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

World Journal of *Gastroenterology*

World J Gastroenterol 2021 December 28; 27(48): 8201-8377

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 27 Number 48 December 28, 2021

FRONTIER

REVIEW

ORIGINAL ARTICLE

Basic Study

Case Control Study

Retrospective Study

LETTER TO THE EDITOR

ABOUT COVER

Editorial Board Member of *World Journal of Gastroenterology*, Jakob Robert Izbicki, MD, Professor, Chairman, Department of General, Visceral and Thoracic Surgery, University of Hamburg, Martinistr. 52, Hamburg D-20246, Germany. izbicki@uke.de

AIMS AND SCOPE

The primary aim of *World Journal of Gastroenterology* (*WJG*, *World J Gastroenterol*) is to provide scholars and readers from various fields of gastroenterology and hepatology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. *WJG* mainly publishes articles reporting research results and findings obtained in the field of gastroenterology and hepatology and covering a wide range of topics including gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, gastrointestinal oncology, and pediatric gastroenterology.

INDEXING/ABSTRACTING

The *WJG* 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, and Scopus. The 2021 edition of Journal Citation Report® cites the 2020 impact factor (IF) for *WJG* as 5.742; Journal Citation Indicator: 0.79; IF without journal self cites: 5.590; 5-year IF: 5.044; Ranking: 28 among 92 journals in gastroenterology and hepatology; and Quartile category: Q2. The *WJG*'s CiteScore for 2020 is 6.9 and Scopus CiteScore rank 2020: Gastroenterology is 19/136.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Jia-Hui Li*; **Production Department Director:** *Yu-Jie Ma*; **Editorial Office Director:** *Ze-Mao Gong*.

NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORS-IN-CHIEF
Andrzej S Tarnawski

EDITORIAL BOARD MEMBERS
http://www.wjgnet.com/1007-9327/editorialboard.htm

PUBLICATION DATE
December 28, 2021

INSTRUCTIONS TO AUTHORS
https://www.wjgnet.com/bpg/gerinfo/204

GUIDELINES FOR ETHICS DOCUMENTS
https://www.wjgnet.com/bpg/GerInfo/287

GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH
https://www.wjgnet.com/bpg/gerinfo/240

PUBLICATION ETHICS
https://www.wjgnet.com/bpg/GerInfo/288

PUBLICATION MISCONDUCT
https://www.wjgnet.com/bpg/gerinfo/208

ARTICLE PROCESSING CHARGE
https://www.wjgnet.com/bpg/gerinfo/242

STEPS FOR SUBMITTING MANUSCRIPTS
https://www.wjgnet.com/bpg/GerInfo/239

ONLINE SUBMISSION
https://www.f6publishing.com

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 December 28; 27(48): 8201-8215

DOI: 10.3748/wjg.v27.i48.8201

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

FRONTIER

Palmitoylation in Crohn's disease: Current status and future directions

Wei-Xin Cheng, Yue Ren, Miao-Miao Lu, Ling-Ling Xu, Jian-Guo Gao, Dong Chen, Farhin Shaheed Kalyani, Zi-Yan Lv, Chun-Xiao Chen, Feng Ji, He-Ning Lin, Xi Jin

ORCID number: Wei-Xin Cheng 0000-0003-1764-0779; Yue Ren 0000-0001-9071-2738; Miao-Miao Lu 0000-0002-9328-8239; Ling-Ling Xu 0000-0002-6668-3057; Jian-Guo Gao 0000-0002-9414-5395; Dong Chen 0000-0003-3892-9248; Farhin Shaheed Kalyani 0000-0001-8276-8125; Zi-Yan Lv 0000-0003-3987-1009; Chun-Xiao Chen 0000-0001-7536-1191; Feng Ji 0000-0003-4252-8850; He-Ning Lin 0000-0002-0255-2701; Xi Jin 0000-0001-9985-3035.

Author contributions: Cheng WX, Ren Y, Lu MM, Xu LL, Gao JG, Chen D, and Lv ZY conducted the literature review and wrote the section on Crohn's disease; Lin H conceived and wrote the section on palmitoylation; Kalyani FS assisted with the writing and edited the review for language; Chen CX and Ji F conceived the review of the topic of Crohn's disease and assisted with the literature review; Jin X conceived the overall concept of the review and supervised the research and writing processes.

Conflict-of-interest statement: Lin H is founder and consultant for Sedec Therapeutics. All other authors declare that they have no conflict of interest.

Supported by National Science Foundation of China, No. 81770574; and Zhejiang Provincial

Wei-Xin Cheng, Yue Ren, Miao-Miao Lu, Ling-Ling Xu, Jian-Guo Gao, Farhin Shaheed Kalyani, Chun-Xiao Chen, Feng Ji, Xi Jin, Department of Gastroenterology, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, Zhejiang Province, China

Dong Chen, Department of Colorectal Surgery, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, Zhejiang Province, China

Zi-Yan Lv, Wenzhou Medical University Renji College, Wenzhou 325035, Zhejiang Province, China

He-Ning Lin, Department of Chemistry and Chemical Biology, Howard Hughes Medical Institute, Cornell University, Ithaca, NY 14853, United States

Corresponding author: Xi Jin, PhD, Professor, Department of Gastroenterology, The First Affiliated Hospital, Zhejiang University School of Medicine, No. 79 Qingchun Road, Hangzhou 310003, Zhejiang Province, China. jxfl007@zju.edu.cn

Abstract

S-palmitoylation is one of the most common post-translational modifications in nature; however, its importance has been overlooked for decades. Crohn's disease (CD), a subtype of inflammatory bowel disease (IBD), is an autoimmune disease characterized by chronic inflammation involving the entire gastrointestinal tract. Bowel damage and subsequent disabilities caused by CD are a growing global health issue. Well-acknowledged risk factors for CD include genetic susceptibility, environmental factors, such as a westernized lifestyle, and altered gut microbiota. However, the pathophysiological mechanisms of this disorder are not yet comprehensively understood. With the rapidly increasing global prevalence of CD and the evident role of S-palmitoylation in CD, as recently reported, there is a need to investigate the relationship between CD and S-palmitoylation. In this review, we summarize the concept, detection, and function of S-palmitoylation as well as its potential effects on CD, and provide novel insights into the pathogenesis and treatment of CD.

Key Words: S-palmitoylation; Crohn's disease; STING; Pathogenesis; Signaling pathway; Drug therapy

National Science Foundation, No. LZ21H030002.

Country/Territory of origin: China

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: March 19, 2021
Peer-review started: March 19, 2021
First decision: May 1, 2021
Revised: May 8, 2021
Accepted: December 2, 2021
Article in press: December 2, 2021
Published online: December 28, 2021

P-Reviewer: Flemming S, Liakina V
S-Editor: Ma YJ
L-Editor: A
P-Editor: Ma YJ

Core Tip: S-palmitoylation is one of the most common post-translational modifications in nature; however, its importance has been overlooked for decades. Crohn's disease (CD) is an autoimmune disease characterized by chronic inflammation of the entire gastrointestinal tract, whose underlying mechanisms of action remain poorly understood. Recent studies have revealed a key role of S-palmitoylation in CD; therefore, there is a need to elucidate the relationship between CD and S-palmitoylation. This review summarizes the basic facts of S-palmitoylation and its potential effect on CD to provide novel insights into the pathogenesis and treatment of CD.

Citation: Cheng WX, Ren Y, Lu MM, Xu LL, Gao JG, Chen D, Kalyani FS, Lv ZY, Chen CX, Ji F, Lin HN, Jin X. Palmitoylation in Crohn's disease: Current status and future directions. *World J Gastroenterol* 2021; 27(48): 8201-8215
URL: https://www.wjgnet.com/1007-9327/full/v27/i48/8201.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i48.8201

INTRODUCTION

Cysteine palmitoylation or S-palmitoylation is the process of adding a 16-carbon saturated fatty acyl chain to the sulfhydryl group of cysteine residues of proteins *via* a labile thioester bond[1,2]. Initial reports on the modification of proteins by palmitate using ^{14}C-labeled palmitic acid date back to the 1970s. Evidence supporting the modification of cysteine residues emerged in the 1980s[3]. Since then, accumulating evidence has shown that over 2000 proteins are S-palmitoylated in mammals, as documented in SwissPalm, an S-palmitoylation database (https://swisspalm.org/). Nevertheless, although S-palmitoylation widely occurs in nature, similar to phosphorylation, acetylation, and ubiquitination, its importance in human health and disease has been overlooked over the years. In fact, there are currently no approved drugs known to target S-palmitoylation. Crohn's disease (CD), a subtype of inflammatory bowel disease (IBD), is characterized by chronic inflammation of the gastrointestinal tract with or without systemic symptoms, leading to bowel damage and disability[4]. Currently, genetic susceptibility, environmental factors, such as a western lifestyle, and an altered gut microbiota are well-known risk factors for CD[5]. However, the detailed mechanism underlying this disorder has yet to be elucidated. With the rapidly increasing global prevalence of CD[6] and recent reports on the effect of S-palmitoylation on CD[7], evaluating the relationship between CD and S-palmitoylation is a meaningful effort to gain insights into pathogenesis and treatment of CD. In this review, we summarize the concept, measurement, and function of S-palmitoylation, as well as its potential effect on CD, with the aim of providing insights into the pathogenesis and treatment of CD.

OVERVIEW OF PROTEIN CYSTEINE PALMITOYLATION

Enzymes controlling S-palmitoylation

The addition of S-palmitoylation is catalyzed by palmitoyltransferases. Known palmitoyltransferases belong to the zinc finger aspartate-histidine-histidine-cysteine (ZDHHC) family[2]. There are 23 ZDHHC proteins in humans and mice, using palmitoyl-CoA as the major palmitoyl donor to acylate substrate proteins[8]. It should be noted that even though proteins prefer palmitoyl-CoA, they are also able to utilize other similar acyl-CoA molecules as substrates; therefore, some researchers prefer to use S-acylation over S-palmitoylation as a more general term to reflect the use of several different long-chain fatty acyl groups. Here, the term S-palmitoylation is used to represent all similar long-chain acylations on cysteine catalyzed by ZDHHCs. In most cases, these acylations are likely to have similar functions; thus, there is no need to specifically differentiate them for the purpose of this review.

ZDHHC proteins are integral membrane proteins with at least four transmembrane helices (Figure 1A). The conserved DHHC cysteine-rich domain is present in the intracellular loop between transmembrane domains 2 and 3[1]. The cysteine residue in

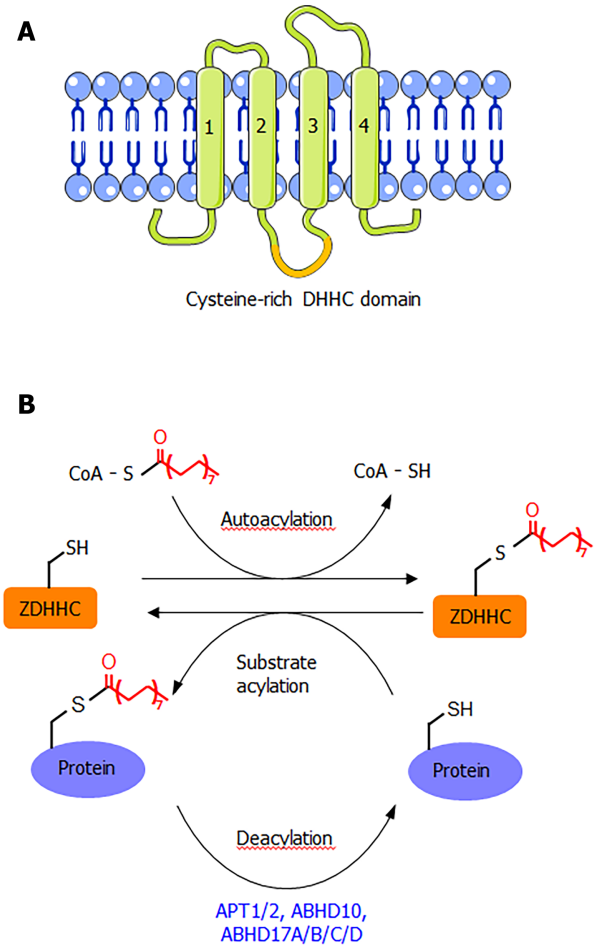

Figure 1 Protein S-palmitoylation. A: The ZDHHC-type palmitoyltransferases are integral membrane proteins with at least four transmembrane helices. The cysteine rich domain containing the DHHC motif is between the second and third transmembrane helices; B: Scheme showing the palmitoylation and depalmitoylation process. ZDHHC are self-palmitoylated first before transferring the palmitoyl group to substrate proteins. Depalmitoylation is catalyzed by the alpha/beta hydrolases.

the conserved DHHC motif is known to serve as a catalytic nucleophile that reacts with the thioester bond in palmitoyl-CoA, forming a palmitoyl-enzyme intermediate, which then relays the palmitoyl group to the cysteine residues in the substrate proteins [1,2,8]. The crystal structure of DHHC20 has been reported[9], providing a structural basis for understanding this class of enzymes. Although S-palmitoylation is not a very stable modification due to the chemically labile nature of the thioester bond, the removal of S-palmitoylation is known to be catalyzed by several depalmitoylases (Figure 1B), including acyl protein thioesterase (APT1 and APT2), α/β-hydrolase domain 17 (ABHD17A/B/C/D), and α/β-hydrolase domain 10 (ABHD10)[1,10]. These enzymes belong to the alpha-beta hydrolase family, with a catalytic serine residue in the active site.

Functions of S-palmitoylation

The most common function of S-palmitoylation is to promote the membrane localization of proteins. This can be easily appreciated from a recent review that listed many S-palmitoylated proteins and the function of S-palmitoylation[1]. This function is consistent with the hydrophobic nature of the palmitoyl group, which is especially true for peripheral membrane proteins (proteins without integral transmembrane domains). One well-known example is the small GTPases of the Ras subfamily, H-Ras, N-Ras, and K-Ras4a[1,11]. These proteins are soluble cytosolic proteins, but function at the plasma membrane or intracellular membranes. Their targeting to the plasma membrane requires prenylation and palmitoylation at the C-terminal sequence. Interestingly, it has been shown that the palmitoylation-depalmitoylation cycle helps to actively promote the trafficking of Ras to the plasma membrane. Several non-receptor tyrosine kinases, such as Fyn and Lyn, also require palmitoylation to target the plasma membrane.

Many integral membrane proteins are also palmitoylated. Integral membrane proteins contain transmembrane domains; thus, in principle, they should not require palmitoylation for membrane targeting. Instead, many reports indicate that palmitoylation promotes the targeting of these proteins to lipid rafts, which are specific membrane microdomains. This phenomenon requires further exploration in future studies. Other functional effects of S-palmitoylation have also been reported, including the regulation of protein stability and the aggregation of proteins[1,8]. However, the exact mechanism of these effects is unclear and may be indirectly caused by the membrane-targeting effect of S-palmitoylation.

Methods for detecting S-palmitoylation

Many convenient tools have been developed for the study of S-palmitoylation, making it relatively easy to study compared to other post-translational modifications. The chemically labile nature of S-palmitoylation has enabled the development of several methods for its detection, including acyl-biotin exchange (ABE)[12,13], acyl-resin-assisted capture (Acyl-Rac)[14], and acyl-PEG exchange (APE)[1,15] (Figure 2A). A common procedure for these methods is to first cap free cysteine residues using a cysteine alkylation reagent, such as iodoacetamide or N-ethyl maleimide. Next, hydroxylamine is used to break down palmitoyl cysteine and release it as a free cysteine. The newly released free cysteine is then captured using a thiol-reactive group (HPDP-biotin in ABE, thiol-reactive resin in acyl-RAC, and thiol-reactive PEG in APE). In ABE, the biotinylation of palmitoylated proteins allows for affinity pulldown using streptavidin beads, and the palmitoylated proteins can then be detected after protein electrophoresis and western blotting, or analyzed by mass spectrometry (MS) in proteomic studies. In acyl-RAC, the palmitoylated proteins are pulled down using a resin and then analyzed using MS in proteomic studies. In a typical procedure for MS detection, the modified peptide is usually not detected by mass spectrometers because it is modified with a large biotin molecule or retained on the resin. However, certain modifications to this procedure can facilitate the detection of palmitoylated peptide. In APE, a large PEG molecule is attached to the palmitoylated protein of interest, which can change the protein size, which in turn can determine the number of palmitoyl cysteine modifications on the protein.

The ABE and acyl-RAC methods have the advantage of being able to detect S-palmitoylation in animal tissues as they reflect the endogenous palmitoylation level of endogenous proteins. A disadvantage of these methods is that there is no information on the identity of the acyl group on the cysteine residues as the hydroxylamine treatment removes all acyl modifications on cysteine residues. Theoretically, a short-chain acyl group modification could mistakenly be identified as palmitoylation; however, we are not aware of any such report for any protein. Another potential disadvantage is that a certain protein's S-palmitoylation may be hydroxylamine-resistant and, therefore, may affect the outcome in acyl exchange assays[16].

A complementary method that could address the limitations of these acyl-exchange methods is metabolic labeling with labeled fatty acid analogs (Figure 2B). Although ^{14}C-labeled palmitic acid was commonly used in early studies, a more convenient and sensitive method that has become commonly used in recent decades is that of clickable fatty acid analogs. This method typically uses an alkyne-tagged fatty acid, such as Alk14, which has 16 carbons similar to palmitic acid, but ends with a C-C triple bond at the end[17,18]. The structure of Alk14 is very similar to that of palmitic acid and can be efficiently utilized by cellular machinery to convert to the corresponding acyl-CoA and acylate proteins. The Alk14-modified protein can then be conjugated to an azide-containing fluorescent or biotin tag using a highly efficient copper-catalyzed cyclo-addition reaction. The conjugation of a fluorescent dye allows for the in-gel fluorescent detection of the Alk14 modification, while the conjugation of biotin allows for affinity purification and MS identification in proteomic studies.

The Alk14 Labeling method is in many ways comparable to ABE, as both allow for the gel-based detection of the S-palmitoylation of proteins of interest and proteomic studies. Alk14 Labeling does not require S-palmitoylation to be sensitive to hydroxylamine and can readily label proteins with dynamic palmitoylation. In contrast to ABE, Alk14 Labeling reflects the ability of a protein to be palmitoylated, but it is technically not the endogenous palmitoylation and is rarely used in animal studies. Generally, Alk14 Labeling and ABE are highly complementary to each other, with Alk14 demonstrating the palmitoylation of target proteins and ABE able to determine endogenous modifications on endogenous proteins. These two methods are often used simultaneously to confirm the S-palmitoylation of a protein of interest.

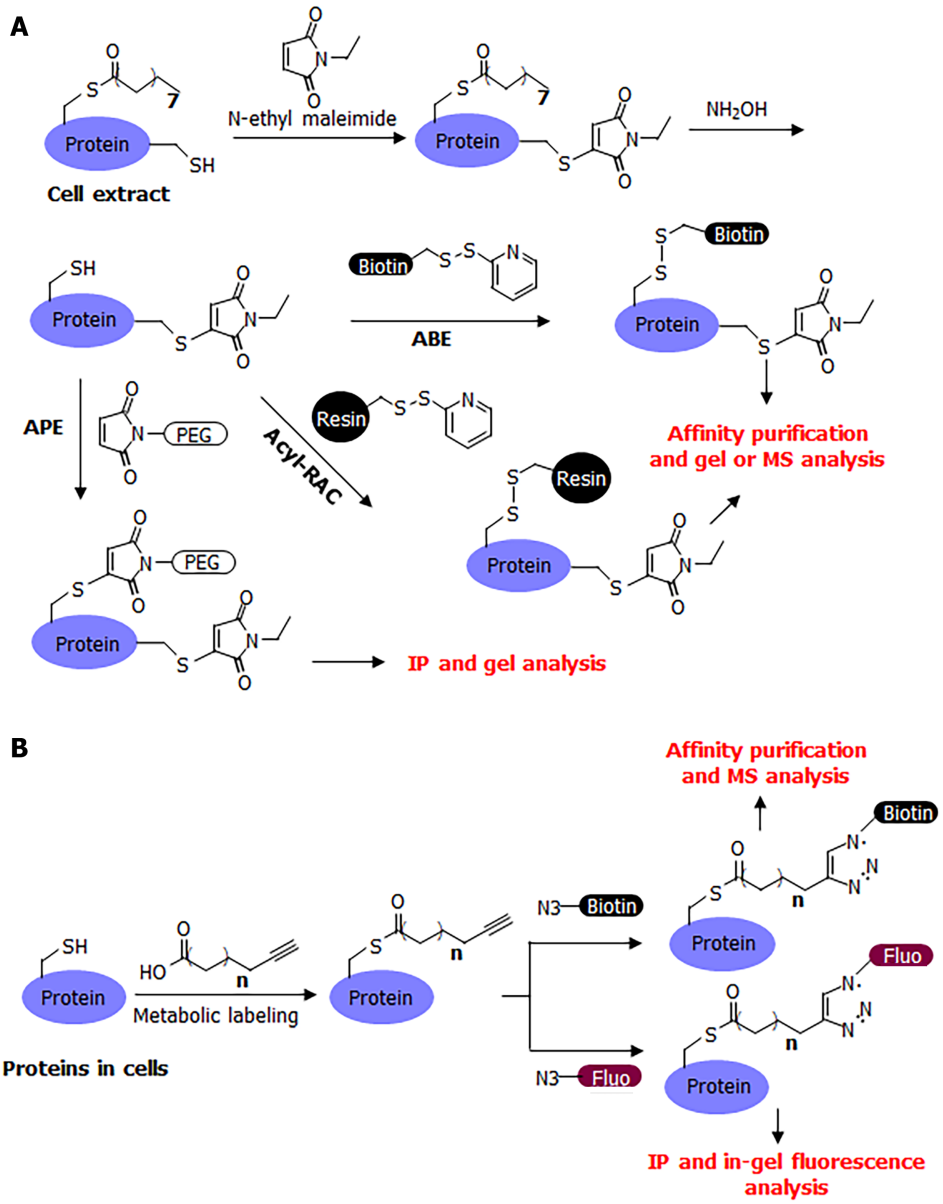

Figure 2 Commonly used methods for detecting S-palmitoylation. A: Scheme showing how acyl-biotin exchange (ABE), acyl-resin assisted capture (acyl-RAC), and acyl-PEG exchange (APE) work; B: Scheme showing how metabolic labeling with alkyne-tagged fatty acid analogs works. ABE: Acyl-biotin exchange; acyl-RAC: Acyl-resin assisted capture; APE: Acyl-PEG exchange.

In comparison to other modifications, such as lysine acetylation, these detection methods make S-palmitoylation relatively straightforward to study. For lysine acetylation, a pan-acetyl-lysine antibody is typically used for affinity pull-down modified proteins, which are then subjected to MS analysis[19]. For a given protein of interest, it can be pulled down using immunoprecipitation, and then acetyl-lysine modification can be detected using western blotting with pan-acetyl-lysine antibodies. These studies rely heavily on the pan-acetyl-lysine antibody, which is expensive and may not work for all acetyl-lysine peptides. For S-palmitoylation, there is no antibody currently available, but acyl exchange methods and metabolic labeling methods have been found to work extremely well.

Methods for studying the functional effects of S-palmitoylation

To investigate the function of palmitoylation on a particular protein, the most common method involves the identification of the site of palmitoylation, followed by the evaluation of the effect of cysteine to serine or alanine mutations on protein function. Typically, the occurrence of cysteine residues in proteins is less frequent compared to other modified residues, such as lysine, making the task of mutating all cysteine residues in a protein of interest much more practical than mutating all lysine residues. If a cysteine residue of a protein is the major palmitoylation site, then mutating it to

Ser/Ala would markedly decrease the S-palmitoylation of the protein (detected by ABE or Alk14 Labeling). Subsequently, the same mutant can be used to observe whether the mutation affects protein localization, stability, and interaction with other proteins, as well as other biochemical activities.

The mutagenesis method, although powerful, has limitations. The mutated cysteine residue may have other functions (structural function or other modifications), which in turn can affect the palmitoylation of the protein; therefore, complementary methods to further confirm its function should be used. These complementary methods include identifying the ZDHHC enzyme that is responsible for the S-palmitoylation of the protein and determining whether the knockdown or knockout of ZDHHC produces the same effect as mutating the modified Cys residues. Similarly, identifying and disrupting the depalmitoylase also produces results consistent with the mutation of palmitoylated Cys. Recently, a method using amber suppression technology and click chemistry to insert a palmitoyl cysteine mimic on proteins in live HEK293T cells has been reported[20]. This method may allow for the gain-of-function analysis of S-palmitoylation. However, how closely the palmitoyl cysteine mimic can replicate the functional effect of S-palmitoylation remains unclear and will need to be tested in more proteins in future studies.

PATHOPHYSIOLOGY AND MOLECULAR PATHWAYS OF CROHN'S DISEASE

A brief review of the pathophysiology of Crohn's disease

It is widely acknowledged that the pathophysiology of CD involves multiple factors, including genetic, environmental, microbial, immunologic, epithelial, and gut mucosal factors[21-23]. These factors are explored in detail in this section.

Genetic factors: Genome-wide association studies (GWAS) have identified over 240 risk variants that affect the recognition of microbial products by intracellular pathways [such as nucleotide oligomerization domain (NOD)–like receptors 2 (NOD2)], autophagy pathways that promote intracellular organelle circulation and the clearance of intracellular microorganisms [such as autophagy-related protein 16 Like 1 (ATG16L1) and immunity-related GTPase M (IRGM)], genes that regulate epithelial barrier function [such as extracellular matrix protein 1 (ECM1)], and pathways that regulate innate and adaptive immunity [such as interleukin (IL)-23R and IL-10][21,22]. Interestingly, known associations between CD and NOD2 gene variants are mainly found in patients of European or Jewish origin, but not in patients of Japanese or Chinese origin[22,24,25]. Another GWAS study supports this, additionally revealing that the tumor necrosis factor superfamily member 15 (TNFSF15) variant is dominant in East Asian populations[26]. These results conclusively indicate that different genetic factors contribute to CD through different inflammatory pathways.

Environmental factors: A series of environmental factors have been reported to affect the incidence of CD, including breastfeeding, living on farms, childhood contact with animals, smoking, antibiotic exposure, and dietary pattern[4,27-29]. Although inconsistent, breastfeeding, living on farms, and childhood contact with animals are believed to represent protective factors for CD[4]. Smoking is one of the most consistently reported risk factors for CD and is associated with a two-fold increase in the risk of developing CD (OR = 1.76, 95%CI: 1.40–2.22)[4,27,28]. A meta-analysis revealed that exposure to antibiotics also markedly increased the risk of CD, especially in children (OR = 2.75, 95%CI: 1.724.38)[29]. Low dietary fiber and an increased intake of saturated fats are also associated with an increased risk of developing CD[4].

Microbial factors: Although the gut host-microbial relationship is symbiotic, close contact between a rich bacterial community and intestinal tissue poses a great risk to health. In humans, in excess of $10^{12}/cm^3$ of bacteria over a span of approximately 200 m^2 are separated from the intestinal tissues by a mere 10-μm epithelial layer[30]. Therefore, it is crucial to maintain homeostasis between the microbiota and mucosal immunity in the gut. Mucus, defensins, IgA, and RegIIIγ are products of the epithelial and immune cells that control the gut microbiota. Certain microbes are beneficial to the growth of various T cell subsets, promoting the induction of type 17 T helper (Th17), regulatory T (Treg), and type 1 T helper (Th1) cells, and regulate mucosal immunity[22,30]. In addition, gut microbes can produce essential components, such as vitamin K, an important coagulation cofactor, and short-chain fatty acids, which are

energy sources for colon epithelial cells[21,31]. Numerous studies have shown that changes in the microbial community result in a dysregulation of homeostasis[31,32]. In these studies, CD was associated with a decrease in the total number, diversity, and richness of microbial species.

Immune factors: CD arises as a result of chronic gastrointestinal inflammation and is associated with tissue destruction *via* the aberrant expression of pro-and anti-inflammatory molecules in response to innate and adaptive immune systems[33,34]. Amongst the numerous immune cells involved, Th17 cells regulated by IL-23 play an important role in immune regulation in the progression of CD[34-36]. IL-23 not only acts on members of the innate immune system but also promotes the proliferation and maintenance of Th17 cells. It is generally acknowledged that Th17 cells promote tissue inflammation, while Treg cells suppress autoimmunity, which suggests that the balance of Th17/Treg cells is crucial in the pathogenesis of CD[37,38]. With the development of GWAS, evidence is increasingly supporting the role of the innate immune response in the pathological process of CD, which includes epithelial barrier integrity, innate microbial sensing, autophagy, and unfolded protein response[34]. Other factors, such as injuries of epithelial and mesenchymal cells, changes in intestinal permeability, and obesity, also contribute to the pathophysiology of CD.

Important pathways in the pathogenesis of CD

In recent decades, complex molecular pathways have been reported to be involved in the pathogenesis of CD. The identification of the main pathways and key factors may provide novel therapeutic targets. Current clinical therapies for IBD include anti-tumor necrosis factor (TNF) antibodies (such as infliximab, adalimumab, and certolizumab pegol), anti-IL-12/23 antibodies (such as ustekinumab), anti-sense oligonucleotides inhibiting SMAD7 transcription (such as mongersen), Janus kinase (JAK) inhibitors (such as tofacitinib and filgotinib), and anti-adhesion molecules (such as vedolizumab, etrolizumab, and anti-MAdCAM1 antibody)[4,39,40]. The main pathways and key factors are discussed in detail in this section (Table 1).

Nuclear factor kappa B signaling pathway: The targeting of TNF-α is a first-line treatment for CD, as well as for several other autoimmune diseases[40]. TNF-α is a pro-inflammatory mediator that plays a crucial role in the immune response to CD. It can induce T cell activation, inflammatory cell recruitment to local inflammatory sites, edema, coagulation, and granuloma formation[41]. Nuclear factor kappa B (NF-κB) signaling is considered the key pathway in lieu of TNF-α. Previous studies have shown that CD patients with high NF-κB activation have specific clinical manifestations, such as a higher frequency of ileocolonic involvement and higher histologic scores, compared to patients with low NF-κB activation[42]. The NF-κB signaling pathway also regulates the expression of IL-1, IL-6, IL-12, and IL-23[43-45] which are involved in mucosal damage within the inflammatory parts of the intestine. Furthermore, the differentiation of Th1 influenced by IL-12, IL-23, and TNF-α is actively involved in CD [45-47]. Corticosteroids, another first-line drug for the treatment of CD, have immunosuppressive effects and can induce the increased expression of IκBα, a key factor in the NF-κB pathway. These findings indicate that the NF-κB pathway plays a central role in the pathogenesis of CD.

Transforming growth factor-β/SMAD signaling pathway: Transforming growth factor-β (TGF-β) is an immunosuppressive cytokine produced by a variety of cells and activated by integrins. The role of TGF-β in intestinal immunity has been intensively investigated in previous studies[48]. Tregs have been suggested to produce anti-inflammatory cytokines, such as IL-10 and TGF-β. IL-10 promotes Treg cell proliferation by activating the signal transducer and activator of transcription (STAT)3, while TGF-β inhibits the proinflammatory responses of macrophages and effector T cells by activating SMAD3 and SMAD4[22]. Therefore, the upregulation of the Treg cell population and the reduction of effector T cells in CD indicate that the TGF-β /SMAD pathway plays a crucial role. In addition, SMAD7 is a downstream target of the TGF-β pathway, inhibiting the TGF-β pathway through negative feedback. In CD, the expression of SMAD7 is increased, leading to a reduction in SMAD3 phosphorylation and the suppression of TGF-β signaling, which may contribute to CD pathogenesis[48].

JAK/STAT signaling pathway: Although clinical trials using tofacitinib for the treatment of CD were canceled due to poor results, the efficacy of filgotinib, a selective JAK1 inhibitor, was confirmed in a randomized, double-blind, placebo-controlled

Table 1 Primary signaling pathways and relative drug applications of Crohn's disease

Signaling pathway	Relative function	Targeted factor	Drug application
NF-κB	Maintenance of epithelial integrity and intestinal immune homeostasis	TNF-α	infliximab, adalimumab, and certolizumab
		IL-12/23	ustekinumab
		IκBa	corticosteroids
TGF-β/SMAD3	Immunosuppression and fibrosis	SMAD7	mongerson
JAK/STAT	Immunoregulation, anti-inflammation and epithelial barrier function	JAK	tofacitinib and filgotinib
Chemokines/integrins	Leukocytes trafficking to targeted location	α4β7 integrin	vedolizumab
		α4β7 and αEβ7 integrins	etrolizumab
		MAdCAM1	PF-00547659
Wnt	Regulation of epithelial proliferation and gut mucosal homeostasis	NA	NA

NF-κB: Nuclear factor kappa B; TGF-β: Transforming growth factor-β; JAK: Janus kinase; STAT: Signal transducer and activator of transcription; Wnt: Wingless/Int1; TNF-α: Tumor necrosis factor-α; IL: Interleukin; NA: Not available.

phase II trial[49,50]. JAK tyrosine kinases and STAT DNA-binding proteins mediate signal transduction and downstream biological effects in response to cytokine receptor binding, some of which are associated with CD pathology. The cytokines mentioned above, which play essential roles in immunoregulation and the maintenance of epithelial barrier function (such as IL-6, IL-10, IL-12, and IL-23) are all dependent on JAK/STAT signaling[51]. STAT3 has also been reported to be crucial for the differentiation of Th17 cells and Th17 cell-dependent colitis, such as CD[37,52]. Interestingly, there is crosstalk between TNF and the JAK/STAT signaling pathway: TNF can amplify JAK-dependent receptor signal transduction by upregulating the expression of STAT[53]. Therefore, the role of JAK/STAT in the pathology of CD should be emphasized.

Wingless/Int1 signaling pathway: The Wingless/Int1 (Wnt) pathway is a key regulator of epithelial proliferation and gut mucosal homeostasis[54,55]. Wnt signaling is crucial for maintaining the stability of epithelial homeostasis, where the inhibition of this pathway leads to crypt loss and tissue degradation[56]. This pathway stimulates the differentiation and maturation of Paneth cells and regulates the expression of the α-defensins HD5 and HD6, in addition to mediating the stabilization of β-catenin[57]. Recently, Courth *et al*[58] found that the relationship between Paneth cells and bone marrow-derived monocytes participates in the mechanism of CD, which is characterized by the reduction of Wnt ligand expression in peripheral blood mononuclear cells (PBMCs) to attenuate intestinal barrier function. Furthermore, Wnt signaling is involved in various inflammatory signaling pathways, including NF-κB, mitogen-activated protein kinase (MAPK), protein kinase B (PKB/AKT), and STAT signaling. This complex network of signaling pathways may explain the contribution of Wnt to inflammatory injury repair[54].

Chemokines and integrins: In CD, chemokines induce the recruitment of immune cells to inflamed and epithelial-damaged sites. A highly effective and sequential adhesion system is involved in this process, in which integrins are activated by chemokines and interact with the addressins on the endothelium. For example, the antibody blockade of CCL25/CCR9 has been found to reduce early chronic ileitis in mice[59]. In addition, the ligation of CCR9 by CCL25 can result in a conformational change in α4β7 integrin, subsequently leading to the stable adhesion of MAdCAM-1 [60]. Collectively, these results indicate that anti-adhesion molecules can be used clinically for CD therapy.

PALMITOYLATION OF MOLECULAR PATHWAYS IN CD

Many of the molecular pathways associated with CD have been reported to be modulated by S-palmitoylation. For example, a high frequency of mutations in NOD1/2 are found in IBD patients, and ZDHHC5-mediated NOD1/2 palmitoylation is responsible for normal gut functions. However, most reported CD-associated pathways in which palmitoylation occurs don't specifically connect CD to palmitoylated factors such as Myd88. Myd88 is a component of TLR signaling that has been reported to be palmitoylated by ZDHHC6, but its palmitoylation hasn't been associated with a gut phenotype. In CD, Myd88 participates in the recognition of extracellular and/or vacuolar intracellular pathogen-associated molecular patterns (PAMPs), which mediate sensing of microbial antigens[34]. The effects of palmitoylation on function of CD-associated factors need further exploration. Whether the effects of palmitoylation on CD symptoms are positive or negative might depend on a varied array of factors. Present opinion suggests that the functional effects of palmitoylation predominantly act to retain normal gut structures and functions. However, it is too early to conclude that all instances of palmitoylation exert positive effects. For instance, palmitoylation-mediated NF-kB activation probably results in negative consequences for CD patients. In this section, we summarize the S-palmitoylation events that have been reported to be associated with signaling pathways implicated in CD.

Palmitoylation in STING signaling

In the presence of damaged DNA, cyclic GMP-AMP synthase (cGAS) is activated and catalyzes the synthesis of cyclic GMP–AMP (cGAMP), which binds and activates its receptor stimulator of interferon genes (STING). STING is a membrane protein typically associated with endoplasmic reticulum (ER) stress. Upon activation, it translocates to the Golgi apparatus, where it is palmitoylated on Cys88 and Cys91, most likely by ZDHHC3, ZDHHC7, or ZDHHC15. Cysteine palmitoylation is important for its ability to activate TANK-binding kinase 1 (TBK1), which in turn phosphorylates interferon regulatory factor 3 (IRF3), which subsequently activates the transcription of immune response genes. STING palmitoylation has been proposed to promote the localization of STING to lipid rafts in the Golgi apparatus, which recruits both TBK1 and IRF3 to allow for downstream signal propagation[61]. Small molecules that can covalently label the palmitoylated Cys residues of STING have been identified and shown to suppress inflammation[62]. Interestingly, 9- or 10-nitro-oleic acid, which can be produced endogenously under inflammation, can also covalently modify the Cys residue of STING and inhibit its palmitoylation. This is likely to be a negative feedback regulation that inhibits STING signaling[62].

Palmitoylation of NOD1/2

NOD1/2 are receptors for pathogen-associated molecular patterns, sensing bacterial peptidoglycans and initiating immune signaling, mainly by activating NF-κB. They are cytosolic proteins associated with bacteria-containing phagosomes upon bacterial infection. The cysteine palmitoylation of NOD1/2 is important for the phagosome translocation of NOD1/2. Palmitoylation occurs on multiple cysteine residues and is catalyzed by ZDHHC5[63]. NOD1/2 mutations are also associated with IBD. Interestingly, several of these mutations decrease the palmitoylation of NOD1/2 and inhibit NF-κB activation[63]. Therefore, methods to modulate the palmitoylation and signaling NOD1/2 hold potential for use in the treatment of CD.

Palmitoylation in TNF/TNFR signaling

Intriguingly, both the ligand TNF-α and its receptor TNFR1 are known to be regulated by cysteine palmitoylation. TNF-α is palmitoylated on Cys47, however, the enzymes regulating palmitoylation have not been reported[64]. Palmitoylation promotes the targeting of membrane TNF-α (before cleavage and secretion) to lipid rafts. TNF-α palmitoylation does not affect the secretion of soluble TNF-α, but affects the stability of the N-terminal intracellular domain[65]. A recent report showed that TNFR1 is palmitoylated and that palmitoylation is regulated by APT2 and TNF-α[66]. However, the site of modification, the exact ZDHHC responsible for palmitoylation, and the effect of palmitoylation on TNF signaling requires further exploration. Given the importance of anti-TNF therapy in CD, the palmitoylation of TNF and TNFR1 deserves further investigation in future studies.

