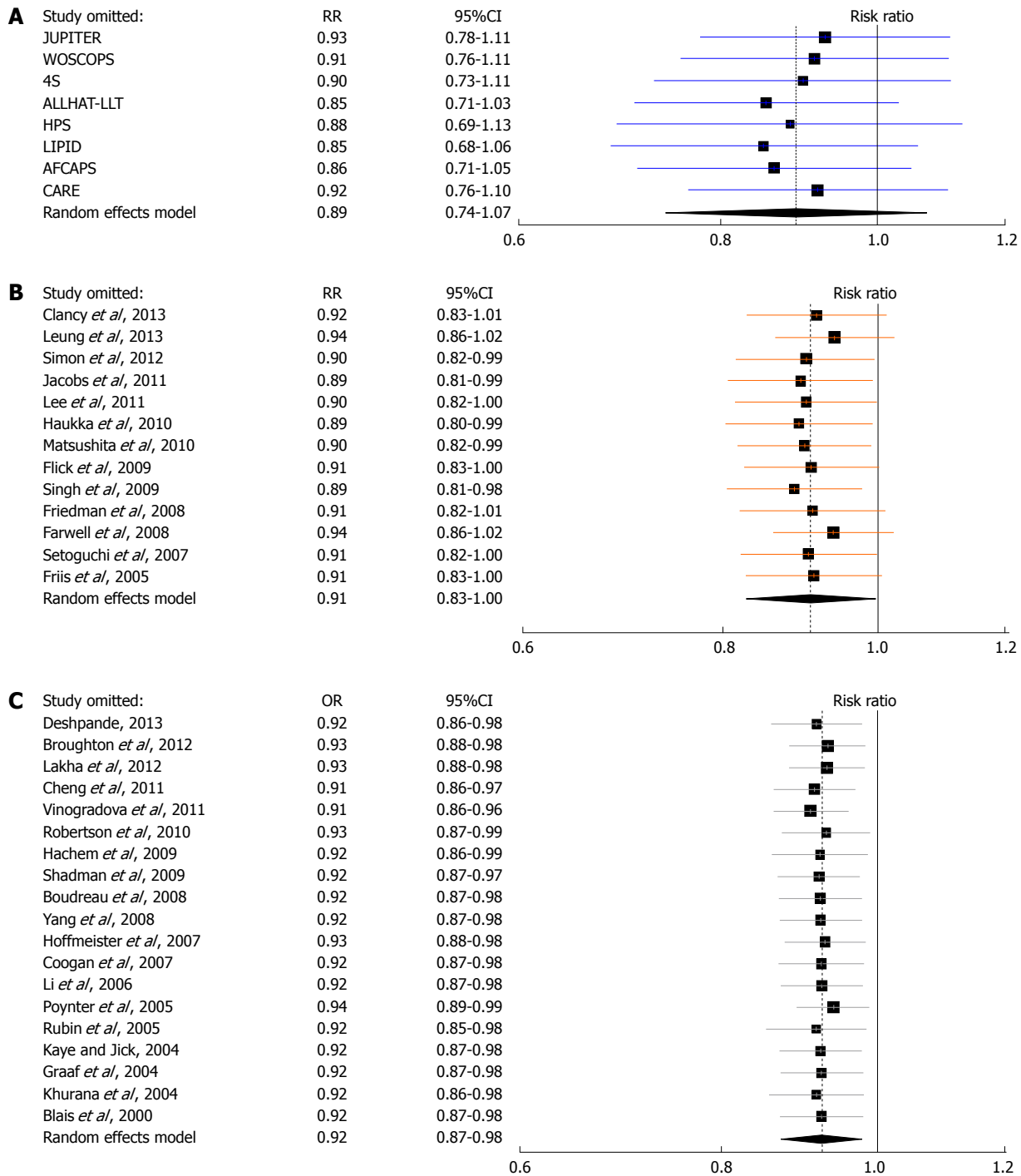


Figure 5 “Leave-one-out” sensitivity analysis for the three meta-analyses: pooled estimates are from random-effects models with one study omitted at a time. A: Randomized controlled trials, B: Cohort studies, C: Case-control studies.

5C); removal of any single study did not alter the statistical significance of the pooled estimate.

Combined analysis

We compared pairwise the pooled RR estimates derived from the three separate analyses with a test of interaction^[31]. We found no statistically significant differences between estimates, either between those assuming a fixed-effects model (RCTs *vs* cohort studies, $\chi^2 = 0.81$, $P = 0.42$; RCTs *vs* case-control studies, $\chi^2 = 0.49$, $P = 0.62$;

cohort studies *vs* case-control studies, $\chi^2 = 1.23$, $P = 0.22$) or those assuming a random-effects model (RCTs *vs* cohort studies, $\chi^2 = 0.18$, $P = 0.86$; RCTs *vs* case-control studies, $\chi^2 = 0.36$, $P = 0.72$; cohort studies *vs* case-control studies, $\chi^2 = 0.30$, $P = 0.76$).

In addition, we performed a combined analysis of observational studies, *i.e.*, cohort and case-control studies (Table 4). Statin use was again associated with a modest reduction in the risk of colorectal cancer, which was statistically significant assuming either a fixed-effects

model (RR = 0.94, 95%CI: 0.92-0.96) or a random-effects model (RR = 0.92, 95%CI: 0.87-0.96; $n = 32$). The Cochran's Q test had a P -value lower than 0.001 and the corresponding I^2 statistic was 75%, indicating substantial between-studies variability. The P -values for the Begg's and the Egger's tests were $P = 0.36$ and $P = 0.16$, respectively, both suggesting a very low probability of publication bias.

Combining all 40 studies for analysis yielded very similar results (Table 4). This is expected, as this particular analysis was dominated by the observational studies (36 studies; 8.1 million participants). These studies accounted for 92.3% and the 98.5% of the weight in the fixed- and the random-effects model, respectively.

Finally, we attempted to analyze the effect of statins separately in colon and rectal cancer. Six cohort studies (out of 13) and five case-control studies (out of 19) provided results by colorectal cancer subsite. In these 11 studies, statins did not appear to have an effect on colon cancer, assuming either a fixed-effects model (RR = 0.97, 95%CI: 0.93-1.02), or a random-effects model (RR = 0.95, 95%CI: 0.87-1.04). The Cochran's Q test had a P -value of 0.02, and the corresponding I^2 was 54%. The p -values for the Begg's and the Egger's tests were $P = 0.31$ and $P = 0.39$, respectively. As regards rectal cancer, the fixed-effects model suggested no effect of statins (RR = 0.98, 95%CI: 0.91-1.05) but the random-effects model suggested a statistically significant effect (RR = 0.78, 95%CI: 0.62-0.97). The Cochran's Q test had a P -value lower than 0.001 and the corresponding I^2 was 79%, indicating substantial heterogeneity. The P -values for the Begg's and the Egger's tests were $P = 0.59$ and $P = 0.06$, respectively, which highlights a significant potential for selective outcome reporting bias in this analysis.

DISCUSSION

Cancer chemoprevention is an area of research that focuses on cancer prevention through pharmacological, biological, and nutritional interventions^[80,81]. In recent years, a growing body of studies suggests that statins may have chemopreventive potential against cancer^[14,15]. However, these hypotheses have not been confirmed by meta-analyses on the association between statin use and most site-specific cancers^[82-86]. On the other hand, concerns have also been raised about the safety of statins, especially among elderly patients^[87-89].

Meta-analysis is a systematic and quantitative integration of the results of a set of independent studies. It allows for an objective appraisal of the epidemiological evidence, which may lead to resolution of uncertainty and disagreement^[90]. We undertook this updated meta-analysis to examine the latest evidence on the association of statin use and colorectal cancer risk. Our results again exclude the strong protective effect (47% risk reduction) of statins first noted in the study by Poynter *et al.*^[25]. However, a more mixed picture emerges from the 40 studies included in the analysis; a modest (on the order

of 10% risk reduction) protective effect of statin use at therapeutic doses against colorectal cancer cannot be excluded by these data.

The point estimates from the three individual meta-analyses were almost identical (RCTs, RR = 0.89; cohort studies, RR = 0.91; case-control studies, OR = 0.92; results from random-effects models), with the effect reaching statistical significance for cohort and case-control studies, but not for RCTs. This is not unexpected, as these were RCTs with cardiovascular primary outcomes and mean follow-up was short (range: 2.0-10.4 years) with only two studies exceeding 6 years; in comparison, a single pre-existing adenomatous colorectal polyp typically requires 10-15 years to evolve into clinically invasive cancer^[91]. As a result, there were few incident colorectal cancer cases and low statistical power in these trials to detect any effect of statin use. More importantly, however, the follow-up time may be insufficient in order for statins to meaningfully affect the neoplastic process and demonstrate an effect on colorectal cancer incidence. For this reason, any potential such effect of statins in these trials might reflect a slower evolution of pre-existing premalignant lesions, rather than a lower incidence of new lesions.

Our result for RCTs is not inconsistent with that of a recent individual patient data meta-analysis from the Cholesterol Treatment Trialists' (CTT) Collaboration^[22], which showed a lack of effect of statin use on colorectal cancer incidence (RR = 0.97, 95%CI: 0.87-1.09). That study included 27 RCTs that had the same limitations as the eight we included, *i.e.*, short follow-up time (mean: 4.1 years), few colorectal cancer cases (1114 in total, overall rate of 0.64%) and ascertainment of colorectal cancer as a secondary outcome. It should also be noted that our findings for RCTs are consistent with those corresponding to the association between fibrates and colorectal cancer risk (RR = 0.98, 95%CI: 0.71-1.34), reported in a recent systematic review and meta-analysis of our research group^[92].

Because of the limitations of RCTs, it is important to also examine the association of statin use and colorectal cancer risk through observational evidence. In our study, meta-analysis of both cohort and case-control studies revealed statistically significant effects, but high between-studies heterogeneity. This heterogeneity is important to consider, because it may point to a variable effect of statin use in different populations and in different colorectal cancer subtypes. Colorectal cancer is a heterogeneous disease in terms of molecular subtypes^[93], and inherited genetic susceptibility plays a role in a significant proportion of cases^[94]. The observational studies analyzed were performed in diverse populations from eleven countries, each of whom might have a different susceptibility profile and different molecular epidemiology of colorectal cancer. Pharmacogenomics might play an important role, and indeed evidence has emerged about a particular gene polymorphism that modifies the effect of statins on colorectal cancer^[95]. Therefore, substantial

differences may underlie the overall effect observed in our meta-analysis.

When undertaking a meta-analysis of observational studies, bias and confounding may always be an alternative explanation for the results. The 13 cohort and 19 case-control studies were statistically controlled for a large number of potential confounders, but adjustment for too many factors can itself introduce bias^[96], and adjustment for different factors in different studies may also explain the substantial heterogeneity observed. In any event, the possibility of residual confounding cannot be excluded, either from unknown or unmeasured factors, or from imperfectly adjusted real confounders.

In this context, one such confounder of particular interest is the socioeconomic status of patients^[97], which may underlie important differences between statin users and non-users as regards lifestyle choices and health-seeking behaviors. Notably, despite the large number of potential confounders controlled for by the 32 observational studies included in our analysis, only two studies^[26,56] adjusted their results for socioeconomic status.

Selection bias and publication bias is another possibility that could affect both randomized and observational studies. Our literature search was as fully inclusive as possible, and we did not exclude any study because of methodological characteristics or subjective quality criteria. Nevertheless, we did not search for unpublished studies or original data. The Begg's and the Egger's tests for all three study types did not show a high likelihood of selective outcome reporting or publication bias, and the funnel plot showed no obvious asymmetry for RCTs and cohort studies, but a slight asymmetry for case-control studies. Thus selective publication of smaller case-control studies with statistically significant results might have occurred to some extent. It should be noted, however, that the result of our meta-analysis of case-control studies was fairly robust in the sensitivity analysis.

By similar reasoning, our sub-analyses of the effect of statins on colon and rectal cancer should be interpreted with caution. A differential effect of statins by colorectal cancer subsite is biologically plausible, due to differences in embryology and physiology. In our analysis, however, available data were scarce (only 11 of 32 observational studies) and there is no way to evaluate and control for outcome reporting bias.

In conclusion, it is safe to say that statins do not appear to strongly reduce the overall risk of colorectal cancer in the general population, at the low doses for managing hypercholesterolemia. However, there is some evidence to suggest a modest overall risk reduction, which could be a composite of an effect of statins in some populations and some colorectal cancer types, and lack of effect in others. Therefore, we believe it is not the end of the road, as has been suggested^[98], for statins and colorectal cancer; rather a new approach is needed. One needs to focus more on basic research and pharmacogenomics, and perform epidemiological studies and clinical trials on high risk populations that might

be more likely to benefit from statins, either as primary chemoprevention or as an adjuvant to treatment. In the meantime, the use of statins should remain restricted to the approved indications.

COMMENTS

Background

Statins are some of the most widely prescribed drugs worldwide, as a result of their proven efficacy in the primary and secondary prevention of cardiovascular morbidity and mortality. Besides their main mechanism of action through the reduction of serum cholesterol, by means of competitive inhibition of hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase, statins exert a variety of "pleiotropic" effects. It has been suggested that statins might play a role in cancer chemoprevention, and data from in vitro and animal model studies have been encouraging.

Research frontiers

The relation between statins and colorectal cancer has been the focus of a growing body of epidemiological research, with often inconsistent results ranging from very protective to moderately harmful.

Innovations and breakthroughs

The authors sought to update their previous systematic review and meta-analysis to reflect the current totality of evidence on statins and colorectal cancer risk.

Applications

The authors believe it is not the end of the road, as has been suggested, for statins and colorectal cancer; rather a new approach is needed. One needs to focus more on basic research and pharmacogenomics, and perform epidemiological studies and clinical trials on high risk populations that might be more likely to benefit from statins, either as primary chemoprevention or as an adjuvant to treatment. In the meantime, the use of statins should remain restricted to the approved indications.

Terminology

Cancer chemoprevention is an area of research that focuses on cancer prevention through pharmacological, biological, and nutritional interventions. Meta-analysis is a systematic and quantitative integration of the results of a set of independent studies. It allows for an objective appraisal of the epidemiological evidence, which may lead to resolution of uncertainty and disagreement.