Palmitoylation in TLR signaling

Toll-like receptors (TLRs) and transmembrane proteins initiate immune signaling by sensing PAMPs. A proteomic study identified several TLRs as palmitoylated proteins. TLR2 palmitoylation was found to occur on a membrane-proximal cysteine residue, Cys609. Palmitoylation is important for TLR2 and NF-κB activation[67]. TLR signaling requires an adaptor protein, Myd88, which is palmitoylated on Cys113 and Cys274 by ZDHHC6. The palmitoylation of Cys113 is important for the recruitment of interleukin-1 receptor-associated kinase 4 (IRAK4) and NF-κB activation. The palmitoylation of Myd88 is also affected by the fatty acid synthase (FASN). Small molecule inhibitors of FASN reduce Myd88 palmitoylation and NF-κB activation[68]. Though no report indicates that Myd88 palmitoylation influences CD, it may exert effects related to sensing of microbial antigens, which is mediated by Myd88. However, as NF-kB activation displayed a high correlation to clinical CD manifestations, impaired palmitoylation resulting in NF-kB inhibition could be beneficial for CD patients.

Palmitoylation in JAK-STAT3 signaling

STAT3-mediated Th17 differentiation is important for IBD. STAT3 is a transcription factor that, when phosphorylated by JAK in response to cytokines, such as IL-6, activates the transcription of genes that promote Th17 cell differentiation. Recently, STAT3 was reported to be regulated by S-palmitoylation of Cys108[7]. Palmitoylation is regulated by ZDHHC7 and depalmitoylated by APT2. Interestingly, the palmitoylation-depalmitoylation cycle has been found to be important for the activation of STAT3. Palmitoylation promotes STAT3 membrane localization and phosphorylation by JAK2. However, to translocate to the nucleus, phosphorylated STAT3 needs to be depalmitoylated. Therefore, APT2 is required for STAT3 activation. Interestingly, APT2 seems to prefer phosphorylated STAT3 over unphosphorylated STAT3, which ensures that the palmitoylation-depalmitoylation cycle moves in one direction, that which promotes STAT3 activation. Accordingly, the deletion or inhibition of either ZDHHC7 or APT2 decreases STAT3 activation, Th17 differentiation, and colitis in a mouse model. Furthermore, APT2 and ZDHHC7 are upregulated in human IBD patients, and the levels of IL-17 are closely correlated with the levels of APT2. This study provides strong evidence that the palmitoylation of STAT3 is a promising target for the treatment of IBD.

STAT3 activation occurs downstream of IL-6 receptor activation. Interestingly, one subunit of the IL-6 receptor, IL6ST (also called Gp130), is also regulated by palmitoylation. In neurons, ZDHHC5 and ZDHHC8 can palmitoylate IL6ST and promote JAK-STAT3 signaling[69]. Thus, it is possible that other proteins in the IL-6 signaling pathway, in addition to IL6ST and STAT3, could be regulated by cysteine palmitoylation. Future studies in this direction could identify additional targets, which would prove useful for advances in the treatment of IBD. Currently, there are no reports regarding the palmitoylation of the SMAD signaling pathway. However, SMAD2 has been reported to work with STAT3 to affect Th17 differentiation[70]. Therefore, SMAD signaling may be indirectly affected by STAT3 palmitoylation.

Palmitoylation in chemokine signaling

Inflammation involves the migration of various immune cells to the site of infection or inflammation. Thus, the inhibition of immune cell migration is an effective strategy to inhibit inflammation and autoimmune responses. Immune cell migration is typically mediated by chemotactic chemokine signaling. Multiple components of the chemokine signaling pathway can be regulated by cysteine palmitoylation. Chemotactic signaling is initiated by the binding of chemotactic ligands to the cell surface G protein-coupled receptors (GPCRs). Sphingosine 1-phosphate (S1P) receptor 1 (S1PR1), which binds to S1P, is important for the migration of mature T cells from the thymus into the blood stream and peripheral lymphoid organs. S1PR1 is palmitoylated by ZDHHC5 on multiple Cys residues at the C-terminus, and palmitoylation is important for its downstream signaling, which is mediated by trimeric G proteins[71,72]. Similarly, another chemotactic receptor, CCR5, is also regulated by cysteine palmitoylation[73]. Despite this, there are currently no reports on the palmitoylation of CCR9, which has been implicated in CD.

Chemotactic GPCR signaling requires coupling with the downstream trimeric G proteins. Interestingly, many trimeric G proteins are known to be regulated by palmitoylation[74,75]. Similarly, RGS proteins, which are regulators of G protein signaling, are also reported to be regulated by palmitoylation[75-77]. However, most of these examples have been reported in neuronal systems, and their role in the

regulation of the immune system has yet to be studied extensively. The Rac1 small GTPase is important for cytoskeletal reorganization, which is required for immune cell adhesion and migration. Rac1 is palmitoylated on Cys176, which promotes its targeting to lipid rafts and inhibits its oligomerization, and is required for its signaling function. Palmitoylation-deficient Rac1 mutant cells are defective in cell spreading and migration[78]. However, the enzymes responsible for regulating Rac1 palmitoylation have yet to be identified. Targeting Rac1 palmitoyltransferases may potentially inhibit immune cell migration, thus representing a potential strategy for the treatment of autoimmune diseases.

POTENTIAL OF PALMITOYLATION SITES AS DRUG TARGETS IN CD

Accumulating evidence has recently provided novel insights into the role of palmitoylation in the pathological mechanism of CD, highlighting potential drug targets for the control of palmitoylation. Since STING signaling is associated with palmitoylation, it is reasonable to assume that STING-associated autoimmune diseases, such as systemic lupus erythematosus (SLE) and Aicardi–Goutières syndrome (AGS), are related to the process of palmitoylation[79]. However, the contribution of STING to CD requires further study. If this relationship is confirmed, a novel promising drug target for the treatment of CD could be identified based on STING-related factors. Other factors related to CD have also been found to undergo palmitoylation during normal functional processes. These findings support the potential of palmitoylation as drug targets in CD, and we hope this area will attract more intensive research in the future.

CONCLUSION

S-palmitoylation is one of the most common post-translational modifications in nature which has been overlooked for decades. With the rapidly increasing global prevalence of CD and recent reports on the effect of S-palmitoylation on CD, elucidating the relationship between CD and S-palmitoylation becomes an urgent task. The basic facts of S-palmitoylation and its potential effect on CD summarized by this review will provide novel insights into the pathogenesis and treatment of CD.

ACKNOWLEDGEMENTS

We thank Dr. Zhou XX from the Department of Gastroenterology, The First Affiliated Hospital, Zhejiang University School of Medicine for her constructive suggestions for the section on Crohn's disease. We also thank Dr. Zhang MM for his elegant work revealing the effect of the STAT3 palmitoylation circle on CD under the supervision of Dr. Lin H, which constitutes one of the major bases for this paper.

REFERENCES

1 **Jiang H**, Zhang X, Chen X, Aramsangtienchai P, Tong Z, Lin H. Protein Lipidation: Occurrence, Mechanisms, Biological Functions, and Enabling Technologies. *Chem Rev* 2018; **118**: 919-988 [PMID: 29292991 DOI: 10.1021/acs.chemrev.6b00750]

2 **Linder ME**, Jennings BC. Mechanism and function of DHHC S-acyltransferases. *Biochem Soc Trans* 2013; **41**: 29-34 [PMID: 23356254 DOI: 10.1042/BST20120328]

3 **Chen ZQ**, Ulsh LS, DuBois G, Shih TY. Posttranslational processing of p21 ras proteins involves palmitylation of the C-terminal tetrapeptide containing cysteine-186. *J Virol* 1985; **56**: 607-612 [PMID: 2997480 DOI: 10.1128/JVI.56.2.607-612.1985]

4 **Torres J**, Mehandru S, Colombel JF, Peyrin-Biroulet L. Crohn's disease. *Lancet* 2017; **389**: 1741-1755 [PMID: 27914655 DOI: 10.1016/S0140-6736(16)31711-1]

5 **Li N**, Shi RH. Updated review on immune factors in pathogenesis of Crohn's disease. *World J Gastroenterol* 2018; **24**: 15-22 [PMID: 29358878 DOI: 10.3748/wjg.v24.i1.15]

6 **Ng SC**, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, Panaccione R, Ghosh S, Wu JCY, Chan FKL, Sung JJY, Kaplan GG. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet* 2017; **390**: 2769-2778 [PMID: 29050646 DOI: 10.1016/S0140-6736(17)32448-0]

7 **Zhang M**, Zhou L, Xu Y, Yang M, Komaniecki GP, Kosciuk T, Chen X, Lu X, Zou X, Linder ME, Lin H. A STAT3 palmitoylation cycle promotes T_H17 differentiation and colitis. *Nature* 2020; **586**: 434-439 [PMID: 33029007 DOI: 10.1038/s41586-020-2799-2]

8 **Linder ME**, Deschenes RJ. Palmitoylation: policing protein stability and traffic. *Nat Rev Mol Cell Biol* 2007; **8**: 74-84 [PMID: 17183362 DOI: 10.1038/nrm2084]

9 **Rana MS**, Kumar P, Lee CJ, Verardi R, Rajashankar KR, Banerjee A. Fatty acyl recognition and transfer by an integral membrane *S*-acyltransferase. *Science* 2018; **359** [PMID: 29326245 DOI: 10.1126/science.aao6326]

10 **Dekker FJ**, Rocks O, Vartak N, Menninger S, Hedberg C, Balamurugan R, Wetzel S, Renner S, Gerauer M, Schölermann B, Rusch M, Kramer JW, Rauh D, Coates GW, Brunsveld L, Bastiaens PI, Waldmann H. Small-molecule inhibition of APT1 affects Ras localization and signaling. *Nat Chem Biol* 2010; **6**: 449-456 [PMID: 20418879 DOI: 10.1038/nchembio.362]

11 **Ko PJ**, Dixon SJ. Protein palmitoylation and cancer. *EMBO Rep* 2018; **19** [PMID: 30232163 DOI: 10.15252/embr.201846666]

12 **Drisdel RC**, Green WN. Labeling and quantifying sites of protein palmitoylation. *Biotechniques* 2004; **36**: 276-285 [PMID: 14989092 DOI: 10.2144/04362RR02]

13 **Wan J**, Roth AF, Bailey AO, Davis NG. Palmitoylated proteins: purification and identification. *Nat Protoc* 2007; **2**: 1573-1584 [PMID: 17585299 DOI: 10.1038/nprot.2007.225]

14 **Forrester MT**, Thompson JW, Foster MW, Nogueira L, Moseley MA, Stamler JS. Proteomic analysis of S-nitrosylation and denitrosylation by resin-assisted capture. *Nat Biotechnol* 2009; **27**: 557-559 [PMID: 19483679 DOI: 10.1038/nbt.1545]

15 **Percher A**, Ramakrishnan S, Thinon E, Yuan X, Yount JS, Hang HC. Mass-tag labeling reveals site-specific and endogenous levels of protein S-fatty acylation. *Proc Natl Acad Sci U S A* 2016; **113**: 4302-4307 [PMID: 27044110 DOI: 10.1073/pnas.1602244113]

16 **Aramsangtienchai P**, Spiegelman NA, Cao J, Lin H. *S*-Palmitoylation of Junctional Adhesion Molecule C Regulates Its Tight Junction Localization and Cell Migration. *J Biol Chem* 2017; **292**: 5325-5334 [PMID: 28196865 DOI: 10.1074/jbc.M116.730523]

17 **Charron G**, Zhang MM, Yount JS, Wilson J, Raghavan AS, Shamir E, Hang HC. Robust fluorescent detection of protein fatty-acylation with chemical reporters. *J Am Chem Soc* 2009; **131**: 4967-4975 [PMID: 19281244 DOI: 10.1021/ja810122f]

18 **Martin BR**, Cravatt BF. Large-scale profiling of protein palmitoylation in mammalian cells. *Nat Methods* 2009; **6**: 135-138 [PMID: 19137006 DOI: 10.1038/nmeth.1293]

19 **Kim SC**, Sprung R, Chen Y, Xu Y, Ball H, Pei J, Cheng T, Kho Y, Xiao H, Xiao L, Grishin NV, White M, Yang XJ, Zhao Y. Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. *Mol Cell* 2006; **23**: 607-618 [PMID: 16916647 DOI: 10.1016/j.molcel.2006.06.026]

20 **Li Y**, Wang S, Chen Y, Li M, Dong X, Hang HC, Peng T. Site-specific chemical fatty-acylation for gain-of-function analysis of protein S-palmitoylation in live cells. *Chem Commun (Camb)* 2020; **56**: 13880-13883 [PMID: 33094750 DOI: 10.1039/d0cc06073a]

21 **Chang JT**. Pathophysiology of Inflammatory Bowel Diseases. *N Engl J Med* 2020; **383**: 2652-2664 [PMID: 33382932 DOI: 10.1056/NEJMra2002697]

22 **de Souza HS**, Fiocchi C. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol* 2016; **13**: 13-27 [PMID: 26627550 DOI: 10.1038/nrgastro.2015.186]

23 **Coufal S**, Galanova N, Bajer L, Gajdarova Z, Schierova D, Jiraskova Zakostelska Z, Kostovcikova K, Jackova Z, Stehlikova Z, Drastich P, Tlaskalova-Hogenova H, Kverka M. Inflammatory Bowel Disease Types Differ in Markers of Inflammation, Gut Barrier and in Specific Anti-Bacterial Response. *Cells* 2019; **8** [PMID: 31337064 DOI: 10.3390/cells8070719]

24 **Inoue N**, Tamura K, Kinouchi Y, Fukuda Y, Takahashi S, Ogura Y, Inohara N, Núñez G, Kishi Y, Koike Y, Shimosegawa T, Shimoyama T, Hibi T. Lack of common NOD2 variants in Japanese patients with Crohn's disease. *Gastroenterology* 2002; **123**: 86-91 [PMID: 12105836 DOI: 10.1053/gast.2002.34155]

25 **Leong RW**, Armuzzi A, Ahmad T, Wong ML, Tse P, Jewell DP, Sung JJ. NOD2/CARD15 gene polymorphisms and Crohn's disease in the Chinese population. *Aliment Pharmacol Ther* 2003; **17**: 1465-1470 [PMID: 12823148 DOI: 10.1046/j.1365-2036.2003.01607.x]

26 **Liu JZ**, van Sommeren S, Huang H, Ng SC, Alberts R, Takahashi A, Ripke S, Lee JC, Jostins L, Shah T, Abedian S, Cheon JH, Cho J, Dayani NE, Franke L, Fuyuno Y, Hart A, Juyal RC, Juyal G, Kim WH, Morris AP, Poustchi H, Newman WG, Midha V, Orchard TR, Vahedi H, Sood A, Sung JY, Malekzadeh R, Westra HJ, Yamazaki K, Yang SK; International Multiple Sclerosis Genetics Consortium; International IBD Genetics Consortium, Barrett JC, Alizadeh BZ, Parkes M, Bk T, Daly MJ, Kubo M, Anderson CA, Weersma RK. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nat Genet* 2015; **47**: 979-986 [PMID: 26192919 DOI: 10.1038/ng.3359]

27 **Ananthakrishnan AN**. Epidemiology and risk factors for IBD. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 205-217 [PMID: 25732745 DOI: 10.1038/nrgastro.2015.34]

28 **Mahid SS**, Minor KS, Soto RE, Hornung CA, Galandiuk S. Smoking and inflammatory bowel disease: a meta-analysis. *Mayo Clin Proc* 2006; **81**: 1462-1471 [PMID: 17120402 DOI: 10.4065/81.11.1462]

29 **Ungaro R**, Bernstein CN, Gearry R, Hviid A, Kolho KL, Kronman MP, Shaw S, Van Kruiningen H, Colombel JF, Atreja A. Antibiotics associated with increased risk of new-onset Crohn's disease but

not ulcerative colitis: a meta-analysis. *Am J Gastroenterol* 2014; **109**: 1728-1738 [PMID: 25223575 DOI: 10.1038/ajg.2014.246]

30 **Hooper LV**, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science* 2012; **336**: 1268-1273 [PMID: 22674334 DOI: 10.1126/science.1223490]

31 **Schirmer M**, Garner A, Vlamakis H, Xavier RJ. Microbial genes and pathways in inflammatory bowel disease. *Nat Rev Microbiol* 2019; **17**: 497-511 [PMID: 31249397 DOI: 10.1038/s41579-019-0213-6]

32 **Marchesi JR**, Adams DH, Fava F, Hermes GD, Hirschfield GM, Hold G, Quraishi MN, Kinross J, Smidt H, Tuohy KM, Thomas LV, Zoetendal EG, Hart A. The gut microbiota and host health: a new clinical frontier. *Gut* 2016; **65**: 330-339 [PMID: 26338727 DOI: 10.1136/gutjnl-2015-309990]

33 **Park JH**, Peyrin-Biroulet L, Eisenhut M, Shin JI. IBD immunopathogenesis: A comprehensive review of inflammatory molecules. *Autoimmun Rev* 2017; **16**: 416-426 [PMID: 28212924 DOI: 10.1016/j.autrev.2017.02.013]

34 **Geremia A**, Biancheri P, Allan P, Corazza GR, Di Sabatino A. Innate and adaptive immunity in inflammatory bowel disease. *Autoimmun Rev* 2014; **13**: 3-10 [PMID: 23774107 DOI: 10.1016/j.autrev.2013.06.004]

35 **Cătană CS**, Berindan Neagoe I, Cozma V, Magdaş C, Tăbăran F, Dumitraşcu DL. Contribution of the IL-17/IL-23 axis to the pathogenesis of inflammatory bowel disease. *World J Gastroenterol* 2015; **21**: 5823-5830 [PMID: 26019446 DOI: 10.3748/wjg.v21.i19.5823]

36 **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]

37 **Yan JB**, Luo MM, Chen ZY, He BH. The Function and Role of the Th17/Treg Cell Balance in Inflammatory Bowel Disease. *J Immunol Res* 2020; **2020**: 8813558 [PMID: 33381606 DOI: 10.1155/2020/8813558]

38 **Noack M**, Miossec P. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. *Autoimmun Rev* 2014; **13**: 668-677 [PMID: 24418308 DOI: 10.1016/j.autrev.2013.12.004]

39 **Sandborn WJ**, Vermeire S, Tyrrell H, Hassanali A, Lacey S, Tole S, Tatro AR; Etrolizumab Global Steering Committee. Etrolizumab for the Treatment of Ulcerative Colitis and Crohn's Disease: An Overview of the Phase 3 Clinical Program. *Adv Ther* 2020; **37**: 3417-3431 [PMID: 32445184 DOI: 10.1007/s12325-020-01366-2]

40 **Cushing K**, Higgins PDR. Management of Crohn Disease: A Review. *JAMA* 2021; **325**: 69-80 [PMID: 33399844 DOI: 10.1001/jama.2020.18936]

41 **Berns M**, Hommes DW. Anti-TNF-α therapies for the treatment of Crohn's disease: the past, present and future. *Expert Opin Investig Drugs* 2016; **25**: 129-143 [PMID: 26616476 DOI: 10.1517/13543784.2016.1126247]

42 **Han YM**, Koh J, Kim JW, Lee C, Koh SJ, Kim B, Lee KL, Im JP, Kim JS. NF-kappa B activation correlates with disease phenotype in Crohn's disease. *PLoS One* 2017; **12**: e0182071 [PMID: 28753650 DOI: 10.1371/journal.pone.0182071]

43 **Becker C**, Wirtz S, Blessing M, Pirhonen J, Strand D, Bechthold O, Frick J, Galle PR, Autenrieth I, Neurath MF. Constitutive p40 promoter activation and IL-23 production in the terminal ileum mediated by dendritic cells. *J Clin Invest* 2003; **112**: 693-706 [PMID: 12952918 DOI: 10.1172/JCI17464]

44 **Becker C**, Wirtz S, Ma X, Blessing M, Galle PR, Neurath MF. Regulation of IL-12 p40 promoter activity in primary human monocytes: roles of NF-kappaB, CCAAT/enhancer-binding protein beta, and PU.1 and identification of a novel repressor element (GA-12) that responds to IL-4 and prostaglandin E(2). *J Immunol* 2001; **167**: 2608-2618 [PMID: 11509602 DOI: 10.4049/jimmunol.167.5.2608]

45 **Atreya I**, Atreya R, Neurath MF. NF-kappaB in inflammatory bowel disease. *J Intern Med* 2008; **263**: 591-596 [PMID: 18479258 DOI: 10.1111/j.1365-2796.2008.01953.x]

46 **Oppmann B**, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, Vega F, Yu N, Wang J, Singh K, Zonin F, Vaisberg E, Churakova T, Liu M, Gorman D, Wagner J, Zurawski S, Liu Y, Abrams JS, Moore KW, Rennick D, de Waal-Malefyt R, Hannum C, Bazan JF, Kastelein RA. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 2000; **13**: 715-725 [PMID: 11114383 DOI: 10.1016/s1074-7613(00)00070-4]

47 **Neurath MF**, Finotto S, Glimcher LH. The role of Th1/Th2 polarization in mucosal immunity. *Nat Med* 2002; **8**: 567-573 [PMID: 12042806 DOI: 10.1038/nm0602-567]

48 **Ihara S**, Hirata Y, Koike K. TGF-β in inflammatory bowel disease: a key regulator of immune cells, epithelium, and the intestinal microbiota. *J Gastroenterol* 2017; **52**: 777-787 [PMID: 28534191 DOI: 10.1007/s00535-017-1350-1]

49 **Panés J**, Sandborn WJ, Schreiber S, Sands BE, Vermeire S, D'Haens G, Panaccione R, Higgins PDR, Colombel JF, Feagan BG, Chan G, Moscariello M, Wang W, Niezychowski W, Marren A, Healey P, Maller E. Tofacitinib for induction and maintenance therapy of Crohn's disease: results of two phase IIb randomised placebo-controlled trials. *Gut* 2017; **66**: 1049-1059 [PMID: 28209624 DOI: 10.1136/gutjnl-2016-312735]

50 **Vermeire S**, Schreiber S, Petryka R, Kuehbacher T, Hebuterne X, Roblin X, Klopocka M, Goldis A, Wisniewska-Jarosinska M, Baranovsky A, Sike R, Stoyanova K, Tasset C, Van der Aa A, Harrison P. Clinical remission in patients with moderate-to-severe Crohn's disease treated with filgotinib (the FITZROY study): results from a phase 2, double-blind, randomised, placebo-controlled trial. *Lancet* 2017; **389**: 266-275 [PMID: 27988142 DOI: 10.1016/S0140-6736(16)32537-5]

51 **Salas A**, Hernandez-Rocha C, Duijvestein M, Faubion W, McGovern D, Vermeire S, Vetrano S, Vande Casteele N. JAK-STAT pathway targeting for the treatment of inflammatory bowel disease. *Nat Rev Gastroenterol Hepatol* 2020; **17**: 323-337 [PMID: 32203403 DOI: 10.1038/s41575-020-0273-0]

52 **Lu D**, Liu L, Ji X, Gao Y, Chen X, Liu Y, Zhao X, Li Y, Jin Y, Zhang Y, McNutt MA, Yin Y. The phosphatase DUSP2 controls the activity of the transcription activator STAT3 and regulates TH17 differentiation. *Nat Immunol* 2015; **16**: 1263-1273 [PMID: 26479789 DOI: 10.1038/ni.3278]

53 **Rogler G**. Efficacy of JAK inhibitors in Crohn's Disease. *J Crohns Colitis* 2020; **14**: S746-S754 [PMID: 31781755 DOI: 10.1093/ecco-jcc/jjz186]

54 **Moparthi L**, Koch S. Wnt signaling in intestinal inflammation. *Differentiation* 2019; **108**: 24-32 [PMID: 30718056 DOI: 10.1016/j.diff.2019.01.002]

55 **Armbruster NS**, Stange EF, Wehkamp J. In the Wnt of Paneth Cells: Immune-Epithelial Crosstalk in Small Intestinal Crohn's Disease. *Front Immunol* 2017; **8**: 1204 [PMID: 29018451 DOI: 10.3389/fimmu.2017.01204]

56 **Kuhnert F**, Davis CR, Wang HT, Chu P, Lee M, Yuan J, Nusse R, Kuo CJ. Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. *Proc Natl Acad Sci U S A* 2004; **101**: 266-271 [PMID: 14695885 DOI: 10.1073/pnas.2536800100]

57 **van Es JH**, Jay P, Gregorieff A, van Gijn ME, Jonkheer S, Hatzis P, Thiele A, van den Born M, Begthel H, Brabletz T, Taketo MM, Clevers H. Wnt signalling induces maturation of Paneth cells in intestinal crypts. *Nat Cell Biol* 2005; **7**: 381-386 [PMID: 15778706 DOI: 10.1038/ncb1240]

58 **Courth LF**, Ostaff MJ, Mailänder-Sánchez D, Malek NP, Stange EF, Wehkamp J. Crohn's disease-derived monocytes fail to induce Paneth cell defensins. *Proc Natl Acad Sci U S A* 2015; **112**: 14000-14005 [PMID: 26512113 DOI: 10.1073/pnas.1510084112]

59 **Rivera-Nieves J**, Ho J, Bamias G, Ivashkina N, Ley K, Oppermann M, Cominelli F. Antibody blockade of CCL25/CCR9 ameliorates early but not late chronic murine ileitis. *Gastroenterology* 2006; **131**: 1518-1529 [PMID: 17101325 DOI: 10.1053/j.gastro.2006.08.031]

60 **Thomas S**, Baumgart DC. Targeting leukocyte migration and adhesion in Crohn's disease and ulcerative colitis. *Inflammopharmacology* 2012; **20**: 1-18 [PMID: 22205271 DOI: 10.1007/s10787-011-0104-6]

61 **Mukai K**, Konno H, Akiba T, Uemura T, Waguri S, Kobayashi T, Barber GN, Arai H, Taguchi T. Activation of STING requires palmitoylation at the Golgi. *Nat Commun* 2016; **7**: 11932 [PMID: 27324217 DOI: 10.1038/ncomms11932]

62 **Haag SM**, Gulen MF, Reymond L, Gibelin A, Abrami L, Decout A, Heymann M, van der Goot FG, Turcatti G, Behrendt R, Ablasser A. Targeting STING with covalent small-molecule inhibitors. *Nature* 2018; **559**: 269-273 [PMID: 29973723 DOI: 10.1038/s41586-018-0287-8]

63 **Lu Y**, Zheng Y, Coyaud É, Zhang C, Selvabaskaran A, Yu Y, Xu Z, Weng X, Chen JS, Meng Y, Warner N, Cheng X, Liu Y, Yao B, Hu H, Xia Z, Muise AM, Klip A, Brumell JH, Girardin SE, Ying S, Fairn GD, Raught B, Sun Q, Neculai D. Palmitoylation of NOD1 and NOD2 is required for bacterial sensing. *Science* 2019; **366**: 460-467 [PMID: 31649195 DOI: 10.1126/science.aau6391]

64 **Utsumi T**, Takeshige T, Tanaka K, Takami K, Kira Y, Klostergaard J, Ishisaka R. Transmembrane TNF (pro-TNF) is palmitoylated. *FEBS Lett* 2001; **500**: 1-6 [PMID: 11434916 DOI: 10.1016/s0014-5793(01)02576-5]

65 **Poggi M**, Kara I, Brunel JM, Landrier JF, Govers R, Bonardo B, Fluhrer R, Haass C, Alessi MC, Peiretti F. Palmitoylation of TNF alpha is involved in the regulation of TNF receptor 1 signalling. *Biochim Biophys Acta* 2013; **1833**: 602-612 [PMID: 23159491 DOI: 10.1016/j.bbamcr.2012.11.009]

66 **Zingler P**, Särchen V, Glatter T, Caning L, Saggau C, Kathayat RS, Dickinson BC, Adam D, Schneider-Brachert W, Schütze S, Fritsch J. Palmitoylation is required for TNF-R1 signaling. *Cell Commun Signal* 2019; **17**: 90 [PMID: 31382980 DOI: 10.1186/s12964-019-0405-8]

67 **Chesarino NM**, Hach JC, Chen JL, Zaro BW, Rajaram MV, Turner J, Schlesinger LS, Pratt MR, Hang HC, Yount JS. Chemoproteomics reveals Toll-like receptor fatty acylation. *BMC Biol* 2014; **12**: 91 [PMID: 25371237 DOI: 10.1186/s12915-014-0091-3]

68 **Kim YC**, Lee SE, Kim SK, Jang HD, Hwang I, Jin S, Hong EB, Jang KS, Kim HS. Toll-like receptor mediated inflammation requires FASN-dependent MYD88 palmitoylation. *Nat Chem Biol* 2019; **15**: 907-916 [PMID: 31427815 DOI: 10.1038/s41589-019-0344-0]

69 **Collura KM**, Niu J, Sanders SS, Montersino A, Holland SM, Thomas GM. The palmitoyl acyltransferases ZDHHC5 and ZDHHC8 are uniquely present in DRG axons and control retrograde signaling *via* the Gp130/JAK/STAT3 pathway. *J Biol Chem* 2020; **295**: 15427-15437 [PMID: 32958558 DOI: 10.1074/jbc.RA120.013815]

70 **Yoon JH**, Sudo K, Kuroda M, Kato M, Lee IK, Han JS, Nakae S, Imamura T, Kim J, Ju JH, Kim DK, Matsuzaki K, Weinstein M, Matsumoto I, Sumida T, Mamura M. Phosphorylation status determines the opposing functions of Smad2/Smad3 as STAT3 cofactors in TH17 differentiation. *Nat Commun* 2015; **6**: 7600 [PMID: 26194464 DOI: 10.1038/ncomms8600]

71 **Ohno Y**, Ito A, Ogata R, Hiraga Y, Igarashi Y, Kihara A. Palmitoylation of the sphingosine 1-phosphate receptor S1P is involved in its signaling functions and internalization. *Genes Cells* 2009; **14**: 911-923 [PMID: 19619245 DOI: 10.1111/j.1365-2443.2009.01319.x]

72 **Badawy SMM**, Okada T, Kajimoto T, Ijuin T, Nakamura SI. DHHC5-mediated palmitoylation of S1P receptor subtype 1 determines G-protein coupling. *Sci Rep* 2017; **7**: 16552 [PMID: 29185452 DOI: 10.1038/s41598-017-16457-4]

73 **Blanpain C**, Wittamer V, Vanderwinden JM, Boom A, Renneboog B, Lee B, Le Poul E, El Asmar L, Govaerts C, Vassart G, Doms RW, Parmentier M. Palmitoylation of CCR5 is critical for receptor trafficking and efficient activation of intracellular signaling pathways. *J Biol Chem* 2001; **276**: 23795-23804 [PMID: 11323418 DOI: 10.1074/jbc.M100583200]

74 **Tsutsumi R**, Fukata Y, Noritake J, Iwanaga T, Perez F, Fukata M. Identification of G protein alpha subunit-palmitoylating enzyme. *Mol Cell Biol* 2009; **29**: 435-447 [PMID: 19001095 DOI: 10.1128/MCB.01144-08]

75 **Lin H**. Protein cysteine palmitoylation in immunity and inflammation. *FEBS J* 2021 [PMID: 33506611 DOI: 10.1111/febs.15728]

76 **Castro-Fernández C**, Janovick JA, Brothers SP, Fisher RA, Ji TH, Conn PM. Regulation of RGS3 and RGS10 palmitoylation by GnRH. *Endocrinology* 2002; **143**: 1310-1317 [PMID: 11897687 DOI: 10.1210/endo.143.4.8713]

77 **Wang J**, Xie Y, Wolff DW, Abel PW, Tu Y. DHHC protein-dependent palmitoylation protects regulator of G-protein signaling 4 from proteasome degradation. *FEBS Lett* 2010; **584**: 4570-4574 [PMID: 21035448 DOI: 10.1016/j.febslet.2010.10.052]

78 **Navarro-Lérida I**, Sánchez-Perales S, Calvo M, Rentero C, Zheng Y, Enrich C, Del Pozo MA. A palmitoylation switch mechanism regulates Rac1 function and membrane organization. *EMBO J* 2012; **31**: 534-551 [PMID: 22157745 DOI: 10.1038/emboj.2011.446]

79 **Lee-Kirsch MA**, Gong M, Chowdhury D, Senenko L, Engel K, Lee YA, de Silva U, Bailey SL, Witte T, Vyse TJ, Kere J, Pfeiffer C, Harvey S, Wong A, Koskenmies S, Hummel O, Rohde K, Schmidt RE, Dominiczak AF, Gahr M, Hollis T, Perrino FW, Lieberman J, Hübner N. Mutations in the gene encoding the 3'-5' DNA exonuclease TREX1 are associated with systemic lupus erythematosus. *Nat Genet* 2007; **39**: 1065-1067 [PMID: 17660818 DOI: 10.1038/ng2091]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 December 28; 27(48): 8216-8226

DOI: 10.3748/wjg.v27.i48.8216

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

FRONTIER

New era of electrochemotherapy in treatment of liver tumors in conjunction with immunotherapies

Blaž Trotovšek, Mihajlo Djokić, Maja Čemažar, Gregor Serša

ORCID number: Blaž Trotovšek 0000-0002-0791-2083; Mihajlo Djokić 0000-0002-5678-1315; Maja Čemažar 0000-0002-1418-1928; Gregor Serša 0000-0002-7641-5670.

Author contributions: Trotovšek B and Serša G were equally involved in the conceptualization and writing of the manuscript; Djokić M helped write and edit the manuscript; Čemažar M edited the manuscript; and all authors read and approved the final manuscript, met the criteria for authorship established by the International Committee of Medical Journal Editors, and verified the validity of the reported results.

Conflict-of-interest statement: We have no conflict-of-interest to declare. Editing services provided by American Journal Experts was financed through the funding of Slovenian Research Agency - grant P3-0003.

Supported by the Slovenian Research Agency (ARRS) (P3-0003). The investment was co-financed by the Republic of Slovenia and the European Regional Development Fund (Project SmartGene.Si).

Country/Territory of origin: Slovenia

Specialty type: Gastroenterology

Blaž Trotovšek, Mihajlo Djokić, Department of Abdominal Surgery, University Medical Centre Ljubljana, Ljubljana 1000, Slovenia

Blaž Trotovšek, Mihajlo Djokić, Medical Faculty Ljubljana, University of Ljubljana, Ljubljana 1000, Slovenia

Maja Čemažar, Gregor Serša, Department of Experimental Oncology, Institute of Oncology, Ljubljana 1000, Slovenia

Maja Čemažar, Faculty of Health Sciences, University of Primorska, Izola 6310, Slovenia

Gregor Serša, Faculty of Health Sciences, University of Ljubljana, Ljubljana 1000, Slovenia

Corresponding author: Blaž Trotovšek, MD, PhD, Associate Professor, Department of Abdominal Surgery, University Medical Centre Ljubljana, Zaloska cesta 7, Ljubljana 1000, Slovenia. blaz.trotovsek@kclj.si

Abstract

Electrochemotherapy is a local ablative therapy that increases the cytotoxicity of either bleomycin or cisplatin by applying electric pulses (electroporation) to tumors. It has already been widely used throughout Europe for the treatment of various types of human and veterinary cutaneous tumors, with an objective response rate ranging from 70%-90%, depending on the tumor histotype. Recently, electrochemotherapy was introduced for the treatment of primary liver tumors, such as hepatocellular carcinoma (HCC). The complete response rate was 85% per treated lesion, with a durable response. Therefore, electrochemotherapy could become a treatment of choice for HCC, especially after achieving a transition from an open surgery approach to a percutaneous approach that uses dedicated electrodes. Electrochemotherapy elicits a local immune response and can be considered an in situ vaccination. HCC, among others, is a potentially immunogenic tumor; thus, electrochemotherapy could boost adjuvant immunotherapy to achieve a better and longer-lasting antitumor response. Therefore, therapeutic strategies that combine electrochemotherapy with immune checkpoint inhibitors or adjuvant treatment with cytokines are indicated for HCC. Immunogene therapy using electroporation as a delivery system for plasmid DNA coding for interleukin-12 is a highly promising approach. This electroporation approach has shown efficacy in preclinical settings and veterinary oncology and is awaiting translation for the treatment of liver tumors, *i.e.*, HCC.

and hepatology

Provenance and peer review: Invited article; Externally peer reviewed

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: March 26, 2021
Peer-review started: March 26, 2021
First decision: October 16, 2021
Revised: October 28, 2021
Accepted: December 16, 2021
Article in press: December 16, 2021
Published online: December 28, 2021

P-Reviewer: Morganti AG
S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

Key Words: Electroporation; Electrochemotherapy; Gene therapy; Gene electrotransfer; Interleukin-12; Immune checkpoint inhibitors; Hepatocellular carcinoma

Core Tip: Electrochemotherapy was found to be feasible, safe and highly effective for the treatment of hepatocellular carcinoma (HCC). A local immune response is induced through the destruction of tumor cells; therefore, the electrochemotherapy approach can be considered an in situ vaccination. Electrochemotherapy combined with immune checkpoint inhibitors had an interactive effect on melanoma tumors and HCC. Furthermore, electrochemotherapy can be combined with immunostimulation with cytokines. Electrochemotherapy involving the gene electrotransfer of a plasmid DNA coding for interleukin-12 (IL-12) has already been shown to have clinical value. The combination of electrochemotherapy and immunogene therapy with IL-12 *via* electroporation might be a feasible new treatment strategy for HCC that is also potentially applicable to other liver tumors.

Citation: Trotovšek B, Djokić M, Čemažar M, Serša G. New era of electrochemotherapy in treatment of liver tumors in conjunction with immunotherapies. *World J Gastroenterol* 2021; 27(48): 8216-8226
URL: https://www.wjgnet.com/1007-9327/full/v27/i48/8216.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i48.8216

INTRODUCTION

Liver tumors represent a group of tumors that arise in liver tissue. Hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed tumor, the fourth leading cause of cancer-related death worldwide and is responsible for over 850000 deaths annually. Outcomes are poor overall, with an estimated 5-year survival rate of approximately 20%[1].

The most common type of primary liver tumor is HCC, which represents approximately 90% of all primary liver tumors, followed by intrahepatic cholangiocarcinoma. The incidence of liver tumors varies from Europe to Asia, mostly because of regional differences in the prevalence of risk factors. This difference is most clearly seen in HCC. HCC generally occurs in the presence of liver cirrhosis or liver disease. The incidence of HCC in eastern Asia is 3.5-fold higher than the incidence in Europe, mainly because of the difference in the incidence of hepatitis B/C in Asia and Europe [2].

In addition to hepatitis B/C infection, one of the most common risk factors for HCC is alcohol abuse. Other risk factors include dietary aflatoxin exposure, smoking, nonalcoholic fatty liver disease associated with obesity, and diabetes, which is increasingly emerging as a key contributor to the incidence of HCC in the United States and other western countries[2,3].

Therapy options are individualized and based on the stage of disease, liver function, and performance status of the patient.

Therapy options can be divided into three categories as follows: (1) Curative options for early-stage tumors are surgery, liver transplantation and ablation, *e.g.*, microwave ablation (MWA) or radiofrequency ablation (RFA); MWA is more convenient for the treatment of larger lesions, especially those in close proximity to blood vessels[4]; (2) Locoregional therapy, such as transarterial chemoembolization (TACE) and transarterial radioembolization (TARE), for intermediate-stage tumor; TACE is the standard of care for patients without curative treatment options with liver-only disease and without macrovascular invasion or for patients listed for liver transplantation as "bridging" to transplantation; and (3) Systemic therapy for advanced tumors (atezolizumab and bevacizumab, sorafenib, levatinib, regorafenib, cabozantinib, and ramucirumab). Surgical and locoregional therapies are not covered in this review, as they have been reviewed extensively elsewhere[1,2,5-9].

LOCAL THERAPIES FOR LIVER TUMORS

Local therapies are particularly appropriate for the treatment of liver tumors, mostly due to the feasibility of the percutaneous approach. Thermal ablative therapies have been the most rapidly adopted local therapy approaches. Tumors up to 3 cm in diameter can be successfully ablated with either RFA or MWA. Tumor control is achieved with complete responses (CRs) ranging from 40%-80%[10]. However, the local and locoregional recurrence rates in the liver following thermal ablative therapies are significant due to the localized nature of their efficacy. A local recurrence rate of up to 20% has been reported during follow-up after RFA. Similar results have been seen in patients treated with MWA, considering that patients treated with MWA had larger and more lesions or lesions in the vicinity of blood vessels[11]. Another thermal ablative technique is cryoablation, which is based on repetitive cycles of freezing (argon gas) and thawing (helium gas) of tumors, causing the formation of intracellular ice crystals that lead to cell death. The efficacy of cryoablation is similar to that reported for RFA[12].

There are some nonthermal ablative therapies available in addition to these thermal ablative therapies. Electroporation-based treatment is one of them. Irreversible electroporation is a relatively well-established treatment approach, and the use of electrochemotherapy is on the rise. Irreversible electroporation is a well-accepted therapy for liver tumors, including HCC[13]. This percutaneously performed approach has been practiced in renowned centers, and their reports have demonstrated the feasibility and safety of the approach. Irreversible electroporation is based on the delivery of a long train of up to 100 electric pulses of 1000 V per cm of the distance between the electrodes to destabilize the cell membrane and induce necrotic cell death. Some reports have also indicated the induction of immunogenic cell death following irreversible electroporation[14]. The drawback of irreversible electroporation is that it takes a considerable amount of time to deliver all the electric pulses, and the repetitive delivery of electric pulses increases the temperature of the area around the electrodes; therefore, irreversible electroporation cannot be considered a completely nonthermal technique. Electrochemotherapy is a nonthermal therapy since only 8 electric pulses are delivered between the electrodes to permeabilize the cell membrane, which leads to reversible electroporation[15]. The train of 8 pulses induces permeable structures in the cell membrane, which immediately start to reseal after the pulses are delivered. The application of electric pulses does not affect cell viability per se. The cytotoxic effect is exerted by the drug, which is delivered into the cells due to the permeabilization of the cell membrane. The cytotoxic effect of bleomycin or cisplatin on tumor cells is slowly exerted by the induction of apoptotic, mitotic, and immunogenic cell death[16]. Therefore, the advantage of electrochemotherapy is the slow reaction and the exertion of a cytotoxic effect from the drug only, which avoids the clinical problem of massive necrosis[14]. The drug dosage needed to exert the cytotoxic action is very small due to the increased intracellular delivery of the drugs by electroporation; therefore, there are no severe systemic side effects even when the drug is delivered systemically. Electrochemotherapy can prevent tumor bleeding through the disruption of small tumor vessels; furthermore, electrochemotherapy can promote hemostasis in bleeding tumors[15].

ELECTROCHEMOTHERAPY FOR THE TREATMENT OF HCC

The first study of electrochemotherapy for the treatment of liver tumors was a preliminary study on colorectal liver metastases that indicated the safety and feasibility of the approach[17]. The approach was described from a technical point of view during open surgery. The standard operating procedures for the electrochemotherapy of cutaneous tumors were followed but adapted for the specifics of the liver tumors, especially for tumors larger than 3 cm in diameter[18]. These protocols were then followed in the subsequent application of electrochemotherapy for the treatment of colorectal liver metastases and HCC. The pilot and subsequent phase II study of the treatment of colorectal liver metastases with electrochemotherapy demonstrated a significant benefit of electrochemotherapy as a treatment for patients for whom electrochemotherapy was the only remaining treatment option. A 75% CR rate of metastases was achieved. Effective treatment provided long-term local tumor control as well as a long, progression-free survival rate. The success of electrochemotherapy enabled patients to receive successive treatments and consequently a prolonged life expectancy[19].

Electrochemotherapy was also performed on HCC tumors in patients for whom other curative treatment options had been exhausted. We observed slow resolution of the treated tumors, those associated with cirrhotic livers, and in situations when tumors were adjacent to or embraced major liver vessels or bile ducts. We took advantage of the nonthermal action of electrochemotherapy and demonstrated the feasibility of the approach in patients with difficult-to-treat situations[20,21]. It was demonstrated in a separate study on pig livers that electrochemotherapy does not affect the function and architecture of larger tumor vessels[22]. Furthermore, in that study, no specific pathological effects of electrochemotherapy on healthy liver parenchyma, vessels, or bile ducts were observed, which provided a good starting point for the use of electrochemotherapy in the treatment of HCC, especially in cases where tumors are in contact with larger hollow structures of the liver.

The results obtained for the treated tumors described above demonstrated that electrochemotherapy has similar effectiveness to other ablative therapies. We achieved CR in 84.4% of treated lesions in the phase II trial with a median follow-up time of 50 mo. Thus, the effectiveness of electrochemotherapy is comparable to the effectiveness of MWA, which achieves disease-free survival in 67.2% of patients at 36 mo and 49.1% at 60 mo[11]. Early reports for percutaneous irreversible electroporation show an efficacy of 72%-100% across different studies[13,23].

The main limitation of electrochemotherapy for liver tumors in previous studies was that the procedure was performed intraoperatively during open surgery. This was necessary to maximally control the execution of the treatment and explore the limits of the treatment. Based on the experience gained and the results obtained, we can now claim that electrochemotherapy could produce equally beneficial treatment effects for HCC tumors as other ablative therapies and could be used for the treatment of other liver tumors and metastases. The limitation of not being a percutaneous technique has been recently overcome[21,24].

The percutaneous application of electrochemotherapy was enabled by the development of a new pulse generator Cliniporator®VITAE (IGEA SpA, Carpi, Italy), which can generate sufficient power to treat deep-seated tumors. Additionally, long needle electrodes are available, which are similar to those used for irreversible electroporation[15]. The first attempt to treat HCC with percutaneous electrochemotherapy was performed in Ljubljana and demonstrated the feasibility, safety and efficacy of the percutaneous approach to electrochemotherapy for the treatment of HCC (Figure 1)[21]. We are currently gaining new experience in the percutaneous approach, and the process of transition from intraoperative to percutaneous electrochemotherapy is underway. Additionally, other authors have reported the feasibility of percutaneous electrochemotherapy for the treatment of HCC portal vein tumor thrombus at the hepatic hilum in six patients[24].

Another percutaneous electrochemotherapy application was performed for the treatment of cholangiocarcinoma in the hepatic hilum[25]. The treatment proved to be safe and effective in five patients and improved the prognosis and quality of life of patients with unresectable perihilar cholangiocarcinoma.

The design and production of new multineedle electrodes for percutaneous use will enable easier and reliable placement of electrodes, avoiding the tedious and laborious placement of single needle electrodes. Currently, needle electrodes need to be placed in the right position with the prerequisite of being in a parallel position to obtain adequate electric field distribution. The treatment plan needs to be prepared for the placement of the electrodes to cover the whole tumor with an electric field sufficient to permeabilize the whole tumor mass. Electrodes are placed at the edge of the tumor or in normal tissue to ensure appropriate safety margins[26]. Minimally invasive endoscopic and laparoscopic electrodes were recently developed as an alternative to this procedure of placing single needles and are now available for clinical use. The shaft of the electrode is inserted in the abdomen, and then the electrode array is inserted into the tumor, extending in an umbrella-like fashion[21]. Endoscopic electrodes have also been developed and are available on the market. The electrode is mounted on the endoscope. The electrodes are parallel plates in a chamber in which the tumor tissue is pulled for injection with bleomycin followed by electroporation. The results of the pilot study using these electrodes have already been published[27]. Seven patients with colorectal tumors who were deemed ineligible for or had declined standard treatment were included. They were treated with bleomycin either intratumorally or intravenously, and the electric pulses were delivered through the endoscopic electrode device. Safety and efficacy were assessed clinically and by scans immediately after treatment, and adverse events were reported. This first-in-human study showed that electrochemotherapy for colorectal tumors using an endoscopic electrode device can induce a local tumor response and is safe for fragile elderly

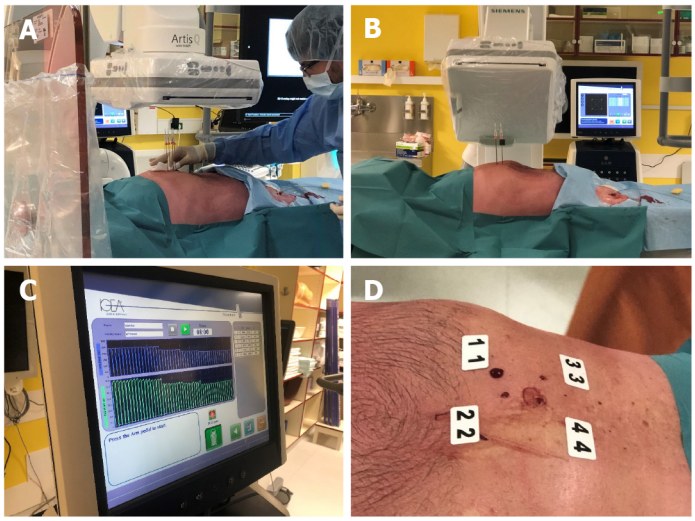

Figure 1 Electrochemotherapy using long needle electrodes in the percutaneous procedure. A: Positioning of the electrodes; B: Verification of the electrode positioning with computerized tomography; C: Treatment of the tumor, electric pulse delivery; D: Posttreatment.

patients with comorbidities.

The intraoperative approach might still be an option in surgical situations in which an unexpected, difficult-to-surgically treat situation occurs; in such a situation, electrochemotherapy can represent a viable treatment option.

CURRENT DEVELOPMENTS IN ABLATIVE THERAPIES COMBINED WITH IMMUNOTHERAPY

The current paradigm is that local and locoregional ablative therapies can elicit a local immune response that can be boosted by immunotherapeutic approaches. This approach is currently being explored, predominantly with a combination of radiotherapy and immune checkpoint inhibitors; however, other ablative techniques are already in clinical trials in combination with immune therapies. These clinical trials explored which tumors would benefit the most and the optimal timing, sequence, dose of immune therapy, and the number of fractions and dose per fraction for radiotherapy. Radiotherapy can stimulate a proinflammatory environment by killing tumor cells and stimulating the infiltration of immune cells, thus turning immunologically cold tumors into immunologically hot tumors. Radiation damage resulting in micronuclei in cells stimulates cytosolic nucleic acid sensor pathways, such as cyclic GMP-AMP synthase, which is a stimulator of interferon genes. Additionally, irradiation modulates neoantigen expression, which impacts immune surveillance and sets the stage for combined treatment with immune checkpoint inhibitors[28,29]. However, as stated, questions arise regarding the appropriate doses and fractionation of tumor irradiation to elicit an adequate immunogenic response. Clinical studies indicate that stereotactic body radiation therapy is more powerful in enhancing antitumor immunity and works better with immune checkpoint inhibitors than fractionated conventional radiotherapy. This effect was observed when this combination was tested in non-small cell lung cancer, melanoma, head and neck cancer, HCC, pancreatic cancer, and genital tumors[30].

Several clinical studies have been initiated on the treatment of HCC with the combination of locoregional and local ablative therapies with immune checkpoint inhibitors based on promising results from studies testing immune checkpoint inhibitors in advanced HCC. It is known from retrospective studies of other tumor types that the clinical efficacy of immune checkpoint inhibitors correlates with tumor burden; therefore, it is better to treat smaller tumors with this approach. Another reason for combining electrochemotherapy with immune checkpoint inhibitors is that although immune therapies are also combined with surgical approaches, the immunological effects that are observed after local and locoregional therapies favor such combinations. Current clinical studies are evaluating immune checkpoint inhibitors as an adjuvant therapy with RFA in neoadjuvant settings and are investigating whether the combination with immune checkpoint inhibitors in tumors larger than 3 cm can be

performed with curative intent (NCT03847428 and NCT03630640). Furthermore, the role of anti-vascular endothelial growth factor (VEGF) therapies in combination with immune checkpoint inhibitors and local ablative therapies should be determined in the future. It has been shown that anti-VEGF therapies overcome intrinsic resistance to immune checkpoint inhibitors (Figure 2). Additionally, an increase in VEGF after RFA was observed in patients with HCC; thus, inhibiting VEGF can enhance the effect of immune therapy in combination with the local ablative therapies required to achieve a complete response of HCC[9].

CAN ELECTROCHEMOTHERAPY FOR IN SITU VACCINATION IN COMBINATION WITH IMMUNE CHECKPOINT INHIBITORS BE EXPLOITED?

Similar findings to those outlined above were found with electroporation-based treatments. The results of these reports showed that both irreversible electroporation and electrochemotherapy could induce immunogenic cell death[14]. A recent study on electrochemotherapy in mice compared the response of different tumors to electrochemotherapy and correlated it with the immune status of those tumors. The response of tumors correlated with the immune status; specifically, more immunogenic tumors responded significantly better than less immunogenic tumors. Furthermore, the response varied according to the drug used for electrochemotherapy. The study indicated that intratumoral cisplatin electrochemotherapy seems to be very effective for immunogenic tumors. All these data indicate that electrochemotherapy elicits immunogenic cell death in situ by releasing ATP and high-mobility group box and calreticulin translocation, which is dependent on tumor immunogenicity and the drug used for electrochemotherapy[14,16,31].

The results following electrochemotherapy performed for patients with melanoma during therapy with immune checkpoint inhibitors against either cytotoxic T-lymphocyte antigen or programmed cell death ligand 1 were published in a retrospective study[32]. The local response rate was higher than the reported local response rate for electrochemotherapy only. Ipilimumab combined with electrochemotherapy was feasible, tolerable, and showed a high systemic response rate. The second report to date is a case report, where a symptomatic melanoma lesion that was refractory to nivolumab was successfully treated with electrochemotherapy and achieved a 4-year durable response[33].

One question that remains is how the combined treatment affects the local recurrence-free interval and systemic progression-free interval or even influences overall survival. Another question is whether the combined treatment increases long-term survival. The retrospective analysis of the combined electrochemotherapy and pembrolizumab treatment of patients with melanoma demonstrated that all these parameters were increased[34]. This study proved that electrochemotherapy can be considered an in situ vaccination. However, the question arises as to whether this holds true for all tumor types and treatment parameters. Some preclinical data indicate that not all tumors are equally susceptible to electrochemotherapy. Their responses are dependent on some immune response-related parameters in addition to intrinsic sensitivity to chemotherapeutic drugs and vascularization, such as major histocompatibility complex I expression and mutational burden[16]. This is the so-called "immunogenicity" of the tumors. Therefore, the treatment induces immunogenic cell death and the in situ vaccination effect to different degrees in different tumors. This was demonstrated by the adjuvant effects of immunotherapy with the cytokine interleukin-12 (IL-12). The adjuvant effect was more pronounced in less immunogenic tumors, indicating less responsiveness to electrochemotherapy, and less pronounced potentiation was observed in more immunogenic tumors that were more responsive to electrochemotherapy[16].

Therefore, we can expect that not all tumors in the liver will respond equally to adjuvant immunotherapy either with immune checkpoint inhibitors or other immunotherapies. However, a comparison between the responses of colorectal liver metastases and HCC to electrochemotherapy showed that HCC responds better[19,20]. Does this mean that HCC is more immunogenic than colorectal liver metastases and that adjuvant immunotherapy would not contribute significantly? It is well established that HCC is an immunologically hot tumor, and it was demonstrated that HCC is responsive to immune checkpoint inhibitors in clinical trials[35,36]. However, the combination with electrochemotherapy needs to be investigated for all liver cancers.

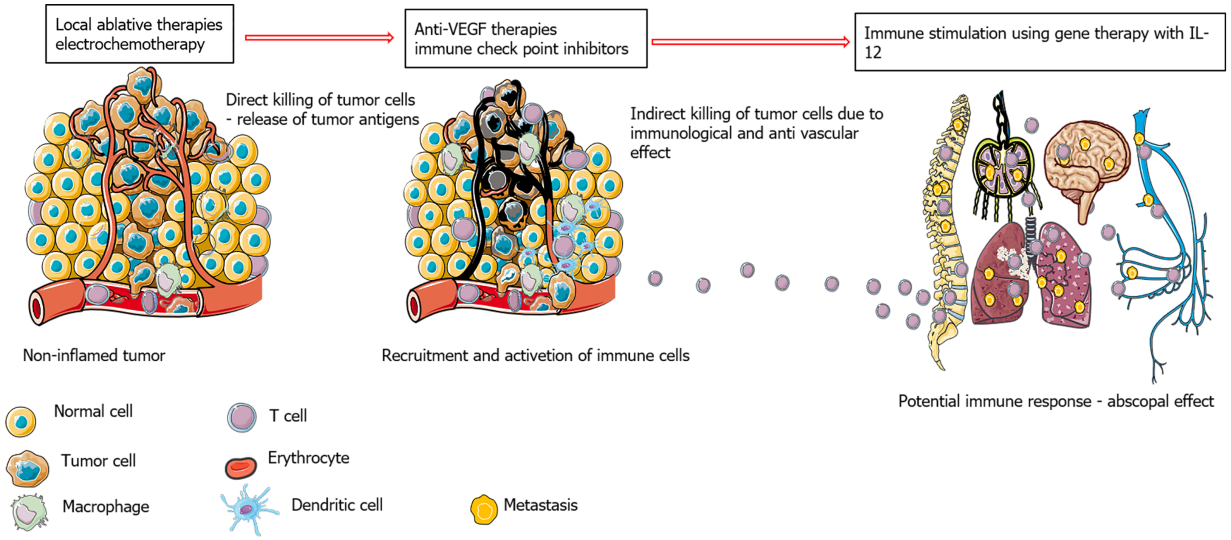

Figure 2 Potential benefits of combining local ablative therapies with immune checkpoint inhibitors and anti-vascular therapies. IL-12: Interleukin-12.

The other aspect is that HCC is better vascularized than colorectal liver metastases; therefore, the disruptive vascular effect of electrochemotherapy is more pronounced and could also account for the overall antitumor effectiveness[19-21].

IS THERE A POSSIBILITY FOR ADJUVANT IMMUNOSTIMULATION?

If ongoing clinical trials on ablative therapies will meet expectations in combination with immune checkpoint inhibitors and other systemic treatments (tyrosine kinase inhibitors and anti-VEGF), then a new line of treatment will be available for cancer patients. The effects will certainly vary between the patients according to the tumor type, the type of ablative technique, and the degree to which the tumors need to be destroyed for the best vaccination effect. These aspects need to be explored, but first, reliable markers are needed for the measurement of immune effects *in vivo*[36].

Nevertheless, if the current combination of immune checkpoint inhibitors and other drugs does not provide optimal treatment outcomes, we will need to explore additional ways to boost the immune responses of the organism. Adjuvant immunostimulation with cytokines has been explored as one option. Historically, some interleukins, such as IL-2 and IL-12, were evaluated in clinical trials in the form of recombinant proteins. These combinations with radiotherapy have yielded promising results, but the cytokine side effects were overwhelming in some cases. Therefore, this approach was abandoned[37].

There are now new techniques that can provide targeted and controllable expression of the desired molecules. Gene therapy is one such method that is gaining attention, especially with the development of genetically based coronavirus 2019 vaccines. Special attention has been given to naked DNA plasmids that can be delivered to targeted tissues by nonviral delivery techniques, such as electroporation [38]. This technique is called gene electrotransfer and can be used to deliver genes to either healthy tissues or tumors. In healthy tissues, such as muscle or skin, the expression is either systemic or localized. Therefore, if transfection occurs in normal cells, the expression lasts until the cells start to divide, as the plasmid is expressed episomally and is not retained as the cells divide. However, the encoded protein works in a paracrine fashion.

This technique is also gaining recognition because clinical studies in the United States have demonstrated the feasibility, safety, and efficacy of similar gene therapies for cancer treatment using a plasmid coding for IL-12. IL-12 is a potent proinflammatory cytokine with pleiotropic activity[39]. Most importantly, it can engage in multiple effector mechanisms and reverse tumor immunosuppression. Numerous localized delivery strategies are being explored to maximize its effectiveness, among which naked plasmid delivery with electroporation is promising. This approach has already been proven safe and effective for the treatment of cutaneous melanoma, and clinical trials for other tumors are underway[40,41].

Therefore, the immune-gene therapy approach might be the next step in immunotherapy. The approach could be exploited for skin tumors and liver tumors and be used as a monotherapy or in combination with ablative techniques.

COMBINED ELECTROCHEMOTHERAPY AND GENE ELECTROTRANSFER OF PLASMID DNA CODING FOR IL-12

There are two options for the combined electrochemotherapy and gene electrotransfer approach for the treatment of HCC. The first involves combined treatment delivered during the same electroporation session since both drug and gene delivery is based on electroporation. Therefore, the same electroporation session could be exploited to perform both electrochemotherapy and gene electrotransfer. In theory, the two treatments require different electric pulse parameters for optimal/high delivery, but preclinical data indicate that gene electrotransfer can occur with the same electric pulses that are used for electrochemotherapy[42]. Therefore, gene delivery of IL-12 coding plasmids to tumors could be performed during electrochemotherapy. The problem of how to deliver the plasmid into the tumor needs to be resolved. One option could be to adjust the new percutaneous electrodes with a syringe to deliver the plasmid into tumors.

The second approach for combining electrochemotherapy with gene electrotransfer for the treatment of HCC is to perform gene electrotransfer into distant muscle or skin for systemic transgene delivery. For example, localized transfection into the muscle could result in the shedding of IL-12 from the muscle into the bloodstream[43-45]. The shedding of the transgene would be controllable, sustainable and without pharmacological peaks that can produce severe side effects. This approach would provide a more prolonged action of the transgene and could also provide a good treatment effect.

CONCLUSION

Local ablative therapies that destroy tumor cells activate localized immune reactions; thus, these therapies can be considered in situ vaccines. Electrochemotherapy is an ablative therapy that elicits this in situ vaccination effect. Electrochemotherapy has been used for the treatment of HCC tumors in patients where other treatment options have been exhausted. This approach has been proven to be feasible, safe, and highly effective. Its limits were explored in the open surgery approach; however, with the development of new percutaneous electrodes, electrochemotherapy could be performed in a similar percutaneous way to other ablative therapies used for the treatment of liver tumors. Electrochemotherapy combined with immune checkpoint inhibitors has been shown to have an interactive effect as a treatment for melanoma tumors. Similar to the combination of inhibitors with other ablative therapies, the combination of immune checkpoint inhibitors and electrochemotherapy could also be effective for the treatment of HCC. Furthermore, electrochemotherapy could be combined with cytokine immunostimulation methods. The combination of electrochemotherapy with gene electrotransfer of a naked plasmid coding for IL-12 has already proven its value in preclinical work. Therefore, the combination of electrochemotherapy with IL-12 immunogene therapy, which are both delivered *via* electroporation, could be a new treatment approach for HCC tumors and possibly other liver tumors.

ACKNOWLEDGEMENTS

We gratefully thank American Journal Experts for linguistic editing of the manuscript.

REFERENCES

1 **Llovet JM**, Zucman-Rossi J, Pikarsky E, Sangro B, Schwartz M, Sherman M, Gores G. Hepatocellular carcinoma. *Nat Rev Dis Primers* 2016; **2**: 16018 [PMID: 27158749 DOI: 10.1038/nrdp.2016.18]

2 **Kulik L**, El-Serag HB. Epidemiology and Management of Hepatocellular Carcinoma. *Gastroenterology* 2019; **156**: 477-491.e1 [PMID: 30367835 DOI: 10.1053/j.gastro.2018.08.065]

3 **Yang JD**, Hainaut P, Gores GJ, Amadou A, Plymoth A, Roberts LR. A global view of hepatocellular carcinoma: trends, risk, prevention and management. *Nat Rev Gastroenterol Hepatol* 2019; **16**: 589-604 [PMID: 31439937 DOI: 10.1038/s41575-019-0186-y]

4 **Tan W**, Deng Q, Lin S, Wang Y, Xu G. Comparison of microwave ablation and radiofrequency ablation for hepatocellular carcinoma: a systematic review and meta-analysis. *Int J Hyperthermia* 2019; **36**: 264-272 [PMID: 30676100 DOI: 10.1080/02656736.2018.1562571]

5 **European Association for the Study of the Liver**. ; European Association for the Study of the Liver. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* 2018; **69**: 182-236 [PMID: 29628281 DOI: 10.1016/j.jhep.2018.03.019]

6 **Marrero JA**, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, Roberts LR, Heimbach JK. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. *Hepatology* 2018; **68**: 723-750 [PMID: 29624699 DOI: 10.1002/hep.29913]

7 **Ohri N**, Dawson LA, Krishnan S, Seong J, Cheng JC, Sarin SK, Kinkhabwala M, Ahmed MM, Vikram B, Coleman CN, Guha C. Radiotherapy for Hepatocellular Carcinoma: New Indications and Directions for Future Study. *J Natl Cancer Inst* 2016; **108** [PMID: 27377923 DOI: 10.1093/jnci/djw133]

8 **Vibert E**, Schwartz M, Olthoff KM. Advances in resection and transplantation for hepatocellular carcinoma. *J Hepatol* 2020; **72**: 262-276 [PMID: 31954491 DOI: 10.1016/j.jhep.2019.11.017]

9 **Llovet JM**, De Baere T, Kulik L, Haber PK, Greten TF, Meyer T, Lencioni R. Locoregional therapies in the era of molecular and immune treatments for hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol* 2021; **18**: 293-313 [PMID: 33510460 DOI: 10.1038/s41575-020-00395-0]

10 **Tiong L**, Maddern GJ. Systematic review and meta-analysis of survival and disease recurrence after radiofrequency ablation for hepatocellular carcinoma. *Br J Surg* 2011; **98**: 1210-1224 [PMID: 21766289 DOI: 10.1002/bjs.7669]

11 **Sun Q**, Shi J, Ren C, Du Z, Shu G, Wang Y. Survival analysis following microwave ablation or surgical resection in patients with hepatocellular carcinoma conforming to the Milan criteria. *Oncol Lett* 2020; **19**: 4066-4076 [PMID: 32391107 DOI: 10.3892/ol.2020.11529]

12 **Song KD**. Percutaneous cryoablation for hepatocellular carcinoma. *Clin Mol Hepatol* 2016; **22**: 509-515 [PMID: 28081593 DOI: 10.3350/cmh.2016.0079]

13 **Sugimoto K**, Abe M, Yoshimasu Y, Takeuchi H, Kasai Y, Itoi T. Irreversible electroporation of hepatocellular carcinoma: the role of ultrasonography. *Ultrasonography* 2020; **39**: 229-237 [PMID: 32450674 DOI: 10.14366/usg.20023]

14 **Brock RM**, Beitel-White N, Davalos RV, Allen IC. Starting a Fire Without Flame: The Induction of Cell Death and Inflammation in Electroporation-Based Tumor Ablation Strategies. *Front Oncol* 2020; **10**: 1235 [PMID: 32850371 DOI: 10.3389/fonc.2020.01235]

15 **Sersa G**, Ursic K, Cemazar M, Heller R, Bosnjak M, Campana LG. Biological factors of the tumour response to electrochemotherapy: Review of the evidence and a research roadmap. *Eur J Surg Oncol* 2021; **47**: 1836-1846 [PMID: 33726951 DOI: 10.1016/j.ejso.2021.03.229]

16 **Ursic K**, Kos S, Kamensek U, Cemazar M, Miceska S, Markelc B, Bucek S, Staresinic B, Kloboves Prevodnik V, Heller R, Sersa G. Potentiation of electrochemotherapy effectiveness by immunostimulation with IL-12 gene electrotransfer in mice is dependent on tumor immune status. *J Control Release* 2021; **332**: 623-635 [PMID: 33705828 DOI: 10.1016/j.jconrel.2021.03.009]

17 **Edhemovic I**, Gadzijev EM, Brecelj E, Miklavcic D, Kos B, Zupanic A, Mali B, Jarm T, Pavliha D, Marcan M, Gasljevic G, Gorjup V, Music M, Vavpotic TP, Cemazar M, Snoj M, Sersa G. Electrochemotherapy: a new technological approach in treatment of metastases in the liver. *Technol Cancer Res Treat* 2011; **10**: 475-485 [PMID: 21895032 DOI: 10.7785/tcrt.2012.500224]

18 **Mir LM**, Gehl J, Sersa G, Collins CG, Garbay J-R, Billard V, Geertsen PF, Rudolf Z, O'sullivan GC, Marty M. Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the Cliniporator TM by means of invasive or non-invasive electrodes. *EJC Sup* 2006 [DOI: 10.1016/J.EJCSUP.2006.08.003]

19 **Edhemovic I**, Brecelj E, Gasljevic G, Marolt Music M, Gorjup V, Mali B, Jarm T, Kos B, Pavliha D, Grcar Kuzmanov B, Cemazar M, Snoj M, Miklavcic D, Gadzijev EM, Sersa G. Intraoperative electrochemotherapy of colorectal liver metastases. *J Surg Oncol* 2014; **110**: 320-327 [PMID: 24782355 DOI: 10.1002/jso.23625]

20 **Djokic M**, Cemazar M, Popovic P, Kos B, Dezman R, Bosnjak M, Zakelj MN, Miklavcic D, Potrc S, Stabuc B, Tomazic A, Sersa G, Trotovsek B. Electrochemotherapy as treatment option for hepatocellular carcinoma, a prospective pilot study. *Eur J Surg Oncol* 2018; **44**: 651-657 [PMID: 29402556 DOI: 10.1016/j.ejso.2018.01.090]

21 **Djokic M**, Dezman R, Cemazar M, Stabuc M, Petric M, Smid LM, Jansa R, Plesnik B, Bosnjak M, Tratar UL, Trotovsek B, Kos B, Miklavcic D, Sersa G, Popovic P. Percutaneous image guided electrochemotherapy of hepatocellular carcinoma: technological advancement. *Radiol Oncol* 2020; **54**: 347-352 [PMID: 32562533 DOI: 10.2478/raon-2020-0038]

22 **Zmuc J**, Gasljevic G, Sersa G, Edhemovic I, Boc N, Seliskar A, Plavec T, Brloznik M, Milevoj N, Brecelj E, Kos B, Izlakar J, Jarm T, Snoj M, Stukelj M, Miklavcic D, Cemazar M. Large Liver Blood Vessels and Bile Ducts Are Not Damaged by Electrochemotherapy with Bleomycin in Pigs. *Sci Rep*

2019; **9**: 3649 [PMID: 30842517 DOI: 10.1038/s41598-019-40395-y]
23 **Zimmerman A**, Grand D, Charpentier KP. Irreversible electroporation of hepatocellular carcinoma: patient selection and perspectives. *J Hepatocell Carcinoma* 2017; **4**: 49-58 [PMID: 28331845 DOI: 10.2147/JHC.S129063]
24 **Tarantino L**, Busto G, Nasto A, Fristachi R, Cacace L, Talamo M, Accardo C, Bortone S, Gallo P, Tarantino P, Nasto RA, Di Minno MN, Ambrosino P. Percutaneous electrochemotherapy in the treatment of portal vein tumor thrombosis at hepatic hilum in patients with hepatocellular carcinoma in cirrhosis: A feasibility study. *World J Gastroenterol* 2017; **23**: 906-918 [PMID: 28223736 DOI: 10.3748/wjg.v23.i5.906]
25 **Tarantino L**, Busto G, Nasto A, Nasto RA, Tarantino P, Fristachi R, Cacace L, Bortone S. Electrochemotherapy of cholangiocellular carcinoma at hepatic hilum: A feasibility study. *Eur J Surg Oncol* 2018; **44**: 1603-1609 [PMID: 30017329 DOI: 10.1016/j.ejso.2018.06.025]
26 **Miklavcic D**, Snoj M, Zupanic A, Kos B, Cemazar M, Kropivnik M, Bracko M, Pecnik T, Gadzijev E, Sersa G. Towards treatment planning and treatment of deep-seated solid tumors by electrochemotherapy. *Biomed Eng Online* 2010; **9**: 10 [PMID: 20178589 DOI: 10.1186/1475-925X-9-10]
27 **Falk Hansen H**, Bourke M, Stigaard T, Clover J, Buckley M, O'Riordain M, Winter DC, Hjorth Johannesen H, Hansen RH, Heebøll H, Forde P, Jakobsen HL, Larsen O, Rosenberg J, Soden D, Gehl J. Electrochemotherapy for colorectal cancer using endoscopic electroporation: a phase 1 clinical study. *Endosc Int Open* 2020; **8**: E124-E132 [PMID: 32010744 DOI: 10.1055/a-1027-6735]
28 **McLaughlin M**, Patin EC, Pedersen M, Wilkins A, Dillon MT, Melcher AA, Harrington KJ. Inflammatory microenvironment remodelling by tumour cells after radiotherapy. *Nat Rev Cancer* 2020; **20**: 203-217 [PMID: 32161398 DOI: 10.1038/s41568-020-0246-1]
29 **Jesenko T**, Bosnjak M, Markelc B, Sersa G, Znidar K, Heller L, Cemazar M. Radiation Induced Upregulation of DNA Sensing Pathways is Cell-Type Dependent and Can Mediate the Off-Target Effects. *Cancers (Basel)* 2020; **12** [PMID: 33202881 DOI: 10.3390/cancers12113365]
30 **Li S**, Shen L. Radiobiology of stereotactic ablative radiotherapy (SABR): perspectives of clinical oncologists. *J Cancer* 2020; **11**: 5056-5068 [PMID: 32742453 DOI: 10.7150/jca.44408]
31 **Calvet CY**, Famin D, André FM, Mir LM. Electrochemotherapy with bleomycin induces hallmarks of immunogenic cell death in murine colon cancer cells. *Oncoimmunology* 2014; **3**: e28131 [PMID: 25083316 DOI: 10.4161/onci.28131]
32 **Heppt MV**, Eigentler TK, Kähler KC, Herbst RA, Göppner D, Gambichler T, Ulrich J, Dippel E, Loquai C, Schell B, Schilling B, Schäd SG, Schultz ES, Matheis F, Tietze JK, Berking C. Immune checkpoint blockade with concurrent electrochemotherapy in advanced melanoma: a retrospective multicenter analysis. *Cancer Immunol Immunother* 2016; **65**: 951-959 [PMID: 27294607 DOI: 10.1007/s00262-016-1856-z]
33 **Karaca B**, Yayla G, Erdem M, Gürler T. Electrochemotherapy with anti-PD-1 treatment induced durable complete response in heavily pretreated metastatic melanoma patient. *Anticancer Drugs* 2018; **29**: 190-196 [PMID: 29271783 DOI: 10.1097/CAD.0000000000000580]
34 **Campana LG**, Peric B, Mascherini M, Spina R, Kunte C, Kis E, Rozsa P, Quaglino P, Jones RP, Clover AJP, Curatolo P, Giorgione R, Cemazar M, Terlizzi F, Bosnjak M, Sersa G. Combination of Pembrolizumab with Electrochemotherapy in Cutaneous Metastases from Melanoma: A Comparative Retrospective Study from the InspECT and Slovenian Cancer Registry. *Cancers (Basel)* 2021; **13** [PMID: 34503099 DOI: 10.3390/cancers13174289]
35 **Pinato DJ**, Guerra N, Fessas P, Murphy R, Mineo T, Mauri FA, Mukherjee SK, Thursz M, Wong CN, Sharma R, Rimassa L. Immune-based therapies for hepatocellular carcinoma. *Oncogene* 2020; **39**: 3620-3637 [PMID: 32157213 DOI: 10.1038/s41388-020-1249-9]
36 **Arora S**, Velichinskii R, Lesh RW, Ali U, Kubiak M, Bansal P, Borghaei H, Edelman MJ, Boumber Y. Existing and Emerging Biomarkers for Immune Checkpoint Immunotherapy in Solid Tumors. *Adv Ther* 2019; **36**: 2638-2678 [PMID: 31410780 DOI: 10.1007/s12325-019-01051-z]
37 **Car BD**, Eng VM, Lipman JM, Anderson TD. The toxicology of interleukin-12: a review. *Toxicol Pathol* 1999; **27**: 58-63 [PMID: 10367675 DOI: 10.1177/019262339902700112]
38 **Keller H**. Malta kENUP F. covidX. 2021 [cited 17 March 2021]. In: covidX [Internet]. Kalkara (Malta) - . Available from: https://www.covidx.eu/covid-evax
39 **Nguyen KG**, Vrabel MR, Mantooth SM, Hopkins JJ, Wagner ES, Gabaldon TA, Zaharoff DA. Localized Interleukin-12 for Cancer Immunotherapy. *Front Immunol* 2020; **11**: 575597 [PMID: 33178203 DOI: 10.3389/fimmu.2020.575597]
40 **Algazi A**, Bhatia S, Agarwala S, Molina M, Lewis K, Faries M, Fong L, Levine LP, Franco M, Oglesby A, Ballesteros-Merino C, Bifulco CB, Fox BA, Bannavong D, Talia R, Browning E, Le MH, Pierce RH, Gargosky S, Tsai KK, Twitty C, Daud AI. Intratumoral delivery of tavokinogene telseplasmid yields systemic immune responses in metastatic melanoma patients. *Ann Oncol* 2020; **31**: 532-540 [PMID: 32147213 DOI: 10.1016/j.annonc.2019.12.008]
41 **Bhatia S**, Longino NV, Miller NJ, Kulikauskas R, Iyer JG, Ibrani D, Blom A, Byrd DR, Parvathaneni U, Twitty CG, Campbell JS, Le MH, Gargosky S, Pierce RH, Heller R, Daud AI, Nghiem P. Intratumoral Delivery of Plasmid IL12 Via Electroporation Leads to Regression of Injected and Noninjected Tumors in Merkel Cell Carcinoma. *Clin Cancer Res* 2020; **26**: 598-607 [PMID: 31582519 DOI: 10.1158/1078-0432.CCR-19-0972]
42 **Cemazar M**, Golzio M, Sersa G, Hojman P, Kranjc S, Mesojednik S, Rols MP, Teissie J. Control by pulse parameters of DNA electrotransfer into solid tumors in mice. *Gene Ther* 2009; **16**: 635-644

[PMID: 19212425 DOI: 10.1038/gt.2009.10]

43 **Chiarella P**, Massi E, De Robertis M, Sibilio A, Parrella P, Fazio VM, Signori E. Electroporation of skeletal muscle induces danger signal release and antigen-presenting cell recruitment independently of DNA vaccine administration. *Expert Opin Biol Ther* 2008; **8**: 1645-1657 [PMID: 18847301 DOI: 10.1517/14712598.8.11.1645]

44 **Tevz G**, Kranjc S, Cemazar M, Kamensek U, Coer A, Krzan M, Vidic S, Pavlin D, Sersa G. Controlled systemic release of interleukin-12 after gene electrotransfer to muscle for cancer gene therapy alone or in combination with ionizing radiation in murine sarcomas. *J Gene Med* 2009; **11**: 1125-1137 [PMID: 19777440 DOI: 10.1002/jgm.1403]

45 **Tevz G**, Pavlin D, Kamensek U, Kranjc S, Mesojednik S, Coer A, Sersa G, Cemazar M. Gene electrotransfer into murine skeletal muscle: a systematic analysis of parameters for long-term gene expression. *Technol Cancer Res Treat* 2008; **7**: 91-101 [PMID: 18345697 DOI: 10.1177/153303460800700201]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 December 28; 27(48): 8227-8241

DOI: 10.3748/wjg.v27.i48.8227

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

FRONTIER

Magnetic challenge against gastroesophageal reflux

Mauro Bortolotti

ORCID number: Mauro Bortolotti 0000-0002-5273-9013.

Author contributions: Bortolotti M drafted the manuscript and revised the manuscript.

Conflict-of-interest statement: The author declares no conflict of interest.