Peer review

The article is interesting and welcome. It is written by a team with experience in the meta-analysis and who has published scientific papers in the field of colorectal cancer. Choice trials were correctly made and motivated. Statistical analysis is laborious and thorough. The results are analyzed and discussed with competence. The risk reduction of colon cancer is properly justified, as the need for future studies. Figures are properly made, and the bibliography is correctly written.

REFERENCES

- 1 Walley T, Folino-Gallo P, Schwabe U, van Ganse E. Variations and increase in use of statins across Europe: data from administrative databases. *BMJ* 2004; **328**: 385-386 [PMID: 14962875 DOI: 10.1136/bmj.328.7436.385]
- 2 Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet* 1994; **344**: 1383-1389 [PMID: 7968073]
- 3 Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, McKillop JH, Packard CJ. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. *N Engl J Med* 1995; **333**: 1301-1307 [PMID: 7566020 DOI: 10.1056/NEJM199511163332001]
- 4 Sacks FM, Pfeffer MA, Moye LA, Rouleau JL, Rutherford JD, Cole TG, Brown L, Warnica JW, Arnold JM, Wun CC, Davis BR, Braunwald E. The effect of pravastatin on coronary

- events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators. *N Engl J Med* 1996; **335**: 1001-1009 [PMID: 8801446 DOI: 10.1056/NEJM199610033351401]
- 5 **Downs JR**, Clearfield M, Weis S, Whitney E, Shapiro DR, Beere PA, Langendorfer A, Stein EA, Kruyer W, Gotto AM. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. *JAMA* 1998; **279**: 1615-1622 [PMID: 9613910]
 - 6 Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. *N Engl J Med* 1998; **339**: 1349-1357 [PMID: 9841303 DOI: 10.1056/NEJM199811053391902]
 - 7 **Ridker PM**, Danielson E, Fonseca FA, Genest J, Gotto AM, Kastelein JJ, Koenig W, Libby P, Lorenzatti AJ, MacFadyen JG, Nordestgaard BG, Shepherd J, Willerson JT, Glynn RJ. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *N Engl J Med* 2008; **359**: 2195-2207 [PMID: 18997196 DOI: 10.1056/NEJMoa0807646]
 - 8 **Goldstein JL**, Brown MS. Regulation of the mevalonate pathway. *Nature* 1990; **343**: 425-430 [PMID: 1967820 DOI: 10.1038/343425a0]
 - 9 **Witztum JL**, Steinberg D. Role of oxidized low density lipoprotein in atherogenesis. *J Clin Invest* 1991; **88**: 1785-1792 [PMID: 1752940 DOI: 10.1172/JCI115499]
 - 10 **Gazzerro P**, Proto MC, Gangemi G, Malfitano AM, Ciaglia E, Pisanti S, Santoro A, Laezza C, Bifulco M. Pharmacological actions of statins: a critical appraisal in the management of cancer. *Pharmacol Rev* 2012; **64**: 102-146 [PMID: 22106090 DOI: 10.1124/pr.111.004994]
 - 11 **Bonetti PO**, Lerman LO, Napoli C, Lerman A. Statin effects beyond lipid lowering--are they clinically relevant? *Eur Heart J* 2003; **24**: 225-248 [PMID: 12590901]
 - 12 **Davignon J**. Beneficial cardiovascular pleiotropic effects of statins. *Circulation* 2004; **109**: III39-III43 [PMID: 15198965 DOI: 10.1161/01.CIR.0000131517.20177.5a]
 - 13 **Baigent C**, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C, Kirby A, Sourjina T, Peto R, Collins R, Simes R. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. *Lancet* 2005; **366**: 1267-1278 [PMID: 16214597 DOI: 10.1016/S0140-6736(05)67394-1]
 - 14 **Katz MS**. Therapy insight: Potential of statins for cancer chemoprevention and therapy. *Nat Clin Pract Oncol* 2005; **2**: 82-89 [PMID: 16264880 DOI: 10.1038/ncponc0097]
 - 15 **Demierre MF**, Higgins PD, Gruber SB, Hawk E, Lippman SM. Statins and cancer prevention. *Nat Rev Cancer* 2005; **5**: 930-942 [PMID: 16341084 DOI: 10.1038/nrc1751]
 - 16 **Hindler K**, Cleeland CS, Rivera E, Collard CD. The role of statins in cancer therapy. *Oncologist* 2006; **11**: 306-315 [PMID: 16549815 DOI: 10.1634/theoncologist.11-3-306]
 - 17 **Chan KK**, Oza AM, Siu LL. The statins as anticancer agents. *Clin Cancer Res* 2003; **9**: 10-19 [PMID: 12538446]
 - 18 **Boudreau DM**, Yu O, Johnson J. Statin use and cancer risk: a comprehensive review. *Expert Opin Drug Saf* 2010; **9**: 603-621 [PMID: 20377474 DOI: 10.1517/14740331003662620]
 - 19 **Bjerre LM**, LeLorier J. Do statins cause cancer? A meta-analysis of large randomized clinical trials. *Am J Med* 2001; **110**: 716-723 [PMID: 11403756]
 - 20 **Dale KM**, Coleman CI, Henyan NN, Kluger J, White CM. Statins and cancer risk: a meta-analysis. *JAMA* 2006; **295**: 74-80 [PMID: 16391219 DOI: 10.1001/jama.295.1.74]
 - 21 **Bonovas S**, Filioussi K, Tsavaris N, Sitaras NM. Statins and cancer risk: a literature-based meta-analysis and meta-regression analysis of 35 randomized controlled trials. *J Clin Oncol* 2006; **24**: 4808-4817 [PMID: 17001070 DOI: 10.1200/JCO.2006.06.3560]
 - 22 **Emberson JR**, Kearney PM, Blackwell L, Newman C, Reith C, Bhala N, Holland L, Peto R, Keech A, Collins R, Simes J, Baigent C. Lack of effect of lowering LDL cholesterol on cancer: meta-analysis of individual data from 175,000 people in 27 randomised trials of statin therapy. *PLoS One* 2012; **7**: e29849 [PMID: 22276132 DOI: 10.1371/journal.pone.0029849]
 - 23 **Lochhead P**, Chan AT. Statins and colorectal cancer. *Clin Gastroenterol Hepatol* 2013; **11**: 109-118; quiz e13-14 [PMID: 22982096 DOI: 10.1016/j.cgh.2012.08.037]
 - 24 **Bonovas S**, Tsantes A, Drosos T, Sitaras NM. Cancer chemoprevention: a summary of the current evidence. *Anticancer Res* 2008; **28**: 1857-1866 [PMID: 18630472]
 - 25 **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: 10.1056/NEJMoa043792]
 - 26 **Vinogradova Y**, Coupland C, Hippisley-Cox J. Exposure to statins and risk of common cancers: a series of nested case-control studies. *BMC Cancer* 2011; **11**: 409 [PMID: 21943022 DOI: 10.1186/1471-2407-11-409]
 - 27 **Bonovas S**, Filioussi K, Flordellis CS, Sitaras NM. Statins and the risk of colorectal cancer: a meta-analysis of 18 studies involving more than 1.5 million patients. *J Clin Oncol* 2007; **25**: 3462-3468 [PMID: 17687150 DOI: 10.1200/JCO.2007.10.8936]
 - 28 **Greenland S**. Invited commentary: a critical look at some popular meta-analytic methods. *Am J Epidemiol* 1994; **140**: 290-296 [PMID: 8030632]
 - 29 **Emerson JD**, Burdick E, Hoaglin DC, Mosteller F, Chalmers TC. An empirical study of the possible relation of treatment differences to quality scores in controlled randomized clinical trials. *Control Clin Trials* 1990; **11**: 339-352 [PMID: 1963128]
 - 30 **Greenland S**. Quantitative methods in the review of epidemiologic literature. *Epidemiol Rev* 1987; **9**: 1-30 [PMID: 3678409]
 - 31 **Altman DG**, Bland JM. Interaction revisited: the difference between two estimates. *BMJ* 2003; **326**: 219 [PMID: 12543843]
 - 32 **Petitti DB**. Statistical methods in meta-analysis. In: Petitti DB. Meta-Analysis, Decision Analysis, and Cost-Effectiveness Analysis. New York: Oxford University Press, 1999
 - 33 **Team RDC**. R: A Language and Environment for Statistical Computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing, 2013. Available from: URL: <http://www.R-project.org/>
 - 34 **Schwarzer G**. Meta: An R package for meta-analysis [Internet]. 2013. Available from: URL: <http://cran.r-project.org/package=meta>
 - 35 **Mantel N**, Haenszel W. Statistical aspects of the analysis of data from retrospective studies of disease. *J Natl Cancer Inst* 1959; **22**: 719-748 [PMID: 13655060]
 - 36 **DerSimonian R**, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**: 177-188 [PMID: 3802833]
 - 37 **Begg CB**, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. *Biometrics* 1994; **50**: 1088-1101 [PMID: 7786990]
 - 38 **Egger M**, Davey Smith G, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. *BMJ* 1997; **315**: 629-634 [PMID: 9310563]
 - 39 **Higgins JP**, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. *BMJ* 2003; **327**: 557-560 [PMID: 12958120 DOI: 10.1136/bmj.327.7414.557]
 - 40 **Higgins JPT**, Green S. Cochrane handbook for systematic reviews of interventions. Chichester: The Cochrane Collaboration, 2008
 - 41 **Stroup DF**, Berlin JA, Morton SC, Olkin I, Williamson GD, Rennie D, Moher D, Becker BJ, Sipe TA, Thacker SB. Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. *JAMA* 2000; **283**: 2008-2012

- [PMID: 10789670]
- 42 **Liberati A**, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JP, Clarke M, Devereaux PJ, Kleijnen J, Moher D. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. *PLoS Med* 2009; **6**: e1000100 [PMID: 19621070 DOI: 10.1371/journal.pmed.1000100]
 - 43 **Hsia J**, MacFadyen JG, Monyak J, Ridker PM. Cardiovascular event reduction and adverse events among subjects attaining low-density lipoprotein cholesterol < 50 mg/dl with rosuvastatin. The JUPITER trial (Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin). *J Am Coll Cardiol* 2011; **57**: 1666-1675 [PMID: 21492764 DOI: 10.1016/j.jacc.2010.09.082]
 - 44 **Ford I**, Murray H, Packard CJ, Shepherd J, Macfarlane PW, Cobbe SM. Long-term follow-up of the West of Scotland Coronary Prevention Study. *N Engl J Med* 2007; **357**: 1477-1486 [PMID: 17928595 DOI: 10.1056/NEJMoa065994]
 - 45 **Heart Protection Study Collaborative Group**. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet* 2002; **360**: 7-22 [PMID: 12114036 DOI: 10.1016/S0140-6736(02)09327-3]
 - 46 **ALLHAT Officers and Coordinators for the ALLHAT Collaborative Research Group**. The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial. Major outcomes in moderately hypercholesterolemic, hypertensive patients randomized to pravastatin vs usual care: The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT-LLT). *JAMA* 2002; **288**: 2998-3007 [PMID: 12479764]
 - 47 **Strandberg TE**, Pyörälä K, Cook TJ, Wilhelmsen L, Faergeman O, Thorgeirsson G, Pedersen TR, Kjekshus J. Mortality and incidence of cancer during 10-year follow-up of the Scandinavian Simvastatin Survival Study (4S). *Lancet* 2004; **364**: 771-777 [PMID: 15337403 DOI: 10.1016/S0140-6736(04)16936-5]
 - 48 **LIPID Study Group (Long-term Intervention with Pravastatin in Ischaemic Disease)**. Long-term effectiveness and safety of pravastatin in 9014 patients with coronary heart disease and average cholesterol concentrations: the LIPID trial follow-up. *Lancet* 2002; **359**: 1379-1387 [PMID: 11978335 DOI: 10.1016/S0140-6736(02)08351-4]
 - 49 **Leung HW**, Chan AL, Lo D, Leung JH, Chen HL. Common cancer risk and statins: a population-based case-control study in a Chinese population. *Expert Opin Drug Saf* 2013; **12**: 19-27 [PMID: 23199231 DOI: 10.1517/14740338.2013.744392]
 - 50 **Clancy Z**, Keith SW, Rabinowitz C, Ceccarelli M, Gagne JJ, Maio V. Statins and colorectal cancer risk: a longitudinal study. *Cancer Causes Control* 2013; **24**: 777-782 [PMID: 23361340 DOI: 10.1007/s10552-013-0160-x]
 - 51 **Simon MS**, Rosenberg CA, Rodabough RJ, Greenland P, Ockene I, Roy HK, Lane DS, Cauley JA, Khandekar J. Prospective analysis of association between use of statins or other lipid-lowering agents and colorectal cancer risk. *Ann Epidemiol* 2012; **22**: 17-27 [PMID: 22056480 DOI: 10.1016/j.annepidem.2011.10.006]
 - 52 **Lee JE**, Baba Y, Ng K, Giovannucci E, Fuchs CS, Ogino S, Chan AT. Statin use and colorectal cancer risk according to molecular subtypes in two large prospective cohort studies. *Cancer Prev Res (Phila)* 2011; **4**: 1808-1815 [PMID: 21680706 DOI: 10.1158/1940-6207.CAPR-11-0113]
 - 53 **Jacobs EJ**, Newton CC, Thun MJ, Gapstur SM. Long-term use of cholesterol-lowering drugs and cancer incidence in a large United States cohort. *Cancer Res* 2011; **71**: 1763-1771 [PMID: 21343395 DOI: 10.1158/0008-5472.CAN-10-2953]
 - 54 **Matsushita Y**, Sugihara M, Kaburagi J, Ozawa M, Iwashita M, Yoshida S, Saito H, Hattori Y. Pravastatin use and cancer risk: a meta-analysis of individual patient data from long-term prospective controlled trials in Japan. *Pharmacoepidemiol Drug Saf* 2010; **19**: 196-202 [PMID: 19856484 DOI: 10.1002/pds.1870]
 - 55 **Haukka J**, Sankila R, Klaukka T, Lonnqvist J, Niskanen L, Tanskanen A, Wahlbeck K, Tiihonen J. Incidence of cancer and statin usage--record linkage study. *Int J Cancer* 2010; **126**: 279-284 [PMID: 19739258 DOI: 10.1002/ijc.24536]
 - 56 **Singh H**, Mahmud SM, Turner D, Xue L, Demers AA, Bernstein CN. Long-term use of statins and risk of colorectal cancer: a population-based study. *Am J Gastroenterol* 2009; **104**: 3015-3023 [PMID: 19809413 DOI: 10.1038/ajg.2009.574]
 - 57 **Flick ED**, Habel LA, Chan KA, Haque R, Quinn VP, Van Den Eeden SK, Sternfeld B, Orav EJ, Seeger JD, Quesenberry CP, Caan BJ. Statin use and risk of colorectal cancer in a cohort of middle-aged men in the US: a prospective cohort study. *Drugs* 2009; **69**: 1445-1457 [PMID: 19634923 DOI: 10.2165/00003495-200969110-00004]
 - 58 **Friedman GD**, Flick ED, Udaltsova N, Chan J, Quesenberry CP, Habel LA. Screening statins for possible carcinogenic risk: up to 9 years of follow-up of 361,859 recipients. *Pharmacoepidemiol Drug Saf* 2008; **17**: 27-36 [PMID: 17944002 DOI: 10.1002/pds.1507]
 - 59 **Farwell WR**, Scranton RE, Lawler EV, Lew RA, Brophy MT, Fiore LD, Gaziano JM. The association between statins and cancer incidence in a veterans population. *J Natl Cancer Inst* 2008; **100**: 134-139 [PMID: 18182618 DOI: 10.1093/jnci/djm286]
 - 60 **Setoguchi S**, Glynn RJ, Avorn J, Mogun H, Schneeweiss S. Statins and the risk of lung, breast, and colorectal cancer in the elderly. *Circulation* 2007; **115**: 27-33 [PMID: 17179016 DOI: 10.1161/CIRCULATIONAHA.106.650176]
 - 61 **Friis S**, Poulsen AH, Johnsen SP, McLaughlin JK, Fryzek JP, Dalton SO, Sørensen HT, Olsen JH. Cancer risk among statin users: a population-based cohort study. *Int J Cancer* 2005; **114**: 643-647 [PMID: 15578694 DOI: 10.1002/ijc.20758]
 - 62 **Deshpande G**. Association between cardiovascular drugs and colon cancer [Internet]. Cited Aug 13 2013. Available from: URL: <http://archive.hshsl.umaryland.edu/handle/10713/2752>
 - 63 **Lakha F**, Theodoratou E, Farrington SM, Tenesa A, Cetnar-skyj R, Din FV, Porteous ME, Dunlop MG, Campbell H. Statin use and association with colorectal cancer survival and risk: case control study with prescription data linkage. *BMC Cancer* 2012; **12**: 487 [PMID: 23088590 DOI: 10.1186/1471-2407-12-487]
 - 64 **Broughton T**, Singleton J, Beales IL. Statin use is associated with a reduced incidence of colorectal cancer: a colonoscopy-controlled case-control study. *BMC Gastroenterol* 2012; **12**: 36 [PMID: 22530742 DOI: 10.1186/1471-230X-12-36]
 - 65 **Cheng MH**, Chiu HF, Ho SC, Tsai SS, Wu TN, Yang CY. Statin use and the risk of colorectal cancer: a population-based case-control study. *World J Gastroenterol* 2011; **17**: 5197-5202 [PMID: 22215945 DOI: 10.3748/wjg.v17.i47.5197]
 - 66 **Robertson DJ**, Riis AH, Friis S, Pedersen L, Baron JA, Sørensen HT. Neither long-term statin use nor atherosclerotic disease is associated with risk of colorectal cancer. *Clin Gastroenterol Hepatol* 2010; **8**: 1056-1061 [PMID: 20816860 DOI: 10.1016/j.cgh.2010.08.010]
 - 67 **Shadman M**, Newcomb PA, Hampton JM, Wernli KJ, Trentham-Dietz A. Non-steroidal anti-inflammatory drugs and statins in relation to colorectal cancer risk. *World J Gastroenterol* 2009; **15**: 2336-2339 [PMID: 19452574]
 - 68 **Hachem C**, Morgan R, Johnson M, Kuebler M, El-Serag H. Statins and the risk of colorectal carcinoma: a nested case-control study in veterans with diabetes. *Am J Gastroenterol* 2009; **104**: 1241-1248 [PMID: 19352344 DOI: 10.1038/ajg.2009.64]
 - 69 **Yang YX**, Hennessy S, Probert K, Hwang WT, Sarkar M, Lewis JD. Chronic statin therapy and the risk of colorectal cancer. *Pharmacoepidemiol Drug Saf* 2008; **17**: 869-876 [PMID: 18412290 DOI: 10.1002/pds.1599]
 - 70 **Boudreau DM**, Koehler E, Rulyak SJ, Haneuse S, Harrison