Country/Territory of origin: Italy

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

Mauro Bortolotti, Department of Internal Medicine and Gastroenterology, S. Orsola-Malpighi Polyclinic, University of Bologna, Bologna 40138, Italy

Mauro Bortolotti, Via Massarenti 48, Bologna 40138, Italy

Corresponding author: Mauro Bortolotti, MD, Via Massarenti 48, Bologna 40138, Italy. bormau@tin.it

Abstract

Almost 15 years have passed since the first paper on the possibility of using magnets to prevent gastro-esophageal reflux (GER) was published and so it is time to assess the results obtained with the first magnetic device available on the market, the Linx magnetic sphincter augmentation (MSA) and to consider what other options are forthcoming. MSA demonstrated an anti-reflux activity similar to that of Nissen fundoplication, considered the "gold standard" surgical treatment for GER disease, and caused less gas-bloating and a better ability to allow vomiting and belching. However, unlike Nissen fundoplication, this magnetic device is burdened by complications, which are roughly similar to those of the non-magnetic anti-reflux Angelchik prosthesis, that, after considerable use in the eighties, was shelved due to these complications. It is interesting to note that some of these complications show the same pathophysiological mechanism in both devices. The upcoming new magnetic devices should avoid these complications, as their anti-reflux magnetic mechanism is completely different. The experiments in animals regarding these new magnetic appliances were examined, remarking their advantages and drawbacks, but the way to apply them in surgical practice is long and difficult, although worthy, as they represent the future of magnetic surgery.

Key Words: Gastro-esophageal reflux disease; Magnetic sphincter augmentation device; Nissen fundoplication; Angelchik prosthesis; Lower esophageal sphincter; Dysphagia

Core Tip: The idea of a magnetic device aimed to prevent gastroesophageal reflux was conceived and realized more or less 15 years ago, for which it is time to take stock and consider its future. The first and only device available nowadays in the market is the Linx magnetic sphincter augmentation. Its effectiveness was examined and compared

Received: May 27, 2021
Peer-review started: May 27, 2021
First decision: June 21, 2021
Revised: July 7, 2021
Accepted: December 3, 2021
Article in press: December 3, 2021
Published online: December 28, 2021

P-Reviewer: Morozov S, Sawada A, Sweis R, Tustumi F
S-Editor: Wang JJ
L-Editor: Webster JR
P-Editor: Wang JJ

to that of Nissen fundoplication, whereas its complications, similar to those of the Angelchik prosthesis, were described and their pathophysiology discussed. Furthermore, the pros and cons of the upcoming magnetic anti-reflux devices were examined, underlining the fact that, working with a mechanism completely different to that of the first device, many of its complications could be avoided.

Citation: Bortolotti M. Magnetic challenge against gastroesophageal reflux. *World J Gastroenterol* 2021; 27(48): 8227-8241
URL: https://www.wjgnet.com/1007-9327/full/v27/i48/8227.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i48.8227

INTRODUCTION

It is well known that gastrointestinal sphincters may undergo a weakening in their function of blocking the retrograde flux of contents as at the gastro-esophageal junction level, giving rise to the gastro-esophageal reflux (GER) and the antegrade flux, as at the anal level, causing fecal incontinence. Researchers have tried to strengthen these sphincters by means of medical and surgical treatments, with varying success, and in these last few years they have started using magnets.

Until a few years ago magnetic devices were used only in laparoscopic and endoscopic surgery, providing alternatives for retraction, anchoring, compression, mobilization, and anastomosis[1]. In particular, circular magnets applied face to face have been used to create an "anastomosis" between two adjacent intestinal loops, through necrosis of the compressed tissues, in order to bypass the stop caused by scarring stenosis or by an inoperable cancer obstructing the intestinal lumen[2]. This latter appliance of the magnetic force made me think that the reciprocal attraction of a couple of low power magnets placed face to face outside the opposite walls of a sphincter, may squeeze it, thereby closing the lumen. Thus, some fifteen years ago I described in a bench-top experiment this novel idea of strengthening a gut sphincter with magnets and sent the article to the Journal of Biomechanics in 2003, but "oddly" the article was only published in 2006[3]. As illustrated in Figure 1 in this study a couple of magnetic plaques were applied with the opposite polarities facing each other on the opposite sides of a flaccid tube perfused with water by means of a pump at a certain pressure. The plaques, which attract one another, squeeze the lumen of the tube thereby blocking the flux of the content (like a sphincter that prevents reflux). When the endoluminal pressure is increased above the attraction force of the magnets, the plaques detach themselves, allowing the flow to resume (like a sphincter that opens). On the other hand, when the endoluminal pressure is decreased, the attraction force again prevails and the plaques again squeeze the lumen (to prevent reflux). Furthermore, the force of closure of the plaques can be increased or decreased as desired using magnets with a different force of attraction.

A few years after the aforementioned publication, an increasing number of papers from 2008 to today on the use of magnets to strengthen gut sphincters, and in particular the lower esophageal sphincter (LES), became available. The first magnetic device available on the market to strengthen a weak LES, called Linx magnetic sphincter augmentation (MSA), appeared in an article[4] approximately 13 years ago and represented a clever evolution of the first idea previously published in 2006[3]. This paper was followed by many other studies and so today it is time to consider the surgical magnetic story, assess its successes and failures, as well as drawbacks and complications, and look to the future with the upcoming magnetic devices. A literature search was carried out essentially in the PubMed database, with the following search terms: "magnetic sphincter augmentation device"; "Linx reflux management system"; and "antireflux magnetic devices". From the articles thus found, the most significant and representative were chosen to fulfil the aim of the study. However, a systematic review is not the purpose of this study, but, starting from state-of-the art , I have tried to provide a perspective for future research.

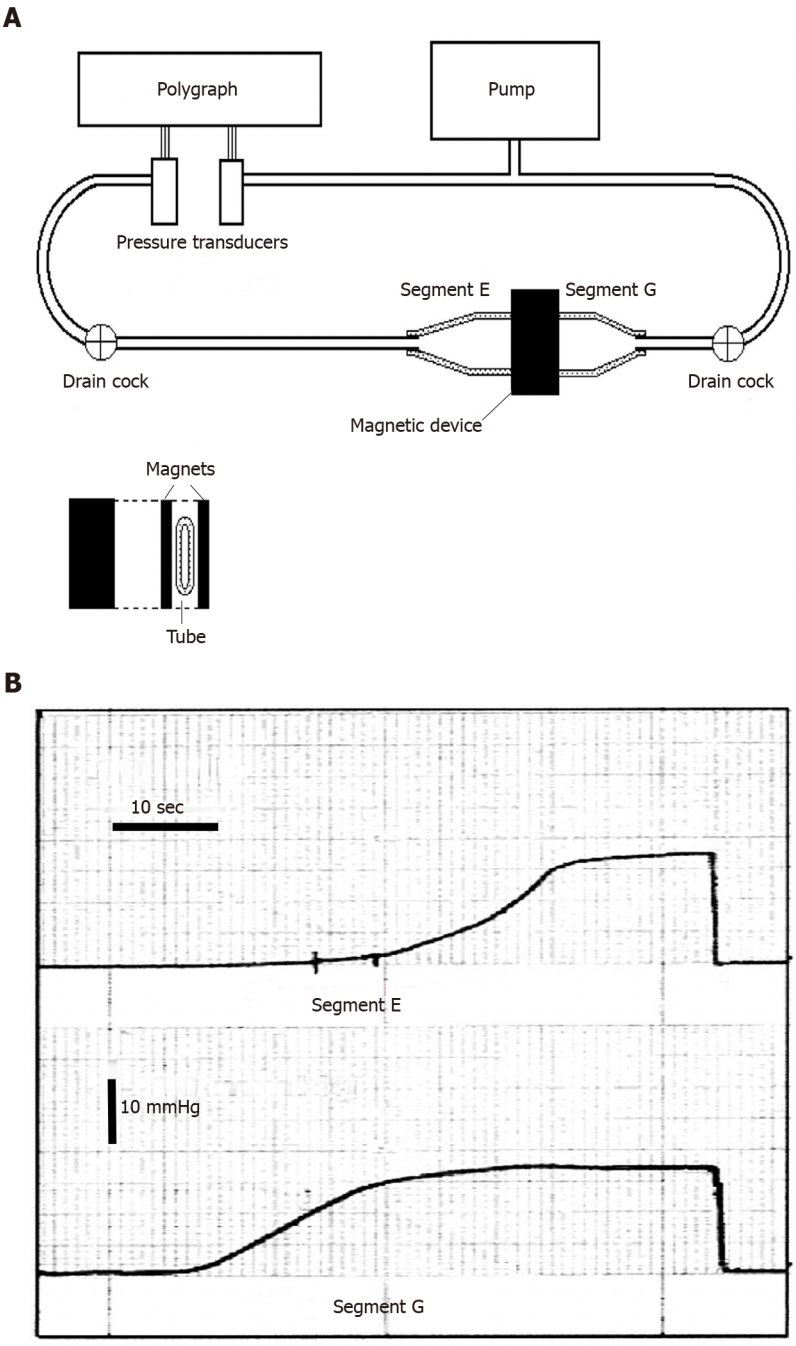

Figure 1 Benchtop experiment to demonstrate the possibility of creating a sphincter with two magnetic plaques. A: Schematic illustration of the bench model used to study the new anti-reflux device based on magnets. On the right there is a flaccid polyethylene tube 2.8 cm in diameter, mimicking the gastro-esophageal junction. It is squeezed perpendicularly by two rectangular magnets made of plastoferrite (Flexo) 2 cm × 4 cm × 0.5 cm with an attraction force of 0.36 N/cm^2, when in contact and 0.16 N/cm^2, at a distance of 7 mm. It creates a high pressure zone 2 cm wide, that divides the tube in segment E (esophagus) and G (stomach). The tube is perfused with water by a pump and the pressure variations in each segment are detected with 2 pressure transducers and recorded by a polygraph; B: Intraluminal pressure variations in segment G (bottom) and E (top). The pressure in segment G (stomach) was progressively increased by the pump and when it reached approximately 11.5 mmHg, the magnets, simulating the sphincter, get detached, so that the pressure in segment E (esophagus) starts to increase, mimicking a gastro-esophageal reflux and reaching the level of the segment G. Once the pump stops the pressure falls and the magnets adhere again, closing the passage. Exchanging the letter E for G and G for E, this sequence of events may represent the passage of a bolus through the zone squeezed by the magnets. A-B: Citation: Bortolotti M. A novel anti-reflux device based on magnets. *J Biomech* 2006; 39: 564-7. Copyright© The Authors 2020. Published by Elsevier. The authors obtained permission for use of the figure from the Elsevier Publishing Group (Supplementary material).

THE MSA DEVICE

The first MSA device to prevent GER (LINX Reflux Management System) was produced by Torax Medical, Inc., Shore View, MN, United States, and was utilized in a 2008 study by Bonavina *et al*[4]. It consisted of a "collar" of titanium beads with a magnetic core of neodymium interlinked along an independent flexible titanium wire

(Figure 2). The magnets were allowed to slide against one another along the wire, self-attracting by their magnetic force and self-detaching under the action of an opposing force, as the expanding pressure that dilates the "collar". In this manner they can attach and detach each other, thereby tightening or widening the collar which, consequently, closes and opens the esophageal lumen below. This "magnetic collar" is placed around the abdominal esophagus at the patient's LES level, by adapting its circumference by increasing or decreasing the number of magnetic beads.

Effectiveness of the Linx MSA device ("magnetic collar") in preventing GER

The first clinical trial[4] with the "magnetic collar" MSA, carried out in 2008 on 38 GERD patients, reported that, after a mean follow-up of 209 d, the GERD-Health Related Quality of Life (HRQL) score significantly decreased from 26.0 to 1.0, whereas, 3 mo after insertion, 89% of patients were off anti-reflux medications, and 79% had a normal 24-h pH recording test. Mild dysphagia occurred in 45% of patients. A subsequent study[5] performed in 2013 on 100 patients showed that at the 1-year follow-up there was a normalization or a 50% or greater reduction in esophageal acid exposure at 24-h pH test in 64% of patients, together with an improvement of 50% or more in GERD-HRQL scores in 92% of patients. In addition, there was a 50% or greater reduction in the use of proton-pump inhibitors (PPIs) and a significant increase in LES pressure. However, 36% of patients did not reach the normal esophageal acid exposure, whereas at the 1-year follow-up, esophagitis was still present in 10% of patients and had developed in 8%.

More or less similar results regarding the effectiveness in preventing GER were obtained by other investigators[6-11] in the following years up to 2020. One of the recent most complete studies from a single referral center was that of Ferrari *et al*[12], who followed up 124 patients for six up to 12 years (median 9 years) after insertion of the MSA device. The mean total GERD-HRQL score significantly improved from 19.9 to 4.01, PPIs were discontinued by 79% of patients, the mean total percent time with pH < 4 at 24-h pH recording significantly decreased from 9.6% to 4.1% and 89% of patients achieved intra-esophageal pH normalization. However, the term normalization is inexact, being only an improvement. In fact, although there was a significant decrease in the total % time pH < 4, the total number of reflux episodes, and particularly of those longer than 5 min, did not significantly decrease (Table 1)[12]. This indicates that the MSA device may not completely seal the gastro-esophageal junction and explains why in this study gastrointestinal endoscopy after a follow-up of 6 years revealed a grade A esophagitis in 4.7% of patients and incomplete intestinal metaplasia in 2.8%. In addition, the fact that the number of reflux episodes was not significantly decreased, whereas the total % time of acid exposure was significantly decreased, indicates that the mean duration of each reflux episode is decreased. However, this short duration does not depend on the closure of the gastro-esophageal junction by the MSA device, but it is due to an improved peristaltic clearance activity of the distal esophagus, which rapidly cleanses the mucosa from the refluxed acid[13]. In conclusion, after MSA device insertion the number of reflux episodes does not change significantly, but esophageal acid exposure after each reflux decreases with some benefits for the mucosa.

Comparison between MSA and Nissen fundoplication

The clinical results of MSA are not overwhelming when compared to those of Nissen fundoplication, which is considered paramount in GER surgical treatment. Nissen fundoplication showed excellent GER symptom control, low rates of complications and reoperations in long-term follow-up studies[14], whereas only 15% of patients reported recurrent symptoms[15]. In a review of studies with a long-term outcome [16], the control of reflux symptoms, such as heartburn and regurgitation, was achieved in 84% to 97% of patients, and in another similar review[17] good and excellent results were reported in 85%-95% of patients, with reflux recurrence in only 1%-8.5%, and dysphagia in 0%-10%.

Of great interest are the comparative studies of MSA *vs* Nissen fundoplication (Table 2). In two studies of a systematic review and meta-analysis, one with 1211 patients[18], and the other with 688 patients[19], postoperative GERD-HRQL and PPI suspension were similar in both the MSA and fundoplication groups, but MSA resulted in less gas-bloating and a greater ability to belch and vomit. Similar results were obtained in other comparative studies[20-23]. However, Riegler *et al*[20] found that the percentage of MSA patients with PPI suspension was significantly higher than that of fundoplication patients, whereas Warren *et al*[23] found the opposite results. Skubleny *et al*[19] noted that the occurrence of gas-bloating was not statistically different between the two treatments. In addition, Aiolfi *et al*[18] reported the

Table 1 Esophageal pH measurements (mean ± SD) off proton pump inhibitors[12]

Measure	Baseline	6-12 yr	*P* value
	n = 124	*n* = 91	
Total time (%)			
pH < 4	9.7 (6.4)	4.2 (4.9)	< 0.001
Upright	9.7 (7.8)	4.6 (4.9)	< 0.001
Supine	8.3 (9.6)	3.3 (7.4)	< 0.001
Reflux episodes			
Total number	92.2 (92.2)	71.5 (67.7)	0.125
Number lasting > 5 min	6.1 (6.0)	4.3 (5.8)	0.036
Longest (min)	32.9 (34.2)	19.6 (31.5)	0.005
DeMeester score	40.7 (26.5)	16.3 (18.8)	< 0.001

occurrence, although not statistically significant, of dysphagia requiring endoscopic dilatation in 9.3% of patients of the MSA group *vs* 6.6% of the fundoplication group, whereas Warren *et al*[23] observed that mild dysphagia was significantly more frequent in MSA patients. Skubleny *et al*[19] found a trend with 24% of MSA patients requiring dilatation *vs* 3.3% in those with fundoplication. In addition, Sheu *et al*[24] stated that dysphagia associated with MSA lasted longer, was more severe and required dilatation more frequently compared with fundoplication. The operative time in patients with MSA was shorter than in those with fundoplication[18,19,21,23]. Finally, both the MSA intervention[25] and fundoplication[26] were followed by the regression of intestinal metaplasia. In conclusion, although there are no randomized controlled trials to more properly compare MSA results with those of Nissen fundoplication, it can be said that both systems are roughly similar in preventing GER. However, on the one hand MSA has the advantage of less gas bloating and greater ability to vomit and belch, while on the other hand it has the disadvantage of a more prolonged and severe dysphagia, requiring more frequent endoscopic dilatation and, in some cases, device removal, as we will see later, along with other complications.

MSA complications and their pathophysiology

The most frequent complication after MSA device insertion was dysphagia; however, its occurrence was highly variable. Ganz *et al*[5] reported that 68% of patients developed dysphagia in the immediate postoperative period, which decreased to 11% after 1 year. Twenty seven percent of these patients underwent esophageal dilatation and 3% required device removal, whereas in the remaining patients dysphagia spontaneously improved after some months. In a review of 35 studies[27], the most common postoperative complication was dysphagia ranging between 6% and 83%, whereas Ayazi *et al*[28] reported a 15.5% rate of persistent postoperative dysphagia in a group of 380 patients who underwent MSA device insertion. Thirty-one percent of these patients required at least one dilatation due to dysphagia or chest pain and the overall positive response rate to this procedure was 67%, whereas 1.8% required device removal. Schwameis *et al*[29] compared to pseudoachalasia the difficult transit at the level of the esophago-gastric junction caused by the MSA device, because it mimics the clinical and pathophysiological manifestations of idiopathic achalasia.

The occurrence of dysphagia or incomplete GER prevention may have various explanations. The length of the "magnetic collar" (MSA) circumference, which must be adapted to each patient by adding or removing some beads, may increase exposure risk due to an incorrect measurement. Furthermore, sometimes by adding a bead, the collar may be too large, giving rise to incomplete GER prevention, whereas, by not adding the bead, the collar may be too tight, causing dysphagia. This phenomenon could occur in patients with smaller esophageal circumferences. Dysphagia and uncontrolled GER, which appear some time after surgery, could also be explained in a different way. The MSA device, as the months go by, may be "encapsulated" by fibrous tissue, as demonstrated by necropsy performed in a porcine model 11 mo after MSA implantation[30]. This "encapsulation" of the MSA device due to a fibrotic reaction was also confirmed in patients, in whom the "magnetic collar" was explanted because of complications[31,32]. The fibrosis around the magnetic mechanisms of the

Table 2 Comparison of magnetic sphincter augmentation and fundoplication

	Aiolfi *et al*[18]			Skubleny *et al*[19]			Riegler *et al*[20]			Reynolds *et al*[21]			Guidozzi *et al*[22]			Warren *et al*[23]		
	MSA	FUNDO	*P* value	MSA	FUNDO	*P* value	MSA	FUNDO	*P* value	MSA	FUNDO	*P* value	MSA	FUNDO	*P* value	MSA	FUNDO	*P* value
Type of study	Systematic review and meta-analysis			Systematic review and meta-analysis			O. prospective multicenter study			O. retrospective review from a single center			Systemic review and meta-analysis			Multi institutional retrospective cohort study		
N. patients (*n*)	686	525		415	273		202	47		52	67		632	467		169	185	
Follow-up (mo)	6-12	6-12		7-12	7-16		12	12		12	12		15.5	15.8		12	12	
GERD-HRQL score	POR = 0.48		0.101	20.5 *vs* 3	19.7 *vs* 3.2	NS	20 *vs* 3	23 *vs* 3.5	0.177	4.3 p.	5.1 p.	0.47	WMD = 0.34		0.525	21 *vs* 3	19 *vs* 4	0.17
PPI suspension	POR = 0.81		0.548	81.4%[1]	81.5%[1]	0.68	81.8%	63.0%	0.009	85%	92%	0.37	POR = 1.08		0.877	76%	88%	0.02
Gas/bloating	POR = 0.39		< 0.001	26.7%[1]	53.4%[1]	0.06	10.0%	31.9%	< 0.001	23%	53%	< 0.01	POR = 0.34		0.004	47%	59%	0.008
Ability to vomit	POR = 10.1		< 0.001	93.5%[1]	49.5%[1]	< 0.0001	91.3%	44.4%	< 0.001	4%	19%	< 0.01				95%	43%	< 0.001
Ability to belch	POR = 5.53		< 0.001	95.2%[1]	65.9%[1]	< 0.00001	98.4%	88.9%	0.007	10.0%	36%	< 0.01	POR = 12.34		< 0.001	96.5%	69.2%	< 0.001
Dysphagia	POR = 1.56		0.119	33.9%[1]	47.1%[1]	0.43	7%	10.6%	0.373	46%	56%	0.25	POR = 0.94		0.822	58%	47%	0.31
Operative time (min)	42-73	76-118		63.7	76.8					66	82	< 0.01				60	76	< 0.001

[1]Weighted mean percent values. MSA: Magnetic sphincter augmentation; FUNDO: Fundoplication; O.: Observational; WMD: Weighted mean difference; POR: Pooled odds ratio; NS: Not statistically significant; *vs*: Signifies preoperative *versus* postoperative score; p.: Postoperative.

MSA device could hamper the detachment and reattachment of the magnetic beads, which should slip along the wires, when the "collar" has to open or close, causing dysphagia or GER, respectively. Another cause of dysphagia is described in the subheading below.

MSA complications similar to those of the Angelchik prosthesis

Even if the "magnetic collar", hypothetically speaking, is blocked in the open position by fibrotic "encapsulation", it could maintain its ability to prevent GER and could continue to perform a sort of barrier function against GER. The explanation of this phenomenon could be sought in a mechanism similar to that of another anti-reflux collar, which is unable to tighten or dilate: The "notorious" Angelchik prosthesis[33]. This prosthesis consisted of a collar with a circular section made of silicone that was surgically placed around the abdominal esophagus to prevent GER in the eighties of last century. The Angelchik prosthesis was used for almost 15 years, due to good results against reflux obtained in several studies[34-36]. Some prospective randomized trials demonstrated that the Angelchik prosthesis was as effective in preventing GER,

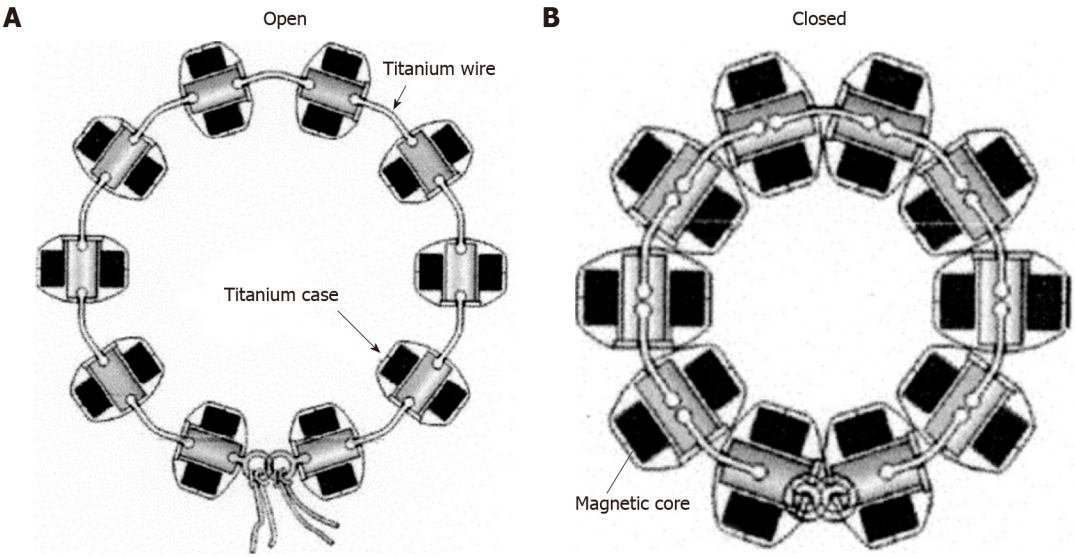

Figure 2 Schematic drawing of the Linx magnetic augmentation device to insert around the abdominal portion of the esophagus in the open and closed position. A: Open position; B: Closed position. A-B: Citation: Bonavina L, Saino GI, Bona D, Lipham J, Ganz RA, Dunn D, DeMeester T. Magnetic augmentation of the lower esophageal sphincter: results of a feasibility clinical trial. *J Gastrointest Surg* 2008; 12: 2133-40. Copyright© The Authors 2020. Published by Springer Nature. The authors obtained permission for use of the figure from Springer Nature (Supplementary material).

as the Nissen fundoplication[37], and with similar 24-h pH monitoring results[38]. The anti-reflux mechanism of this device occurs through the prevention of LES unfolding, when challenged by an increase in intragastric pressure[39] and, mostly, through the "padding" action against the posterior wall of the abdominal esophagus, which creates a barrier to GER[40]. In this way it causes a high pressure zone at the LES level, which can be detected by manometry[34,36]. The "magnetic collar" MSA, just in the hypothesis that its function is hindered by fibrosis, could resemble a sort of Angelchik prosthesis made of metal, which would produce with its weight, a continuous pressure against the posterior wall of the abdominal esophagus, closing the lumen to reflux. However, this mechanism of the Angelchik prosthesis, on the one hand, could help to control GER, but, on the other hand, could represent an obstacle to bolus transit, causing persistent, and sometimes severe dysphagia[41]. This fact required the removal of the prosthesis in some cases[36,42] and was also responsible for some other more severe complications. In fact, a continuous compression of the plastic collar, leaning on the esophageal wall, in some cases also caused erosions, fistulas and perforations of the esophagus and stomach, that sometimes were followed by migration of the device into the gastric lumen[43-48]. These complications began to appear years after insertion of the prosthesis, but despite this, it continued to be implanted for years. In the first decade of the current century the Angelchik prosthesis, which had seemed to be a good alternative to Nissen fundoplication, was definitely shelved.

In a manner similar to that of the Angelchik prosthesis the MSA "magnetic collar" too, leaning on the distal esophageal wall, being also heavier, may induce ischemia and consequently may cause erosion of the wall. The latter complication may be revealed by persistent severe dysphagia[49,50] or odinophagia[51]. In some cases the device may protrude more or less deeply into the esophageal lumen[50-54]. The appearance of these complications requires device removal. In addition, a prolonged leaning of the MSA device against the esophageal wall was suspected, but without clear proof of being responsible, probably through a chronic foreign body reaction, for an adenocarcinoma found in the distal esophagus of a patient with the MSA device [55].

Causes and timing of MSA device removal

MSA device removal, however, has been performed not only for the occurrence of erosions and device protrusion, but also for severe dysphagia, recurrent GER and epigastric pain. In a retrospective review[54], 5.5% of 435 patients undergoing MSA device implantation from 2009 to 2017 in a single institution, required removal, the most common reasons being recurrent GER (54%), dysphagia (38%), or erosion (8%). In a single referral center[12], 124 patients were followed up for 6 up to 12 years (median 9 years) after insertion of the MSA device, and 9.2% of patients required laparoscopic device removal for various reasons: The most frequent were erosions,

regurgitation, heartburn, and dysphagia, but also foreign body sensation, odinophagia, pharyngodynia, chronic cough and even the need for a magnetic resonance study. In another retrospective single center cohort study[31], after a median follow-up of 48 mo 6.7% of 164 patients were explanted. In almost half of cases this occurred due to recurrence of heartburn or regurgitation, followed by dysphagia, and, in the remainder of cases, due to chest pain and full-thickness erosion of the esophageal wall with partial penetration of the device. The majority of the removals occurred within two years after implantation[31], whereas for other investigators most cases of removal for erosion occurred between 1 and 4 years after device placement [56]. According to the commercial registries in the United States and Europe, the worldwide clinical experience of 497 magnetic implants established that the median duration was 2.9 years[5]. In another study[57], the median duration was 274 d in the first 1000 MSA implanted patients in 82 institutions, whereas Smith *et al*[58], consulting the MAUDE database from 2012 to 2016 regarding 3283 implanted patients, found that the median duration was 1.4 years and more than half of the removals occurred within the first year. In conclusion, removal was required in 5% to 9.2% of patients and occurred in the first few years after device placement mainly for dysphagia, recurrence of GERD symptoms and erosions and the duration varied from 274 d to 2.9 years. These differences in implant removal, as well as in the occurrence of adverse events, may be due to the fact that the number increases with time, and therefore the real number in retrospective reviews, likely depends on the follow-up duration. Moreover, it should also be kept in mind that different sizing protocols may play an important role in producing important data differences.

Procedures and consequences of the removal

Furthermore, the operative management of the MSA device removal and especially its pathophysiologic consequences must be considered. The removal of the device was carried out using a single stage procedure[31], or, more rarely, in two stages: First endoscopically for the visible beads, then laparoscopically for the remaining beads within 3 mo after complete healing[51]. Tatum *et al*[53] reported that the MSA devices were removed through laparotomy (4%), laparoscopically (88%), or through a combination of endoscopy and laparoscopy (8%). After removal, these patients underwent repeated MSA (33%), fundoplication (21%), gastrectomy (4%), or no additional procedure (42%). Symptoms prompting removal of the MSA device were eliminated in 52% of patients and improved in an additional 35%, whereas in 13% of cases the symptoms persisted. As removal of the MSA device is followed not only by recurrent GER, but also by a delayed gastric emptying, prokinetics should be added to the medical therapy with a PPI[49] or surgical treatment with fundoplication[52]. The onset of delayed gastric emptying after removal may be easily explained by damage to the right branch of the vagus nerve, which runs along the posterior part of the abdominal esophagus. This is the region where the penetration and removal of the MSA device usually takes place. Apart from the occurrence of erosion and removal, the continuous friction and pressure of the rather heavy MSA "collar", as well as the creation of the tunnel around the abdominal esophagus to insert it, both could damage or irritate the area of vagus nerve passage, with possible motor dysfunction of the stomach and intestine. A delay in gastric emptying induced by a lesion to the vagus nerve was found at the 6 mo follow-up in 125 patients after anti-reflux surgery[59]. Consequently, it would be interesting to perform a gastric emptying test before and 6 mo after the insertion of the MSA device in a group of patients undergoing the procedure, or at least in those complaining of dysphagia.

In conclusion, in patients subjected to MSA device insertion there are complications and adverse events, the occurrence of which shows great variability from one study to another. A possible explanation for this can be found in the different sizing protocols as well as in the duration of the follow-up. Some complications, such as dysphagia or GER could be considered related to a not so perfect adjustment of the MSA collar length or, when they appear or worsen after months, might perhaps be due to wrapping of the working mechanism of the device by a coating of fibrous tissue, which stiffens with time. Dysphagia may also be linked to the "collar" shape of the MSA device pressing with its weight on the posterior wall of the distal esophagus, as the Angelchik prosthesis does. This leaning of the "magnetic collar" on the distal esophageal wall may be responsible for more severe complications, which manifest themselves over time, such as erosions and device penetration through the esophageal wall. The consequent MSA device removal also leaves a functional aftermath at the gastro-esophageal junction as well as the stomach. Considering the trend over time of these latter complications, which in some way could recall to mind those of the Angelchik prosthesis, although much less severe, one might wonder if there may be a

risk that the the story of the latter will repeat itself with the "magnetic collar", as was feared in an article in 2014[60]. However, I do not think this could happen, as the power of technology will not allow it.

OTHER MAGNETIC TECHNIQUES TO PREVENT GER

As previously mentioned, another way of exploiting magnetic force to prevent GER was devised in a bench-top study published in 2006[3]. As previously described, this system consisted of two small magnetic plaques, that, when applied in opposite positions around the abdominal esophagus, should attract each other, squeezing the LES, to prevent GER. These magnetic plaques are also capable of detaching themselves, when the endoluminal pressure increases above a determined value, to allow transit of the bolus. The pair of plaques should be surgically inserted at the LES level to form a magnetic valve with a dynamic closure that should be sufficient to prevent the reflux of contents, without the risk of fibrosis that blocks them in the open or closed position, since they are separated by the esophageal lumen.

Another experimental study was subsequently performed to evaluate the feasibility of this method[61]. Two small magnetic plaques (5 mm × 20 mm × 1.5 mm) made of plastoferrite were implanted by means of a special endoesophageal device (Figure 3) in two submucosal longitudinal tunnels in the opposite parts of the distal esophagus of esophago-gastric specimens taken from an "*ex vivo*" swine. The magnetic plaques with the opposite polarities facing, through a reciprocal attraction closed the esophageal lumen (Figure 4), creating a high-pressure zone. The latter was measured by a manometric catheter passed through the gastroesophageal junction, showing after five pull-throughs, a mean pressure ± SD of 14.2 ± 1.27 mmHg, which was significantly higher than the basal pressure of 1.5 ± 0.26 mmHg. This preliminary study suggests that it could be possible to create functional closure at the LES level with a pressure sufficient to prevent GER with a couple of magnetic plaques with various attraction forces, using a safe and simple endoscopic procedure.

A technique inspired by the one just described was devised by Dobashi *et al*[62]. In porcine models first "*ex vivo*" and then "*in vivo*", two magnets of neodymium (3 mm × 12 mm) were endoscopically inserted with opposite polarities into two opposite subadventitial tunnels of the distal esophagus, with the aim of closing the lumen with their reciprocal attraction (Figure 5). The tunnels were created with the aid of blunt dissection by means of a biliary balloon catheter. Unfortunately, the tunnels "*in vivo*" were successful in only five of 10 pigs and the magnet augmentation device was functionally active in only 4 of them. In another study by the same investigator[63] neodymium ring magnets (4.8 OD × 1.6 ID mm and 1.6 mm thick) were endoscopically anchored to the esophageal mucosa with a suture anchor from a needle arm fixed full-thickness to the esophageal wall, to create a flap. Two to three magnets were placed in opposite positions at the LES level, to induce closure of the lumen with reciprocal attraction. This procedure was performed both in nine cadaveric and six surviving pigs. In the latter animals the mean LES pressure increased from 8.4 to 32.4 mmHg just after device placement. Repeated endoscopy after two weeks showed intact magnets in four of 6 animals with a persistent increase in LES pressure. These magnets can be easily removed, but low durability is expected and it is not known whether these magnetic rings are really capable of completely sealing the lumen. In conclusion, the first applications "*in vivo*" of these different endoluminal magnetic systems did not yield outstanding results and they clearly require further development. This deserves to be performed, as they present various advantages with respect to the "magnetic collar".

Advantages and shortcomings of the "two plaques system"

With regard to the working mechanism, the system based on a "collar" of magnets in the MSA device seems perfect at the work-bench, but, once inserted into a living organism, things change. In fact, the biological reaction could trouble its perfect functioning, wrapping the device by a coating of fibrous tissue, that with time become stiffer and could cause thus dysphagia or GER. The mechanism of the two magnetic plaques, instead is not subject to this possible drawback, because it does not have mechanical sliding parts, which could be blocked by the deposition of fibrin, possibly hindering the to and fro movements of the magnets. In fact, the attraction force acts through the lumen of the esophagus, so that the magnets are free to approach and separate. The fibrous coating on the magnets may also contribute to securing them in their crevice in the esophageal wall. Naturally, the magnetic plaques should be

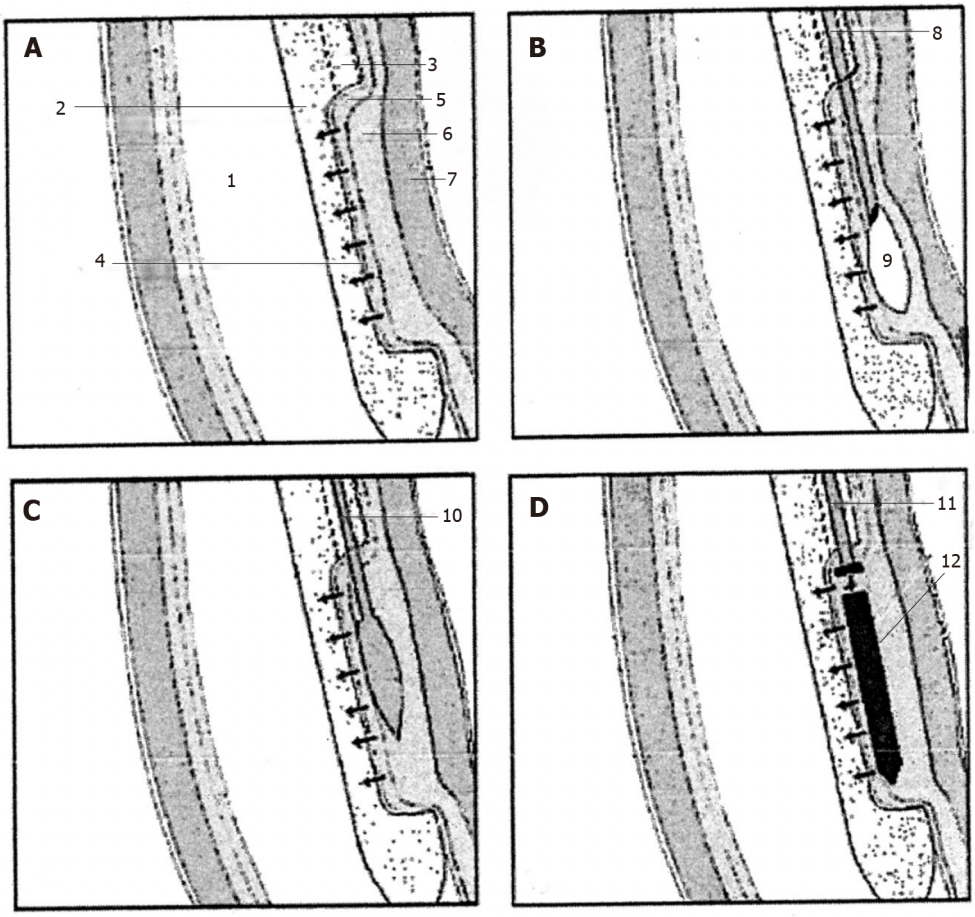

Figure 3 Extremity of the special endoesophageal probe positioned at the LES level in a sequence of operations for the deployment of a magnetic plaque seen in profile. A: The mucosa of the distal esophagus is sucked onto the perforated wall of the operative chamber; B: The needle injects milliliters of saline solution to create a blister in the submucosa; C: The end of the catheter with a blunted bolt creates a pouch in the submucosa; D: The magnetic plaque (seen in profile) is pushed into the pouch. 1: Esophageal lumen; 2: Delivery probe; 3: Deployment channel; 4: Perforated wall of the aspiration chamber; 5: Mucosal layer; 6: Submucosal layer; 7: Muscular layer; 8: Needle-catheter; 9: Saline solution; 10: Bolt-catheter; 11: Magnetic plaque seen in profile. A-D: Citation: Bortolotti M, Grandis A, Mazzero G. A novel endoesophageal magnetic device to prevent gastroesophageal reflux. *Surg Endosc* 2009; 4: 885-9. Copyright© The Authors 2020. Published by Springer Nature. The authors obtained permission for use of the figure from Springer Nature (Supplementary material).

covered by a soft biomaterial to avoid undesirable reactions of the surrounding tissues and must have an appropriate force of attraction to close the lumen without causing ischemia and erosions of the underlying compressed tissues.

In this regard another advantage of the "two plaques system", unlike the "magnetic collar" MSA, lies in the possibility of accurately establishing the force of closure by choosing magnets with different attraction forces for different conditions. In fact, the distance between the two plaques may vary from patient to patient and, therefore, their force of attraction varies with the square of the distance. Consequently, plaques with greater attraction force are required for greater distances, and *vice versa*. This system offers the possibility of choosing, even during insertion, the most suitable plaques by measuring with a manometric probe or other systems the endoluminal pressure obtained. The MSA "magnetic collar", instead, always exerting the same force of attraction between the beads, could become less effective when the area to surround is large, thus facilitating reflux. The reverse could occur for small circumferences, with the creation of an obstacle to content transit and consequent dysphagia.

Furthermore, with the "two plaques system" it is possible to realize an anti-reflux device that can be inserted endoscopically, as described above. This possibility, assuming it works with the magnetic plaques, would cost much less than laparoscopy and the MSA device.

The drawback of this system lies in the fact that at the present time it is difficult to obtain a stable insertion of the plaques in the esophageal wall. The system by Dobashi *et al*[62] with a sub-adventitial tunnel seems to provide excellent fixing, but was followed by functional success in only four of 10 cases. The other system by Dobashi *et al*[63] with 2-3 ring magnets anchored to the distal esophageal wall like a flap was

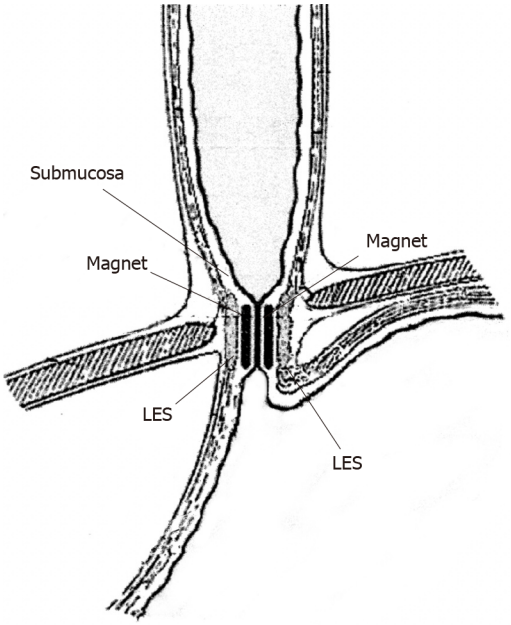

Figure 4 Schematic section following a vertical frontal plane through the lower portion of the esophago-gastric wall showing in profile the two magnetic plaques inserted face to face in the submucosal position at the lower esophageal sphincter level; these attracting each other close the gastro-esophageal junction. Citation: Bortolotti M, Grandis A, Mazzero G. A novel endoesophageal magnetic device to prevent gastroesophageal reflux. *Surg Endosc* 2009; 4: 885-9. Copyright© The Authors 2020. Published by Springer Nature. The authors obtained permission for use of the figure from Springer Nature (Supplementary material).

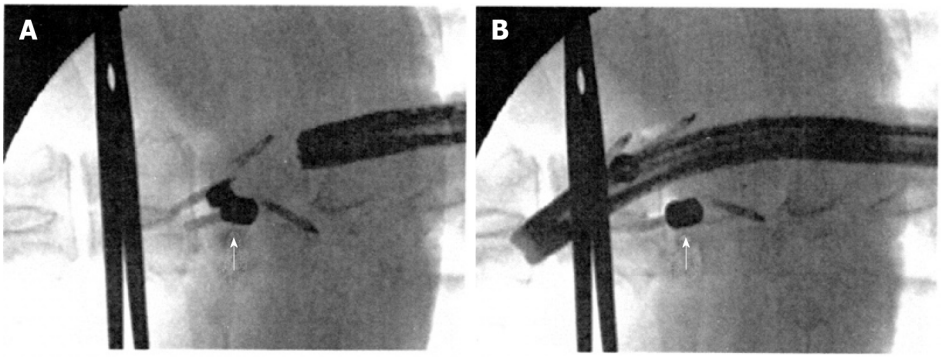

Figure 5 Fluoroscopic view after insertion of the magnets. A: Magnets in the sub-adventitial space opposing the respective esophageal walls. A surgical clamp indicates the level of the esophago-gastric junction and the arrow indicates the magnets attracted to one another and closing the lumen; B: Magnets separated by the passage of the endoscope. The arrow indicates one of the magnets separated from the other. A-B: Citation: Modified from: Dobashi A, Wu SW, Deters JL, Miller CA, Knipschield MA, Cameron GP, Lu L, Rajan E, Gostout CJ. Endoscopic magnet placement into subadventitial tunnels for augmenting the lower esophageal sphincter using submucosal endoscopy: ex vivo and in vivo study in a porcine model (with video). *Gastrointest Endosc* 2019; 89: 422-428. Copyright© The Authors 2020. Published by Elsevier. The authors obtained permission for use of the figure from Elsevier (Supplementary material).

successful in only four of 6 surviving pigs after 2 wk. Furthermore, although it obtained a high endoluminal pressure, it may give the impression of not completely seal the esophageal lumen against reflux. The insertion of magnets in submucosal tunnels, chosen by Bortolotti *et al*[60], by means of a special endoesophageal device is easy to perform, but it requires a more stable fixing of the devices to the esophageal wall. A biologic glue and closure of the proximal mucosal opening by a surgical stitch, could avoid loss of the magnets. To date, no one has attempted to apply the couple of magnetic plaques outside the esophageal wall, in areas where the vagus nerve does not pass. Indeed, this idea poses considerable problems in fixing these plaques. The solution could be obtained by various expedients, such as suture anchors, surgical stitches and biological glue, whereas the magnetic plaques should have particular shapes, with hooks, holes for surgical threads *etc*. I am confident that a good solution for fixing the plaques outside the esophageal wall will be found by a skilled surgeon.

Please note that this system with two plaques could also be easily used to prevent fecal incontinence. A couple of the plaques may be surgically positioned on the right and left sides of the incontinent anal sphincter, with the opposite polarities facing each other, so that, by self-attracting they could keep the anal canal closed[64].

CONCLUSION

Considering the clinical effectiveness and occurrence of more or less severe complications, one might wonder whether the magnetic anti-reflux device MSA actually represents an extraordinary progress with respect to Nissen fundoplication. One of the major criticisms to MSA studies is that up to now there has not been any randomized controlled trial which correctly compared the MSA results with those of Nissen fundoplication. However, considering the available studies, it can be said that the MSA system achieves a GER control roughly similar to that of fundoplication with the advantage of less gas bloating and a greater ability to vomit and belch. On the other hand, it has the disadvantage of more prolonged and severe dysphagia, requiring endoscopic dilatation more frequently and, in some cases, device removal[11]. The latter may also be necessary for some other severe complications, which are fortunately infrequent, such as mucosal erosions and device penetration through the esophageal wall.

It would be of concern if this "magnetic way" for GER treatment could met the same fate as the Angelchik prosthesis, which tried to replace fundoplication, but after 15 years it was shelved due to numerous and severe complications. I believe this will not happen in this case, as "magnetic sphincters" represent a real progress in the surgical treatment of GER. I am convinced that the magnetic technique is not a spark in the dark followed by the full return of fundoplication for the following reasons: The MSA device is relatively easier to insert, whereas fundoplication, on the other hand, requires an expert surgeon for its perfect realization. In addition, I also believe that the upcoming "two magnetic plaques system" with submucosal or sub-adventitial tunnels, could be the future of the magnetic era. It is unfortunate that this magnetic system, which presents many advantages, is not yet available and calls for further experiments on animals and clinical trials in selected patients, to achieve sufficient reliability in order to enter into surgical practice. This new road appears to be a long one filled with obstacles, but I think it is worthwhile trying to continue, unless one wants to go further into the future by studying the possibility of biocompatible magnetic nanoparticles to be injected into two longitudinal sections of a weak sphincter facing one another and then magnetically oriented for the purpose to attract themselves along with the surrounding muscle, thus closing the lumen. Unfortunately this is still a dream, but dreams can sometimes come true.

ACKNOWLEDGEMENTS

The author thanks Romano Bragaglia MD and Henry Monaco BA (Hons) for assistance in the English language and Andrea Lugli MSc for help with Tables and Figures composition.

REFERENCES

1 **Diaz R**, Davalos G, Welsh LK, Portenier D, Guerron AD. Use of magnets in gastrointestinal surgery. *Surg Endosc* 2019; **33**: 1721-1730 [PMID: 30805789 DOI: 10.1007/s00464-019-06718-w]

2 **Chen H**, Ma T, Wang Y, Zhu HY, Feng Z, Wu RQ, Lv Y, Dong DH. Fedora-type magnetic compression anastomosis device for intestinal anastomosis. *World J Gastroenterol* 2020; **26**: 6614-6625 [PMID: 33268950 DOI: 10.3748/wjg.v26.i42.6614]

3 **Bortolotti M**. A novel antireflux device based on magnets. *J Biomech* 2006; **39**: 564-567 [PMID: 16309688 DOI: 10.1016/j.jbiomech.2004.10.035]

4 **Bonavina L**, Saino GI, Bona D, Lipham J, Ganz RA, Dunn D, DeMeester T. Magnetic augmentation of the lower esophageal sphincter: results of a feasibility clinical trial. *J Gastrointest Surg* 2008; **12**: 2133-2140 [PMID: 18846406 DOI: 10.1007/s11605-008-0698-1]

5 **Ganz RA**, Peters JH, Horgan S, Bemelman WA, Dunst CM, Edmundowicz SA, Lipham JC, Luketich JD, Melvin WS, Oelschlager BK, Schlack-Haerer SC, Smith CD, Smith CC, Dunn D, Taiganides PA. Esophageal sphincter device for gastroesophageal reflux disease. *N Engl J Med* 2013; **368**: 719-727 [PMID: 23425164 DOI: 10.1056/NEJMoa1205544]

6 **Saino G**, Bonavina L, Lipham JC, Dunn D, Ganz RA. Magnetic Sphincter Augmentation for Gastroesophageal Reflux at 5 Years: Final Results of a Pilot Study Show Long-Term Acid Reduction and Symptom Improvement. *J Laparoendosc Adv Surg Tech A* 2015; **25**: 787-792 [PMID: 26437027 DOI: 10.1089/lap.2015.0394]

7 **Ganz RA**, Edmundowicz SA, Taiganides PA, Lipham JC, Smith CD, DeVault KR, Horgan S, Jacobsen G, Luketich JD, Smith CC, Schlack-Haerer SC, Kothari SN, Dunst CM, Watson TJ, Peters J, Oelschlager BK, Perry KA, Melvin S, Bemelman WA, Smout AJ, Dunn D. Long-term Outcomes of Patients Receiving a Magnetic Sphincter Augmentation Device for Gastroesophageal Reflux. *Clin Gastroenterol Hepatol* 2016; **14**: 671-677 [PMID: 26044316 DOI: 10.1016/j.cgh.2015.05.028]

8 **Rona KA**, Reynolds J, Schwameis K, Zehetner J, Samakar K, Oh P, Vong D, Sandhu K, Katkhouda N, Bildzukewicz N, Lipham JC. Efficacy of magnetic sphincter augmentation in patients with large hiatal hernias. *Surg Endosc* 2017; **31**: 2096-2102 [PMID: 27553803 DOI: 10.1007/s00464-016-5204-3]

9 **Schwameis K**, Nikolic M, Morales Castellano DG, Steindl A, Macheck S, Kristo I, Zörner B, Schoppmann SF. Results of Magnetic Sphincter Augmentation for Gastroesophageal Reflux Disease. *World J Surg* 2018; **42**: 3263-3269 [PMID: 29619511 DOI: 10.1007/s00268-018-4608-8]

10 **Louie BE**, Smith CD, Smith CC, Bell RCW, Gillian GK, Mandel JS, Perry KA, Birkenhagen WK, Taiganides PA, Dunst CM, McCollister HM, Lipham JC, Khaitan LK, Tsuda ST, Jobe BA, Kothari SN, Gould JC. Objective Evidence of Reflux Control After Magnetic Sphincter Augmentation: One Year Results From a Post Approval Study. *Ann Surg* 2019; **270**: 302-308 [PMID: 29697454 DOI: 10.1097/SLA.0000000000002789]

11 **Sheu EG**, Rattner DW. Evaluation of the LINX antireflux procedure. *Curr Opin Gastroenterol* 2015; **31**: 334-338 [PMID: 26039726 DOI: 10.1097/MOG.0000000000000189]

12 **Ferrari D**, Asti E, Lazzari V, Siboni S, Bernardi D, Bonavina L. Six to 12-year outcomes of magnetic sphincter augmentation for gastroesophageal reflux disease. *Sci Rep* 2020; **10**: 13753 [PMID: 32792508 DOI: 10.1038/s41598-020-70742-3]

13 **Kahrilas PJ**, Dodds WJ, Hogan WJ. Effect of peristaltic dysfunction on esophageal volume clearance. *Gastroenterology* 1988; **94**: 73-80 [PMID: 3335301 DOI: 10.1016/0016-5085(88)90612-9]

14 **Simorov A**, Ranade A, Jones R, Tadaki C, Shostrom V, Boilesen E, Oleynikov D. Long-term patient outcomes after laparoscopic anti-reflux procedures. *J Gastrointest Surg* 2014; **18**: 157-62; discussion 162 [PMID: 24234243 DOI: 10.1007/s11605-013-2401-4]

15 **Bathla L**, Legner A, Tsuboi K, Mittal S. Efficacy and feasibility of laparoscopic redo fundoplication. *World J Surg* 2011; **35**: 2445-2453 [PMID: 21915744 DOI: 10.1007/s00268-011-1250-0]

16 **Richards KF**, Fisher KS, Flores JH, Christensen BJ. Laparoscopic Nissen fundoplication: cost, morbidity, and outcome compared with open surgery. *Surg Laparosc Endosc* 1996; **6**: 140-143 [PMID: 8680637]

17 **Fuchs KH**, Breithaupt W, Fein M, Maroske J, Hammer I. Laparoscopic Nissen repair: indications, techniques and long-term benefits. *Langenbecks Arch Surg* 2005; **390**: 197-202 [PMID: 15235916 DOI: 10.1007/s00423-004-0489-4]

18 **Aiolfi A**, Asti E, Bernardi D, Bonitta G, Rausa E, Siboni S, Bonavina L. Early results of magnetic sphincter augmentation versus fundoplication for gastroesophageal reflux disease: Systematic review and meta-analysis. *Int J Surg* 2018; **52**: 82-88 [PMID: 29471155 DOI: 10.1016/j.ijsu.2018.02.041]

19 **Skubleny D**, Switzer NJ, Dang J, Gill RS, Shi X, de Gara C, Birch DW, Wong C, Hutter MM, Karmali S. LINX® magnetic esophageal sphincter augmentation versus Nissen fundoplication for gastroesophageal reflux disease: a systematic review and meta-analysis. *Surg Endosc* 2017; **31**: 3078-3084 [PMID: 27981382 DOI: 10.1007/s00464-016-5370-3]

20 **Riegler M**, Schoppman SF, Bonavina L, Ashton D, Horbach T, Kemen M. Magnetic sphincter augmentation and fundoplication for GERD in clinical practice: one-year results of a multicenter, prospective observational study. *Surg Endosc* 2015; **29**: 1123-1129 [PMID: 25171881 DOI: 10.1007/s00464-014-3772-7]

21 **Reynolds JL**, Zehetner J, Nieh A, Bildzukewicz N, Sandhu K, Katkhouda N, Lipham JC. Charges, outcomes, and complications: a comparison of magnetic sphincter augmentation versus laparoscopic Nissen fundoplication for the treatment of GERD. *Surg Endosc* 2016; **30**: 3225-3230 [PMID: 26541730 DOI: 10.1007/s00464-015-4635-6]

22 **Guidozzi N**, Wiggins T, Ahmed AR, Hanna GB, Markar SR. Laparoscopic magnetic sphincter augmentation versus fundoplication for gastroesophageal reflux disease: systematic review and pooled analysis. *Dis Esophagus* 2019; **32** [PMID: 31069388 DOI: 10.1093/dote/doz031]

23 **Warren HF**, Reynolds JL, Lipham JC, Zehetner J, Bildzukewicz NA, Taiganides PA, Mickley J, Aye RW, Farivar AS, Louie BE. Multi-institutional outcomes using magnetic sphincter augmentation versus Nissen fundoplication for chronic gastroesophageal reflux disease. *Surg Endosc* 2016; **30**: 3289-3296 [PMID: 26541740 DOI: 10.1007/s00464-015-4659-y]

24 **Sheu EG**, Nau P, Nath B, Kuo B, Rattner DW. A comparative trial of laparoscopic magnetic sphincter augmentation and Nissen fundoplication. *Surg Endosc* 2015; **29**: 505-509 [PMID: 25012804 DOI: 10.1007/s00464-014-3704-6]

25 **Alicuben ET**, Tatum JM, Bildzukewicz N, Samakar K, Samaan JS, Silverstein EN, Sandhu K, Houghton CC, Lipham JC. Regression of intestinal metaplasia following magnetic sphincter augmentation device placement. *Surg Endosc* 2019; **33**: 576-579 [PMID: 30046950 DOI: 10.1007/s00464-018-6367-x]

26 **Simonka Z**, Paszt A, Abrahám S, Pieler J, Tajti J, Tiszlavicz L, Németh I, Izbéki F, Rosztóczy A,

Wittmann T, Rárosi F, Lázár G. The effects of laparoscopic Nissen fundoplication on Barrett's esophagus: long-term results. *Scand J Gastroenterol* 2012; **47**: 13-21 [PMID: 22150083 DOI: 10.3109/00365521.2011.639081]

27 **Schizas D**, Mastoraki A, Papoutsi E, Giannakoulis VG, Kanavidis P, Tsilimigras D, Ntourakis D, Lyros O, Liakakos T, Moris D. LINX® reflux management system to bridge the "treatment gap" in gastroesophageal reflux disease: A systematic review of 35 studies. *World J Clin Cases* 2020; **8**: 294-305 [PMID: 32047777 DOI: 10.12998/wjcc.v8.i2.294]

28 **Ayazi S**, Zheng P, Zaidi AH, Chovanec K, Chowdhury N, Salvitti M, Komatsu Y, Omstead AN, Hoppo T, Jobe BA. Magnetic Sphincter Augmentation and Postoperative Dysphagia: Characterization, Clinical Risk Factors, and Management. *J Gastrointest Surg* 2020; **24**: 39-49 [PMID: 31388888 DOI: 10.1007/s11605-019-04331-9]

29 **Schwameis K**, Ayazi S, Zaidi AH, Hoppo T, Jobe BA. Development of pseudoachalasia following magnetic sphincter augmentation (MSA) with restoration of peristalsis after endoscopic dilation. *Clin J Gastroenterol* 2020; **13**: 697-702 [PMID: 32472375 DOI: 10.1007/s12328-020-01140-5]

30 **Ganz RA**, Gostout CJ, Grudem J, Swanson W, Berg T, DeMeester TR. Use of a magnetic sphincter for the treatment of GERD: a feasibility study. *Gastrointest Endosc* 2008; **67**: 287-294 [PMID: 18226691 DOI: 10.1016/j.gie.2007.07.027]

31 **Asti E**, Siboni S, Lazzari V, Bonitta G, Sironi A, Bonavina L. Removal of the Magnetic Sphincter Augmentation Device: Surgical Technique and Results of a Single-center Cohort Study. *Ann Surg* 2017; **265**: 941-945 [PMID: 27163959 DOI: 10.1097/SLA.0000000000001785]

32 **Harnsberger CR**, Broderick RC, Fuchs HF, Berducci M, Beck C, Gallo A, Jacobsen GR, Sandler BJ, Horgan S. Magnetic lower esophageal sphincter augmentation device removal. *Surg Endosc* 2015; **29**: 984-986 [PMID: 25119542 DOI: 10.1007/s00464-014-3757-6]

33 **Angelchik JP**, Cohen R. A new surgical procedure for the treatment of gastroesophageal reflux and hiatal hernia. *Surg Gynecol Obstet* 1979; **148**: 246-248 [PMID: 154176]

34 **Starling JR**, Reichelderfer MO, Pellett JR, Belzer FO. Treatment of symptomatic gastroesophageal reflux using the Angelchik prosthesis. *Ann Surg* 1982; **195**: 686-691 [PMID: 7082060 DOI: 10.1097/00000658-198206000-00002]

35 **Eyre-Brook IA**, Codling BW, Gear MW. Results of a prospective randomized trial of the Angelchik prosthesis and of a consecutive series of 119 patients. *Br J Surg* 1993; **80**: 602-604 [PMID: 8518898 DOI: 10.1002/bjs.1800800517]

36 **Maddern GJ**, Myers JC, McIntosh N, Bridgewater FH, Jamieson GG. The effect of the Angelchik prosthesis on esophageal and gastric function. *Arch Surg* 1991; **126**: 1418-1422 [PMID: 1747057 DOI: 10.1001/archsurg.1991.01410350112018]

37 **Deakin M**, Mayer D, Temple JG. Surgery for gastro-oesophageal reflux: the Angelchik prosthesis compared to the floppy Nissen fundoplication. Two-year follow-up study and a five-year evaluation of the Angelchik prosthesis. *Ann R Coll Surg Engl* 1989; **71**: 249-252 [PMID: 2774454]

38 **Hill AD**, Walsh TN, Bolger CM, Byrne PJ, Hennessy TP. Randomized controlled trial comparing Nissen fundoplication and the Angelchik prosthesis. *Br J Surg* 1994; **81**: 72-74 [PMID: 8313128 DOI: 10.1002/bjs.1800810124]

39 **Bonavina L**, DeMeester T, Mason R, Stein HJ, Feussner H, Evander A. Mechanical effect of the Angelchik prosthesis on the competency of the gastric cardia: pathophysiologic implications and surgical perspectives. *Dis Esophagus* 1997; **10**: 115-118 [PMID: 9179481 DOI: 10.1093/dote/10.2.115]

40 **Benjamin SB**, Knuff TK, Fink M, Woods E, Castell DO. The Angelchik antireflux prosthesis. Effects on the lower esophageal sphincter of primates. *Ann Surg* 1983; **197**: 63-67 [PMID: 6848055]

41 **Varshney S**, Kelly JJ, Branagan G, Somers SS, Kelly JM. Angelchik prosthesis revisited. *World J Surg* 2002; **26**: 129-133 [PMID: 11898046 DOI: 10.1007/s00268-001-0192-3]

42 **Stuart RC**, Dawson K, Keeling P, Byrne PJ, Hennessy TP. A prospective randomized trial of angelchik prosthesis versus Nissen fundoplication. *Br J Surg* 1989; **76**: 86-89 [PMID: 2645016 DOI: 10.1002/bjs.1800760127]

43 **Albin J**, Noel T, Allan K, Khalil KG. Intrathoracic esophageal perforation with the Angelchik antireflux prosthesis: report of a new complication. *Gastrointest Radiol* 1985; **10**: 330-332 [PMID: 4054497 DOI: 10.1007/BF01893123]

44 **Massaioli N**, Bertero D, Buzio M, Mecozzi B, Albertino B, Mosca A. [Endogastric migration of an Angelchik prosthesis. A case report and review of the literature]. *Minerva Chir* 1990; **45**: 189-194 [PMID: 2192307]

45 **Pence MM**, Hubbard M, Singla MB, Young PE. Esophagogastric Fistula Caused by an Angelchik Antireflux Prosthesis. *ACG Case Rep J* 2015; **2**: 213-215 [PMID: 26203442 DOI: 10.14309/crj.2015.62]

46 **Kauten JR**, Mansour KA. Complications of the Angelchik prosthesis in the management of gastroesophageal reflux. *Am Surg* 1986; **52**: 208-213 [PMID: 3954273]

47 **Benjamin SB**, Kerr R, Cohen D, Motaparthy V, Castell DO. Complications of the Angelchik antireflux prosthesis. *Ann Intern Med* 1984; **100**: 570-5 [PMID: 6367581 DOI: 10.7326/0003-4819-100-4-570]

48 **Smith RS**, Chang FC, Hayes KA, deBakker J. Complications of the Angelchik antireflux prosthesis. A community experience. *Am J Surg* 1985; **150**: 735-738 [PMID: 4073367 DOI: 10.1016/0002-9610(85)90419-2]

49 **Stetler JL**, Gill S, Patel A, Davis SS Jr, Lin E. Surgical Technique for Laparoscopic Removal of a

Magnetic Lower Esophageal Sphincter Augmentation Device. *J Laparoendosc Adv Surg Tech A* 2015; 1025-1028 [PMID: 26584252 DOI: 10.1089/lap.2015.0460]

50 **Bauer M**, Meining A, Kranzfelder M, Jell A, Schirren R, Wilhelm D, Friess H, Feussner H. Endoluminal perforation of a magnetic antireflux device. *Surg Endosc* 2015; **29**: 3806-3810 [PMID: 25877789 DOI: 10.1007/s00464-015-4145-6]

51 **Parmar AD**, Tessler RA, Chang HY, Svahn JD. Two-Stage Explantation of a Magnetic Lower Esophageal Sphincter Augmentation Device Due to Esophageal Erosion. *J Laparoendosc Adv Surg Tech A* 2017; **27**: 829-833 [PMID: 28488920 DOI: 10.1089/lap.2017.0153]

52 **Salvador R**, Costantini M, Capovilla G, Polese L, Merigliano S. Esophageal Penetration of the Magnetic Sphincter Augmentation Device: History Repeats Itself. *J Laparoendosc Adv Surg Tech A* 2017; **27**: 834-838 [PMID: 28586287 DOI: 10.1089/lap.2017.0182]

53 **Yeung BPM**, Fullarton G. Endoscopic removal of an eroded magnetic sphincter augmentation device. *Endoscopy* 2017; **49**: 718-719 [PMID: 28558405 DOI: 10.1055/s-0043-109236]

54 **Tatum JM**, Alicuben E, Bildzukewicz N, Samakar K, Houghton CC, Lipham JC. Removing the magnetic sphincter augmentation device: operative management and outcomes. *Surg Endosc* 2019; **33**: 2663-2669 [PMID: 30386987 DOI: 10.1007/s00464-018-6544-y]

55 **Stadlhuber RJ**, Dubecz A, Meining A, Stein HJ. Adenocarcinoma of the Distal Esophagus in a Patient With a Magnetic Sphincter Augmentation Device: First of Many to Come? *Ann Thorac Surg* 2015; **99**: e147-e148 [PMID: 26046907 DOI: 10.1016/j.athoracsur.2015.03.063]

56 **Alicuben ET**, Bell RCW, Jobe BA, Buckley FP 3rd, Daniel Smith C, Graybeal CJ, Lipham JC. Worldwide Experience with Erosion of the Magnetic Sphincter Augmentation Device. *J Gastrointest Surg* 2018; **22**: 1442-1447 [PMID: 29667094 DOI: 10.1007/s11605-018-3775-0]

57 **Lipham JC**, Taiganides PA, Louie BE, Ganz RA, DeMeester TR. Safety analysis of first 1000 patients treated with magnetic sphincter augmentation for gastroesophageal reflux disease. *Dis Esophagus* 2015; **28**: 305-311 [PMID: 24612509 DOI: 10.1111/dote.12199]

58 **Smith CD**, Ganz RA, Lipham JC, Bell RC, Rattner DW. Lower Esophageal Sphincter Augmentation for Gastroesophageal Reflux Disease: The Safety of a Modern Implant. *J Laparoendosc Adv Surg Tech A* 2017; **27**: 586-591 [PMID: 28430558 DOI: 10.1089/lap.2017.0025]

59 **van Rijn S**, Rinsma NF, van Herwaarden-Lindeboom MY, Ringers J, Gooszen HG, van Rijn PJ, Veenendaal RA, Conchillo JM, Bouvy ND, Masclee AA. Effect of Vagus Nerve Integrity on Short and Long-Term Efficacy of Antireflux Surgery. *Am J Gastroenterol* 2016; **111**: 508-515 [PMID: 26977759 DOI: 10.1038/ajg.2016.42]

60 **Bortolotti M**. The "magnetic collar": the ultimate solution for gastroesophageal reflux? *Scand J Gastroenterol* 2014; **49**: 511-512 [PMID: 24460023 DOI: 10.3109/00365521.2013.878383]

61 **Bortolotti M**, Grandis A, Mazzero G. A novel endoesophageal magnetic device to prevent gastroesophageal reflux. *Surg Endosc* 2009; **23**: 885-889 [PMID: 19116748 DOI: 10.1007/s00464-008-0244-y]

62 **Dobashi A**, Wu SW, Deters JL, Miller CA, Knipschield MA, Cameron GP, Lu L, Rajan E, Gostout CJ. Endoscopic magnet placement into subadventitial tunnels for augmenting the lower esophageal sphincter using submucosal endoscopy: ex vivo and in vivo study in a porcine model (with video). *Gastrointest Endosc* 2019; **89**: 422-428 [PMID: 30261170 DOI: 10.1016/j.gie.2018.09.015]

63 **Dobashi A**, Deters JL, Miller CA, Lavey CJ, Rajan E. Magnet-assist endoscopic augmentation of the lower esophageal sphincter for treatment of gastroesophageal reflux disease: cadaveric and survival studies in a porcine model (with video). *Surg Endosc* 2021; **35**: 4478-4484 [PMID: 33048232 DOI: 10.1007/s00464-020-07954-1]

64 **Bortolotti M**, Ugolini G, Grandis A, Montroni I, Mazzero G. A novel magnetic device to prevent fecal incontinence (preliminary study). *Int J Colorectal Dis* 2008; **23**: 499-501 [PMID: 18231796 DOI: 10.1007/s00384-008-0437-9]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 December 28; 27(48): 8242-8261

DOI: 10.3748/wjg.v27.i48.8242

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

REVIEW

Emerging therapeutic options in inflammatory bowel disease

Jesus K Yamamoto-Furusho, Norma N Parra-Holguín

ORCID number: Jesus K Yamamoto-Furusho 0000-0002-5247-5812; Norma N Parra-Holguín 0000-0002-0570-1127.

Author contributions: Yamamoto-Furusho JK provided the research idea, search information, selection of the papers, write and edit the final manuscript; Parra-Holguín NN searched the information and write the manuscript.

Conflict-of-interest statement: Yamamoto-Furusho JK is a member of the advisory board, an opinion leader and speaker for Abbvie Laboratories de México, Abbvie (international), Takeda Mé xico, Pfizer (international and regional), and Janssen Cilag (international and Mexico). He is an opinion leader and speaker for Farmasa, Ferring, and Farmasa Schwabe and a research advisor for UCB México. He has received funds for research studies from the Shire, Bristol Myers Squib, Pfizer, Takeda, and Celgene laboratories. Parra-Holguín NN declares no conflict of interest.

Country/Territory of origin: Mexico

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed

Peer-review model: Single blind

Jesus K Yamamoto-Furusho, Norma N Parra-Holguín, Gastroenterology Unit, Inflammatory Bowel Disease Clinic, Instituto Nacional de Ciencias Medicas y Nutricion, Mexico City 14080, Mexico

Corresponding author: Jesus K Yamamoto-Furusho, MD, MS, PhD, Chief Doctor, Director, Full Professor, Gastroenterology Unit, Inflammatory Bowel Disease Clinic, Instituto Nacional de Ciencias Medicas y Nutricion, Vasco de Quiroga 15, Colonia Belisario Domínguez sección XVI, Alcaldía Tlalpan, Mexico City 14080, Mexico. kazuofurusho@hotmail.com

Abstract

Inflammatory bowel disease (IBD) is a chronic disease that requires chronic treatment throughout the evolution of the disease, with a complex physiopathology that entails great challenges for the development of new and specific treatments for ulcerative colitis and Crohn´s disease. The anti-tumor necrosis factor alpha therapy has impacted the clinical course of IBD in those patients who do not respond to conventional treatment, so there is a need to develop new therapies and markers of treatment response. Various pathways involved in the development of the disease are known and the new therapies have focused on blocking the inflammatory process at the gastrointestinal level by oral, intravenous, subcutaneous, and topical route. All these new therapies can lead to more personalized treatments with higher success rates and fewer relapses. These treatments have not only focused on clinical remission, but also on achieving macroscopic changes at the endoscopic level and microscopic changes by achieving mucosal healing. These treatments are mainly based on modifying signaling pathways, by blocking receptors or ligands, reducing cell migration and maintaining the integrity of the epithelial barrier. Therefore, this review presents the efficacy and safety of the new treatments that are currently under study and the advances that have been made in this area in recent years.

Key Words: Inflammatory bowel disease; Review; Emerging; Treatment; Ulcerative colitis; Crohn´s disease

Core Tip: This review is to present the efficacy and safety of novel treatments for inflammatory bowel disease. The new treatments that may be available in the future are new anti-tumor necrosis factor alpha, anti-integrines, anti-interleukines, modulation of

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: March 2, 2021
Peer-review started: March 2, 2021
First decision: April 17, 2021
Revised: June 4, 2021
Accepted: November 30, 2021
Article in press: November 30, 2021
Published online: December 28, 2021

P-Reviewer: Ji G
S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H

sphingosine-1-phosphate, janus kinase inhibitors, toll like receptor agonist, therapy on the integrity of the epithelial barrier, phosphodiesterase-4 inhibitors and antisense oligonucleotide therapy, currently in clinical studies. Many of them with encouraging results in clinical studies, while others have not been able to maintain significant results in the final phases.

Citation: Yamamoto-Furusho JK, Parra-Holguín NN. Emerging therapeutic options in inflammatory bowel disease. *World J Gastroenterol* 2021; 27(48): 8242-8261
URL: https://www.wjgnet.com/1007-9327/full/v27/i48/8242.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i48.8242

INTRODUCTION

The pathogenesis of inflammatory bowel disease (IBD) is multifactorial and involves a series of factors specific to the patient and the environment. The chronic inflammatory process in ulcerative colitis (UC) and Crohn´s disease (CD) is causing damage to the intestinal mucosa with gastrointestinal and systemic symptoms. The anti-tumor necrosis factor alpha (TNF-α) therapy has impacted in the clinical course of IBD in those patients who do not respond to conventional treatment. Up to 30.0% of patients may not respond to initial anti-TNF alfa therapy and up to 46.0% may lose response during disease evolution[1]. Therefore, there is a need to innovate with the development of new treatments to be able to modify the clinical course of IBD including fewer clinical relapses, hospitalizations, surgeries and better quality of life. Currently, the approved biological treatments have great limitations such as their route of administration and adverse events. In recent years, new therapies have been developed to reduce the inflammatory process through different signaling pathways. There are several new mechanisms of action available such as anti-integrines, anti-interleukines, modulation of sphingosine-1-phosphate (S1P1), janus kinase (JAK) inhibitors, toll like receptor (TLR) agonist, phosphatidylcholin, phosphodiesterase-4 (PDE4) inhibitors and antisense oligonucleotide therapy, which are promising therapies currently in clinical studies. The mechanisms of action of the new biological treatments are illustrated in Figure 1. The purpose of this review is to present the efficacy and safety of novel treatments for IBD.

PATHOGENESIS OF IBD

IBD is now recognized as an immune-mediated disease that occurs in genetically susceptible hosts and can be described as chronic perturbations in homeostasis between the host and the external environment. The interface of these interactions can be divided into three critical elements: intestinal epithelium, immune cells, and commensal microbiota.

One consensus hypothesis is that each of these three major host compartments that functions as an integrated supraorganism is affected by specific environmental (enteropathogens, antibiotics, smoking *etc.*) and genetic factors that come together in a susceptible host and lead to chronic dysregulation and development of inflammation [2]. Thus, in both UC and CD, an inflammatory pathway likely emerges from the genetic predisposition that is associated with inappropriate innate immune and epithelial sensing and reactivity to commensal microbiota that secrete inflammatory mediators, together with inadequate regulatory pathways that lead to activated CD4+ T cells within the intestinal epithelium and lamina propria, secreting excessive quantities of inflammatory cytokines relative to anti-inflammatory cytokines. Some activate other inflammatory cells (macrophages and B cells) and others act indirectly to recruit other lymphocytes, inflammatory leukocytes, and mononuclear cells from the vasculature into the gut, through interactions between homing receptors on leukocytes (*e.g.*, α4β7 integrin) and addressins on the vascular endothelium (*e.g.*, MadCAM1). Neutralization of TNF or α4β7 integrin is consistent with an effective treatment of IBD. There are three major types of CD4+ T cells that promote inflammation in the gut, all of which are possibly associated with colitis in animal models and humans: TH1 cells (secrete interferon, TNF), TH2 cells [secrete interleukin (IL)-4, IL-5, IL-13] and TH17

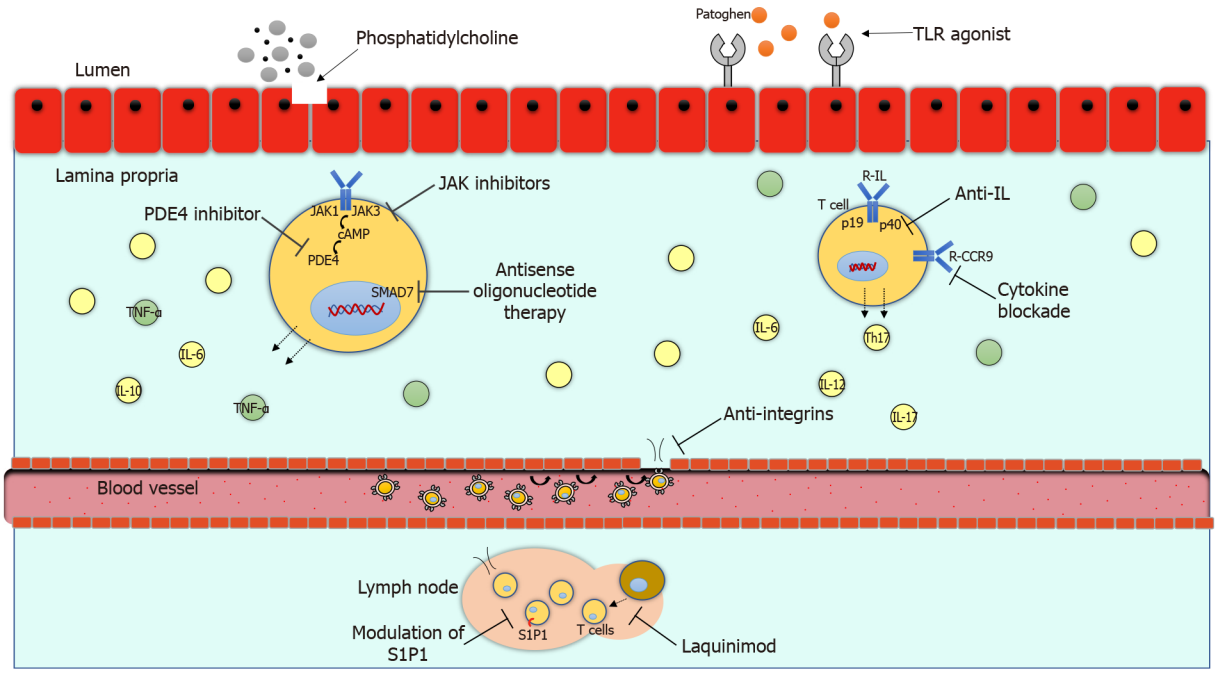

Figure 1 Mechanism of action of new therapies in inflammatory bowel disease. JAK: Janus kinase; TLR: Toll like receptor; IL: Interleukin; S1P1: Sphingosine-1-phosphate; PDE4: Phosphodiesterase-4; TNF: Tumor necrosis factor.

cells (secrete IL-17, IL-21). Each of these subsets of T cells cross-regulate each other. The TH1 cytokine pathway is initiated by IL-12, a key cytokine in the pathogenesis of experimental models of mucosal inflammation. IL-4 and IL-23, together with Il-6 and transforming growth factor beta (TGF-β), induce TH2 and TH17 cells, respectively. IL-23 also inhibits the suppressive functions of regulatory T cells[3]. Activated macrophages secrete TNF and IL-6.

Understanding inflammatory pathways has led to the development of new therapies, such as monoclonal antibodies that block pro-inflammatory cytokines or the signaling by their receptors (*e.g.*, anti-TNF-α anti-IL-12, anti-IL-23, anti-IL-6 or JAK inhibitors); molecules associated with leukocyte recruitment (*e.g.*, anti-α4β7); and the use of cytokines that inhibit inflammation (*e.g.*, IL-10) or promote intestinal barrier function (*e.g.*, epidermal growth factor), which may be beneficial to humans with intestinal inflammation.