- R, Mandelson MT. Cardiovascular medication use and risk for colorectal cancer. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 3076-3080 [PMID: 18957524 DOI: 10.1158/1055-9965.EPI-08-0095]
- 71 **Hoffmeister M**, Chang-Claude J, Brenner H. Individual and joint use of statins and low-dose aspirin and risk of colorectal cancer: a population-based case-control study. *Int J Cancer* 2007; **121**: 1325-1330 [PMID: 17487832 DOI: 10.1002/ijc.22796]
 - 72 **Coogan PF**, Smith J, Rosenberg L. Statin use and risk of colorectal cancer. *J Natl Cancer Inst* 2007; **99**: 32-40 [PMID: 17202111 DOI: 10.1093/jnci/djk003]
 - 73 **Li L**, Thompson C, Tucker T. No association between lipid-lowering statin use and risk of colon cancer (abstract CC7). In: Proceedings of the 34th Annual Meeting of the North American Primary Care Research Group (NAPCRG). AZ: Tucson, 2006
 - 74 **Rubin DT**, Blumentals WA, Sheer RL, Steinbuch M, Law L. Statins and risk of colorectal cancer: Results from a large case-control study. *Am J Gastroenterol* 2005; **100**: S394
 - 75 **Khurana V**, Jaganmohan S, Chalasani R, Singh T, Roy P, Caldito G, Fort C. Statins do not reduce colon cancer risk in humans: A case-control study in half million veterans. *Am J Gastroenterol* 2004; **99**: S242
 - 76 **Kaye JA**, Jick H. Statin use and cancer risk in the General Practice Research Database. *Br J Cancer* 2004; **90**: 635-637 [PMID: 14760377 DOI: 10.1038/sj.bjc.6601566]
 - 77 **Graaf MR**, Beiderbeck AB, Egberts AC, Richel DJ, Guchelaar HJ. The risk of cancer in users of statins. *J Clin Oncol* 2004; **22**: 2388-2394 [PMID: 15197200 DOI: 10.1200/JCO.2004.02.027]
 - 78 **Blais L**, Desgagné A, LeLorier J. 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors and the risk of cancer: a nested case-control study. *Arch Intern Med* 2000; **160**: 2363-2368 [PMID: 10927735]
 - 79 **L'Abbé KA**, Detsky AS, O'Rourke K. Meta-analysis in clinical research. *Ann Intern Med* 1987; **107**: 224-233 [PMID: 3300460]
 - 80 **Sporn MB**. Approaches to prevention of epithelial cancer during the preneoplastic period. *Cancer Res* 1976; **36**: 2699-2702 [PMID: 1277177]
 - 81 **Bonovas S**. Cancer chemoprevention: progress and perspectives. *Curr Drug Targets* 2011; **12**: 1871-1873 [PMID: 21158713]
 - 82 **Bonovas S**, Nikolopoulos G, Filioussi K, Peponi E, Bagos P, Sitaras NM. Can statin therapy reduce the risk of melanoma? A meta-analysis of randomized controlled trials. *Eur J Epidemiol* 2010; **25**: 29-35 [PMID: 19844794 DOI: 10.1007/s10654-009-9396-x]
 - 83 **Bonovas S**, Filioussi K, Sitaras NM. Statins are not associated with a reduced risk of pancreatic cancer at the population level, when taken at low doses for managing hypercholesterolemia: evidence from a meta-analysis of 12 studies. *Am J Gastroenterol* 2008; **103**: 2646-2651 [PMID: 18684187 DOI: 10.1111/j.1572-0241.2008.02051.x]
 - 84 **Bonovas S**, Filioussi K, Sitaras NM. Statin use and the risk of prostate cancer: A metaanalysis of 6 randomized clinical trials and 13 observational studies. *Int J Cancer* 2008; **123**: 899-904 [PMID: 18491405 DOI: 10.1002/ijc.23550]
 - 85 **Bonovas S**, Filioussi K, Tsantes A, Sitaras NM. Use of statins and risk of haematological malignancies: a meta-analysis of six randomized clinical trials and eight observational studies. *Br J Clin Pharmacol* 2007; **64**: 255-262 [PMID: 17578480 DOI: 10.1111/j.1365-2125.2007.02959.x]
 - 86 **Bonovas S**, Filioussi K, Tsavaris N, Sitaras NM. Use of statins and breast cancer: a meta-analysis of seven randomized clinical trials and nine observational studies. *J Clin Oncol* 2005; **23**: 8606-8612 [PMID: 16260694 DOI: 10.1200/JCO.2005.02.7045]
 - 87 **Bonovas S**, Sitaras NM. Does pravastatin promote cancer in elderly patients? A meta-analysis. *CMAJ* 2007; **176**: 649-654 [PMID: 17325332 DOI: 10.1503/cmaj.060803]
 - 88 **Weverling-Rijnsburger AW**, Blauw GJ, Lagaay AM, Knook DL, Meinders AE, Westendorp RG. Total cholesterol and risk of mortality in the oldest old. *Lancet* 1997; **350**: 1119-1123 [PMID: 9343498]
 - 89 **Bonovas S**, Nikolopoulos G, Sitaras NM. Efficacy and safety of more intensive lowering of LDL cholesterol. *Lancet* 2011; **377**: 715; author reply 715-716 [PMID: 21353894 DOI: 10.1016/S0140-6736(11)60261-4]
 - 90 **Nikolopoulos GK**, Bagos PG, Bonovas S. Developing the evidence base for cancer chemoprevention: use of meta-analysis. *Curr Drug Targets* 2011; **12**: 1989-1997 [PMID: 21158703]
 - 91 **Jänne PA**, Mayer RJ. Chemoprevention of colorectal cancer. *N Engl J Med* 2000; **342**: 1960-1968 [PMID: 10874065 DOI: 10.1056/NEJM200006293422606]
 - 92 **Bonovas S**, Nikolopoulos GK, Bagos PG. Use of fibrates and cancer risk: a systematic review and meta-analysis of 17 long-term randomized placebo-controlled trials. *PLoS One* 2012; **7**: e45259 [PMID: 23028888 DOI: 10.1371/journal.pone.0045259]
 - 93 **Shen L**, Toyota M, Kondo Y, Lin E, Zhang L, Guo Y, Hernandez NS, Chen X, Ahmed S, Konishi K, Hamilton SR, Issa JP. Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. *Proc Natl Acad Sci U S A* 2007; **104**: 18654-18659 [PMID: 18003927 DOI: 10.1073/pnas.0704652104]
 - 94 **Markowitz SD**, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. *N Engl J Med* 2009; **361**: 2449-2460 [PMID: 20018966 DOI: 10.1056/NEJMra0804588]
 - 95 **Lipkin SM**, Chao EC, Moreno V, Rozek LS, Rennert H, Pinchev M, Dizon D, Rennert G, Kopelovich L, Gruber SB. Genetic variation in 3-hydroxy-3-methylglutaryl CoA reductase modifies the chemopreventive activity of statins for colorectal cancer. *Cancer Prev Res (Phila)* 2010; **3**: 597-603 [PMID: 20403997 DOI: 10.1158/1940-6207.CAPR-10-0007]
 - 96 **Schisterman EF**, Cole SR, Platt RW. Overadjustment bias and unnecessary adjustment in epidemiologic studies. *Epidemiology* 2009; **20**: 488-495 [PMID: 19525685 DOI: 10.1097/EDE.0b013e3181a819a1]
 - 97 **Bonovas S**, Sitaras NM. Statins and cancer risk: a confounded association. *Gastroenterology* 2009; **137**: 740; author reply 740-741 [PMID: 19563833 DOI: 10.1053/j.gastro.2009.02.088]
 - 98 **Ahnen DJ**, Byers T. Editorial: Colorectal cancer and statins: reflections from the end of the road. *Am J Gastroenterol* 2009; **104**: 3024-3026 [PMID: 19956119 DOI: 10.1038/ajg.2009.572]

P- Reviewers: Friis S, Mihaila RG

S- Editor: Zhai HH L- Editor: A E- Editor: Liu XM



Effectiveness of acupuncture to treat irritable bowel syndrome: A meta-analysis

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Author contributions: Zhang S designed the research and revised the manuscript; Chao GQ and Zhang S performed the research and analyzed the data; Chao GQ wrote the manuscript. Chao GQ and Zhang S contributed equally to this work as co-first authors.

Supported by the Youth Fund of National Natural Science Foundation of China, No. 81202828; and the Natural Science Foundation of Zhejiang Province, China, No. LY12H03013; and Academic Climbing Project of the Youth Discipline Leader of Universities in Zhejiang Province (pd2013209)

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Received: September 26, 2013 Revised: November 25, 2013

Accepted: December 12, 2013

Published online: February 21, 2014

Abstract

AIM: To evaluate the efficacy of acupuncture for treatment of irritable bowel syndrome (IBS) through meta-analysis of randomized controlled trials.

METHODS: We searched MEDLINE, PubMed, Scopus, Web of Science, and Cochrane Central Register of Controlled Trials from 1966 to February 2013 for double-blind, placebo-controlled trials investigating the efficacy of acupuncture in the management of IBS. Studies were screened for inclusion based on randomization, controls, and measurable outcomes reported. We used the modified Jadad score for assessing the quality of the articles. STATA 11.0 and Revman 5.0 were used for meta-analysis. Publication bias was assessed by Begg's and Egger's tests.

RESULTS: Six randomized, placebo-controlled clinical trials met the criteria and were included in the meta-analysis. The modified Jadad score of the articles was > 3 , and five articles were of high quality. We analyzed the heterogeneity and found that these studies did not cause heterogeneity in our meta-analysis. Begg's test showed $P = 0.707$ and Egger's test showed $P = 0.334$. There was no publication bias in our meta-analysis (Begg's test, $P = 0.707$; Egger's test, $P = 0.334$). From the forest plot, the diamond was on the right side of the vertical line and did not intersect with the line. The pooled relative risk for clinical improvement with acupuncture was 1.75 (95%CI: 1.24-2.46, $P = 0.001$). Using the two different systems of STATA 11.0 and Revman 5.0, we confirmed the significant efficacy of acupuncture for treating IBS.