RESEARCH METHODS

We performed an exhaustive search, encompassing the last 10 years, in the Medline/PubMed, the Cochrane Database, EMBASE (Ovid), and LILACS databases, using the following MeSH terms: ulcerative colitis, Crohn's disease, inflammatory bowel disease, pathogenesis, biologic therapy, new anti-TNF-α agents, anti-integrin therapy, vedolizumab, etrolizumab, abrilumab, ontamalimab, cytokine blockade, anti-interleukin therapy, vercirnon, anti-interlukin 23, eldelumab, rizankizumab, mirikizumab, brazikumab, guselkumab, briakinumab, anti-interleukin 17, secukinumab, brodalumab, anti-interleukin 6, interleukin 22, JAK inhibitors, upadacitinib, filgotinib, peficitinib, modulation of SIP1, ozanimod, etrasimod, amiselimod, laquinimod, toll like receptor agonist, cobitolimob, phosphatidylcholine, PDE4 inhibitor, apremilast, antisense oligonucleotide therapy, mongersen, GATA3 DNAzyme, alicaforsen. The search was limited to randomized controlled trials (RCTs) conducted on human subjects. Language: English. We also searched for any relevant RCTs included in the IBD Group Specialized Trials Register, the World Health Organization International Clinical Trials Registry, the European Union Clinical Trials Register, and the ClinicalTrials.gov to ensure identification of all eligible studies; and recent conference proceedings (European Crohn's and Colitis Organisation, United European Gastroenterology Week, and Digestive Disease Week). Finally, we conducted supplemental searches of the regulatory authorities' websites (European Medicines Agency: www.ema.europa.eu; United States Food and Drug Adminis-

tration: www.fda.gov) to obtain details on study characteristics or outcomes.

NEW ANTI-TNF-α THERAPY

AVX-470

This is a polyclonal anti-TNF antibody, currently in development and it has been tested in patients with moderate to severe disease UC activity. There is few information about its mechanism of action, it has been proposed to act locally in the gastrointestinal tract named AVX-470 has shown to inhibit gut inflammation in mice [4]. It is considered a large weight molecule of 160–900 kDa, with an oral administration which can avoid systemic adverse events. In phase 1 clinical trial, patients receive AVX-470 at doses of 0.2, 1.6 or 3.5 g a day, clinical response was an achievement in 7 (25.9%) with AVX-470 groups *vs* 1 (11.1%) in the placebo group and a significant reduction in serum C reactive protein (CRP) and IL-6. Low levels of anti-TNF antibodies were observed in patients who received this treatment, the antibody levels were lower compared to other anti-TNF therapies, having less immunogenicity avoiding future loss of response to this treatment, with a good safety profile, there were no serious adverse events in this human trial[5]. The phases of clinical trials of these new treatments are listed in Table 1.

ANTI-INTEGRIN THERAPY

Integrins are receptors found on the cell surface for cell proliferation, signaling, and migration, its subunits binds to cell adhesion molecules (CAMs). The α4β1 integrin heterodimer binds VCAM-1 or fibronectin, α4β7 integrin heterodimer binds mucosal vascular addressin (MAd) CAM-1 and the αEβ7 integrin heterodimer binds E-cadherin [6]. Inhibiting these molecules have a therapeutic effect since it decreases the cell migration of pro-inflammatory cells in the gastrointestinal tract[7].

Ontamalimab (SHP647, PF-00547659)

This is a fully human anti-MAdCAM-1 antibody, reducing lymphocyte migration. In a phase 2 study (TURANDOT trial) in patients with moderate to severe UC who failed conventional treatment, were randomized to receive ontamalimab subcutaneously (SC) at a dose of 7.5 mg, 22.5 mg, 75 mg, 225 mg or placebo every 4 wk, clinical remission was presented in 8 (11.3%), 12 (16.7%), 11 (15.5%) and 4 (5.7%) in the groups respectively and in the placebo group only in 2.7% of patients[8]. In the open label study for UC patients (TURANDOT II trial) mucosal healing increased from 20.3% from baseline to 28.5% at week 16 and was maintained until week 144 of follow-up[9]. The phase 3 study for patients with UC is currently recruiting patients[10]. In the phase 2 study (OPERA) in patients with CD, the results did not show significant differences compared to the placebo group[11], therefore, the phase 3 study in CD was suspended by the sponsor[12].

Etrolizumab (rhuMAbBeta7)

This is a humanized IgG1 monoclonal antibody (mAb) for the β7 integrin subunit and blocks the interactions of α4β7 with MAdCAM-1 and αEβ7 with E-cadherin[13]. This therapy suppresses the trafficking of lymphocytes in the intestine and the retention of lymphocytes in the intraepithelial compartment. In a phase 2 study, its efficacy for induction of remission in patients with UC was demonstrated previously with subcutaneous administration[11]. Currently, the phase 3 study is underway for patients with UC and CD with moderate to severe activity, it is composed of multiple randomized control trials HIBISCUS I and II, GARDENIA, LAUREL, HICKORY, ERGAMOT and open-label extension trials COTTONWOOD and JUNIPER. Also the purpose of these studies is not only to verify its efficacy and safety, but to compare with other biological treatments such as adalimumab and infliximab[14].

Abrilumab (AMG 181)

This is a fully humanized IgG2 mAb, with the same mechanism of action like vedolizumab, against the integrin α4β7[15]. A phase 2 study was conducted in patients with moderate to severe UC refractory to anti-TNF alpha and immunomodulatory therapy, were randomized to receive abrilumab SC at doses of 7, 21 or 70 mg on day 1, week 2 and 4, then every 4 wk, abrilumab 210 mg on day 1 or placebo. The clinical

Table 1 Phase of clinical trials for emerging therapeutic options for inflammatory bowel

Treatment	UC	CD	Treatment	UC	CD	Treatment	UC	CD	Treatment	UC	CD
Anti-IL			Anti-integrin			JAK inhibitors			Other therapies		
Rizankinumab	III	III	Ontamalimab	III	II	Upadacitinib	III	III	AVX-470	I	
Mirikizumab	III	II	Etrolizumab	III	III	Filgotinib	III	III	Laquinimod	-	II
Brazikumab	II	III	Abrilumab	II	II	Peficitinib	II	-	Cobitolimod	III	-
Guselkumab	II	III	AJM300	III	-	TD-1473	II	II	BL-7040	II	-
Briakinumab	-	II	Cytokine blockade			Modulation of SIP1			Phosphatidylcholine	III	-
PTG□200	-	II	Vercirnon	-	III	Ozanimod	III	III	Apremilast	II	-
Secukinumab	-	II	Eldelumab	II	II	Etrasimod	III	-	Mongersen	-	II
Brodalumab	-	II	GSK3050002	I	-	Amiselimod	-	II	GATA3 DNAzyme	II	-
PF-04236921	-	II				KRP-203	II	-	STNM01	II	

UC: Ulcerative colitis; CD: Crohn´s disease; IL: Interleukin; JAK: Janus kinase; S1P1: Sphingosine-1-phosphate.

remission rates were 98 (13.3%), 79 (12.7%) and 116 (4.3%) ($P \leq 0.05$) for abrilumab 70 mg, 210 mg and for placebo respectively at week 8. No serious adverse events occurred during the study. The most frequent adverse events reported for both groups was the reaction at the injection site, nasopharyngitis, headache, and arthralgias[16] . For patients with CD, a phase 2 study was conducted and were randomized to receive placebo or abrilumab at doses of 21 mg or 70 mg SC on day 1, weeks 2 and 4, and every 4 wk for 24 wk or only one dose of 210 mg SC on day 1, the primary endpoint was not reached and there were no significant differences in clinical remission compared to the placebo group[17].

AJM300

AJM300 is an oral small molecule antagonist of α4 and target α4β7 and α4β1 integrin. Previous studies have demonstrated, a significant decrease in the number of T lymphocytes in the lamina propria in mice[18]. The therapeutic efficacy and safety of AJM300 were tested in a phase 2a study with 102 UC patients and were administered 960 mg orally for 8 wk, 3 times a day or placebo, to evaluate the induction to clinical remission. Clinical response rates were 32 (62.7%) and 13 (25.5%) ($P = 0.0002$), clinical remission in 12 (23.5%) and 2 (3.9%) ($P = 0.0099$), mucosal healing in 30 (58.8%) and 15 (29.4%) ($P = 0.0014$) at week 8 in the AJM300 and placebo group, respectively. This study demonstrated a significant improvement in clinical response, endoscopic remission, and histological response. No serious adverse effects were documented and only the most common adverse event was nasopharyngitis[19]. A phase 3 study with the same doses is currently being conducted to evaluate the efficacy and safety in patients with UC[20].

CYTOKINE BLOCKADE

Vercirnon (CCX282-B)

This is an antagonist against the receptor CCR9, inhibiting leukocyte traffic to the small intestine[21]. In a study phase 2 in patients with CD, subjects received 250 mg once daily, 250 mg twice daily, or 500 mg once daily of vercirnon or placebo for 12 wk as induction therapy and then they receive 250 mg of vercirnon through week 16 if they response were randomly assigned to receive 250 mg of vercirnon twice a day or placebo for 36 wk. Response rates for the induction therapy at week 12 was about 55 (56.0%, $P = 0.168$), 47 (49.0%, $P = 0.792$), 59 (61.0%, $P = 0.039$) in vercinon groups and 68 (47%) in the placebo group. In the maintenance period, 68 (47%) of subjects on vercirnon were in remission *vs* 29 (31%) in the placebo group ($P = 0.012$)[22] During the phase 3 study, patients were randomized into three groups to receive vercirnon 500 mg once a day, 500 twice a day, or placebo, clinical response at week 12 was in 56 (27.6%, $P = 0.546$), 55 (27.2%, $P = 0.648$) and 51 (25.1%), respectively. The most frequent adverse events were headache, worsening of CD and abdominal pain. This treatment

failed to show the effectiveness of previous studies and no significant differences between the all study groups[23], so subsequent studies were canceled.

Eldelumab (BMS-936557)

Eldelumab is a fully human mAb against the chemokine CXCL10, this chemokine is also involved in the traffic of leukocytes to the colon, its receptor CXCR3 is expressed on most T cells. In a phase 2 study in patients with UC, they receive 10 mg/kg of eldelumab or placebo intravenously (IV) every other week. The primary and secondary endpoints of clinical response, clinical remission and mucosal healing at day 57 were not met, but the clinical response and clinical remission rates were associated with higher drug exposure[24]. A phase 2 trial in patients with CD receives eldelumab 10 mg, 20 mg or placebo at days 1 and 8 and alternate weeks. Clinical remission was 29.3%, 22.5% and 20.0% in the 20 mg/kg, 10 mg/kg and placebo groups at week 11, but they were not significantly superior to the placebo group[25]. Despite the encouraging results of the clinical response related to drug exposure and a good safety profile, the response rates were lower, so further studies were not continued in IBD.

GSK3050002

This is a mAb IgG1 with affinity to chemokine CCL20, binds to its receptor CCR6 expressed mainly in dendritic cells and B cells. The chemokine CCL20 is up-regulated in active IBD[26]. Currently, there are only phase 1 studies focused on patients with UC. In a study with healthy volunteers, they were administered, dose escalation of IV GSK3050002. With a half-life time of 2 wk, with a dose dependent decrease in CCR6, and a good safety profile at doses from 0.1 to 20 mg/kg[27].

ANTI-IL THERAPY

Anti- IL-23

In genome association studies, a strong association with the production of IL-17 and IL-23 has been shown, especially in patients with CD[28,29], as well as an increase in the expression of messenger RNA of these molecules and their intracellular proteins in the lamina propria of the gastrointestinal tract of patients with IBD[30,31].

Risankizumab (BI-655066)

This is a mAb that targets the p19 subunit, specific for IL-23. In the phase 2 studies for the induction of clinical remission in patients with moderate to severe CD, risankizumab was administered at doses of 200 and 600 mg IV where clinical remission was obtained in 12 (31%) *vs* 6 (15%) patients in the placebo group at week 12[32]. The maintenance of clinical remission with risankizumab in patients with CD, it was maintained in 44 (71%) of patients, 50 (81%) patients had a clinical response, 22 (35%) obtained endoscopic remission, 15 (24%) mucosal healing and 18 (29%) achieved clinical and endoscopic (deep) remission at week 52[33]. A phase 2 and 3 studies are currently recruiting patients with moderate to severe UC activity, with IV induction doses and subcutaneous maintenance SC doses[34], a phase 3 study of maintenance of remission is planned for patients who achieved clinical response and remission in the induction study[35]. A phase 3 study for induction of remission in CD and its maintenance until week 52[36].

Mirikizumab (LY3074828)

This is a mAb that blocks selectively the p19 subunit of IL-23. In the phase 2 study in patients with moderate to severe activity of UC were randomized into four groups to receive doses at 50 mg, 200 mg, 600 mg and placebo SC at 4 and 8 wk. Clinical remission was obtained in 10 (15.9%), 14 (22.6%) and in 7 (11.5%) patients, respectively, compared with only 3 (4.8%) patients in the placebo group at week 12. The maintenance of clinical remission at doses of 200 mg every 4 wk, 200 mg every 12 wk and placebo, with 22 (46.8%), 17 (37.0%) and 1 (7.7%) of patients at week 52 in the maintenance of clinical remission[37]. The most frequently reported adverse effects were nasopharyngitis, nausea and worsening of UC. A phase 3 study (LUCENT 1) for induction of remission in 12 wk for UC patients with moderate to severe activity is currently under recruitment[38], as well as maintenance of remission (LUCENT 3)[39]. A phase 2 study for patients with CD (SERENITY) and a phase 3 study with an active arm for ustekinumab[40].

Brazikumab (MEDI2070)

This is a mAb selectively directed to the p19 subunit of IL-23. Efficacy was evaluated in patients with CD and moderate to severe activity, who had a failure to anti-TNF-α, they were randomized with a dose of brazikumab of 700 mg IV or placebo at weeks 0 and 4. Followed by maintenance doses of 210 mg SC every 4 wk from weeks 12 to 112. Clinical response was measured in 29 (49.2%) *vs* 16 (26.7%) response from the placebo group at week 8. At week 24, the clinical response of 28 (53.8%) in the brazikumab group *vs* 30 (57.7%) in patients in the placebo group. A secondary outcome was to measure the expression of IL-22, a pro-inflammatory cytokine induced by the action of IL-23. Patients with a higher expression of IL-22 at the start of treatment was associated with a higher probability of response to brazikumab compared to the placebo group. The most frequently adverse effects were headache, nasopharyngitis, abdominal pain, arthralgia and proctalgia[41]. In patients with UC with moderate to severe activity named the EXPEDITION, which is a long-term phase 2 study of brazikumab in patients with UC with moderate to severe activity, is underway with IV brazikumab on days 1, 15 and 43, followed by brazikumab SC starting on day 71 every 4 wk[42]. It is also being evaluated in CD patients in a phase 3 study with severe activity, with IV brazikumab on days 1, 29, and 57, followed by SC brazikumab. For CD, a phase 3 study with an active arm is being recruited to compare adalimumab in which IL-22 was also included as a prognostic factor of response to treatment[43].

Guselkumab

This is a mAb against the p19 subunit, whose efficacy has been proven and was approved for psoriasis treatment[44]. There are no data available so far on its efficacy and safety in patients with IBD, data are only available in patients with psoriasis and psoriatic arthritis where it has shown successful results with few adverse effects. There is an ongoing phase 2 study with combined therapy with guselkumab and golimumab in patients with moderate to severe UC activity. Participants will receive guselkumab at first dose as an IV infusion and the second one as a SC injection in addition to golimumab two doses as an SC injection and placebo[45]. For CD, a phase 2 study (GALAXI 1) is underway, participants will be assigned to five treatment groups, where groups 1 to 3will receive two doses of guselkumab IV and SC; group 4 will receive ustekinumab IV infusion followed by SC dosing, and group 5 will receive IV placebo at week 12. Those patients who do not respond will receive two doses of ustekinumab IV and SC. In GALAXI 2 and 3 studies, participants will be randomized to guselkumab, ustekinumab, or placebo[46]. A phase 3 study, is ongoing in patients with moderate to severe CD activity with IV guselkumab (3 doses) followed by SC guselkumab[47].

Briakinumab

This is a mAb antibody against the p40 subunit of IL-12 and 23. Early studies, showed significant decreased in Th1 Lymphocytes in the gastrointestinal tract[48]. Currently it is only being evaluated for the treatment of psoriasis. In a phase 2 study, patients with CD were included in four treatment groups, they received briakinumab doses of 200 mg, 400 mg, 700 mg and placebo at weeks 0, 4 and 8. Patients who responded with doses of 400 mg and 700 mg were included in the maintenance phase at doses of 200 mg, 400 mg, 700 mg and placebo at weeks 12, 16 and 20. At week 24, 21 (43%), 21 (48%), 21 (57%) and 14 (29%) patients were in remission in the respectively groups. The most frequent adverse effect reported were respiratory infections in 20.7%, nausea in 17.3%, abdominal pain and headache 14.3%[49]. No current studies are undergoing in patients with CD and briakinumab.

PTG200 (JNJ67864238)

This is a selective inhibitor blocks the IL-23 receptor, it has the main advantage of oral administration. In vivo studies, have demonstrated that a high concentration of this molecule at the gastrointestinal level and a minimum concentration at the systemic level. Phase 1 trials in healthy volunteers showed few adverse effects, none of them serious, with a half-life of approximately 1.5 h[50]. A phase 2 study is currently underway in patients with CD with moderate to severe activity to evaluate the efficacy and safety for 12 wk, with daily oral administration of PTG-200[51].

Anti-IL-17

The IL-23 is involved in the signaling pathway of Th17 cells, these lymphocytes are producers of cytokines that enhance or regulate immune responses by interacting with other inflammatory cells such as macrophages, neutrophils, eosinophils, and

basophils. These cells participate in the expression of subsets regulatory T cells and Th1, Th2, and Th17 lymphocytes[52]. Stimulation of neutrophil activation and IL-23-mediated induction of IL-17 and IL-22 production by neutrophils. All IL-17 producing cells predominate in patients with UC, mainly in the lamina propria, and CD transmurally[30].

Secukinumab (AIN457)

Is a mAb of the IgG type which binds selectively to IL-17, preventing its union with its receptor, with this action the inflammatory process caused by this cytokine. In a phase 2 study carried out in patients with CD with moderate to severe active disease in which 59 patients were included who received IV secukinumab or placebo, 31% of patients in the secukinumab group discontinued the study prematurely due to lack of response to treatment. Higher rates of adverse effects were observed compared to the placebo group, 29 (74.4%) *vs* 10 (50%) patients. The most frequent adverse event were infections, worsening of CD, abdominal pain and arthralgias[53]. Secukinumab was approved for the treatment of psoriasis, but have been reported cases of IBD after the application of these biological in this group of patients[54,55], therefore, its use in patients with known IBD is not recommended and no new studies are undergoing.

Brodalumab (AMG 827)

Is a mAb that acts directed against the IL-17 receptor, inhibiting the inflammatory activity of this interleukin with high affinity[56]. Its availability is limited to psoriasis patients with moderate to severe disease. In the phase 2 study, patients with moderate to severe CD were enrolled to receive different doses of brodalumab 210, 350 and 700 mg at weeks 0 and 4 compared to a placebo group. This study was interrupted for aggravation of CD activity. Only 130 patients were randomized to receive treatment groups with clinical response in 1 (3.1%), 5 (15.2%), 3 (9.1%) and 1 (3.1%) in the brodalumab at 210 mg, 350 mg, 700 mg and placebo respectively at week 6. The most frequent adverse effect was worsening activity of CD[57]. There are no ongoing studies for Brodalumab in IBD.

Anti-IL-6

This cytokine has inflammatory effects and inhibits apoptosis of T lymphocytes in the gastrointestinal mucosa[58]. Serum IL-6 concentrations are elevated in patients, with CD and correlates with CRP levels[59].

PF-04236921

The PF-04236921 molecule is a IgG2 mAb that inhibits the action of IL-6, it has an approximate half-life of 36 to 51 d. The induction of clinical remission was evaluated with doses of 10 mg, 50 mg, 200 mg and placebo. The response rate at dose of 50 mg was 49.3% *vs* 30.6% ($P \leq 0.05$) in the placebo group at week 8 and 27.4% and 10.9% ($P \leq 0.05$) respectively at week 12. Common adverse effects were headache, abdominal pain and nasopharyngitis while serious adverse effects were presented in 3 (4.5%), in 7 (9.9%), in 8 (20%) patients in the 10 mg, 50 mg and 200 mg groups respectively, which include perforation and abscess formation[60].

IL-22 THERAPY

Unlike the previous interleukins, IL-22 has an anti-inflammatory mechanism, it is elevated during inflammatory processes, with multiple functions such as regulation of the interaction between bacteria-host, protection and healing of the mucosa[59]. In patients with CD, it is higher compared to patients with UC, since previous studies have shown greater expression in the small intestine[61,62] and patients with active UC[63].

UTTR1147A

In a phase 1 stage in healthy volunteers, ascending doses of this molecule were used by IV and SC routes where they showed adequate pharmacokinetics with a good level of safety[64]. A phase 2 study is currently being recruited in patients with moderate to severe active UC, which will also include active arms with vedolizumab for the induction of clinical remission at week 8 as well as a maintenance phase will be evaluated as the primary objective until week 30[65].

JAK INHIBITORS

Upadacitinib

This is a selective oral inhibitor of JAK1 compared to JAK2, JAK3 and TYK-2[66,67]. Upadacitinib down-regulates multiple pro-inflammatory cytokines, including the following interleukins: IL-2, 4, 6, 7, 9, 15, 21, and interferon gamma that are relevant to the pathogenesis of IBD[68]. A total of 220 patients were included to evaluate the induction of clinical remission in patients with CD who received upadacitinib orally twice a day, the clinical remission was reach in 39 (13%) with 3 mg, in 37 (27%, $P < 0.1$) with 6 mg, in 36 (11%) with 12 mg, 35 (14%) with 24 mg and 37 (11%) in the placebo group once a day at week 16. Endoscopic remission was greater the higher the dose, but not the clinical remission[66]. These results are similar for UC at doses of 7.5 mg, 15 mg, 30 mg or 45 mg once a day, with clinical remission in 4 (8.5%, $P = 0.052$), in (14.3%, $P = 0.013$), in 7 (13.5%, $P = 0.011$), in 11 (19.6%, $P = 0.002$) respectively and 0% in the placebo group at week 8[69]. Currently are conducting phase 3 studies for both diseases[70,71].

Filgotinib

This is an inhibitor with higher selectivity for JAK1 over JAK2 and JAK3[72] in order to assess the induction of remission in patients with moderate to severe CD, 200 mg orally was administered once daily against placebo over a period of 10 wk, in 60 patients (47%) who received filgotinib achieved clinical remission at week 10 *vs* 10 (23%, $P = 0.0077$) patients in the placebo group, the most frequent adverse effects were: nasopharyngitis and urinary tract infections[73]. It is currently in recruitment in phase 3 study for patients with CD[74] and UC[75] with moderate to severe activity naïve to biological therapy or who had failure or intolerance to any other biological treatment.

Peficitinib

Peficitinib inhibits selectively for JAK3 over JAK1, JAK2, and TYK2[76]. In phase 2 with UC patients, it was evaluated the efficacy at doses of 25 mg, 75 mg, 150 mg once a day, 75 mg twice a day and placebo orally. The primary endpoint of dose-response was not reached at week 8, but the clinical response, clinical remission and mucosal healing were higher at doses of ≥ 75 mg. Biochemical markers like fecal calprotectin and CRP were not significantly reduced with peficitinib. The most frequent adverse events were worsening of UC, increased blood creatine phosphokinase and anemia [77].

TD-1473

TD-1473 is a gut-selective pan-JAK inhibitor, administered orally, inhibits cytokine signaling directly in the gastrointestinal tract avoiding systemic effects. Phase 1 in mice and healthy volunteers show high intestinal drug exposure compared with plasma. The Phase 1 study was done in UC with moderate to severe active disease, and evaluate 3 doses 20 mg, 80 mg and 270 mg orally once a day after an overnight fast for 28 d, no efficacy analysis was carried out but tendencies to decrease UC activity were found[78]. A phase 2 study is currently being carried out in patients with CD (DIONE) [79] and a phase 2 and 3 for patients with UC (RHEA)[80].

MODULATION OF SIP1

Small molecule drugs have intrinsic properties that distinguish them from biological therapies: they are administered orally, have a short half-life and a low risk of immunogenicity[81].

Ozanimod

This is an oral agonist of the S1P1 and 5 receptors, decreasing the number of activated lymphocytes circulating to the gastrointestinal tract. The clinical remission occurred in 11 (16%, $P = 0.048$) who received 1 mg ozanimod and in 9 (14%, $P = 0.14$) who received 0.5 mg ozanimod, compared with 4 (6%) patients who received placebo at week 8. In the maintenance period, the clinical remission was in 14 (21%, $P = 0.01$) in the ozanimod 1 mg group, 17 (26%, $P = 0.002$) in the 0.5 mg group, and 6% in the placebo group at week 32. The main adverse effects presented were anemia and headache[82]. Preliminary results in CD receiving ozanimod 1 mg orally daily showed improvement in mucosal healing in patients with moderate to severe CD treated for 12 wk[83]. A

phase 3 study, is currently being carried out to evaluate the induction and maintenance of clinical remission for CD and a phase 3 for UC is completed pending publication of official results[84,85].

Etrasimod (APD334)

This is a selective modulator of the S1P1, S1P4 and S1P5 sphingosine receptors, decreasing the production of several cytokines[86]. After treatment with etrasimod 2 mg once daily, an approximately 53% decreased in mean lymphocyte count was observed in healthy volunteer patients on day 3, with a continuous decrease in 69% of patients by day 21. In a phase 2 study in UC, were randomized in 3 groups: 1 mg, 2 mg and placebo for 12 wk orally once a day, the primary endpoint was an improvement in the modified Mayo index that evaluates the frequency of stools, rectal bleeding and endoscopic findings. Clinical remission was observed in 33.0% ($P \leq 0.001$) of the etrasimod 2 mg group compared with 8.1% of the placebo group. Endoscopic improvement occurred in 41.8% ($P = 0.003$) in the 2 mg group. No significant differences were found concerning adverse effects compared with the placebo group [87]. A phase 3 study is recruiting patients, with UC for the administration of etrasimod 2 mg orally for 52 wk[88].

Amiselimod (MT-1303)

This is a S1P1 receptor modulator, with more favorable cardiac safety profile than other S1P1 receptor modulators[89]. It was evaluated in patients with CD, with clinically active disease and elevated biomarkers, in patients who were previously treated with steroids, immunomodulators and/or anti-TNF-α treatment. The dose evaluated was 0.4 mg orally once a day for 14 wk. The primary endpoint of CDAI100 was achieved in 19 (48.7%) in the amiselimod group *vs* 20 (54.1%) patients in the placebo group. Adverse effects were observed in both groups, infections occurred in 26% *vs* 13% of the placebo group. Cardiac disorders such as ventricular tachycardia, bradycardia, ventricular extrasystoles were observed[90].

KRP-203

This is a S1P1, 4, 5 receptor agonist and partial agonist of S1P3 receptor. In a phase 2 with moderate UC activity and 5-aminosalicylate refractory patients. They received 1.2 mg of KRP203 or placebo daily for 8 wk. No statistically significant differences were found between both groups, but the frequency of clinical remission was 14% and 0% in the placebo group. No adverse cardiac events were reported during the study, the most frequent adverse events were gastrointestinal disorders and headache[91].

OTHER MECANISM OF ACTION

Laquinimod

This an oral small-molecule with a direct inhibitory effect on T cells and causes a decreased pro-inflammatory cytokines in the gastrointestinal tract[92]. In a phase 2 study in patients with active CD, they receive 0.5 mg, 1.5 mg, or 2 mg a day of laquinimod or a placebo, for 8 wk. The primary endpoint was a clinical response of 70 or 100 points of CDAI reduction from baseline or remission and no treatment failure. A dose of 0.5 mg showed improvement on remission rates in 14 patients (48.3%) *vs* 10 patients (15.9%), a response of 100 CDAI of 55.2% *vs* 31.7% and response CDAI 70 in 62.1% *vs* 34.9% in the placebo group. The most frequents adverse events were headache and abdominal pain[93].

TLR agonist

The TLR-9 is mainly expressed on dendritic cells and macrophages, the TLR recognize pathogenic molecules to release anti-inflammatory mechanisms. TLR-9 expression is upregulated in the mucosa of the rectum in UC patients with active disease compared with healthy controls and patients with UC in remission. Activation of the TLR-9 receptor has been proposed to stimulate intestinal mucosal healing[94].

Cobitolimod (DIMS0150)

This is a TLR-9 agonist which is a synthetic oligonucleotide that induced the production of IL-10 and other anti-inflammatory cytokines[95]. Furthermore, it has been seen in cell studies to increase steroid sensitivity in patients with steroid-resistant UC patients[96]. In UC patients refractory to conventional treatment and anti-TNF-α

therapy, were included to receive rectally DIMS0150 30 mg or placebo. No statistical differences between 30 mg and placebo were found, with the induction of clinical remission at week 12 in 44.4% and 46.5% respectively. With symptomatic remission in 32.1% *vs* 14.0% in the 30 mg and placebo group (P = 0.020) at week 4, and 44.4% *vs* 27.9% at week 8 (P = 0.061). Mucosal healing at week 4 in 21.0% *vs* 4.7% (P = 0.01), there were no major safety events during study development[97]. A phase 2 trial (CONDUCT study) patients were randomized to receive rectal enemas at doses of 31 mg, 125 mg or 250 mg at weeks 0 and 3, and cobitolimod at doses of 125 mg or placebo at week 0, 1, 2 and 3. There were statistically significant differences for clinical remission at week 6 in the 250 mg group in the 21.0% *vs* 7% in placebo (P = 0.0025)[98].

BL-7040

This is a TLR-9 modulator, in phase 2, in UC with moderate clinical activity, received BL-7040 orally, 12 mg for 19–21 d followed by 40 mg for an additional 14 d, clinical remission was achieved in 12.5%, mucosal healing was achieved in 50%, and was well tolerated with one serious adverse event not related to the study[99].

FOCUSED THERAPY ON THE INTEGRITY OF THE EPITHELIAL BARRIER

Phosphatidylcholine (LT-02)

Is usually found in the intestinal barrier, maintaining its integrity, it is decreased in patients with UC and cause epithelial permeability[100], these changes have developed in mice models and a probable role in the pathogenesis of IBD development has been demonstrated[101]. In a phase 2 study in UC patients, the treatment was administered orally with pellets, four times daily at doses of 0, 0.8, 1.6, or 3.2 g. Clinical remission was achieved in the 31.4% of 3.2 g *vs* 15.0% in the placebo group (P = 0.089). Mucosal healing was achieved in 47.4% *vs* 32.5% (P = 0.098), histologic remission in 47 (40.5%) *vs* 8 (20.0%) respectively (P = 0.016)[102]. A phase 3 study was recently conducted (PROTECT-2) compared with mesalamine and placebo for the maintenance of remission in patients with UC, but the results have not been published so far[103]. The study for induction of remission (PROTECT-3) in UC was terminated because it did not show any efficacy for achieving induction of remission[104].

PDE4 inhibitor

Apremilast: This an oral small molecule that specifically inhibits PDE4[105] ,with activation of intracellular cAMP levels and an increase the production of anti-inflammatory cytokines with effects on innate inmmunity[106] and is currently approved for the use in psoriasis. In the phase 2 study in patients with UC, patients were randomized to receive apremilast 30 mg, 40 mg or placebo twice daily for 12 wk and subsequently randomized to receive 30 or 40 mg for 40 wk. Clinical remission was achieved in 31.6% and in 12.1%, (P = 0.01) in the groups of 30 mg and placebo, respectively at week 12, without significant differences for the group of 40 mg. During the maintenance period, clinical remission was achieved in 40.4% in the 30 mg group *vs* 32.7% in the 40 mg group[107].

Antisense oligonucleotide therapy

Mongersen GED0301: TGF-β is an important cytokine with an anti-inflammatory functions, with a regulatory function of T cells[108]. The activation of this factor causes a phosphorylation of the SMAD2/3complex complex, in this pathway SMAD7 acts, which is responsible for downregulating TGF-B, blocking the activation of the SMAD2/3complex complex. TGF-B is normally produced in patients with IBD but it did not achieve its anti-inflammatory effect due to the high production of SMAD7 in these patients[109]. Mongersen is an anti-SMAD7 oligonucleotide, against SMAD7 mRNA, decreasing the production of this inhibitor[110]. Mongersen is for oral use and binds to the TGF-β receptor inhibiting the signal of SMAD2 and 3[111], and reduce pro inflammatory cytokines[112]. A phase 2 study of Mongersen was conducted in CD patients with doses of 10, 40, 160 and placebo, clinical remission at 2 wk was archived in 55% and 65% in the groups of 40 and 160 mg respectively ($P \leq 0.001$), with no significant differences in the 10 mg group[113]. A subsequent study was performed, with a dose of 160 mg in three groups 4, 8 and 12 wk of follow-up with clinical remission in 32%, 35% and 48% respectively[114]. In the phase 3 study was cancelled for findings of non-effectiveness in this group of CD patients[115].

GATA3 DNAzyme (SB010)

The inflammatory process is regulated by lymphocytes Th2 and the production of IL-4, 5 and 13 in UC. In CD the response is characterized by Th1 and release of interferon gamma and TNF. This treatment was first studied in patients with asthma and the evidence was shown a decrease in IL production[116]. GATA3 is a transcription factor for the transcription of cytokines of Th2 response[117], and GATA3 RNA transcripts are higher in colonic UC biopsies[116]. Animal models treated with a DNAzyme anti-GATA3 with intrarectal administration showed a reduction in the production of pro-inflammatory cytokines[118]. Phase 2 was conducted to evaluate the efficacy and safety of a topical formulation by enema in patients with moderate to severe active UC, but results have not been published yet[119].

STNM01

In patients with CD, the development of fibrotic stenoses is common due to the chronic inflammation that causes a remodeling process. The treatment of this issue is endoscopic or surgical resection. In recent years, the enzyme carbohydrate sulfotransferase 15 (CHST15) was discovered, is responsible for regulating the production of glycosaminoglycans that cause the fibrotic process in patients with CD[109]. STNM01 is an RNA oligonucleotide against CHST15, inhibits the expression of mRNA with less production of glycosaminoglycans in the colon. The first studies in mice were carried out using direct submucosal injections into the colon[120]. The study in CD patients with ulcerative lesions was randomized to receive a single submucosal injection by endoscopic route or placebo, in the largest ulcerated lesion that was visualized by colonoscopy. A decrease in the extent of fibrosis was documented by histology, and no adverse effects were documented during the study[121]. A phase 2a study was conducted in patients with refractory and left-sided UC in 24 patients. They were randomized into 3 groups to receive a single dose of 25 nM, 250 nM or placebo by submucosal injection. The primary endpoint was mucosal healing on days 14 and 25, which was achieved in 62.5% *vs* 28.6% in the 250 nM and placebo group, respectively. Clinical response was shown by 62.5% in the STNM01 250 nM group (P = 0.3200) *vs* 28.6% in the placebo group and clinical remission in 50.0% in the 250 nM *vs* 14.3% in the placebo group (P = 0.04), with a good safety profile[122].

Alicaforsen

This a 20-base ICAM-1 human antisense oligonucleotide that targets the mRNA of ICAM-1 and causes its inactivation[123]. Initially, it was used in patients with CD, IV and SC with few results, in recent years alicaforsen was reformulate to its use in enemas for patients with UC and pouchitis. A randomized phase 2 study was carried out in patients with UC with mild to moderate distal disease, they received a 60 mL enema with 0.1, 0.5, 2 or 4 mg/mL or placebo once daily for 28 d. Alicaforsen improves the disease activity index in 70% *vs* 28% patients in the placebo group (P = 0.004) at day 29. The most frequent adverse events were asthenia, infections, and nausea. No serious adverse events related to the medical treatment[124]. In another phase 2 clinical trial, no significant difference was observed between treatment arms and placebo in the primary endpoint[125]. In a case series in patients with refractory pouchitis, clinical improvement was achieved in 84.6%, but 81.8% patients had a relapse after a median of 16 wk[126]. A phase 3 study was performed in patients with pouchitis who failed at least one course of antibiotics and received alicaforsen 240 mg or placebo once daily for 6 wk. Preliminary results showed reduction in the stool frequency in 33.8% and 26.2% in the treatment group *vs* placebo, respectively[127].

CONCLUSION

The clinical course of the disease in IBD may change in the coming years with the evolution of the new therapies that are being studied at this time. Most of these new therapies are in advanced phases of study with promising results, with similar response rates to currently approved therapies. The purpose of these new therapeutic targets will allow us to personalize medicine to treat IBD, according to the characteristic pathogenesis of each patient. More studies are needed to verify their efficacy and safety, as well as studies comparing these therapies with emerging or approved therapies to have accurate results.

REFERENCES

1 **Roda G**, Jharap B, Neeraj N, Colombel JF. Loss of Response to Anti-TNFs: Definition, Epidemiology, and Management. *Clin Transl Gastroenterol* 2016; **7**: e135 [PMID: 26741065 DOI: 10.1038/ctg.2015.63]

2 **Xavier RJ**, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 2007; **448**: 427-434 [PMID: 17653185 DOI: 10.1038/nature06005]

3 **Nascimento Santos L**, Carvalho Pacheco LG, Silva Pinheiro C, Alcantara-Neves NM. Recombinant proteins of helminths with immunoregulatory properties and their possible therapeutic use. *Acta Trop* 2017; **166**: 202-211 [PMID: 27871775 DOI: 10.1016/j.actatropica.2016.11.016]

4 **Burton RE**, Kim S, Patel R, Hartman DS, Tracey DE, Fox BS. Structural features of bovine colostral immunoglobulin that confer proteolytic stability in a simulated intestinal fluid. *J Biol Chem* 2020; **295**: 12317-12327 [PMID: 32665404 DOI: 10.1074/jbc.RA120.014327]

5 **Harris MS**, Hartman D, Lemos BR, Erlich EC, Spence S, Kennedy S, Ptak T, Pruitt R, Vermeire S, Fox BS. AVX-470, an Orally Delivered Anti-Tumour Necrosis Factor Antibody for Treatment of Active Ulcerative Colitis: Results of a First-in-Human Trial. *J Crohns Colitis* 2016; **10**: 631-640 [PMID: 26822613 DOI: 10.1093/ecco-jcc/jjw036]

6 **Dotan I**, Allez M, Danese S, Keir M, Tole S, McBride J. The role of integrins in the pathogenesis of inflammatory bowel disease: Approved and investigational anti-integrin therapies. *Med Res Rev* 2020; **40**: 245-262 [PMID: 31215680 DOI: 10.1002/med.21601]

7 **Slack RJ**, Macdonald SJF, Roper JA, Jenkins RG, Hatley RJD. Emerging therapeutic opportunities for integrin inhibitors. *Nat Rev Drug Discov* 2021 [PMID: 34535788 DOI: 10.1038/s41573-021-00284-4]

8 **Vermeire S**, Sandborn WJ, Danese S, Hébuterne X, Salzberg BA, Klopocka M, Tarabar D, Vanasek T, Greguš M, Hellstern PA, Kim JS, Sparrow MP, Gorelick KJ, Hinz M, Ahmad A, Pradhan V, Hassan-Zahraee M, Clare R, Cataldi F, Reinisch W. Anti-MAdCAM antibody (PF-00547659) for ulcerative colitis (TURANDOT): a phase 2, randomised, double-blind, placebo-controlled trial. *Lancet* 2017; **390**: 135-144 [PMID: 28527704 DOI: 10.1016/S0140-6736(17)30930-3]

9 **Shire**. Long-Term Safety of PF-00547659 In Ulcerative Colitis (TURANDOT II). [accessed 2020 Dec 27]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT01771809 ClinicalTrials.gov Identifier: NCT01771809

10 **Takeda**. A Safety Extension Study of Ontamalimab in Participants With Moderate to Severe Ulcerative Colitis or Crohn's Disease (AIDA). [accessed 2020 Dec 27]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03283085 ClinicalTrials.gov Identifier: NCT03283085

11 **Saruta M**, Park DI, Kim YH, Yang SK, Jang BI, Cheon JH, Im JP, Kanai T, Katsuno T, Ishiguro Y, Nagaoka M, Isogawa N, Li Y, Banerjee A, Ahmad A, Hassan-Zahraee M, Clare R, Gorelick KJ, Cataldi F, Watanabe M, Hibi T. Anti-MAdCAM-1 antibody (PF-00547659) for active refractory Crohn's disease in Japanese and Korean patients: the OPERA study. *Intest Res* 2020; **18**: 45-55 [PMID: 32013314 DOI: 10.5217/ir.2019.00039]

12 **Takeda**. Efficacy and Safety Study of Ontamalimab as Induction Therapy in Participants With Moderate to Severe Crohn's Disease (CARMEN CD 306) (CARMEN CD 306). [accessed 2020 Dec 27]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03566823 ClinicalTrials.gov Identifier: NCT03566823

13 **Vermeire S**, O'Byrne S, Keir M, Williams M, Lu TT, Mansfield JC, Lamb CA, Feagan BG, Panes J, Salas A, Baumgart DC, Schreiber S, Dotan I, Sandborn WJ, Tew GW, Luca D, Tang MT, Diehl L, Eastham-Anderson J, De Hertogh G, Perrier C, Egen JG, Kirby JA, van Assche G, Rutgeerts P. Etrolizumab as induction therapy for ulcerative colitis: a randomised, controlled, phase 2 trial. *Lancet* 2014; **384**: 309-318 [PMID: 24814090 DOI: 10.1016/S0140-6736(14)60661-9]

14 **Sandborn WJ**, Vermeire S, Tyrrell H, Hassanali A, Lacey S, Tole S, Tatro AR; Etrolizumab Global Steering Committee. Etrolizumab for the Treatment of Ulcerative Colitis and Crohn's Disease: An Overview of the Phase 3 Clinical Program. *Adv Ther* 2020; **37**: 3417-3431 [PMID: 32445184 DOI: 10.1007/s12325-020-01366-2]

15 **Pan WJ**, Köck K, Rees WA, Sullivan BA, Evangelista CM, Yen M, Andrews JM, Radford-Smith GL, Prince PJ, Reynhardt KO, Doherty DR, Patel SK, Krill CD, Zhou K, Shen J, Smith LE, Gow JM, Lee J, Treacy AM, Yu Z, Platt VM, Borie DC. Clinical pharmacology of AMG 181, a gut-specific human anti-α4β7 monoclonal antibody, for treating inflammatory bowel diseases. *Br J Clin Pharmacol* 2014; **78**: 1315-1333 [PMID: 24803302 DOI: 10.1111/bcp.12418]

16 **Hibi T**, Motoya S, Ashida T, Sai S, Sameshima Y, Nakamura S, Maemoto A, Nii M, Sullivan BA, Gasser RA Jr, Suzuki Y. Efficacy and safety of abrilumab, an α4β7 integrin inhibitor, in Japanese patients with moderate-to-severe ulcerative colitis: a phase II study. *Intest Res* 2019; **17**: 375-386 [PMID: 30739435 DOI: 10.5217/ir.2018.00141]

17 **Amgen**. Abrilumab (AMG 181) in Adults With Moderate to Severe Crohn´s Disease. [accessed 2020 Dec 27]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/results/NCT01696396 ClinicalTrials.gov Identifier: NCT01696396

18 **Sugiura T**, Kageyama S, Andou A, Miyazawa T, Ejima C, Nakayama A, Dohi T, Eda H. Oral treatment with a novel small molecule alpha 4 integrin antagonist, AJM300, prevents the

development of experimental colitis in mice. *J Crohns Colitis* 2013; 7: e533-42 [PMID: 23623333 DOI: 10.1016/j.crohns.2013.03.014]

19 **Yoshimura N**, Watanabe M, Motoya S, Tominaga K, Matsuoka K, Iwakiri R, Watanabe K, Hibi T; AJM300 Study Group. Safety and Efficacy of AJM300, an Oral Antagonist of α4 Integrin, in Induction Therapy for Patients With Active Ulcerative Colitis. *Gastroenterology* 2015; **149**: 1775-1783.e2 [PMID: 26327130 DOI: 10.1053/j.gastro.2015.08.044]

20 **Eisai Inc**. A Study to Evaluate the Safety and Efficacy of AJM300 in Participants with Active Ulcerative Colitis. [accessed 2020 Dec 27]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03531892 ClinicalTrials.gov Identifier: NCT03531892

21 **Misselwitz B**, Juillerat P, Sulz MC, Siegmund B, Brand S; Swiss IBDnet, an official working group of the Swiss Society of Gastroenterology. Emerging Treatment Options in Inflammatory Bowel Disease: Janus Kinases, Stem Cells, and More. *Digestion* 2020; **101** Suppl 1: 69-82 [PMID: 32570252 DOI: 10.1159/000507782]

22 **Keshav S**, Vaňásek T, Niv Y, Petryka R, Howaldt S, Bafutto M, Rácz I, Hetzel D, Nielsen OH, Vermeire S, Reinisch W, Karlén P, Schreiber S, Schall TJ, Bekker P; Prospective Randomized Oral-Therapy Evaluation in Crohn's Disease Trial-1 PROTECT-1 Study Group. A randomized controlled trial of the efficacy and safety of CCX282-B, an orally-administered blocker of chemokine receptor CCR9, for patients with Crohn's disease. *PLoS One* 2013; **8**: e60094 [PMID: 23527300 DOI: 10.1371/journal.pone.0060094]

23 **Feagan BG**, Sandborn WJ, D'Haens G, Lee SD, Allez M, Fedorak RN, Seidler U, Vermeire S, Lawrance IC, Maroney AC, Jurgensen CH, Heath A, Chang DJ. Randomised clinical trial: vercirnon, an oral CCR9 antagonist, vs. placebo as induction therapy in active Crohn's disease. *Aliment Pharmacol Ther* 2015; **42**: 1170-1181 [PMID: 26400458 DOI: 10.1111/apt.13398]

24 **Ragusa F**. Th1 chemokines in ulcerative colitis. *Clin Ter* 2015; **166**: e126-31 [PMID: 25945446 DOI: 10.7417/CT.2015.1835]

25 **Sandborn WJ**, Rutgeerts P, Colombel JF, Ghosh S, Petryka R, Sands BE, Mitra P, Luo A. Eldelumab [anti-interferon-γ-inducible protein-10 antibody] Induction Therapy for Active Crohn's Disease: a Randomised, Double-blind, Placebo-controlled Phase IIa Study. *J Crohns Colitis* 2017; **11**: 811-819 [PMID: 28333187 DOI: 10.1093/ecco-jcc/jjx005]

26 **Skovdahl HK**, Damås JK, Granlund AVB, Østvik AE, Doseth B, Bruland T, Mollnes TE, Sandvik AK. C-C Motif Ligand 20 (CCL20) and C-C Motif Chemokine Receptor 6 (CCR6) in Human Peripheral Blood Mononuclear Cells: Dysregulated in Ulcerative Colitis and a Potential Role for CCL20 in IL-1β Release. *Int J Mol Sci* 2018; **19** [PMID: 30347808 DOI: 10.3390/ijms19103257]

27 **Bouma G**, Zamuner S, Hicks K, Want A, Oliveira J, Choudhury A, Brett S, Robertson D, Felton L, Norris V, Fernando D, Herdman M, Tarzi R. CCL20 neutralization by a monoclonal antibody in healthy subjects selectively inhibits recruitment of $CCR6^+$ cells in an experimental suction blister. *Br J Clin Pharmacol* 2017; **83**: 1976-1990 [PMID: 28295451 DOI: 10.1111/bcp.13286]

28 **Barrett JC**, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, Brant SR, Silverberg MS, Taylor KD, Barmada MM, Bitton A, Dassopoulos T, Datta LW, Green T, Griffiths AM, Kistner EO, Murtha MT, Regueiro MD, Rotter JI, Schumm LP, Steinhart AH, Targan SR, Xavier RJ; NIDDK IBD Genetics Consortium, Libioulle C, Sandor C, Lathrop M, Belaiche J, Dewit O, Gut I, Heath S, Laukens D, Mni M, Rutgeerts P, Van Gossum A, Zelenika D, Franchimont D, Hugot JP, de Vos M, Vermeire S, Louis E; Belgian-French IBD Consortium; Wellcome Trust Case Control Consortium, Cardon LR, Anderson CA, Drummond H, Nimmo E, Ahmad T, Prescott NJ, Onnie CM, Fisher SA, Marchini J, Ghori J, Bumpstead S, Gwilliam R, Tremelling M, Deloukas P, Mansfield J, Jewell D, Satsangi J, Mathew CG, Parkes M, Georges M, Daly MJ. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet* 2008; **40**: 955-962 [PMID: 18587394 DOI: 10.1038/ng.175]

29 **Sedda S**, Bevivino G, Monteleone G. Targeting IL-23 in Crohn's disease. *Expert Rev Clin Immunol* 2018; **14**: 907-913 [PMID: 30223688 DOI: 10.1080/1744666X.2018.1524754]

30 **Fonseca-Camarillo G**, Mendivil EJ, Furuzawa-Carballeda J, Yamamoto-Furusho JK. Interleukin 17 gene and protein expression are increased in patients with ulcerative colitis. *Inflamm Bowel Dis* 2011; **17**: E135-E136 [PMID: 21761512 DOI: 10.1002/ibd.21816]

31 **Siakavellas SI**, Bamias G. Role of the IL-23/IL-17 axis in Crohn's disease. *Discov Med* 2012; **14**: 253-262 [PMID: 23114581]

32 **Feagan BG**, Sandborn WJ, D'Haens G, Panés J, Kaser A, Ferrante M, Louis E, Franchimont D, Dewit O, Seidler U, Kim KJ, Neurath MF, Schreiber S, Scholl P, Pamulapati C, Lalovic B, Visvanathan S, Padula SJ, Herichova I, Soaita A, Hall DB, Böcher WO. Induction therapy with the selective interleukin-23 inhibitor risankizumab in patients with moderate-to-severe Crohn's disease: a randomised, double-blind, placebo-controlled phase 2 study. *Lancet* 2017; **389**: 1699-1709 [PMID: 28411872 DOI: 10.1016/S0140-6736(17)30570-6]

33 **Feagan BG**, Panés J, Ferrante M, Kaser A, D'Haens GR, Sandborn WJ, Louis E, Neurath MF, Franchimont D, Dewit O, Seidler U, Kim KJ, Selinger C, Padula SJ, Herichova I, Robinson AM, Wallace K, Zhao J, Minocha M, Othman AA, Soaita A, Visvanathan S, Hall DB, Böcher WO. Risankizumab in patients with moderate to severe Crohn's disease: an open-label extension study. *Lancet Gastroenterol Hepatol* 2018; **3**: 671-680 [PMID: 30056030 DOI: 10.1016/S2468-1253(18)30233-4]

34 **AbbVie**. A Multicenter, Randomized, Double-Blind, Placebo Controlled Induction Study to

Evaluate the Efficacy and Safety of Risankizumab in Participants With Moderately to Severely Active Ulcerative Colitis. [accessed 2020 Dec 27]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://www.clinicaltrials.gov/ct2/show/NCT03398148 ClinicalTrials.gov Identifier: NCT03398148

35 **AbbVie**. A Study to Assess the Efficacy and Safety of Risankizumab in Participants With Ulcerative Colitis. [accessed 2020 Dec 27]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://www.clinicaltrials.gov/ct2/show/NCT03398135 ClinicalTrials.gov Identifier: NCT03398135

36 **AbbVie**. A Study of the Efficacy and Safety of Risankizumab in Participants With Crohn's Disease. [accessed 2020 Dec 27]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://www.clinicaltrials.gov/ct2/show/NCT03105128 ClinicalTrials.gov Identifier: NCT03105128

37 **Sandborn WJ**, Ferrante M, Bhandari BR, Berliba E, Hibi T, D'Haens GR, Tuttle JL, Krueger K, Friedrich S, Durante M, Arora V, Naegeli AN, Schmitz J, Feagan BG. Efficacy and Safety of Continued Treatment With Mirikizumab in a Phase 2 Trial of Patients With Ulcerative Colitis. *Clin Gastroenterol Hepatol* 2020 [PMID: 32950748 DOI: 10.1016/j.cgh.2020.09.028]

38 **Eli Lilly and Company**. An Induction Study of Mirikizumab in Participants With Moderately to Severely Active Ulcerative Colitis (LUCENT 1). [accessed 2020 Dec 28]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://www.clinicaltrials.gov/ct2/show/NCT03518086 ClinicalTrials.gov Identifier: NCT03518086

39 **Eli Lilly and Company**. A Study to Evaluate the Long-Term Efficacy and Safety of Mirikizumab in Participants With Moderately to Severely Active Ulcerative Colitis (LUCENT 3). [accessed 2020 Dec 28]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://www.clinicaltrials.gov/ct2/show/NCT03519945 ClinicalTrials.gov Identifier: NCT03519945

40 **Eli Lilly and Company**. A Study of Mirikizumab (LY3074828) in Participants With Crohn's Disease (VIVID-1). [accessed 2020 Dec 28]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://www.clinicaltrials.gov/ct2/show/NCT03926130 ClinicalTrials.gov Identifier: NCT03926130

41 **Sands BE**, Chen J, Feagan BG, Penney M, Rees WA, Danese S, Higgins PDR, Newbold P, Faggioni R, Patra K, Li J, Klekotka P, Morehouse C, Pulkstenis E, Drappa J, van der Merwe R, Gasser RA Jr. Efficacy and Safety of MEDI2070, an Antibody Against Interleukin 23, in Patients With Moderate to Severe Crohn's Disease: A Phase 2a Study. *Gastroenterology* 2017; **153**: 77-86.e6 [PMID: 28390867 DOI: 10.1053/j.gastro.2017.03.049]

42 **Allergan**. An Active and Placebo-Controlled Study of Brakizumab in Participants with Moderately to Severely Active Ulcerative Colitis [EXPEDITION]. [accessed 2020 Dec 28]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03616821 ClinicalTrials.gov Identifier: NCT0361682

43 **Allergan**. An Active and Placebo-Controlled Study of Brakizumab in Participant With Moderately to Severely Active Crohn´s Disease. [accessed 2020 Dec 29]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03759288 ClinicalTrials.gov Identifier: NCT03759288

44 **MacDonald JK**, Nguyen TM, Khanna R, Timmer A. Anti-IL-12/23p40 antibodies for induction of remission in Crohn's disease. *Cochrane Database Syst Rev* 2016; **11**: CD007572 [PMID: 27885650 DOI: 10.1002/14651858.CD007572]

45 **Janssen Research & Development**, LLC. A Study of Efficacy and Safety of Combination Therapy With Guselkumab and Golimumab in Participants With Moderately to Severely Active Ulcerative Colitis (VEGA). [accessed 2020 Dec 29]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03662542 ClinicalTrials.gov Identifier: NCT03662542

46 **Janssen Research & Development**, LLC. A Study of the Efficacy and Safety of Guselkumab in Participants With Moderately to Severely Active Crohn's Disease (GALAXI). [accessed 2020 Dec 29]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://www.clinicaltrials.gov/ct2/show/NCT0346641 ClinicalTrials.gov Identifier: NCT0346641

47 **Janssen Pharmaceutical K**. K. A Study of Guselkumab in Participants With Moderately to Severely Active Crohn's Disease. [accessed 2020 Dec 29]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: clinicaltrials.gov/ct2/show/NCT04397263 ClinicalTrials.gov Identifier: NCT04397263

48 **Mannon PJ**, Fuss IJ, Mayer L, Elson CO, Sandborn WJ, Present D, Dolin B, Goodman N, Groden C, Hornung RL, Quezado M, Yang Z, Neurath MF, Salfeld J, Veldman GM, Schwertschlag U, Strober W; Anti-IL-12 Crohn's Disease Study Group. Anti-interleukin-12 antibody for active Crohn's disease. *N Engl J Med* 2004; **351**: 2069-2079 [PMID: 15537905 DOI: 10.1056/NEJMoa033402]

49 **Panaccione R**, Sandborn WJ, Gordon GL, Lee SD, Safdi A, Sedghi S, Feagan BG, Hanauer S, Reinisch W, Valentine JF, Huang B, Carcereri R. Briakinumab for treatment of Crohn's disease: results of a randomized trial. *Inflamm Bowel Dis* 2015; **21**: 1329-1340 [PMID: 25989338 DOI: 10.1097/MIB.0000000000000366]

50 **Potagonist Therapeutics**, Inc. Pharmacokinetics and Pharmacodynamics of Differente PTG-300 Regimen in Healthy Volunteers. [accessed 2020 Dec 29]. In: ClinicalTrials.gov [Internet]. Bethseda

(MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT04516382 ClinicalTrials.gov Identifier: NCT04516382

51 **Janssen Research & Development**, LLC. A Study Evaluating Participants With Moderately to Severely Active Crohn's Disease (PRISM). [accessed 2020 Dec 29]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://www.clinicaltrials.gov/ct2/show/NCT04102111 ClinicalTrials.gov Identifier: NCT04102111

52 **de Souza HS**, Fiocchi C. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol* 2016; **13**: 13-27 [PMID: 26627550 DOI: 10.1038/nrgastro.2015.186]

53 **Hueber W**, Sands BE, Lewitzky S, Vandemeulebroecke M, Reinisch W, Higgins PD, Wehkamp J, Feagan BG, Yao MD, Karczewski M, Karczewski J, Pezous N, Bek S, Bruin G, Mellgard B, Berger C, Londei M, Bertolino AP, Tougas G, Travis SP; Secukinumab in Crohn's Disease Study Group. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut* 2012; **61**: 1693-1700 [PMID: 22595313 DOI: 10.1136/gutjnl-2011-301668]

54 **Ojeda Gómez A**, Madero Velázquez L, Buendía Sanchez L, Pascual Sánchez I, Pérez Rabasco E, García Monsalve A, González Ferrández JA, García Sepulcre MF. Inflammatory bowel disease new-onset during secukinumab therapy: real-world data from a tertiary center. *Rev Esp Enferm Dig* 2021 [PMID: 34696593 DOI: 10.17235/reed.2021.8397/2021]

55 **Wang J**, Bhatia A, Krugliak Cleveland N, Gupta N, Dalal S, Rubin DT, Sakuraba A. Rapid Onset of Inflammatory Bowel Disease after Receiving Secukinumab Infusion. *ACG Case Rep J* 2018; **5**: e56 [PMID: 30105273 DOI: 10.14309/crj.2018.56]

56 **Papp KA**, Reid C, Foley P, Sinclair R, Salinger DH, Williams G, Dong H, Krueger JG, Russell CB, Martin DA. Anti-IL-17 receptor antibody AMG 827 leads to rapid clinical response in subjects with moderate to severe psoriasis: results from a phase I, randomized, placebo-controlled trial. *J Invest Dermatol* 2012; **132**: 2466-2469 [PMID: 22622425 DOI: 10.1038/jid.2012.163]

57 **Targan SR**, Feagan B, Vermeire S, Panaccione R, Melmed GY, Landers C, Li D, Russell C, Newmark R, Zhang N, Chon Y, Hsu YH, Lin SL, Klekotka P. A Randomized, Double-Blind, Placebo-Controlled Phase 2 Study of Brodalumab in Patients With Moderate-to-Severe Crohn's Disease. *Am J Gastroenterol* 2016; **111**: 1599-1607 [PMID: 27481309 DOI: 10.1038/ajg.2016.298]

58 **Allocca M**, Jovani M, Fiorino G, Schreiber S, Danese S. Anti-IL-6 treatment for inflammatory bowel diseases: next cytokine, next target. *Curr Drug Targets* 2013; **14**: 1508-1521 [PMID: 24102406 DOI: 10.2174/13894501113146660224]

59 **Mitsuyama K**, Tomiyasu N, Suzuki A, Takaki K, Takedatsu H, Masuda J, Yamasaki H, Matsumoto S, Tsuruta O, Toyonaga A, Sata M. A form of circulating interleukin-6 receptor component soluble gp130 as a potential interleukin-6 inhibitor in inflammatory bowel disease. *Clin Exp Immunol* 2006; **143**: 125-131 [PMID: 16367943 DOI: 10.1111/j.1365-2249.2005.02960.x]

60 **Danese S**, Vermeire S, Hellstern P, Panaccione R, Rogler G, Fraser G, Kohn A, Desreumaux P, Leong RW, Comer GM, Cataldi F, Banerjee A, Maguire MK, Li C, Rath N, Beebe J, Schreiber S. Randomised trial and open-label extension study of an anti-interleukin-6 antibody in Crohn's disease (ANDANTE I and II). *Gut* 2019; **68**: 40-48 [PMID: 29247068 DOI: 10.1136/gutjnl-2017-314562]

61 **Mizoguchi A**, Yano A, Himuro H, Ezaki Y, Sadanaga T, Mizoguchi E. Clinical importance of IL-22 cascade in IBD. *J Gastroenterol* 2018; **53**: 465-474 [PMID: 29075900 DOI: 10.1007/s00535-017-1401-7]

62 **Zenewicz LA**, Yancopoulos GD, Valenzuela DM, Murphy AJ, Stevens S, Flavell RA. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. *Immunity* 2008; **29**: 947-957 [PMID: 19100701 DOI: 10.1016/j.immuni.2008.11.003]

63 **Yamamoto-Furusho JK**, Miranda-Pérez E, Fonseca-Camarillo G, Sánchez-Muñoz F, Dominguez-Lopez A, Barreto-Zuñiga R. Colonic epithelial upregulation of interleukin 22 (IL-22) in patients with ulcerative colitis. *Inflamm Bowel Dis* 2010; **16**: 1823 [PMID: 20222141 DOI: 10.1002/ibd.21235]

64 **Rothenberg ME**, Wang Y, Lekkerkerker A, Danilenko DM, Maciuca R, Erickson R, Herman A, Stefanich E, Lu TT. Randomized Phase I Healthy Volunteer Study of UTTR1147A (IL-22Fc): A Potential Therapy for Epithelial Injury. *Clin Pharmacol Ther* 2019; **105**: 177-189 [PMID: 29952004 DOI: 10.1002/cpt.1164]

65 A Study to Evaluate the Efficacy, Safety, and Pharmacokinetics of UTTR1147A Compared With Placebo and With Vedolizumab in Participants With Moderate to Severe Ulcerative Colitis (UC). [accessed 2020 Dec 29]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03558152 ClinicalTrials.gov Identifier: NCT03558152

66 **Lucaciu LA**, Seicean R, Seicean A. Small molecule drugs in the treatment of inflammatory bowel diseases: which one, when and why? *Eur J Gastroenterol Hepatol* 2020; **32**: 669-677 [PMID: 32282548 DOI: 10.1097/MEG.0000000000001730]

67 **Parmentier JM**, Voss J, Graff C, Schwartz A, Argiriadi M, Friedman M, Camp HS, Padley RJ, George JS, Hyland D, Rosebraugh M, Wishart N, Olson L, Long AJ. In vitro and in vivo characterization of the JAK1 selectivity of upadacitinib (ABT-494). *BMC Rheumatol* 2018; **2**: 23 [PMID: 30886973 DOI: 10.1186/s41927-018-0031-x]

68 **McInnes IB**, Byers NL, Higgs RE, Lee J, Macias WL, Na S, Ortmann RA, Rocha G, Rooney TP, Wehrman T, Zhang X, Zuckerman SH, Taylor PC. Comparison of baricitinib, upadacitinib, and tofacitinib mediated regulation of cytokine signaling in human leukocyte subpopulations. *Arthritis Res Ther* 2019; **21**: 183 [PMID: 31375130 DOI: 10.1186/s13075-019-1964-1]

69 **Sandborn WJ**, Ghosh S, Panes J, Schreiber S, D'Haens G, Tanida S, Siffledeen J, Enejosa J, Zhou W, Othman AA, Huang B, Higgins PDR. Efficacy of Upadacitinib in a Randomized Trial of Patients With Active Ulcerative Colitis. *Gastroenterology* 2020; **158**: 2139-2149.e14 [PMID: 32092309 DOI: 10.1053/j.gastro.2020.02.030]

70 **AbbVie**. A Study of the Efficacy and Safety of Upadacitnib (ABT-494) in Participants With Moderately to Severely Active Crohn's Disease Who Have Inadequately Responded to or Are Intolerant to Conventional and/or Biologic. [accessed 2020 Dec 29]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03345849 ClinicalTrials.gov Identifier: NCT03345849

71 **AbbVie**. A Study of the Efficacy and Safety of Upadacitinib (ABT-494) in Participants With Moderately to Severely Active Ulcerative Colitis (U-Accomplish). [accessed 2020 Dec 29]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03653026 ClinicalTrials.gov Identifier: NCT03653026

72 **Van Rompaey L**, Galien R, van der Aar EM, Clement-Lacroix P, Nelles L, Smets B, Lepescheux L, Christophe T, Conrath K, Vandeghinste N, Vayssiere B, De Vos S, Fletcher S, Brys R, van 't Klooster G, Feyen JH, Menet C. Preclinical characterization of GLPG0634, a selective inhibitor of JAK1, for the treatment of inflammatory diseases. *J Immunol* 2013; **191**: 3568-3577 [PMID: 24006460 DOI: 10.4049/jimmunol.1201348]

73 **Vermeire S**, Schreiber S, Petryka R, Kuehbacher T, Hebuterne X, Roblin X, Klopocka M, Goldis A, Wisniewska-Jarosinska M, Baranovsky A, Sike R, Stoyanova K, Tasset C, Van der Aa A, Harrison P. Clinical remission in patients with moderate-to-severe Crohn's disease treated with filgotinib (the FITZROY study): results from a phase 2, double-blind, randomised, placebo-controlled trial. *Lancet* 2017; **389**: 266-275 [PMID: 27988142 DOI: 10.1016/S0140-6736(16)32537-5]

74 **Sciences Gilead**. Filgotinib in the Induction and Maintenance of Remission in Adultos With Moderately to Severely Acrive Crohn´s Disease (Diversity 1). [accessed 2020 Dec 29]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02914561 ClinicalTrials.gov Identifier: NCT02914561

75 **Sciences Gilead**. Filgotinib in the Induction and Maintenance of Remission in Adultos With Moderately to Severely Active Ulcerative Colitis (SELECTION1). [accessed 2020 Dec 29]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02914522 ClinicalTrials.gov Identifier: NCT02914522

76 **Hamaguchi H**, Amano Y, Moritomo A, Shirakami S, Nakajima Y, Nakai K, Nomura N, Ito M, Higashi Y, Inoue T. Discovery and structural characterization of peficitinib (ASP015K) as a novel and potent JAK inhibitor. *Bioorg Med Chem* 2018; **26**: 4971-4983 [PMID: 30145050 DOI: 10.1016/j.bmc.2018.08.005]

77 **D'Amico F**, Fiorino G, Furfaro F, Allocca M, Danese S. Janus kinase inhibitors for the treatment of inflammatory bowel diseases: developments from phase I and phase II clinical trials. *Expert Opin Investig Drugs* 2018; **27**: 595-599 [PMID: 29938545 DOI: 10.1080/13543784.2018.1492547]

78 **Davies SC**, Hussein IM, Nguyen TM, Parker CE, Khanna R, Jairath V. Oral Janus kinase inhibitors for maintenance of remission in ulcerative colitis. *Cochrane Database Syst Rev* 2020; **1**: CD012381 [PMID: 31984480 DOI: 10.1002/14651858.CD012381.pub2]

79 **Biopharma Theravance**. Efficacy and Safety of TD-1473 in Crohn's Disease (DIONE). [accessed 2020 Dec 30]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03635112 ClinicalTrials.gov Identifier: NCT03635112

80 **Biopharma Theravance**. Efficacy & Safety of TD-1473 in Ulcerative Colitis (RHEA). [accessed 2020 Dec 30]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03758443 ClinicalTrials.gov Identifier: NCT03758443

81 **Ma C**, Battat R, Dulai PS, Parker CE, Sandborn WJ, Feagan BG, Jairath V. Innovations in Oral Therapies for Inflammatory Bowel Disease. *Drugs* 2019; **79**: 1321-1335 [PMID: 31317509 DOI: 10.1007/s40265-019-01169-y]

82 **Sandborn WJ**, Feagan BG, Wolf DC, D'Haens G, Vermeire S, Hanauer SB, Ghosh S, Smith H, Cravets M, Frohna PA, Aranda R, Gujrathi S, Olson A; TOUCHSTONE Study Group. Ozanimod Induction and Maintenance Treatment for Ulcerative Colitis. *N Engl J Med* 2016; **374**: 1754-1762 [PMID: 27144850 DOI: 10.1056/NEJMoa1513248]

83 **Celgene**. Efficacy and Safety Trial of RPC1063 for Moderate to Severe Croh´s Diseaase. [accessed 2020 Dec 30]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02531113 ClinicalTrials.gov Identifier: NCT02531113

84 **Celgene**. Safety and Efficacy Trial of RPC1063 for Moderate to Severe Ulcerative Colitis. [accessed 2020 Dec 30]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02435992 ClinicalTrials.gov Identifier: NCT02435992

85 **Celgene**. Induction Study #1 of Oral Oznimod as Induction Therapy for Moderately to Severely Active Crohn's Disease. [accessed 2020 Dec 30]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03440372 ClinicalTrials.gov Identifier: NCT03440372

86 **Buzard DJ**, Kim SH, Lopez L, Kawasaki A, Zhu X, Moody J, Thoresen L, Calderon I, Ullman B,

Han S, Lehmann J, Gharbaoui T, Sengupta D, Calvano L, Montalban AG, Ma YA, Sage C, Gao Y, Semple G, Edwards J, Barden J, Morgan M, Chen W, Usmani K, Chen C, Sadeque A, Christopher RJ, Thatte J, Fu L, Solomon M, Mills D, Whelan K, Al-Shamma H, Gatlin J, Le M, Gaidarov I, Anthony T, Unett DJ, Blackburn A, Rueter J, Stirn S, Behan DP, Jones RM. Discovery of APD334: Design of a Clinical Stage Functional Antagonist of the Sphingosine-1-phosphate-1 Receptor. *ACS Med Chem Lett* 2014; 5: 1313-1317 [PMID: 25516790 DOI: 10.1021/ml500389m]

87 **Sandborn WJ**, Peyrin-Biroulet L, Zhang J, Chiorean M, Vermeire S, Lee SD, Kühbacher T, Yacyshyn B, Cabell CH, Naik SU, Klassen P, Panés J. Efficacy and Safety of Etrasimod in a Phase 2 Randomized Trial of Patients With Ulcerative Colitis. *Gastroenterology* 2020; **158**: 550-561 [PMID: 31711921 DOI: 10.1053/j.gastro.2019.10.035]

88 **Pharmaceuticals Arena**. Etrasimod Versus Placebo for the Treatment of Moderately to Severely Active Ulcerative Colitis (ELEVATE UC 52). [accessed 2020 Dec 30]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT03945188 ClinicalTrials.gov Identifier: NCT03945188

89 **Shimano K**, Maeda Y, Kataoka H, Murase M, Mochizuki S, Utsumi H, Oshita K, Sugahara K. Amiselimod (MT-1303), a novel sphingosine 1-phosphate receptor-1 functional antagonist, inhibits progress of chronic colitis induced by transfer of CD4+CD45RBhigh T cells. *PLoS One* 2019; **14**: e0226154 [PMID: 31805144 DOI: 10.1371/journal.pone.0226154]

90 **Mitsubishi Tanabe Pharma Corporation**. Safety and Efficacy of MT-1303 in Subjects With Moderate to Severe Active Crohn´s Disease. [accessed 2020 Dec 30]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02378688 ClinicalTrials.gov Identifier: NCT02378688

91 **Radeke HH**, Stein J, Van Assche G, Rogler G, Lakatos PL, Muellershausen F, Moulin P, Jarvis P, Colin L, Gergely P, Kruis W. A Multicentre, Double-Blind, Placebo-Controlled, Parallel-Group Study to Evaluate the Efficacy, Safety, and Tolerability of the S1P Receptor Agonist KRP203 in Patients with Moderately Active Refractory Ulcerative Colitis. *Inflamm Intest Dis* 2020; **5**: 180-190 [PMID: 33313070 DOI: 10.1159/000509393]

92 **Brunmark C**, Runström A, Ohlsson L, Sparre B, Brodin T, Aström M, Hedlund G. The new orally active immunoregulator laquinimod (ABR-215062) effectively inhibits development and relapses of experimental autoimmune encephalomyelitis. *J Neuroimmunol* 2002; **130**: 163-172 [PMID: 12225898 DOI: 10.1016/s0165-5728(02)00225-4]

93 **D'Haens G**, Sandborn WJ, Colombel JF, Rutgeerts P, Brown K, Barkay H, Sakov A, Haviv A, Feagan BG; Laquinimod for Crohn's Disease Investigators. A phase II study of laquinimod in Crohn's disease. *Gut* 2015; **64**: 1227-1235 [PMID: 25281416 DOI: 10.1136/gutjnl-2014-307118]

94 **Sánchez-Muñoz F**, Fonseca-Camarillo G, Villeda-Ramírez MA, Miranda-Pérez E, Mendivil EJ, Barreto-Zúñiga R, Uribe M, Bojalil R, Domínguez-López A, Yamamoto-Furusho JK. Transcript levels of Toll-Like Receptors 5, 8 and 9 correlate with inflammatory activity in Ulcerative Colitis. *BMC Gastroenterol* 2011; **11**: 138 [PMID: 22185629 DOI: 10.1186/1471-230X-11-138]

95 **Obermeier F**, Hofmann C, Falk W. Inflammatory bowel diseases: when natural friends turn into enemies-the importance of CpG motifs of bacterial DNA in intestinal homeostasis and chronic intestinal inflammation. *Int J Inflam* 2010; **2010**: 641910 [PMID: 21188217 DOI: 10.4061/2010/641910]

96 **Kuznetsov NV**, Zargari A, Gielen AW, von Stein OD, Musch E, Befrits R, Lofberg R, von Stein P. Biomarkers can predict potential clinical responders to DIMS0150 a toll-like receptor 9 agonist in ulcerative colitis patients. *BMC Gastroenterol* 2014; **14**: 79 [PMID: 24758565 DOI: 10.1186/1471-230X-14-79]

97 **Atreya R**, Reinisch W, Peyrin-Biroulet L, Scaldaferri F, Admyre C, Knittel T, Kowalski J, Neurath MF, Hawkey C. Clinical efficacy of the Toll-like receptor 9 agonist cobitolimod using patient-reported-outcomes defined clinical endpoints in patients with ulcerative colitis. *Dig Liver Dis* 2018; **50**: 1019-1029 [PMID: 30120066 DOI: 10.1016/j.dld.2018.06.010]

98 **Atreya R**, Peyrin-Biroulet L, Klymenko A, Augustyn M, Bakulin I, Slankamenac D, Miheller P, Gasbarrini A, Hébuterne X, Arnesson K, Knittel T, Kowalski J, Neurath MF, Sandborn WJ, Reinisch W; CONDUCT study group. Cobitolimod for moderate-to-severe, left-sided ulcerative colitis (CONDUCT): a phase 2b randomised, double-blind, placebo-controlled, dose-ranging induction trial. *Lancet Gastroenterol Hepatol* 2020; **5**: 1063-1075 [PMID: 33031757 DOI: 10.1016/S2468-1253(20)30301-0]

99 **Dotan I**, Levy-Nissenbaum E, Chowers Y, Fich A, Israeli E, Adar T, Shteingart S, Soreq H, Goldin E. Ameliorating Active Ulcerative Colitis *via* an Orally Available Toll-Like Receptor-9 Modifier: A Prospective Open-Label, Multicenter Phase II Trial. *Dig Dis Sci* 2016; **61**: 3246-3254 [PMID: 27572942 DOI: 10.1007/s10620-016-4276-1]

100 **Stremmel W**, Hanemann A, Braun A, Stoffels S, Karner M, Fazeli S, Ehehalt R. Delayed release phosphatidylcholine as new therapeutic drug for ulcerative colitis--a review of three clinical trials. *Expert Opin Investig Drugs* 2010; **19**: 1623-1630 [PMID: 21105858 DOI: 10.1517/13543784.2010.535514]

101 **Stremmel W**, Hanemann A, Ehehalt R, Karner M, Braun A. Phosphatidylcholine (lecithin) and the mucus layer: Evidence of therapeutic efficacy in ulcerative colitis? *Dig Dis* 2010; **28**: 490-496 [PMID: 20926877 DOI: 10.1159/000320407]

102 **Karner M**, Kocjan A, Stein J, Schreiber S, von Boyen G, Uebel P, Schmidt C, Kupcinskas L, Dina I, Zuelch F, Keilhauer G, Stremmel W. First multicenter study of modified release

phosphatidylcholine "LT-02" in ulcerative colitis: a randomized, placebo-controlled trial in mesalazine-refractory courses. *Am J Gastroenterol* 2014; **109**: 1041-1051 [PMID: 24796768 DOI: 10.1038/ajg.2014.104]

103 **Dr**. Falk Pharma GmBh. Phosphatidylcholine (LT-02) vs. Placebo vs. Mesalamine for Maintenance of Remission in Ulcerative Colitis (PROTECT-2) (PROTECT-2). [accessed 2020 Dec 30]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://www.clinicaltrials.gov/ct2/show/NCT02280629 ClinicalTrials.gov Identifier: NCT02280629

104 **Prometheus Labratories**. A Study to Investigate the Safety and Efficacy of LT-02 in Patients With Mesalamine Refractory Ulcerative Colitis (UC) (PROTECT-3). [accessed 2020 Dec 30]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://www.clinicaltrials.gov/ct2/show/NCT02849951 ClinicalTrials.gov Identifier: NCT02849951

105 **Schafer P**. Apremilast mechanism of action and application to psoriasis and psoriatic arthritis. *Biochem Pharmacol* 2012; **83**: 1583-1590 [PMID: 22257911 DOI: 10.1016/j.bcp.2012.01.001]

106 **Schafer PH**, Parton A, Capone L, Cedzik D, Brady H, Evans JF, Man HW, Muller GW, Stirling DI, Chopra R. Apremilast is a selective PDE4 inhibitor with regulatory effects on innate immunity. *Cell Signal* 2014; **26**: 2016-2029 [PMID: 24882690 DOI: 10.1016/j.cellsig.2014.05.014]

107 **Danese S**, Neurath MF, Kopoń A, Zakko SF, Simmons TC, Fogel R, Siegel CA, Panaccione R, Zhan X, Usiskin K, Chitkara D. Effects of Apremilast, an Oral Inhibitor of Phosphodiesterase 4, in a Randomized Trial of Patients With Active Ulcerative Colitis. *Clin Gastroenterol Hepatol* 2020; **18**: 2526-2534.e9 [PMID: 31926340 DOI: 10.1016/j.cgh.2019.12.032]

108 **Monteleone G**, Boirivant M, Pallone F, MacDonald TT. TGF-beta1 and Smad7 in the regulation of IBD. *Mucosal Immunol* 2008; **1** Suppl 1: S50-S53 [PMID: 19079231 DOI: 10.1038/mi.2008.55]

109 **Coskun M**, Vermeire S, Nielsen OH. Novel Targeted Therapies for Inflammatory Bowel Disease. *Trends Pharmacol Sci* 2017; **38**: 127-142 [PMID: 27916280 DOI: 10.1016/j.tips.2016.10.014]

110 **Monteleone G**, Kumberova A, Croft NM, McKenzie C, Steer HW, MacDonald TT. Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease. *J Clin Invest* 2001; **108**: 601-609 [PMID: 11518734 DOI: 10.1172/JCI12821]