CONCLUSION: Acupuncture exhibits clinically and statistically significant control of IBS symptoms.

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Key words: Irritable bowel syndrome; Functional gastrointestinal disorder; Acupuncture; Meta-analysis

Core tip: Irritable bowel syndrome (IBS) is not life-threatening, but leads to significant impairment of health-related quality of life. There is still no universally accepted satisfactory treatment for IBS. Nowadays, acupuncture is increasingly popular in patients with various diseases. Several studies showed an improvement in quality of life in IBS patients after acupuncture, but other studies showed no improvement. We performed a meta-analysis to establish the therapeutic efficacy of acupuncture for IBS.

Chao GQ, Zhang S. Effectiveness of acupuncture to treat irritable bowel syndrome: A meta-analysis. *World J Gastroenterol* 2014; 20(7): 1871-1877 Available from: URL: <http://www.wjgnet.com>

INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder characterized by chronic or recurrent abdominal pain or discomfort, which is associated with disturbed bowel function and feelings of abdominal distention and bloating^[1]. It is the most common reason that patients seek medical advice from primary care physicians and gastroenterologists. The prevalence of IBS reaches 7%-10% worldwide depending on the criteria used for diagnosis^[2]. It is a common gastrointestinal disorder, affecting 10%-15% of the population in developed countries^[3]. IBS is associated with a significant reduction in health-related quality of life^[4]. Estimates of annual direct and indirect costs associated with IBS exceed 41 billion dollars in major industrial countries^[5].

IBS is caused by multiple factors, although its nosogenesis is not clearly known. The causative factors include intestinal motility and intestinal smooth muscle functional disturbance, visceral paresthesia, alterations in the brain-gut axis, psychological factors, gastrointestinal hormones, and intestinal infection. The pathophysiology of IBS includes alterations in intestinal motility, visceral hypersensitivity, and abnormalities in the processing of visceral information^[6]. IBS affects all age groups and it is believed that factors such as familial aggregation, early life events, diet and psychosocial conditions might drive disease development^[7]. The disease is not life-threatening, but leads to significant impairment of health-related quality of life, which causes physical role limitations as well as pain and a lower perception of general health^[8]. Despite numerous studies targeting treatment of IBS^[9,10], there is still no universally accepted satisfactory treatment for this condition. Systematic reviews of conventional medications for IBS have found that the evidence for drug efficacy is weak^[11], and that no drug is effective in treating all the symptoms of IBS^[12].

Acupuncture has become increasingly popular in patients with various diseases, including IBS^[13]. Several recently published studies of acupuncture showed improvement in quality of life, regardless of whether it was traditional or sham acupuncture^[14-16]. In patients with IBS, no significant difference in quality of life improvement was observed after treatment with true or sham acupuncture^[17,18]. Another study^[19] showed that acupuncture for IBS provided an additional benefit over usual care alone. We performed a meta-analysis of randomized controlled trials to assess whether there was any benefit of acupuncture in improving symptoms or health-related quality of life in patients with IBS.

MATERIALS AND METHODS

Search strategy

We searched MEDLINE, PubMed, Scopus, Web of Sci-

ence, and Cochrane Central Register of Controlled Trials for studies investigating the efficacy of acupuncture for IBS. Data published from 1966 to February 2013 were collected. The search terms were: "acupuncture" or "acupuncture and moxibustion", and "irritable bowel", "functional bowel diseases" or "irritable colon". The search was restricted to English-language literature. We searched the references of reviewed articles for additional articles missed by the computerized database search. All primary and review articles, as well as their references, were reviewed independently in duplicate.

Study selection

All controlled trials investigating the efficacy of acupuncture in patients with IBS were considered. Studies were screened for inclusion, through review of the published article, based on the following criteria: randomization, controls, and measurable outcomes reported. Each article was reviewed in duplicate for inclusion, with substantial inter-rater agreement. Trials were disqualified if they were not controlled or their outcomes did not consider efficacy. Reviewers independently extracted data on country, diagnostic criteria, IBS type, assessment, treatment and time.

Methodology quality assessment

We used the modified Jadad score for assessing the quality of the article. Jadad score, which evaluates studies based on their description of randomization, concealment of allocation, double blinding, and dropouts (withdrawals), was used to assess the methodological quality of the trials. The quality scale ranges from 0 to 7 points with a low quality report of score ≤ 3 and a high quality report of score ≥ 4 .

Statistical analysis

All analyses were performed using STATA 11.0 and Revman 5.0. Data from selected studies were extracted into 2×2 tables. All included studies were weighted and pooled. Relative risk (OR) and 95%CI were calculated and effect size (weighted mean difference) meta-analysis was performed with STATA 11.0 and Revman 5.0. The event rate in the experimental (intervention) group against the event rate in the control group was calculated using a L'Abbe plot as an aid to explore the heterogeneity of effect estimates. In case of homogeneity, a fixed-effect model was used for meta-analysis; otherwise a random-effect model was applied. Publication bias was assessed by Begg's and Egger's tests. $P \geq 0.05$ indicated that there was no publication bias.

RESULTS

Article selection

The literature search identified 64 citations involving acupuncture and IBS, six of which met our inclusion criteria^[6,17,19-22]. Of the 58 excluded articles, 14 were review articles, 10 were not in English, 13 were observational studies from which we could not extract the results, 15

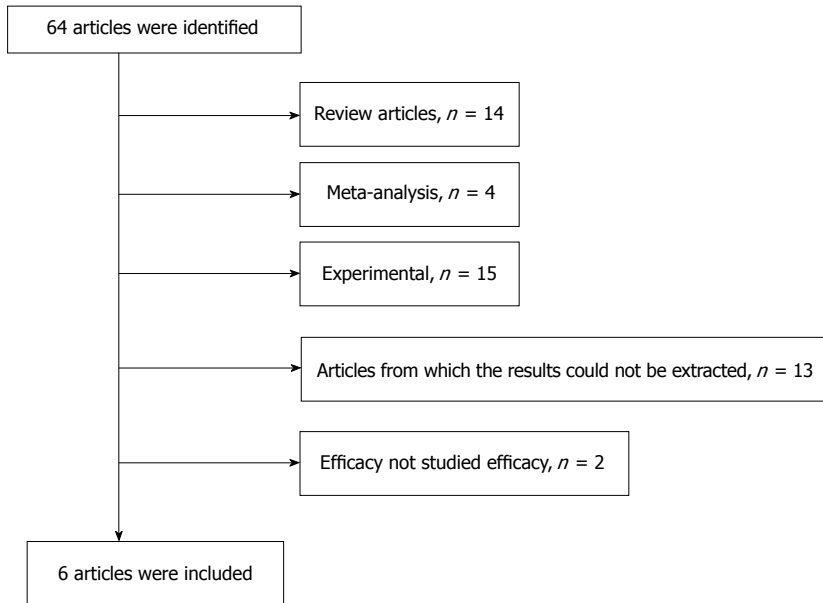


Figure 1 Flow diagram of the study selection process.

Table 1 Modified Jadad score of the articles

Study	Randomization	Concealment of allocation	Double blinding	Withdrawal and dropouts	Total score
MacPherson <i>et al</i> ^[19]	2	2	0	1	5
Forbes <i>et al</i> ^[17]	2	2	2	1	7
Sun <i>et al</i> ^[20]	2	1	0	1	4
Lembo <i>et al</i> ^[6]	1	1	1	1	4
Lowe <i>et al</i> ^[22]	1	2	1	0	4
Anastasi <i>et al</i> ^[21]	0	2	1	0	3

were experimental studies, two did not study efficacy, and four were meta-analyses (Figure 1).

Modified Jadad score assessment

The quality of the six articles was assessed by modified Jadad score (Table 1). We found that the score of two articles was > 4 , the score of three articles was 4, and one article had a score of 3. Therefore, five articles were of high quality.

The total number, country, IBS type, diagnostic criteria, assessment mode, time and treatment for each study are reported in Table 2. All subtypes of IBS (diarrhea-predominant, constipation-predominant, and alternating) were incorporated in the included studies. The Rome criteria were used for diagnosis. The control treatment of four studies^[6,17,21,22] was sham acupuncture, one^[20] was medical treatment, and the other^[19] was usual care only. The six studies were conducted in different countries.

Heterogeneity test

We performed the heterogeneity test using the χ^2 test ($\chi^2 = 3.72$, $P = 0.59$) (Figure 2). We found that the included studies did not cause heterogeneity in our meta-analysis. Therefore, a fixed-effect model was used for meta-analysis.

Publication bias assessment

The reason for publication bias was that positive results could be published easily, whereas negative results could not. Or, negative results caused the researchers to abandon their studies, which meant that the negative results were not published, thus affecting the meta-analysis. In our analysis, we performed Begg's test (Figure 3A) and Egger's test (Figure 3B), and both the tests indicated that there was no publication bias in our meta-analysis (Begg's test, $P = 0.707$; Egger's test, $P = 0.334$).

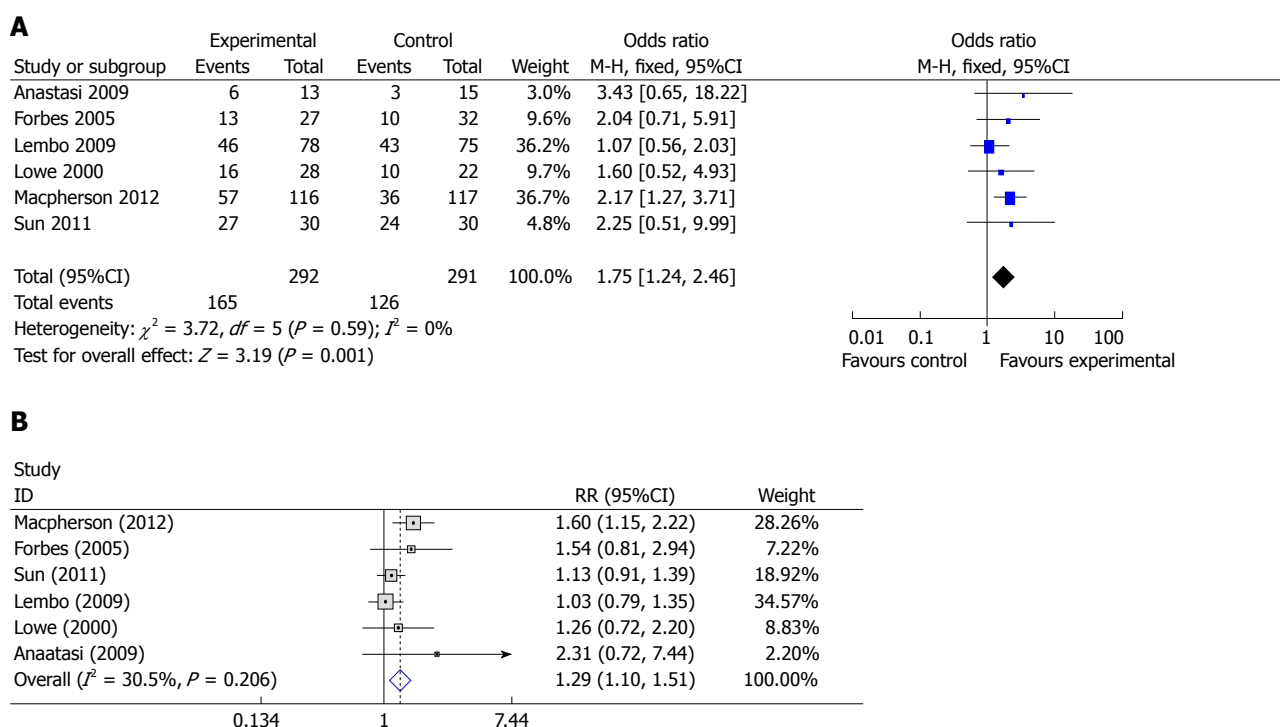
Merging and meta-analysis

The meta-analysis showed that the heterogeneity among the six studies included was not significant ($P = 0.59$), therefore, we used a fixed-effects model for the meta-analysis. From the forest plot, the diamond was on the right side of the vertical line and did not intersect with the line. From Figure 2A, we found that OR was 1.75 (95%CI: 1.24-2.46, $Z = 3.19$, $P = 0.001$) (Figure 2A), which meant that acupuncture had an effect on IBS. However, because the number of included articles was small, further analysis should be done in the future. In order to verify accuracy, we used STATA 11.0 to check the analysis. Figure 2B shows that the diamond was on

Table 2 Characteristics of papers included in the meta-analysis

Study	Country	n	Diagnostic criteria	IBS type	Assessment	Time	Acupuncture	Control treatment
Macpherson <i>et al</i> ^[19]	United Kingdom	233	Rome criteria	IBS	IBS symptom severity score	12 wk (every 3 wk)	Offer 10 weekly individualized acupuncture sessions plus usual care	Usual care only
Forbes <i>et al</i> ^[17]	United Kingdom	59	Rome I criteria and Manning criteria	IBS	Global symptom score based on patient diary	13 wk	Individualized 10 sessions over 10 wk	Sham acupuncture at non-acupoints
Sun <i>et al</i> ^[20]	China	63	Rome III	IBS-D	Symptom score	4 wk	Fixed formula; 20 sessions over 4 wk	Pinaverium bromide (50 mg <i>tid</i>)
Lembo <i>et al</i> ^[6]	United States	230	Rome II	IBS	IBS adequate relief	3 wk	Flexible formula; 10 sessions over 3 wk	Sham acupuncture at non-acupoints
Lowe <i>et al</i> ^[22]	Canada	50	Rome	IBS	Symptom relief	4 wk	Fixed formula; 8 sessions over 4 wk	Sham acupuncture - tapping blunt needle on the skin then tapping the needle in place
Anastasi <i>et al</i> ^[21]	United States	29	Rome II or Rome III	IBS	Clinical global impression scale	4 wk (measured 3 wk)	Flexible formula with moxibustion at all points; 8 sessions over 4 wk	Sham acupuncture - superficial needles 2-3 cm

IBS-D: Irritable bowel syndrome (IBS) with diarrhea.

**Figure 2** Meta-analysis. A: Meta-analysis of acupuncture for treating Irritable bowel syndrome (IBS) with Revman 5.0; B: Acupuncture for treating IBS with STATA 11.0.

the right of the vertical line and did not intersect with the line. Therefore, using the two different systems, we confirmed the significant efficacy of acupuncture for treating IBS.

DISCUSSION

IBS is defined as “abdominal pain or discomfort that occurs in association with altered bowel habits over a period of at least 3 mo”^[23]. The diagnosis of IBS, a highly prevalent functional gastrointestinal disorder, is currently

based on the presence of a characteristic symptom profile (abdominal pain/discomfort, bloating/distension, alterations in defecatory function) in the absence of a demonstrable organic disease of the gastrointestinal tract^[24]. The burden of IBS is significant enough to contribute to considerable impairment of quality of life. Patients with IBS have higher healthcare resource utilization than non-IBS patients in terms of more frequent physician visits, more tests, greater medication use, and increased rates of unnecessary surgery^[25]. Although IBS is common, its pathophysiology is not completely under-

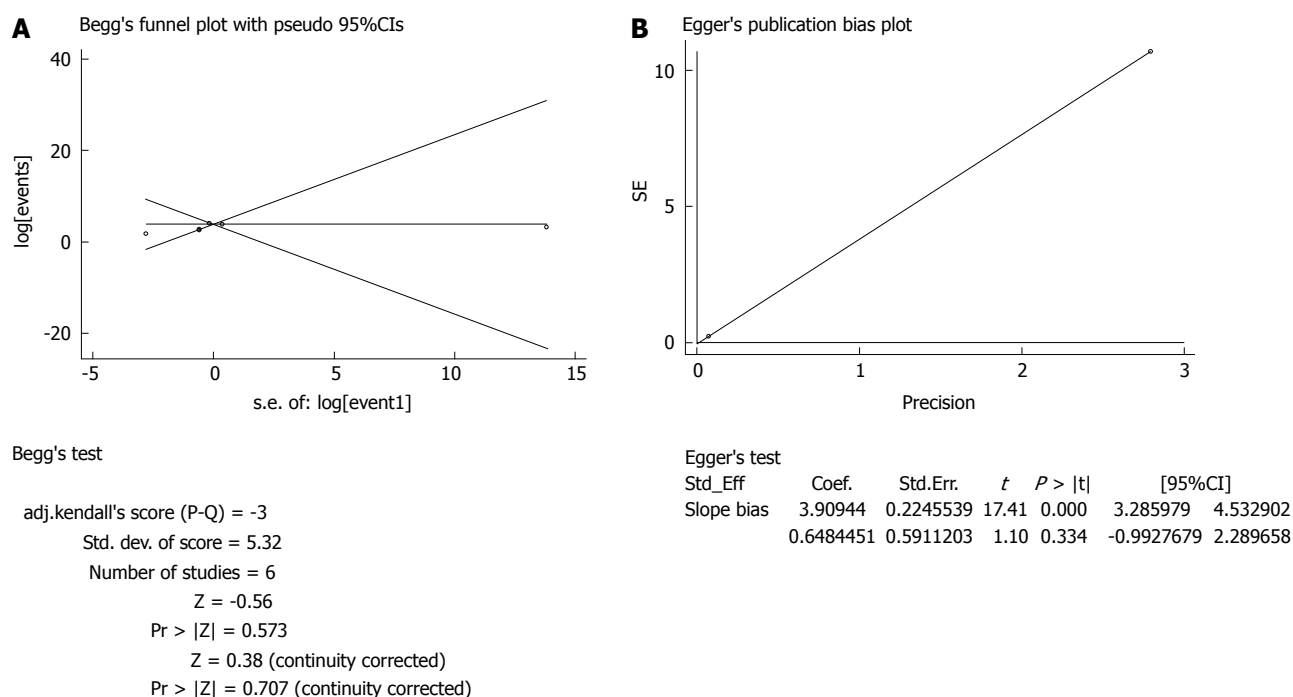


Figure 3 Publication bias. A: Begg's funnel plot for publication bias; B: Egger's publication bias plot.

stood, which poses problems in the search for effective therapeutic approaches.

Although the pathogenesis of IBS is not fully known, a multifactorial involvement of diet, gene mutations, psychosocial factors, and immune-mediated processes is hypothesized^[26]. Visceral hypersensitivity and dysregulation of central pain perception in the brain-gut axis are considered to play a pivotal role in the pathophysiology of IBS. One theory regarding the pathophysiology of IBS involves interference of neurotransmission between the central nervous system and the intestines.

IBS can be classified as either diarrhea predominant, constipation predominant, or a mixed form^[27]. Due to the wide range of symptoms that may be experienced, the available pharmacological treatments are mainly targeted at symptom reduction. In many studies, we found that pharmacological treatment of IBS varied from antidepressants including tricyclic antidepressants and selective serotonin reuptake inhibitors, to antispasmodics, 5-hydroxytryptamine (5-HT)-3 receptor antagonists, 5-HT₄ receptor agonists, antibiotics, probiotics, and melatonin^[28]. Effective treatments for IBS are needed to relieve symptoms, improve quality of life, and reduce healthcare utilization. However, acupuncture, a 3000-year-old traditional Chinese medical practice, is receiving increasing acceptance in Western medicine for treating certain medical conditions.

Acupuncture is one of the prominent methods in alternative medicine that has been tried on various disturbances of the digestive tract. It can affect the visceral system by stimulating the somatic system, in accordance with the visceral hyperalgesia theory of the central nervous system^[29]. However, previous studies of acu-

puncture in IBS have not provided conclusive evidence of its efficacy^[30]. A Cochrane review of six trials, with a median sample size of 54, found insufficient evidence to determine if acupuncture was an effective treatment for IBS^[31]. A systematic review^[32] showed unspecific effects of acupuncture for IBS compared with sham acupuncture. Another meta-analysis^[33] showed that there was no benefits of acupuncture relative to a credible sham acupuncture control for IBS symptom severity or IBS-related quality of life. A recent study^[19] showed that acupuncture for IBS provided an additional benefit. In some studies, acupuncture was believed to alter visceral sensation and motility by stimulating the somatic nervous system and the vagus nerve in IBS^[34-36]. Consequently, the real efficacy of acupuncture for IBS is still unclear because the quality of some of the articles included in the systematic review and meta-analysis was poor.

Nevertheless, our meta-analysis of six randomized controlled trials suggests that acupuncture improves the symptoms of IBS, including abdominal pain and distension, sensation of incomplete defecation, times of defecation per day, and state of stool. One study^[37] reported that acupuncture might modulate pain in IBS by two actions: (1) modulation of serotonin pathway at insula; and (2) modulation of mood and affection in the higher cortical center via the ascending pathway at the pulvinar and medial nucleus of the thalamus. However, in our analysis, only one article^[19] showed a positive effect, so the potential placebo effects of acupuncture should not be dismissed. In our meta-analysis, five of the six articles were of high quality, and there was no publication bias. We used two different operating systems to perform the analysis, and both showed significant differences. No

serious adverse events associated with acupuncture were reported in the articles. The mechanism of action of acupuncture for IBS is unclear at present. Most animal experiments on the mechanism of action have been associated with electro-acupuncture. However, one study^[38] showed that improvement in pain in IBS was positively associated with increased parasympathetic tone in the acupuncture group.

In our meta-analysis, five articles showed no benefit of acupuncture for IBS, and only one^[19] showed a positive effect. However, the meta-analysis showed the benefit of acupuncture. The reason why we obtained a positive end result might have been that the sample size of the positive study was large. The Jadad score of the positive article was 5, and all six included studies were from different countries, therefore, the result was credible. However, the analysis had some limitations. First, the total sample size was not large enough. Second, the treatment mode and the duration were not coincident, thus, we could not confirm how long acupuncture treatment is required to achieve a benefit when treating IBS. Third, because assessment of improvement was not the same and not detailed, it was difficult to assess the effect of acupuncture accurately. Fourth, because the side effects of acupuncture were not recorded in all studies, we could not assess these during treatment of IBS. Fortunately, we did not find any publication bias, which increased the reliability of our meta-analysis. Although our meta-analysis showed that acupuncture was beneficial for IBS patients, we still need further research with larger samples to achieve an accurate result and to explore the functional mechanism of action of acupuncture.

This meta-analysis showed that acupuncture is beneficial for IBS patients. However, this review had some limitations. The data are insufficient to recommend the method as first-line treatment. Moreover, there are insufficient data to establish the long-term results. Therefore, further research is required to assess more accurately the results and mechanism of action of acupuncture for treatment of IBS.

COMMENTS

Background

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder characterized by chronic or recurrent abdominal pain or discomfort. IBS can lead to significant impairment of health-related quality of life, which causes physical role limitations as well as pain and a lower perception of general health. There is still no universally accepted satisfactory treatment for this condition.

Research frontiers

Effective treatments for IBS are needed to relieve symptoms, improve quality of life, and reduce healthcare utilization. This study aimed to confirm the efficacy of acupuncture for treatment of IBS. This may help establish the mechanism of action of acupuncture to treat IBS in further research.

Innovations and breakthroughs

It has been found that pharmacological treatment of IBS varied from antidepressants including tricyclic antidepressants and selective serotonin reuptake inhibitors, to antispasmodics, 5-hydroxytryptamine-3 (5-HT₃) receptor antagonists, 5-HT₄ receptor agonists, antibiotics, probiotics, and melatonin. Despite the numerous studies targeting the treatment of IBS, there is still no universally accepted satisfactory treatment. Nowadays, patients with IBS frequently use

complementary medicine including acupuncture. However, the efficacy of acupuncture to treat IBS is still unknown. Some studies of acupuncture treatment of IBS have shown different results. This study was to confirm the effect of acupuncture by meta-analysis.

Applications

The results suggest that acupuncture reduces the symptoms of IBS, without any serious adverse events. The mechanism of action of acupuncture for IBS is unclear.

Terminology

IBS is defined as "abdominal pain or discomfort that occurs in association with altered bowel habits over a period of at least 3 mo." Acupuncture is one of the prominent methods of alternative medicine that has been tried on various disturbances of the digestive tract. It can affect the visceral system by stimulating the somatic system, in accordance with the visceral hyperalgesia theory of the central nervous system. Jadad score, which evaluates studies based on their description of randomization, concealment of allocation, double blinding, and dropouts (withdrawals), was used to assess the methodological quality of the trials.

Peer review

This is an interesting paper on the effect of acupuncture in IBS. There are few data in this domain. This meta-analysis showed that acupuncture was beneficial for IBS patients. Meta-analysis has some limitations. More research is required so that accurate results can be assessed and the mechanism of action of acupuncture to treat IBS can be confirmed.

REFERENCES

- 1 Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology* 2006; **130**: 1480-1491 [PMID: 16678561]
- 2 Spiegel BM. The burden of IBS: looking at metrics. *Curr Gastroenterol Rep* 2009; **11**: 265-269 [PMID: 19615301]
- 3 Keszthelyi D, Troost FJ, Masclee AA. Irritable bowel syndrome: methods, mechanisms, and pathophysiology. Methods to assess visceral hypersensitivity in irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* 2012; **303**: G141-G154 [PMID: 22595988 DOI: 10.1152/ajpgi.00060]
- 4 Gralnek IM, Hays RD, Kilbourne A, Naliboff B, Mayer EA. The impact of irritable bowel syndrome on health-related quality of life. *Gastroenterology* 2000; **119**: 654-660 [PMID: 10982758]
- 5 Inadomi JM, Fennerty MB, Bjorkman D. Systematic review: the economic impact of irritable bowel syndrome. *Aliment Pharmacol Ther* 2003; **18**: 671-682 [PMID: 14510740]
- 6 Lembo AJ, Conboy L, Kelley JM, Schnyer RS, McManus CA, Quilty MT, Kerr CE, Drossman D, Jacobson EE, Davis RB. A treatment trial of acupuncture in IBS patients. *Am J Gastroenterol* 2009; **104**: 1489-1497 [PMID: 19455132 DOI: 10.1038/ajg.2009.156]
- 7 Rey E, Talley NJ. Irritable bowel syndrome: novel views on the epidemiology and potential risk factors. *Dig Liver Dis* 2009; **41**: 772-780 [PMID: 19665952 DOI: 10.1016/j.dld.2009.07.005]
- 8 Stamuli E, Bloor K, MacPherson H, Tilbrook H, Stuardi T, Brabyn S, Torgerson D. Cost-effectiveness of acupuncture for irritable bowel syndrome: findings from an economic evaluation conducted alongside a pragmatic randomised controlled trial in primary care. *BMC Gastroenterol* 2012; **12**: 149 [PMID: 23095351 DOI: 10.1186/1471-230X-12-149]
- 9 Camilleri M. Management of the irritable bowel syndrome. *Gastroenterology* 2001; **120**: 652-668 [PMID: 11179242]
- 10 Clouse RE. Managing functional bowel disorders from the top down: lessons from a well-designed treatment trial. *Gastroenterology* 2003; **125**: 249-253 [PMID: 12851889]
- 11 Akehurst R, Kaltenthaler E. Treatment of irritable bowel syndrome: a review of randomised controlled trials. *Gut* 2001; **48**: 272-282 [PMID: 11156653]
- 12 Quartero AO, Meineche-Schmidt V, Muris J, Rubin G, de Wit N. Bulking agents, antispasmodic and antidepressant medi-

- cation for the treatment of irritable bowel syndrome. *Cochrane Database Syst Rev* 2005; (2): CD003460 [PMID: 15846668]
- 13 **Ouyang H**, Chen JD. Review article: therapeutic roles of acupuncture in functional gastrointestinal disorders. *Aliment Pharmacol Ther* 2004; **20**: 831-841 [PMID: 15479354]
 - 14 **Linde K**, Jobst K, Panton J. Acupuncture for chronic asthma. *Cochrane Database Syst Rev* 2000; (2): CD000008 [PMID: 10796465]
 - 15 **Linde K**, Streng A, Jürgens S, Hoppe A, Brinkhaus B, Witt C, Wagenpfeil S, Pfaffenrath V, Hammes MG, Weidenhammer W, Willich SN, Melchart D. Acupuncture for patients with migraine: a randomized controlled trial. *JAMA* 2005; **293**: 2118-2125 [PMID: 15870415]
 - 16 **Melchart D**, Streng A, Hoppe A, Brinkhaus B, Witt C, Wagenpfeil S, Pfaffenrath V, Hammes M, Hummelsberger J, Irnich D, Weidenhammer W, Willich SN, Linde K. Acupuncture in patients with tension-type headache: randomised controlled trial. *BMJ* 2005; **331**: 376-382 [PMID: 16055451]
 - 17 **Forbes A**, Jackson S, Walter C, Quraishi S, Jacyna M, Pitcher M. Acupuncture for irritable bowel syndrome: a blinded placebo-controlled trial. *World J Gastroenterol* 2005; **11**: 4040-4044 [PMID: 15996029]
 - 18 **Schneider A**, Enck P, Streitberger K, Weiland C, Bagheri S, Witte S, Friederich HC, Herzog W, Zipfel S. Acupuncture treatment in irritable bowel syndrome. *Gut* 2006; **55**: 649-654 [PMID: 16150852]
 - 19 **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]
 - 20 **Sun JH**, Wu XL, Xia C, Xu LZ, Pei LX, Li H, Han GY. Clinical evaluation of Soothing Gan and invigorating Pi acupuncture treatment on diarrhea-predominant irritable bowel syndrome. *Chin J Integr Med* 2011; **17**: 780-785 [PMID: 22101701 DOI: 10.1007/s11655-011-0875-z]
 - 21 **Anastasi JK**, McMahon DJ, Kim GH. Symptom management for irritable bowel syndrome: a pilot randomized controlled trial of acupuncture/moxibustion. *Gastroenterol Nurs* 2009; **32**: 243-255 [PMID: 19696601 DOI: 10.1097/SGA.0b013e3181b2c920]
 - 22 **Lowe C**, Depew W, Vanner S. A placebo-controlled, double-blind trial of acupuncture in the treatment of irritable bowel syndrome (IBS). *Gastroenterology* 2000; **118**: A3168
 - 23 **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-S5 [PMID: 19521341 DOI: 10.1038/ajg.2008.122]
 - 24 **Drossman DA**, Dumitrascu DL. Rome III: New standard for functional gastrointestinal disorders. *J Gastrointest Liver Dis* 2006; **15**: 237-241 [PMID: 17013448]
 - 25 **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]
 - 26 **Mathew P**, Bhatia SJ. Pathogenesis and management of irritable bowel syndrome. *Trop Gastroenterol* 2009; **30**: 19-25 [PMID: 19624083]
 - 27 **Cash BD**, Chey WD. Diagnosis of irritable bowel syndrome. *Gastroenterol Clin North Am* 2005; **34**: 205-220, vi [PMID: 15862930]
 - 28 **Rahimi R**, Abdollahi M. Herbal medicines for the management of irritable bowel syndrome: a comprehensive review. *World J Gastroenterol* 2012; **18**: 589-600 [PMID: 22363129 DOI: 10.3748/wjg.v18.i7.589]
 - 29 **Fireman Z**, Segal A, Kopelman Y, Sternberg A, Carasso R. Acupuncture treatment for irritable bowel syndrome. A double-blind controlled study. *Digestion* 2001; **64**: 100-103 [PMID: 11684823]
 - 30 **Sung JJ**. Acupuncture for gastrointestinal disorders: myth or magic. *Gut* 2002; **51**: 617-619 [PMID: 12377792]
 - 31 **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]
 - 32 **Schneider A**, Streitberger K, Joos S. Acupuncture treatment in gastrointestinal diseases: a systematic review. *World J Gastroenterol* 2007; **13**: 3417-3424 [PMID: 17659687]
 - 33 **Manheimer E**, Wieland LS, Cheng K, Li SM, Shen X, Berman BM, Lao L. Acupuncture for irritable bowel syndrome: systematic review and meta-analysis. *Am J Gastroenterol* 2012; **107**: 835-847; quiz 848 [PMID: 22488079 DOI: 10.1038/ajg]
 - 34 **Xiao WB**, Liu YL. Rectal hypersensitivity reduced by acupoint TENS in patients with diarrhea-predominant irritable bowel syndrome: a pilot study. *Dig Dis Sci* 2004; **49**: 312-319 [PMID: 15104377]
 - 35 **Cui KM**, Li WM, Gao X, Chung K, Chung JM, Wu GC. Electro-acupuncture relieves chronic visceral hyperalgesia in rats. *Neurosci Lett* 2005; **376**: 20-23 [PMID: 15694267]
 - 36 **Tillisch K**. Complementary and alternative medicine for functional gastrointestinal disorders. *Gut* 2006; **55**: 593-596 [PMID: 16609129]
 - 37 **Chu WC**, Wu JC, Yew DT, Zhang L, Shi L, Yeung DK, Wang D, Tong RK, Chan Y, Lao L, Leung PC, Berman BM, Sung JJ. Does acupuncture therapy alter activation of neural pathway for pain perception in irritable bowel syndrome?: a comparative study of true and sham acupuncture using functional magnetic resonance imaging. *J Neurogastroenterol Motil* 2012; **18**: 305-316 [PMID: 22837879 DOI: 10.5056/jnm]
 - 38 **Schneider A**, Weiland C, Enck P, Joos S, Streitberger K, Maser-Gluth C, Zipfel S, Bagheri S, Herzog W, Friederich HC. Neuroendocrinological effects of acupuncture treatment in patients with irritable bowel syndrome. *Complement Ther Med* 2007; **15**: 255-263 [PMID: 18054727]

P- Reviewers: Bonaz BL, Poli-Neto OB, Pehl C
S- Editor: Gou SX **L- Editor:** Wang TQ **E- Editor:** Liu XM



Recurrent gastrointestinal bleeding and hepatic infarction after liver biopsy

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Received: July 18, 2013 Revised: September 2, 2013

Accepted: September 16, 2013

Published online: February 21, 2014

Abstract

Hepatic artery pseudoaneurysms (HAP) are rare events, particularly after liver biopsy, but can be associated with serious complications. Therefore a high suspicion is necessary for timely diagnosis and appropriate treatment. We report on a case of HAP that potentially formed after a liver biopsy in a patient with sarcoidosis. The HAP in our case was virtually undetectable initially by angiography but resulted in several complications including recurrent gastrointestinal bleeding, hemorrhagic cholecystitis and finally hepatic infarction with abscess formation until it became detectable at a size of 5-mm. The patient remains asymptomatic over a year after endovascular embolization of the HAP. In this report, we demonstrate that a small HAP can avoid detection by angiography at an early stage while being symptomatic for a prolonged course. A high clinical suspicion with a close clinical/radiological follow-up is needed in symptomatic patients with history of liver biopsy despite initial negative work up. Once diagnosed, HAP can be safely and effectively treated by endovas-

cular embolization.

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Key words: Gastrointestinal bleed; Abnormal liver enzymes; Hepatic artery pseudoaneurysms; Liver biopsy; Angiography

Core tip: We describe a case of a 43-year-old woman with a small hepatic artery pseudoaneurysm (HAP) that was persistently symptomatic and avoided radiographic detection at an early stage. High clinical suspicion and close clinical/radiological follow-up is required for patients with risk factors such as previous liver biopsy, even if an initial workup is negative. These small HAPs may cause symptoms as late as several weeks after a liver biopsy, and have the potential to afflict severe complications such as hemobilia and thrombosis of the hepatic artery branches, resulting in hepatic infarction and abscess formation.

Bishehsari F, Ting PS, Green RM. Recurrent gastrointestinal bleeding and hepatic infarction after liver biopsy. *World J Gastroenterol* 2014; 20(7): 1878-1881 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1878.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1878>

INTRODUCTION

Hepatic artery pseudoaneurysms (HAPs) are false aneurysms formed when a tear of a hepatic arterial wall leads to a peri-artery hematoma and HAPs can be caused by medical procedures, trauma, inflammatory or infectious conditions^[1]. Though well described, HAPs are rare occurrences^[2-4], even more so when caused by a liver biopsy and hence few liver biopsy related HAPs have been reported in the literature^[5-8]. HAPs most commonly present

with abdominal pain, hematemesis, anemia, hypovolemia and jaundice^[9,10]. In spite of being rare, HAPs can be a deadly complication if not diagnosed and can lead to massive gastrointestinal (GI) bleeding from hemobilia and aortoenteric fistulas^[4].

Diagnosis of HAPs require a high index of suspicion especially after iatrogenic procedures, and angiography should be performed when HAPs are suspected^[4]. At present, the most effective treatment for HAPs are endovascular embolization, with rare instances of surgeries performed when embolization fails^[1,7,11-14]. We report on a patient with a small HAP following liver biopsy that was initially undetectable by angiography and had been increasingly symptomatic for three months before being detected by angiography. This was treated successfully with endovascular embolization.

CASE REPORT

A 43-year-old woman presented with right upper quadrant (RUQ) pain and hematemesis. She had a past medical history of hypertension, hematemesis one year ago from an endoscopically proven Mallory-Weiss tear and sarcoidosis for several years involving her lungs and liver. A month prior, she had a percutaneous liver biopsy for persistent elevation of her alkaline phosphatase (ALP). The pathology findings were consistent with hepatic sarcoidosis. Subsequently, she was admitted to a local hospital for epigastric pain and hematemesis and was diagnosed by upper endoscopy with a Mallory-Weiss tear. She was treated conservatively and was discharged after a day of observation.

Upon admission, her hemoglobin (Hgb) was 99 g/L, aspartate aminotransferase (AST) 360 IU/L, alanine aminotransferase (ALT) 299 IU/L, ALP 532 IU/L, and total bilirubin (T-BILI) 2.7 mg/dL (direct bilirubin 1.9 mg/dL). Ultrasonography showed heterogenous echogenic material in her gallbladder suggestive of biliary sludge, stones or hemorrhage. Magnetic resonance cholangiopancreatography (MRCP) showed nonenhancing material in the gallbladder with no biliary ductal dilation. Endoscopic retrograde cholangiopancreatography (ERCP) revealed blood draining from the papilla indicative of hemobilia. Hepatic angiography showed no source for hemobilia. The patient's symptoms resolved, her Hgb remained stable and liver enzymes improved. She was discharged to follow-up for a possible elective cholecystectomy.

Three weeks later she presented with RUQ pain and hematemesis. ERCP demonstrated non-bleeding erythematous gastropathy and a normal cholangiogram. MRCP showed heterogenous nonenhancing material within the gallbladder, likely representing blood. Laparoscopic cholecystectomy was performed, her symptoms and liver enzymes improved before discharge.

After one week, she re-presented with intermittent hematochezia. She reported taking nonsteroidal anti-inflammatory drugs (NSAIDs) for intermittent RUQ discomfort and recently discontinuing pantoprazole. She

was afebrile and hemodynamically stable. Her Hgb was 66 g/L, AST 93 IU/L, ALT 137 IU/L, ALP 716 IU/L, T-BILI 1.2 mg/dL. Colonoscopy showed clotted blood in the colon without any bleeding lesions and an upper endoscopy revealed an oozing gastric ulcer that was clipped with successful hemostasis. She was discharged and one week later she presented with severe RUQ pain with fever and chills, but without an overt GI bleed. Computed tomography demonstrated a wedge shaped region of low attenuation in the periphery of segments VI and VII of the liver, suggestive of segmental hepatic infarction. Magnetic resonance imaging (MRI) and angiography (MRA) demonstrated early abscesses within segments VI and VII, with patent hepatic arteries and portal vein branches. The aspirate showed Gram-positive cocci. She was started on antibiotics with an improvement of her symptoms and was subsequently discharged. Abdominal MRI after 1 week revealed improvement in the size of the abscesses, but showed multiple filling defects within the common bile duct (CBD) with mild enhancement of the CBD. She presented with RUQ pain and hematemesis, with elevated liver enzymes, but repeat ERCP was unremarkable. MRA revealed a small 5-mm intrahepatic pseudoaneurysm arising from the superior branch of the anterior division of the right hepatic artery (RHA) with a hypoattenuated lesion in segment VII, suggestive of infarction in the territory supplied by the RHA branch. This was confirmed by subsequent angiography (Figure 1). In retrospect, the review of the prior MRA revealed a less than a 1-mm pseudoaneurysm in the same location. Coil embolization of the pseudoaneurysm was performed with excellent angiographic result. One month later, MRI showed marked improvement in the size and appearance of the abscesses. The patient continued to do well with no further abdominal pain, GI bleed or any rise in her liver enzymes for one and a half years after the embolization.

DISCUSSION

HAPs are rare conditions^[2-4] and are usually due to iatrogenic causes, including liver biopsies, cholecystectomy, transhepatic biliary drainage, and inadvertent surgical injuries^[8,15-17]. Percutaneous liver biopsy (PLB) is a common and safe procedure with low mortality and morbidity rates^[18,19]. There have been only a few case reports on HAPs caused by liver biopsy, but they describe larger pseudoaneurysms that are severely symptomatic shortly after the procedure^[5-7]. Angiography is the most sensitive method that is available to detect HAP^[4]. Our patient, however, remained increasingly symptomatic for three months before the HAP could be detected by imaging and treated successfully. A review of the literature did not reveal any case reports of HAPs as small as 1-mm causing persistent and recurrent symptoms for a prolonged course. This diminutive HAP was complicated by multiple episodes of hemobilia, hemorrhagic cholecystitis requiring cholecystectomy, and RHA thrombosis, which led to abdominal pain, elevation of liver enzymes, and



Figure 1 Angiography findings. A 5-mm intrahepatic pseudoaneurysm (arrow) was detected arising from the superior branch of the anterior division of the right hepatic artery.

hepatic infarction complicated by liver abscess formation.

This case demonstrates that a small HAP can avoid detection by angiography at an early stage. A high clinical suspicion and close clinical/radiological follow up is needed in symptomatic patients with history of liver biopsy despite an initial negative work up. In spite of the rarity of HAPs, the high prevalence of liver biopsies and the severity of the consequences of not detecting them make the recognition of this complication crucial in clinical practice.

Therapeutic modalities to treat HAPs include open surgery and endovascular approach. In light of the rarity of the disease, there have been no randomized comparisons between the two approaches. Open surgical repair is usually associated with complications such as intra-abdominal infection and hepatobiliary diseases, leading to higher morbidity and mortality associated with this approach^[20]. Therefore, the endovascular approach has become the first line treatment for HAPs, leaving surgical repair as the salvage therapy only if the former fails^[21]. The most commonly used endovascular technique is the endovascular ablation of the proximal feeding artery of the HAP either by coil or glue^[14]. After ablation therapy, patients should be closely monitored for sac reperfusion and any potential end-organ ischemia that responds to a repeat endovascular treatment^[22]. However, the risk of end-organ damage is clinically significant only if the embolization procedure involves major arterial supplies^[23]. In these cases, stent placement of the major artery followed by coil embolization of collateral feeding arteries can be used to reduce the risk of ischemia^[24].

REFERENCES

- 1 **Laganà D**, Carrafiello G, Mangini M, Dionigi G, Caronno R, Castelli P, Fugazzola C. Multimodal approach to endovascular treatment of visceral artery aneurysms and pseudoaneurysms. *Eur J Radiol* 2006; **59**: 104-111 [PMID: 16597492 DOI: 10.1016/j.ejrad.2006.02.004]
- 2 **Green MH**, Duell RM, Johnson CD, Jamieson NV. Haemobilia. *Br J Surg* 2001; **88**: 773-786 [PMID: 11412246 DOI: 10.1046/j.1365-2168.2001.01756.x]
- 3 **Harlaftis NN**, Akin JT. Hemobilia from ruptured hepatic artery aneurysm. Report of a case and review of the literature. *Am J Surg* 1977; **133**: 229-232 [PMID: 299994]
- 4 **Tobben PJ**, Zajko AB, Sumkin JH, Bowen A, Fuhrman CR, Skolnick ML, Bron KM, Esquivel CO, Starzl TE. Pseudoaneurysms complicating organ transplantation: roles of CT, duplex sonography, and angiography. *Radiology* 1988; **169**: 65-70 [PMID: 3047790]
- 5 **Ren FY**, Piao XX, Jin AL. Delayed hemorrhage from hepatic artery after ultrasound-guided percutaneous liver biopsy: a case report. *World J Gastroenterol* 2006; **12**: 4273-4275 [PMID: 16830394]
- 6 **Kowdley KV**, Aggarwal AM, Sachs PB. Delayed hemorrhage after percutaneous liver biopsy. Role of therapeutic angiography. *J Clin Gastroenterol* 1994; **19**: 50-53 [PMID: 7930434]
- 7 **Own A**, Balzer JO, Vogl TJ. Bleeding hepatic pseudoaneurysm complicating percutaneous liver biopsy with interventional treatment options. *Eur Radiol* 2005; **15**: 183-185 [PMID: 15007619 DOI: 10.1007/s00330-004-2287-3]
- 8 **Kwauk ST**, Cameron R, Burbridge B, Keith RG. Traumatic pseudoaneurysm of the hepatic artery after percutaneous liver biopsy and laparoscopic cholecystectomy in a patient with biliary cirrhosis: a case report. *Can J Surg* 1998; **41**: 316-320 [PMID: 9711166]
- 9 **Croce MA**, Fabian TC, Spiers JP, Kudsk KA. Traumatic hepatic artery pseudoaneurysm with hemobilia. *Am J Surg* 1994; **168**: 235-238 [PMID: 8080059]
- 10 **Yoon W**, Jeong YY, Kim JK, Seo JJ, Lim HS, Shin SS, Kim JC, Jeong SW, Park JG, Kang HK. CT in blunt liver trauma. *Radiographics* 2005; **25**: 87-104 [PMID: 15653589 DOI: 10.1148/rgr.251045079]
- 11 **Francisco LE**, Asunción LC, Antonio CA, Ricardo RC, Manuel RP, Caridad MH. Post-traumatic hepatic artery pseudoaneurysm treated with endovascular embolization and thrombin injection. *World J Hepatol* 2010; **2**: 87-90 [PMID: 21160978 DOI: 10.4254/wjh.v2.i2.87]
- 12 **Etezadi V**, Gandhi RT, Benenati JF, Rochon P, Gordon M, Benenati MJ, Alehashemi S, Katzen BT, Geisbüsch P. Endovascular treatment of visceral and renal artery aneurysms. *J Vasc Interv Radiol* 2011; **22**: 1246-1253 [PMID: 21741856 DOI: 10.1016/j.jvir.2011.05.012]
- 13 **Gabelmann A**, Görich J, Merkle EM. Endovascular treatment of visceral artery aneurysms. *J Endovasc Ther* 2002; **9**: 38-47 [PMID: 11958324]
- 14 **Sachdev-Ost U**. Visceral artery aneurysms: review of current management options. *Mt Sinai J Med* 2010; **77**: 296-303 [PMID: 20506455 DOI: 10.1002/msj.20181]
- 15 **Tessier DJ**, Fowl RJ, Stone WM, McKusick MA, Abbas MA, Sarr MG, Nagorney DM, Cherry KJ, Gloviczki P. Iatrogenic hepatic artery pseudoaneurysms: an uncommon complication after hepatic, biliary, and pancreatic procedures. *Ann Vasc Surg* 2003; **17**: 663-669 [PMID: 14564553 DOI: 10.1007/s10016-003-0075-1]
- 16 **Finley DS**, Hinojosa MW, Paya M, Imagawa DK. Hepatic artery pseudoaneurysm: a report of seven cases and a review of the literature. *Surg Today* 2005; **35**: 543-547 [PMID: 15976950 DOI: 10.1007/s00595-005-2987-6]
- 17 **Caminiti R**, Rossitto M, Ciccolo A. Pseudoaneurysm of the hepatic artery and hemobilia: a rare complication of laparoscopic cholecystectomy; clinical case and literature review. *Acta Chir Belg* 2011; **111**: 400-403 [PMID: 22299330]
- 18 **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]
- 19 **McGill DB**, Rakela J, Zinsmeister AR, Ott BJ. A 21-year experience with major hemorrhage after percutaneous liver biopsy. *Gastroenterology* 1990; **99**: 1396-1400 [PMID: 2101588]
- 20 **Christie AB**, Christie DB, Nakayama DK, Solis MM. Hepatic artery aneurysms: evolution from open to endovas-

- cular repair techniques. *Am Surg* 2011; **77**: 608-611 [PMID: 21679596]
- 21 **Bulut T**, Yamaner S, Bugra D, Akyuz A, Acarli K, Poyanli A. False aneurysm of the hepatic artery after laparoscopic cholecystectomy. *Acta Chir Belg* 2002; **102**: 459-463 [PMID: 12561154]
 - 22 **Sachdev U**, Baril DT, Ellozy SH, Lookstein RA, Silverberg D, Jacobs TS, Carroccio A, Teodorescu VJ, Marin ML. Management of aneurysms involving branches of the celiac and superior mesenteric arteries: a comparison of surgical and endovascular therapy. *J Vasc Surg* 2006; **44**: 718-724 [PMID: 17011997 DOI: 10.1016/j.jvs.2006.06.027]
 - 23 **Tulsyan N**, Kashyap VS, Greenberg RK, Sarac TP, Clair DG, Pierce G, Ouriel K. The endovascular management of visceral artery aneurysms and pseudoaneurysms. *J Vasc Surg* 2007; **45**: 276-83; discussion 283 [PMID: 17264002 DOI: 10.1016/j.jvs.2006.10.049]
 - 24 **Vainas T**, Klompenhouwer E, Duijm L, Tielbeek X, Teijink J. Endovascular treatment of a hepatic artery pseudoaneurysm associated with gastrointestinal tract bleeding. *J Vasc Surg* 2012; **55**: 1145-1149 [PMID: 22370249 DOI: 10.1016/j.jvs.2011.11.136]

P- Reviewers: Wang DR, Yu B **S- Editor:** Wen LL **L- Editor:** A
E- Editor: Ma S



Pancreatic pseudocystocolonic fistula treated without surgical or endoscopic intervention

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Received: September 11, 2013 Revised: December 9, 2013

Accepted: December 12, 2013

Published online: February 21, 2014

Abstract

We report here a case of pancreatic pseudocystocolic fistula that was treated without surgical or endoscopic intervention. A 76-year-old woman, presenting with a fever and epigastric pain, was referred to our institution. Three months prior to this admission, the patient had been admitted to the hospital for acute pancreatitis. Abdominal computerized tomography (CT) revealed a 9 cm pseudocyst containing air, and a fistular opening was observed *via* colonoscopy. After colonoscopy, the abdominal pain was slightly improved, the fever subsided and laboratory results showed decreased C-reactive protein levels. The observed improvement was likely due to the cleansing of the bowel, which induced spontaneous drainage from the pseudocyst into the colon. Antibiotic therapy was administered and daily bowel cleansing was performed using a polyethylene glycol solution. After three weeks, a follow-up CT

revealed that the size of the pseudocyst had decreased significantly from 9 to 5.3 cm. In addition, laboratory tests were improved. The patient was able to resume a normal diet and was discharged in good overall health from the hospital, without aggravation of the symptoms. A colonoscopy performed 3 mo later and a follow-up CT performed 6 mo later confirmed that both the fistula and pseudocyst had completely disappeared.

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Key words: Acute pancreatitis; Pseudocyst; Pseudocystocolic fistula

Core tip: We present the case of a 76-year-old female who was admitted to our institution with epigastric pain and a fever. Abdominal computed tomography and colonoscopy revealed a pseudocystocolic fistula. The patient was treated with antibiotics and bowel cleansing was administered using a polyethylene glycol solution. Induction of spontaneous drainage into the colon, through bowel cleansing, can treat small pseudocystocolic fistulas.

Kwon JC, Kim BY, Kim AL, Kim TH, Park MI, Jung HJ, Lim JH, Jung JK, Kim HS, Lee DW. Pancreatic pseudocystocolic fistula treated without surgical or endoscopic intervention. *World J Gastroenterol* 2014; 20(7): 1882-1886 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1882.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1882>

INTRODUCTION

The clinical manifestation of acute pancreatitis can range from mild to severe. Most cases of acute pancreatitis are mild and improve within a few days. However, severe

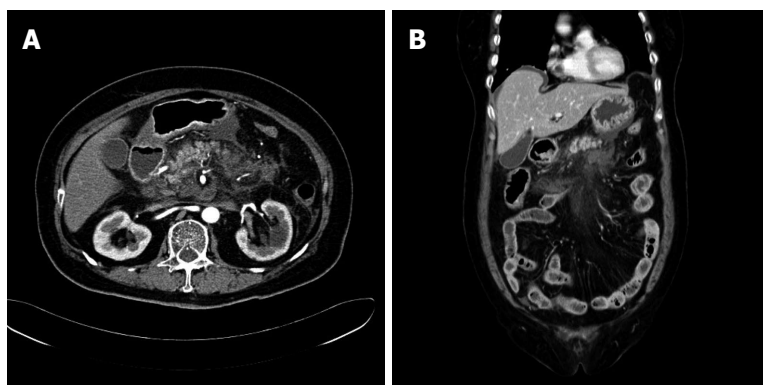


Figure 1 Fatty infiltration and fluid accumulation around the peripancreatic area observed by computerized tomography. Computerized tomography scans taken upon the patient's admission to the hospital. A: Axial view; B: Coronal view.

acute pancreatitis can lead to localized complications such as the formation of a pseudocyst, necrosis or an abscess, but it can also cause systemic complications, such as multi-organ failure or death. In acute pancreatitis complications, colonic involvement has a relatively low incidence of approximately 3%^[1]. Colonic complications range from a localized pseudo-obstruction of the ileus to necrosis, hemorrhage, ischemic colitis and fistula formation^[2]. Pseudocystocolonic fistula formation is especially dangerous when it induces massive lower gastrointestinal bleeding and sepsis, which require rapid diagnoses and appropriate treatments^[3]. Pseudocystocolonic fistulas have primarily been treated by surgery, but many successful cases of nonsurgical intervention have recently been reported^[4-6]. Here, we present a unique case of a patient with acute pancreatitis and a comorbid pseudocystocolonic fistula complication that was treated without any surgical or endoscopic intervention. The treatment and a literature review are reported here.

CASE REPORT

A 76-year-old female patient arrived at the emergency department of our hospital with severe epigastric pain lasting for 3 d. With the exception of maintenance drugs for insulin-independent diabetes mellitus and hypertension, which she had been taking for approximately 20 years, the patient had not taken any medications at the time of admission. The patient had no history of alcohol abuse. When she was admitted, the patient's blood pressure was 112/68 mmHg, her pulse rate was 86 beats/min, her respiratory rate was 23 breaths/min, and she had a body temperature of 38.0 °C.

An examination of peripheral blood at the time of admission yielded the following values: white blood cell (WBC) count of 20740/mm³ (neutrophils: 92.1%); hemoglobin: 15.5 g/dL; C-reactive protein (CRP): 30.52 mg/dL; total bilirubin: 3.84 mg/dL; aspartate aminotransferase (AST): 201 U/L; alanine aminotransferase (ALT): 201 U/L; alkaline phosphatase: 127 IU/L; γ -glutamyltranspeptidase: 403 IU/L; amylase: 178 U/L; lipase: 186 U/L; triglyceride: 149 mg/dL; and calcium: 10.0 mg/dL. Abdominal computerized tomography (CT) revealed extensive accumulation of extrapancreatic fluid along with peripancreatic fatty infiltration, which is typi-

cal of grade E pancreatitis, according to the Balthazar classification system (Figure 1). Because AST and ALT levels were markedly elevated, endoscopic retrograde cholangiopancreatography (ERCP) was performed for suspected gallstone pancreatitis, despite the fact that gallstones were not observed in the CT image^[7]. After endoscopic sphincterotomy and nasobiliary drainage, a plastic stent (5 Fr, 3 cm) was inserted into the pancreatic duct. Seven days after the procedure, the patient no longer complained of abdominal pain and had good overall health. Cholecystectomy was recommended to prevent recurrence, but the patient refused surgical intervention and was subsequently discharged.

Approximately 3 mo after the previous discharge, the patient presented to the hospital again with a 2-wk history of epigastric pain and a mild fever (37.5 °C). Laboratory results showed a WBC count of 9820/mm³ (neutrophils: 74.1%) and a CRP level of 10.27 mg/dL. Amylase, lipase, and other liver function test results were within the normal ranges. An abdominal CT showed an approximately 9.3 cm long pseudocyst that contained air. The presence of air led us to suspect the presence of a fistula on the right side of the colon (Figure 2). A colonoscopy was thus performed to determine if a fistula was present and assess the outcome of the antibiotic treatment. A fistula opening was found in the hepatic flexure (Figure 3). Two days after the colonoscopy, the patient's fever was reduced. Blood laboratory results showed improvements, with decreased CRP levels. The patient's pain was greatly reduced without requiring any invasive treatment or additional oral medications. The antibiotic treatment was continued. To maintain a clean colon and induce colonic drainage of the pseudocyst, a bowel-cleansing regimen was implemented using a polyethylene glycol (PEG) solution. A liter of PEG solution was administered orally every morning and afternoon after which the patient was monitored for the number of bowel movements. If two or less bowel movements were observed, an additional half-liter of the PEG solution was administered in the evening.

After the bowel cleansing treatment, the patient was put on a liquid diet. However, she complained of abdominal pain again and blood tests revealed an increased WBC count and CRP level (Figure 4). A follow-up CT scan showed that although the size of the pseudocyst was

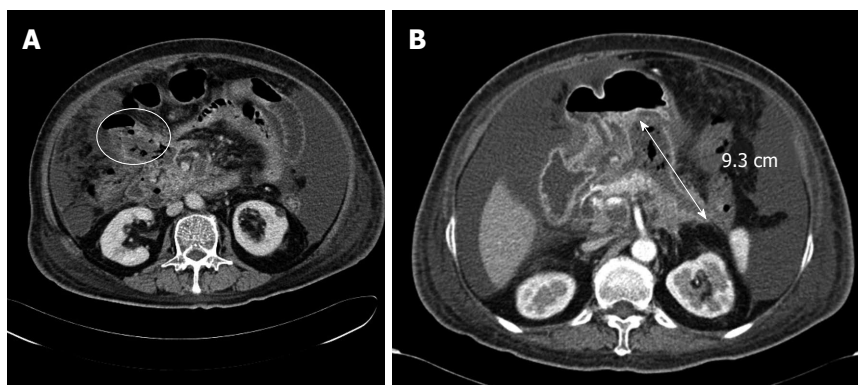


Figure 2 A fistula and a pseudocyst identified through computerized tomography. A: There was a fistula in the right side of the colon (yellow circle); B: There was a pseudocyst approximately 9.3 cm long (demarcated by double-headed arrow) that contained air.

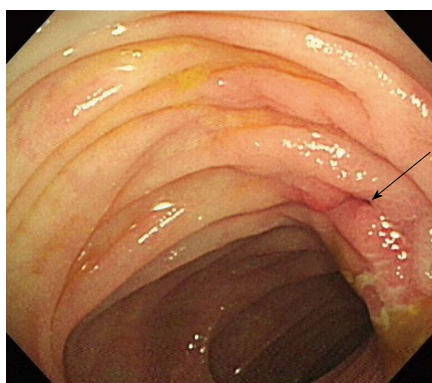


Figure 3 Fistula opening identified by colonoscopy. There was a fistular opening (arrow) that surrounded edematous and hyperemic mucosa at the hepatic flexure.

reduced to approximately 5.3 cm, air was still present inside. Thus, fasting and the bowel-cleansing regimen were continued for an additional 3 wk.

After the 3 wk, the patient resumed a normal diet and was discharged from the hospital in good health, without aggravation of the symptoms. After 3 mo, a follow-up colonoscopy confirmed complete disappearance of the fistula. After 6 mo, a CT confirmed the complete disappearance of the pseudocyst. The patient is currently under outpatient follow-up with subclinical conditions.

DISCUSSION

A pseudocyst, a complication of acute pancreatitis, tends to rupture in the abdominal cavity or form a fistula in the gastrointestinal tract. Pseudocysts occur most frequently in the stomach, accounting for approximately one-third of all cases, followed by the colon and duodenum^[8]. The mechanism underlying fistula formation is thought to be due to elevated internal pressure from fluid accumulation within the pseudocyst and proteolytic enzymes within the fluid that invade adjacent organs or vessels, inducing ischemic changes that enable penetration of the walls of the most vulnerable organs and formation of a fistula^[8]. Most pseudocystoenteric fistulas that form in the upper gastrointestinal tract can be treated conservatively and have a relatively good prognosis. In contrast, fistulas that form in the colon rarely heal spontaneously and tend to be as-

sociated with fatal complications, with a reported mortality of approximately 17%-67%^[9,10]. Such poor prognosis can be attributed to the high possibility of bleeding or sepsis induced by necrosis in peripancreatic tissues and vessels caused by activated pancreatic enzymes mediated by bacteria in the colon^[3].

A CT colonography, performed with water-soluble contrast agents, is a relatively safe and conventional method to diagnose pseudocystocolic fistulas^[1]. However, because colonography has low sensitivity in detecting a fistulous tract compared with ERCP, it is no longer widely used^[11]. Diagnosis can also be achieved using a radiograph obtained after contrast agent injection *via* a nasocystic drainage catheter or percutaneous catheter inserted for pseudocyst drainage^[10,12]. With the recent advent of medical imaging technologies, noninvasive radiologic modalities, such as abdominal CT or magnetic resonance imaging, have also been used for diagnosis^[10,13]. In the present case, the final diagnosis was made based on colonoscopic findings under the impression of a pseudocyst-associated fistula in the right side of the colon, as determined by a 64-channel multidetector CT.

In the past, surgical interventions, such as debridement, wide drainage or externalization of the fistula *via* a temporary colostomy, were the treatment of choice for pseudocystocolic fistulas^[5]. Recent reports, however, have described cases where large fistulas were successfully treated using endoscopic tools, such as an endoloop, clips, and fibrin glue^[6], or fibrin glue alone to treat small fistulas^[14]. Indications for such nonsurgical treatments have not yet been clearly established. Although endoscopic treatments are considered for patients with well-defined pseudocysts that have been complicated by infection, close follow-up and repetitive interventions may be needed and surgical treatments are recommended when sepsis cannot be controlled^[5]. Because the patient in the case described herein had a well-defined pseudocyst with non-life-threatening conditions and a small fistula opening, we chose to treat the patient with a PEG solution. The patient's symptoms and laboratory findings improved after administration of the PEG solution during the colonoscopy. The bowel-cleansing regimen was continued alongside antibiotic therapy without the need for any invasive treatment.

Complete recovery of a pseudocystocolic fistula can be confirmed by the disappearance of the pseudo-

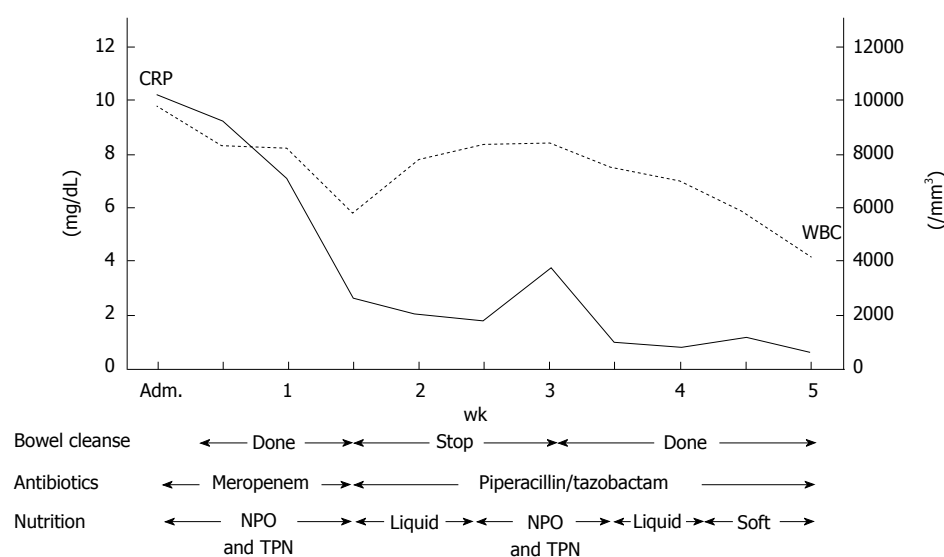


Figure 4 Timetable of bowel cleansing, administration of antibiotics and nutrition. After daily bowel cleansing using a polyethylene glycol solution, white blood cell (WBC) count and C-reactive protein (CRP) value were decreased. After the initial bowel cleanse was stopped, we observed an elevated WBC count and CRP level. The bowel cleanse was continued until 6 wk after admission. Adm: Admission; NPO: Nil per os; TPN: Total parenteral nutrition.

cyst and fistula with a follow-up CT scan and improved clinical symptoms in the patient^[4-6,12]. In this case, the complete disappearance of the fistula was confirmed by follow-up colonoscopy performed 3 mo after treatment. The disappearance of the pseudocyst was confirmed by CT imaging, 6 mo after the patient was admitted.

The present case indicates that when the fistula size is small and the patient's overall health status is good, a pseudocystocolonic fistula can be treated by inducing spontaneous drainage to the colon through a bowel-cleansing regimen, without additional surgical intervention or fistula closure.

COMMENTS

Case characteristics

A 76-year-old female, previously hospitalized for gallstones and pancreatitis 3 mo prior, complained of epigastric pain and fever.

Clinical diagnosis

The patient was diagnosed with a large pseudocyst with a fistula in the right side of the colon.

Differential diagnosis

Abdominal computerized tomography (CT) was performed to identify the cause of the pain and fever.

Laboratory diagnosis

Elevated C-reactive protein was seen in the laboratory results, suggesting an acute inflammatory condition.

Imaging diagnosis

Pseudocyst and fistula in the right of the side colon was seen in an abdominal CT and a fistular opening at the hepatic flexure was confirmed with the colonoscopy.

Treatment

Antibiotics were given to treat the systemic inflammation and polyethylene glycol was administered for colon cleansing.

Related reports

Two previous reports by Will *et al* and Karvonen *et al* showed treatment of a pseudocystocolonic fistula using an endoscopic method.

Experiences and lessons

In patients with good overall health and a small fistula size, a pseudocystocolonic

fistula can be treated by inducing spontaneous drainage to the colon through bowel cleansing, without additional surgical intervention or fistula closure.

Peer review

This case report by Kwon *et al* of a 76-year-old woman presented with acute pancreatitis and as a complication pseudocystocolonic fistula was successfully treated with antibiotics and 3-wk bowel cleansing using polyethylene glycol solution. The disappearance of the pseudocyst and fistula was confirmed by abdominal CT scan and colonoscopy.

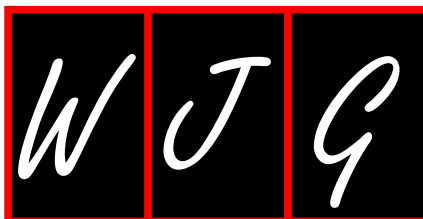
REFERENCES

- 1 **Mohamed SR**, Siriwardena AK. Understanding the colonic complications of pancreatitis. *Pancreatology* 2008; **8**: 153-158 [PMID: 18382101 DOI: 10.1159/000123607]
- 2 **De Backer AI**, Mortelé KJ, Vaneerdeweg W, Ros PR. Pancreatocolonic fistula due to severe acute pancreatitis: imaging findings. *JBR-BTR* 2001; **84**: 45-47 [PMID: 11374629]
- 3 **Santos JC**, Feres O, Rocha JJ, Aracava MM. Massive lower gastrointestinal hemorrhage caused by pseudocyst of the pancreas ruptured into the colon. Report of two cases. *Dis Colon Rectum* 1992; **35**: 75-77 [PMID: 1733688 DOI: 10.1007/BF02053343]
- 4 **Wolfsen HC**, Kozarek RA, Ball TJ, Patterson DJ, Traverso LW, Freeny PC. Pancreaticocenteric fistula: no longer a surgical disease? *J Clin Gastroenterol* 1992; **14**: 117-121 [PMID: 1556424 DOI: 10.1097/00004836-199203000-00009]
- 5 **Howell DA**, Dy RM, Gerstein WH, Hanson BL, Biber BP. Infected pancreatic pseudocysts with colonic fistula formation successfully managed by endoscopic drainage alone: report of two cases. *Am J Gastroenterol* 2000; **95**: 1821-1823 [PMID: 10925992 DOI: 10.1111/j.1572-0241.2000.02162.x]
- 6 **Will U**, Meyer F, Hartmeier S, Schramm H, Bosseckert H. Endoscopic treatment of a pseudocystocolonic fistula by band ligation and endoloop application: case report. *Gastrointest Endosc* 2004; **59**: 581-583 [PMID: 15044905]
- 7 **Tenner S**, Dubner H, Steinberg W. Predicting gallstone pancreatitis with laboratory parameters: a meta-analysis. *Am J Gastroenterol* 1994; **89**: 1863-1866 [PMID: 7942684]
- 8 **Shatney CH**, Sosin H. Spontaneous perforation of a pancreatic pseudocyst into the colon and duodenum. *Am J Surg* 1973; **126**: 433-438 [PMID: 4728935 DOI: 10.1016/S0002-9610(73)80140-0]

- 9 **Wille-Jørgensen P**, Frederiksen HJ. Colonic necrosis or fistula following pancreatitis or gastric surgery. *Eur J Surg* 1991; **157**: 137-139 [PMID: 1676308]
- 10 **Suzuki A**, Suzuki S, Sakaguchi T, Oishi K, Fukumoto K, Ota S, Inaba K, Takehara Y, Sugimura H, Uchiyama T, Konno H. Colonic fistula associated with severe acute pancreatitis: report of two cases. *Surg Today* 2008; **38**: 178-183 [PMID: 18239882 DOI: 10.1007/s00595-007-3593-6]
- 11 **Shim KS**, Suh JM, Yang YS, Choi JY, Park YH. Three-dimensional demonstration and endoscopic treatment of pancreaticoperitoneal fistula. *Am J Gastroenterol* 1993; **88**: 1775-1779 [PMID: 8213724]
- 12 **Urakami A**, Tsunoda T, Hayashi J, Oka Y, Mizuno M. Spontaneous fistulization of a pancreatic pseudocyst into the colon and duodenum. *Gastrointest Endosc* 2002; **55**: 949-951 [PMID: 12024164 DOI: 10.1067/mge.2002.124555]
- 13 **Tüney D**, Altun E, Barlas A, Yegen C. Pancreatico-colonic fistula after acute necrotizing pancreatitis. Diagnosis with spiral CT using rectal water soluble contrast media. *JOP* 2008; **9**: 26-29 [PMID: 18182739]
- 14 **Karvonen J**, Gullichsen R, Salminen P, Grönroos JM. Endoscopic treatment of pseudocystocolonic fistula with fibrin glue. *Gastrointest Endosc* 2010; **72**: 664-665 [PMID: 20421099 DOI: 10.1016/j.gie.2009.12.030]

P- Reviewers: Said SAM, Sumi S **S- Editor:** Song XX
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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 1348 experts in gastroenterology and hepatology from 68 countries.

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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.

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Name of journal

World Journal of Gastroenterology

ISSN

ISSN 1007-9327 (print)

ISSN 2219-2840 (online)

Launch date

October 1, 1995

Frequency

Weekly

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- 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

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- 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]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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- 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

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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>

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- 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

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