111 **Scarozza P**, Schmitt H, Monteleone G, Neurath MF, Atreya R. Oligonucleotides-A Novel Promising Therapeutic Option for IBD. *Front Pharmacol* 2019; **10**: 314 [PMID: 31068803 DOI: 10.3389/fphar.2019.00314]

112 **Monteleone G**, Fantini MC, Onali S, Zorzi F, Sancesario G, Bernardini S, Calabrese E, Viti F, Monteleone I, Biancone L, Pallone F. Phase I clinical trial of Smad7 knockdown using antisense oligonucleotide in patients with active Crohn's disease. *Mol Ther* 2012; **20**: 870-876 [PMID: 22252452 DOI: 10.1038/mt.2011.290]

113 **Monteleone G**, Neurath MF, Ardizzone S, Di Sabatino A, Fantini MC, Castiglione F, Scribano ML, Armuzzi A, Caprioli F, Sturniolo GC, Rogai F, Vecchi M, Atreya R, Bossa F, Onali S, Fichera M, Corazza GR, Biancone L, Savarino V, Pica R, Orlando A, Pallone F. Mongersen, an oral SMAD7 antisense oligonucleotide, and Crohn's disease. *N Engl J Med* 2015; **372**: 1104-1113 [PMID: 25785968 DOI: 10.1056/NEJMoa1407250]

114 **Ardizzone S**, Bevivino G, Monteleone G. Mongersen, an oral Smad7 antisense oligonucleotide, in patients with active Crohn's disease. *Therap Adv Gastroenterol* 2016; **9**: 527-532 [PMID: 27366221 DOI: 10.1177/1756283X16636781]

115 **Bevivino G**, Sedda S, Marafini I, Monteleone G. Oligonucleotide-Based Therapies for Inflammatory Bowel Disease. *BioDrugs* 2018; **32**: 331-338 [PMID: 29948918 DOI: 10.1007/s40259-018-0286-1]

116 **Corren J**. New Targeted Therapies for Uncontrolled Asthma. *J Allergy Clin Immunol Pract* 2019; **7**: 1394-1403 [PMID: 31076057 DOI: 10.1016/j.jaip.2019.03.022]

117 **Tindemans I**, Serafini N, Di Santo JP, Hendriks RW. GATA-3 function in innate and adaptive immunity. *Immunity* 2014; **41**: 191-206 [PMID: 25148023 DOI: 10.1016/j.immuni.2014.06.006]

118 **Ray K**. IBD: A role for GATA3 in ulcerative colitis. *Nat Rev Gastroenterol Hepatol* 2016; **13**: 624 [PMID: 27703228 DOI: 10.1038/nrgastro.2016.163]

119 **Sterna Biologicals GmbH & Co**. KG. Efficacy, Pharmacokinetics, Tolerability, Safety of SB012 Intrarectally Applied in Active Ulcerative Colitis Patients (SECURE). [accessed 2020 Dec 30]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://clinicaltrials.gov/ct2/show/NCT02129439 ClinicalTrials.gov Identifier: NCT02129439

120 **Suzuki K**, Arumugam S, Yokoyama J, Kawauchi Y, Honda Y, Sato H, Aoyagi Y, Terai S, Okazaki K, Suzuki Y, Mizumoto S, Sugahara K, Atreya R, Neurath MF, Watanabe K, Hashiguchi T, Yoneyama H, Asakura H. Pivotal Role of Carbohydrate Sulfotransferase 15 in Fibrosis and Mucosal Healing in Mouse Colitis. *PLoS One* 2016; **11**: e0158967 [PMID: 27410685 DOI: 10.1371/journal.pone.0158967]

121 **Suzuki K**, Yokoyama J, Kawauchi Y, Honda Y, Sato H, Aoyagi Y, Terai S, Okazaki K, Suzuki Y, Sameshima Y, Fukushima T, Sugahara K, Atreya R, Neurath MF, Watanabe K, Yoneyama H, Asakura H. Phase 1 Clinical Study of siRNA Targeting Carbohydrate Sulphotransferase 15 in Crohn's Disease Patients with Active Mucosal Lesions. *J Crohns Colitis* 2017; **11**: 221-228 [PMID: 27484097 DOI: 10.1093/ecco-jcc/jjw143]

122 **Atreya R**, Kühbacher T, Waldner MJ, Hirschmann S, Drvarov O, Abu Hashem R, Maaser C, Kucharzik T, Dinter J, Schramm C, Mertens J, Holler B, Mössner J, Suzuki K, Yokoyama J, Terai S, Yoneyama H, Asakura H, Hibi T, Neurath MF. DOP073 Submucosal injection of the oligonucleotide STNM01 is able to induce clinical remission, mucosal healing and histological response in left-sided ulcerative colitis patients with moderate-to-severe disease. *J Crohn's Colitis*

2017; **11**: S69 [DOI: 10.1093/ecco-jcc/jjx002.110]

123 **Jairath V**, Khanna R, Feagan BG. Alicaforsen for the treatment of inflammatory bowel disease. *Expert Opin Investig Drugs* 2017; **26**: 991-997 [PMID: 28670932 DOI: 10.1080/13543784.2017.1349753]

124 **Greuter T**, Vavricka SR, Biedermann L, Pilz J, Borovicka J, Seibold F, Sauter B, Rogler G. Alicaforsen, an Antisense Inhibitor of Intercellular Adhesion Molecule-1, in the Treatment for Left-Sided Ulcerative Colitis and Ulcerative Proctitis. *Dig Dis* 2018; **36**: 123-129 [PMID: 29207381 DOI: 10.1159/000484979]

125 **van Deventer SJ**, Wedel MK, Baker BF, Xia S, Chuang E, Miner PB Jr. A phase II dose ranging, double-blind, placebo-controlled study of alicaforsen enema in subjects with acute exacerbation of mild to moderate left-sided ulcerative colitis. *Aliment Pharmacol Ther* 2006; **23**: 1415-1425 [PMID: 16669956 DOI: 10.1111/j.1365-2036.2006.02910.x]

126 **Greuter T**, Biedermann L, Rogler G, Sauter B, Seibold F. Alicaforsen, an antisense inhibitor of ICAM-1, as treatment for chronic refractory pouchitis after proctocolectomy: A case series. *United European Gastroenterol J* 2016; **4**: 97-104 [PMID: 26966529 DOI: 10.1177/2050640615593681]

127 **Atlantic Pharmaceuticals Ltd**. Efficacy of Alicaforsen in Pouchitis Patients Who Have Failed to Respond to at Least One Course of Antibiotics. [accessed 2020 Dec 30]. In: ClinicalTrials.gov [Internet]. Bethseda (MD): U.S. National Library of Medicine. Available from: https://www.clinicaltrials.gov/ct2/show/NCT02525523 ClinicalTrials.gov Identifier: NCT02525523

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 December 28; 27(48): 8262-8282

DOI: 10.3748/wjg.v27.i48.8262

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

REVIEW

Issues of origin, morphology and clinical significance of tumor microvessels in gastric cancer

Marina A Senchukova

ORCID number: Marina A Senchukova 0000-0001-8371-740X.

Author contributions: Senchukova MA solely contributed to this paper.

Conflict-of-interest statement: The author declares no conflict of interests for this article.

Country/Territory of origin: Russia

Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Marina A Senchukova, Department of Oncology, Orenburg State Medical University, Orenburg 460021, Russia

Corresponding author: Marina A Senchukova, MD, PhD, Professor, Department of Oncology, Orenburg State Medical University, Sovetskaya Street, Orenburg 460021, Russia.
masenchukova@yandex.com

Abstract

Gastric cancer (GC) remains a serious oncological problem, ranking third in the structure of mortality from malignant neoplasms. Improving treatment outcomes for this pathology largely depends on understanding the pathogenesis and biological characteristics of GC, including the identification and characterization of diagnostic, prognostic, predictive, and therapeutic biomarkers. It is known that the main cause of death from malignant neoplasms and GC, in particular, is tumor metastasis. Given that angiogenesis is a critical process for tumor growth and metastasis, it is now considered an important marker of disease prognosis and sensitivity to anticancer therapy. In the presented review, modern concepts of the mechanisms of tumor vessel formation and the peculiarities of their morphology are considered; data on numerous factors influencing the formation of tumor microvessels and their role in GC progression are summarized; and various approaches to the classification of tumor vessels, as well as the methods for assessing angiogenesis activity in a tumor, are highlighted. Here, results from studies on the prognostic and predictive significance of tumor microvessels in GC are also discussed, and a new classification of tumor microvessels in GC, based on their morphology and clinical significance, is proposed for consideration.

Key Words: Gastric cancer; Angiogenesis; Tumor microvessels; Vascular endothelial growth factor; Hypoxia; Prognosis

Core Tip: In this review, data on the factors associated with the activation of angiogenesis in tumors, the mechanisms of tumor microvessel formation and the features of their morphology, methods for assessing the activity of angiogenesis in a tumor, and their role in the progression of gastric cancer (GC) are discussed. A new

Received: March 25, 2021
Peer-review started: March 25, 2021
First decision: June 26, 2021
Revised: July 2, 2021
Accepted: December 7, 2021
Article in press: December 7, 2021
Published online: December 28, 2021

P-Reviewer: da Costa AC
S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H

classification of tumor microvessels in GC based on their morphology and clinical significance is proposed. Considering the different types of tumor microvessels can have different sensitivities to antiangiogenic therapy, further study of their prognostic and predictive value is undoubtedly relevant.

Citation: Senchukova MA. Issues of origin, morphology and clinical significance of tumor microvessels in gastric cancer. *World J Gastroenterol* 2021; 27(48): 8262-8282
URL: https://www.wjgnet.com/1007-9327/full/v27/i48/8262.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i48.8262

INTRODUCTION

Gastric cancer (GC) remains a serious oncological problem, ranking third in the structure of mortality from malignant neoplasms. The disease is biologically heterogeneous, and the oncogenic mechanisms remain poorly understood[1-3]. In this regard, a deep understanding of the pathogenesis and biological characteristics of GC, including the identification and characterization of diagnostic, prognostic, predictive, and therapeutic biomarkers, is important to improve the results of treatment.

Angiogenesis is a critical process for tumor growth and metastasis, including in GC. Currently, its assessment is considered an important marker of disease prognosis and sensitivity to anticancer therapy[4-9]. The study of angiogenesis is of fundamental importance, not only in terms of predicting disease outcome but also in determining tumor sensitivity to systemic therapy, such as chemotherapy, targeted therapy, and antiangiogenic therapy. In this case, not only is a quantitative assessment of angiogenesis of great importance but also an assessment of the functional adequacy of vessels, in view of the fact that vessels are the pathways for the delivery of anticancer drugs to tumor cells. In connection with the above, this review will discuss modern concepts of the mechanisms of tumor vessel formation and the peculiarities of their morphology, various approaches to the classification of tumor vessels and methods for assessing angiogenesis activity in tumors, and the results of studies on the prognostic and predictive significance of tumor microvessels in GC. Additionally, a new classification of tumor microvessels in GC, based on their morphology and clinical significance, is proposed for consideration.

ACTIVATION FACTORS OF TUMOR ANGIOGENESIS

Vascular endothelial growth factor

The formation of new vessels is associated with the activation of various factors, and among them, vascular endothelial growth factor (VEGF), which is expressed by tumor cells, immune cells, tumor-associated fibroblasts, and endothelial cells (ECs), plays a special role. There are five subtypes of VEGF family proteins, namely, VEGF-A, -B, -C, -D, and placental growth factor, among which VEGF-A is a key protein responsible for the proliferation, survival, and mobilization of endothelial progenitor cells from the bone marrow into the peripheral circulation, as well as for the increased permeability of tumor vessels, which is important for the formation of tumor stroma[10-12]. VEGF-A affects the development of new blood vessels and survival of immature blood vessels[13], while VEGF-C and VEGF-D stimulate the formation, proliferation, and germination of lymphatic ECs[14]. It is believed that ECs of existing lymphatic vessels, bone marrow cells, myeloid progenitors, and finally differentiated macrophages can participate in the formation of tumor lymphatic vessels[15,16].

VEGF signaling is mediated through membrane tyrosine kinase receptors (VEGFR-1, -2 and -3) located on tumor cells and ECs[11,17,18], which leads to the activation of signal transducer and activator of transcription 3 (STAT3), phosphoinositide 3-kinase, extracellular signal-regulated kinase (ERK)/protein kinase B (AKT) and other signaling pathways[8,11,18,19]. An increase in VEGF expression attracts monocytes and macrophages to the tumor stroma, which promotes the activation of matrix metalloproteinases (MMPs) and cell adhesion molecules[20-23] to function in the degradation of the extracellular matrix and initiation of the processes of invasion,

metastasis, and angiogenesis[24-26]. Along the invasive edge of the tumor, the active processes of formation and lysis of the extracellular matrix components proceed, which leads to the formation of channels that facilitate the formation of blood vessels, invasion, and metastasis of tumor cells[27].

Hypoxia

The most powerful stimulant of tumor angiogenesis is hypoxia, which is constantly experienced by cells of growing neoplasms under conditions of insufficient blood supply. One of the key transcription factors responsible for the regulation of gene expression during hypoxia and ischemia is hypoxia-inducible factor-1 alpha (HIF-1α). HIF-1α expression is regulated by the activation of the nuclear factor-kappa B (NF-κB)/HIF-1α/VEGF pathway[28]. Thus, HIF-1α is the main regulator of transcription in the adaptive response to hypoxia, directly participating in the activation of the mechanisms of angiogenesis, invasion, and metastasis of malignant neoplasms, including GC[29].

It has been established that hypoxia can stimulate cells to secrete more exosomes and extracellular vesicles[30,31], containing pro-angiogenic cytokines[30]. Extracellular vesicles originating from cancer cells, under hypoxic conditions, directly transport VEGF or activate the VEGF pathway in ECs, which leads to tumor angiogenesis[31].

Modern technologies of RNA sequencing (RNAseq) have made it possible to create a complete annotation of microRNAs (miRNAs), which are expressed by two-dimensional cultured human ECs under normal[32] or hypoxic[33] conditions. It has been shown that miR-130a is a mediator of the hypoxic response in human primary endothelial colony-forming cells. Under hypoxic conditions of 1% O_2, an increase in the expression and biological activity of miR-130a in ECs was observed, which led to the activation of VEGFR2 and STAT3 and the accumulation of HIF-1α. As a result, there was an increase in the clonogenic potential, proliferative and migratory capacity, and survival of ECs, as well as their ability for two-dimensional migration and tubulogenesis. EC tubulogenesis is also facilitated by the expression of miR-210 associated with hypoxia[34]. Interestingly, under conditions of normoxia, overexpression of miR-130a does not cause such effects[35].

It is important to note that HIF-1α can directly regulate the expression of many molecules associated with vasculogenic mimicry (VM), such as VEGF, twist-related protein, MMP2, and others[36]. The hypoxic microenvironment promotes VM by enhancing the differentiation of cancer stem cells, activating epithelial-endothelial transition (EMT), and remodeling the extracellular matrix[36,37].

In addition to VEGF and HIF-1α, many other proangiogenic factors are known. These include epidermal growth factor, main fibroblast growth factor, platelet growth factor, interleukin-1b (IL-1b), and hepatocyte growth factor (HGF), among others. Table 1 summarizes the role of the most studied factors associated with the activation of angiogenesis[38-67].

The role of exosomes and microRNAs in the regulation of angiogenesis

When assessing the role of various factors in angiogenesis activation, it is important to understand that exosomes are the main mediators of the cross-interaction of tumor cells with ECs, immune cells, fibroblasts, and other stromal cells. Exosomes are involved in the transport of numerous proangiogenic biomolecules, such as VEGF, MMP, microRNAs, and long noncoding RNAs, among others. In addition, exosomes promote angiogenesis by suppressing the expression of factor-inhibiting HIF-1[68].

Currently, miRNAs that both activate and suppress the expression of genes responsible for angiogenesis have been identified. The activation of angiogenesis during hypoxia is associated with the upregulation of miR-26, miR-130a, miR-130b, miR-126, and miR-210[69]. MiR-135b, delivered by exosomes from stomach tumors to ECs, suppresses the expression of the forkhead box O1 protein and promotes angiogenesis in GC[70]. Exosomal miR-155, obtained from GC cells, promotes VEGF expression and the formation of EC tubes. In human umbilical vein endothelial cell culture, miR-155 increases cell proliferation, migration, and ring formation[71]. An oncogenic, long noncoding RNA MALAT1 regulates the expression of VE-cadherin, β-catenin, MMP 2 and 9, MT1-MMP, p-ERK, p-focal adhesion kinase (FAK), and p-paxillin, which have been recognized as classic markers of VM and angiogenesis[72]. IL-1α mRNA enhances the metastatic potential of GC by activating the IL-1α/VEGF signaling pathways[73].

The number of miRNAs associated with angiogenesis suppression is usually reduced in GC patients[74,75]. For example, miR-590 has been shown to inhibit the migration, invasion, and proliferation of GC cells *in vivo* and *in vitro* by targeting VEGFR1/2[75]. Likewise, overexpression of miR-1 in GC cells inhibited proliferation,

Table 1 Factors associated with the activation of tumor angiogenesis

Factor	Signaling pathways	Effects	Ref.
EGF and EGFR	p38 MAPK, HIF-1α, VEGF	Enhanced angiogenesis, increased VEGF expression, and MMP-1	[38]
	EGFR	EMT activation	[39]
	PI3K/Akt/mTOR	EMT activation	[40]
	Notch and MAPK	Enhanced ECs proliferation, vascular growth and development, increased vascular permeability, inhibition of apoptosis	[41]
		Increased expression level in GC patients with peritoneal metastases	[42]
PIGF	VEGF/VEGFR	A high level of PIGF in plasma is associated with enhanced ECs proliferation and decreased survival of GC patients	[4]
Angs (Ang-1, -2, -3, -4)	Ang/Tie	The formation of blood vessels from preexisting, maturation of blood vessels, migration, adhesion, and survival of ECs	[43]
		Plasma Ang-2 level correlated with liver metastases in patients with GC	[44]
		A high level of angiopoietin-like protein 2 in serum is associated with a high risk of early recurrence of GC	[45]
PDGF-β; PDGF-D; PDGF-BB and other		In the intestinal-type GC, higher MVD was correlated to overexpression, intensity, and proportion of PDGF-B, but not of VEGF-A. PDGF-B plays a more important role in angiogenesis in intestinal-type gastric carcinomas than VEGF-A	[46]
	STAT3, AKT, ERK1/2, mTOR and GSK-3β	PDGF-D promoted the migration, proliferation, adhesion, and tube formation of endothelial progenitor cells	[47]
	STAT3, AKT, ERK1/2, mTOR and GSK-3β	PDGF-BB could activate VEGF-A expression	[48]
		A high level of PDGFR-β gene expression in tumor is associated with decreased 5-year overall survival rate in GC patients	[49]
FGFs and FGFR	AKT and Notch	Increased VEGF expression	[50]
	Snail	The effect of FGF-1 on ECs culture is associated with overexpression of Snai1, increased expression of CD31, CD34, and VWF, and formation of tubes	[51]
	WNT and Twist1	EMT activation	[52]
		Serum FGF level was related to MVD, tumor size, infiltration degree, TNM staging, lymph node metastasis, and distant metastasis	[53]
		High levels of FGF2 expression in the tumor is associated with advanced TNM stage and decreased survival of GC patients	[54]
Tryptase	AKT and ERK, PAR-2 and MAPK	The density of mast cells positive to tryptase is associated MVD in GC patients	[55-57]
IL-8	Src/Vav2/Rac1/PAK1	Induction of expression of VEGF-A, VEGFR-1, and VEGFR-2; stimulation of proliferation, survival, and migration of ECs, activation of MMP production	[58]
		Stimulation of ECs migration	[59]
HER2		Expression of HER2 (2+ and 3+) in gastric tumors is associated with an increase in MVD	[60]
		Expression of HER2 in a tumor is associated with an increase in MVD and a decrease in the survival rate of GC patients	[61]
ITGAX	PI3k/Akt	Overexpression of ITGAX in HUVEC is associated with induction of VEGF-A and VEGFR-2 expression, enhanced HUVEC proliferation, migration, and tube	[62]

		formation, as well as promoted angiogenesis and ovarian tumor growth	
IGF2 and IGF1R		Enhances sprouting angiogenesis and affects tip cell phenotype	[63]
MCU		MCU was related with the activation of EMT mechanisms and HIF-1α and VEGF expression. High level of MCU expression in the tumor was associated with the advanced TNM stage and decreased survival of GC patients	[64]
Helicobacter pylori	Wnt/beta-catenin	VEGF and MVD levels were significantly higher in H. pylori-positive tissues	[65]
Epstein-Barr virus	PI3K/AKT/mTOR/HIF-1α	EBV is associated with the formation of vasculogenic mimicry	[66,67]

AKT: Protein kinase B; Ang: Angiopoietin; ECs: Endothelial cells; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; EMT: Epithelial-endothelial transition; ERK: Extracellular signal-regulated kinase; FGF: Fibroblast growth factor; FGFR: Fibroblast growth factor receptor; GC: Gastric cancer; HER2: Human epidermal growth factor receptor 2; HIF: Hypoxia-inducible factor; HUVEC: Human umbilical vein endothelial cells; IGF2: Insulin-like growth factor 2; IGF1R: Insulin-like growth factor 1 receptor; IL-8: Interleukin-8; ITGAX: Integrin alpha x; MAPK: Mitogen-activated protein kinase; MCU: Mitochondrial calcium uniporter; MMP: Matrix metalloproteinase; MVD: Microvessel density; PAR: Protease-activated receptor; PI3K: Phosphoinositide 3-kinase; PIGF: Placental grow factor; PDGF: Platelet-derived growth factors; STAT3: Signal transducer and activator of transcription 3; VEGF: Vascular endothelial growth factor; VWF: Von Willebrand factor.

migration, and formation of EC tubes by suppressing the expression of VEGF-A and endothelin 1[76].

KEY PROANGIOGENIC SIGNALING PATHWAYS

It has been established that proangiogenic and pro-oncogenic pathways are linked to each other. In this context, the activation of these signaling pathways leads to a cascade of interrelated events: proliferation and migration of tumors and ECs, antiapoptosis, EMT, invasion, and tumor metastasis[8]. The most studied proangiogenic and pro-oncogenic signaling pathways are STAT3 and NF-κB. The STAT3 signaling pathway induces angiogenesis by activating VEGF expression[77]. Activation of the signaling pathways can be mediated not only by hypoxia but also by the expression of the cytokines IL-17A and IL-6. For example, the activation of the transcription factor STAT3 by IL-17A promoted an increase in the expression of VEGF and microvessel density (MVD) and was associated with a deterioration in the prognosis of GC[78]. *In vitro* IL-6 increased the levels of JKA, STAT3, p-STAT3, and VEGF-C proteins in GC cells, promoting growth, invasion, and lymphangiogenesis in GC[79]. Macrophages treated with lipopolysaccharides induced the production of tumor necrosis factor (TNF)-α, IL-6, IL-1β, and IL-8 and promoted the activation of the NF-κB and STAT3 signaling pathways[80]. These data are of particular interest since they can contribute to understanding the mechanisms of angiogenesis activation and factors of GC progression in patients with *Helicobacter pylori* and Epstein-Barr virus infections[65-67]. Inhibition of STAT3 decreased VEGF expression[81]. At the same time, it should be noted that in a number of studies, there were no correlations between STAT3 activation and the expression levels of VEGF, HIF-1α, β-catenin, and MVD[82].

NF-κB belongs to a group of transcription factors that form homo and heterodimers and increase or suppress the expression of many genes[83]. NF-κB activation occurs in response to various stimuli, including growth factors, cytokines, hormones, and microbial and chemical compounds, and leads to the synthesis of proangiogenic factors, such as IL-1, IL-8, TNF, IL-6, VEGF, MMP-2, and MMP-9[31].

Signaling pathways associated with the activation of angiogenesis, invasion, EMT, and metastasis also include ITGB1/FAK[84], Wnt/β-catenin[85], NF-κB-MMP-9/VEGF[86], ERK/AKT[11], and other pathways. Knock down of these pathways leads to a decrease in angiogenesis and metastasis.

MECHANISMS OF TUMOR VESSEL FORMATION

It should be noted that the origin of tumor vessels is an important factor affecting their morphology, participation in tumor progression, and tumor sensitivity to antian-

giogenic therapy. Currently, several methods of angiogenesis formation have been described, while different types of pathological vascularization can be observed simultaneously in the tumor stroma[87-89].

Sprouting angiogenesis is the growth of new capillary vessels from pre-existing vessels. This type of angiogenesis is characteristic of all malignant neoplasms, and its routine assessment is carried out by determining the expression of VEGF and MVD in the tumor and adjacent tissues[57,90-94].

The formation of "endothelial sprouts" occurs in several stages and in close interaction with the components of the extracellular matrix. Under the influence of angiogenesis mediators, the basement membrane of the vessels is destabilized, and ECs acquire the ability to proliferate, migrate, and invade. The release of MMPs causes degradation of the basement membrane and leads to directed migration and proliferation of ECs, which differentiate into tip and stalk cells. Within the germinating capillaries, tip cells express high levels of VEGFR2. In response to VEGF, tip cells form characteristic protrusions (filopodia) that are rich in actin. As a result of the polarization of moving ECs, the lumen of the vessel is formed, after which remodeling and maturation occur due to the recruitment of pericytes and synthesis of a new basement membrane[95,96].

It should be noted that the shape and number of this type of vessel depend on the density and composition of the extracellular matrix[97,98], the formation of which is influenced by the permeability of newly formed vessels. Their abnormal permeability increases the density of stromal cells, which leads to an increase in tissue hypoxia and interstitial hypertension, which promotes the entry of cancer cells into the blood and their further spread to distant organs with the formation of metastases[99].

Intussusceptive angiogenesis, this type of angiogenesis is an intravascular process that is invisible under standard light microscopy. It consists of the formation of new capillaries due to the formation of a septum inside their lumen[100-102]. Despite the fact that at present, its role in tumor progression has not been adequately studied, in a number of works, it was noted that in the process of radiation therapy or antiangiogenic therapy, there is a "switch" from sprouting angiogenesis to intussusceptive angiogenesis. The authors believe that the described "switch" can explain the development of tumor resistance to therapy and continued tumor growth after termination of treatment[103,104]. In GC, this type of angiogenesis has not been studied.

Vasculogenesis is a de novo process of blood vessel formation involving progenitor ECs or angioblasts[105]. Its induction in the postnatal period may be due to tissue hypoxia associated with tissue damage or tumor growth. Under physiological conditions, progenitor ECs rest, but under the influence of hypoxia, growth factors, and cytokines, they leave the bone marrow and travel into the peripheral blood, acquiring the ability to circulate, proliferate, and differentiate into mature ECs involved in the formation of new vessels. A number of studies have shown that the number of progenitor ECs in the blood of cancer patients is significantly higher than that in healthy individuals[106,107], and their high content is associated with advanced stages and poor prognosis of the disease[108], including GC[109].

Vessel co-option is a nonangiogenic type of tumor vascularization in which cancer cells use pre-existing blood vessels instead of inducing new blood vessel formation [90]. Thus, the development of a tumor can proceed without the formation of new vessels due to co-option with the vessels of the organ and VM[110]. Currently, vessel co-option, in which the perivascular arrangement of tumor cells is observed[111], is considered the main mechanism for the development of chemoresistance in malignant neoplasms[112].

High endothelial venules (HEVs) are also an example of vessel co-option. HEVs are located in sentinel lymph nodes and serve as a gateway for cancer cells to enter the bloodstream, thereby facilitating distant metastases[87]. HEVs are postcapillary venules characterized by active lymphocyte trafficking and are usually observed in secondary lymphoid organs, excluding the spleen. They are detected using the HEV-specific antibody MECA-79, which is associated with adhesion and transendothelial migration of lymphocytes along the HEV wall[113]. HEVs have been identified in lymphoid infiltrates in breast, ovary, lung, colon, and other carcinomas. In breast cancer and melanoma, high HEV density has been associated with a favorable prognosis, possibly due to an increase in tumor-infiltrating lymphocytes (TILs) and their phenotypes[114,115]. In GC, the number of CD8+ TILs was significantly higher in the HEV-positive group of patients than in the HEV-negative group (P = 0.027), whereas the levels of Foxp3+ and CD20+ TILs did not depend on the presence of HEVs. Overall survival was significantly greater only in the CD8+ TILs- and HEV-positive group. The other combinations were not associated with the survival of

patients with GC[113]. However, in the CD8+ TILs and HEV-positive group, there were significantly fewer patients with lymph node metastases (45.7% and 68.0%, in the CD8+ TILs and HEV-positive group and CD8- TIL and HEV-negative group, respectively; $P = 0.048$). Therefore, it is not entirely clear whether this combination is a sign of a more favorable prognosis of GC or if an improvement in survival is associated with a lower node stage.

VM is the formation of a vessel-like network by tumor cells. This type of angiogenesis is closely associated with extracellular matrix deposition[116]. Originally, the term VM was used to describe the process by which tumor cells form a network of tubular structures with the ability to conduct fluids. Later, VM was understood as any fluid-conducting structures that do not contain ECs (that is, not blood vessels). It is believed that vasculogenesis occurs due to the ability of ECs to self-assemble into a three-dimensional vascular network under the influence of VEGF, FGF-2, and other activators of angiogenesis[117].

In addition to tumor cells, macrophages can take part in the formation of VM structures. Macrophages that form the vasculature have been found to express genes for a variety of cytokines, HIF-1α, and genes commonly associated with ECs, including PECAM-1, endoglin, VE-cadherin, and neuropilins-1, 2. In addition, during the cultivation of lymphatic ECs, tubule-like structures (tubulogenesis) were formed only when cocultivated with macrophages. Macrophages isolated from GC and from metastatic lymph nodes more intensively secrete lymphangiogenic factors, including inflammatory cytokines, MMPs, adhesion molecules, and VEGFs[118]. In GC, patients with PAS+ structures are predisposed to a higher histological class, metastases, distant relapses, and a decrease in overall and disease-free survival[119-121].

Interestingly, VM is associated with the overexpression of MMP-2, MMP-9, VEGF-A, and VEGFR-1 but not with VEGFR-2[122,123], while sprouting angiogenesis is characterized by the overexpression of MMP7, MMP9, and MMP13[124].

At the same time, a number of researchers have questioned the existence of VM in malignant tumors[125]. They argue that the PAS-positive structures observed in VM that do not contain ECs are nothing more than an "artifact", forming as a result of the unstable structure of the tumor endothelium and accumulation of blood originating from microbleeds[125,126]. The reason for the disagreement is believed to be the lack of reliable markers of BM until recently, and the presence of filamentous PAS+ structures in the tumor stroma does not always indicate that these structures are hollow structures capable of performing circulatory functions[116].

FEATURES OF TUMOR VESSELS

In evaluating angiogenesis in malignant growth, it should be considered that tumor vessels have some morphological features distinguishing them from normal vessels:

Tumor vessels are often located chaotically. Tortuosity, the formation of vascular rings and pathological partitions, abnormal arteriovenous shunts, and vascular lacunae are typical. The size of the vessels varies from severe dilatation to sharp narrowing, with possible alternation of expanded and constricted areas[127-129]. Tumor vasculature often has bidirectional blood flow[42,130].

Some authors have noted the absence of pericytes in tumor vessels, which are cells that are functionally related to the vascular endothelium and extremely important for the stabilization and maturation of vascular structures[131,132].

Tumor vessels (mainly of the capillary type) are characterized by increased proliferation of ECs and have impaired endothelial linings and discontinuous basal membranes and abnormal processes[133-135].

Tumor vessels are characterized by increased permeability, which plays an important role in the activation of tumor angiogenesis[99,136].

In the lumen of blood and lymph vessels of the tumor, tumor emboli are often observed, the presence of which is an unfavorable prognostic factor[137-142].

These features determine the oxygen heterogeneity of tumor tissue, which affects the growth and metastasis of malignant tumors[143], as well as the sensitivity of tumor cells to chemotherapy and radiation therapy[144].

RESULTS OF ANGIOGENESIS ACTIVITY ASSESSMENT IN GC

To assess the activity of angiogenesis, *in vitro* and *in vivo* models, as well as immunohistochemical and molecular genetic studies on clinical material, can be used[90,145,

146].

VEGF and VEGFR

Evaluation of the clinical significance of VEGF levels in the blood serum of GC patients showed that these signaling proteins can be used as prognostic, but not diagnostic, biomarkers[147]. Thus, the level of VEGF-C associated with lymphangiogenesis was significantly higher in the serum of GC patients than in the control group[148]. High VEGF-C levels were associated with poorly differentiated cancers, advanced stages, a higher density of lymphatic vessels in the tumor, and the presence of metastases to regional lymph nodes and distant organs[149,150]. In addition, high levels of the marker predicted a decrease in the survival rate of GC patients[148,149], especially in Caucasian patients[151]. However, in contrast, some authors noted lower serum levels of VEGF-C in patients with GC than in the control group[152].

A high level of VEGF-A and a low level of Ang-1 in serum were associated with a decrease in the overall survival of patients with GC, but the differences were not statistically significant. However, a 25% decrease in serum VEGF-A levels after two courses of chemotherapy (docetaxel, cisplatin, and fluorouracil), compared to baseline values, was associated with a better response to treatment and improved overall survival[4,153]. The predictive value of VEGF-A was also noted by other researchers [5]. At the same time, a high level of Ang-2 was associated with a decrease in the overall survival of patients with GC but did not predict the efficacy of bevacizumab alone or in combination with the initial VEGF level[154].

In tumor tissue, the level of VEGF-A expression positively correlated with tumor, node and metastasis (TNM) stage, tumor size, lymph node metastases, and lymphovascular invasion (LVI), as well as a decrease in overall survival[155]. Similar data were obtained by other authors[90-92]. In addition, a positive correlation of VEGF-A with the levels of circulating progenitor ECs and ECs was noted[91]. In turn, the level of VEGF-C expression in a tumor positively correlated with the presence of metastases, MVD, density of lymphatic vessels, and stage of GC but not with age, sex, or grade[156]. Interestingly, although no significant correlations were found between the levels of VEGF and VEGFR-2 expression in tumors, overexpression of VEGFR-2 was associated with a decrease in survival in intestinal GC but not in diffuse GC[157].

MVD

Evaluation of MVD is performed in vascular hotspots using panendothelial immunohistochemistry markers, such as von Willebrand factor, Ulex Europaeus, or antibodies against CD31, CD34 and, less commonly, VE-cadherin, αvβ3-integrin, CD105, or type IV collagen[158,159]. However, it should be noted that these markers do not allow differentiation between mature and immature vessels, which may be important for identifying vessel co-option[160]. In addition, interobserver variability in MVD scoring methods can affect study results, which can be reduced by applying strict scoring rules and consistent training of individual observers[161].

Comparative analysis of MVD in patients with normal gastric mucosa, gastric ulcers, and GC showed that MVD in GC was significantly higher than that in benign processes in the stomach. MVD also correlated with the expression of fibroblast activation protein (FAP) and HGF[53]. FAP, HGF, and MVD were significantly correlated with the depth of tumor invasion and TNM stage.

In GC, endocan-expressing MVD was associated with tumor size, Borrmann type, tumor differentiation, tumor invasion, lymph node metastases, TNM stage and VEGF and VEGFR2 expression. Patients with high levels of endocan-MVD had significantly lower overall survival[6]. Similar results in assessing MVD in patients with GC were obtained by other researchers[57,90,93,94]. However, in patients with a more aggressive diffuse type of GC, there was a decrease in the expression of MVD in the tumor compared with GC of the intestinal type, and this decrease was associated with advanced TNM stage of the disease. There were no differences in VEGF expression in GC of diffuse and intestinal types[162].

For the assessment of lymphatic vessel density, one should consider the fact that lymphatic vessels can play a dual role in malignant tumors[163,164] in that they can promote cancer metastasis, and their high density correlates with a decrease in patient survival[165,166]. Thus, in GC, high lymphatic vessel density was associated with metastases to the lymph nodes and LVI[9]. The presence of functional lymphatic vessels also enhances the antitumor immune response and facilitates the delivery of chemotherapeutic agents, enhancing their action[167,168]. Interestingly, in GC, vessels that stained for both the D2-40 antibody (a marker of lymphatic vessels) and factor VIII (a marker of blood vessels) were identified. The authors noted that MVD in the tumor was higher than in nontumor tissue, but there were no differences in MVD in mucosal

carcinoma and submucosa-invasive carcinoma tissues[169].

Expression of cancer stem cell markers

In GC, upregulated expression of CD44 and CD133 correlated with high TNM stage, high depth of invasion, lymph node metastasis, vascular invasion, distant metastasis, and poor five-year overall survival[170].

LVI and perineural invasion

When assessing LVI, it is important to exclude false-positive and false-negative cases of LVI, which is possible when using the immunohistochemical method of staining tumor tissue[171]. In a group of patients with LVI+/perineural invasion (PNI)+, the overall and relapse-free survival rates were significantly lower than in the group of patients who were LVI-/PNI-[137-140], including in patients with lymph node-negative GC[141,142] and in patients who received neoadjuvant chemotherapy[172]. Interestingly, adjuvant chemotherapy significantly improved overall and disease-free survival in PNI+ but not PNI- patients, and these results were not influenced by disease stage[173].

It is important to note that at present, extravascular mechanisms of tumor cell spread, including PNI, are being considered. Recently, the term angiotropism was introduced, which indicates the tendency of tumor cells to spread through continuous migration along the abluminal surfaces of vessels or other pathways to nearby or more distant sites without entering the vascular channels[174].

VM

In patients with GC, the presence of VM was associated with poor overall and disease-free survival, high tumor grade, advanced stage, lymph node metastasis, deep tumor invasion, and distant metastasis[94,120,123,175-177]. Positive correlations were found between VM and the expression of the stem cell markers CD133 and Lgr5. The cancer stem cells responsible for the formation of VM are believed to be able to determine the chemotherapy and radioresistance of malignant neoplasms[94,175-177].

In experimental oncology, the migration ability of ECs[178-180], the three-dimensional model for calculating MVD[181,182], methods of three-dimensional spheroids for EC cocultivation with monocytes, fibroblasts and other cells of the tumor microenvironment, EC metabolism, identification of progenitor ECs and other methods of analysis are also used to assess angiogenesis. They can be reproduced both *in vitro* and *in vivo*. However, these methods are hardly applicable in wide clinical practice due to the need to perform laborious and complex manipulations using immunodeficient animals and expensive equipment. A detailed analysis of methods for assessing angiogenesis is presented in the "Consensus guidelines for the use and interpretation of angiogenesis assays"[117].

HETEROGENICITY OF TUMOR MICROVESSELS IN GC

The unsatisfactory results of antiangiogenic therapy highlight the relevance of further studies on angiogenesis for disease prognosis and tumor response to therapy, as well as for the search of new directions in the treatment of malignant neoplasms[183]. It should be noted that at present, in clinical practice, preference is given to the quantitative assessment of angiogenesis, which include the determination of MVD, level of VEGF expression, and other markers, in GC[4-7,156]. At the same time, tumor vessels are known to be heterogeneous in their origin and morphology, and various types of vessels may differ not only in clinical significance but also in their sensitivity to antiangiogenic therapy[130,133,184-186].

Despite the fact that heterogeneity of tumor vessels has been confirmed by numerous studies, a standard classification of vessels has not yet been developed, which would consider not only morphological features but also the relationship with the clinical and morphological characteristics of the pathological process, long-term treatment results and sensitivity to therapy. The proposed classifications are aimed primarily at determining the sensitivity of malignant neoplasms to antiangiogenic therapy. Thus, Gee *et al*[187] proposed distinguishing tumor microvessels by their degree of maturity. The authors, depending on the size, perfusion, EC proliferation, and presence of pericytes, identified three types of microvessels: (1) highly proliferative, nonperfused EC sprouts emanating from functional vessels; (2) small, perfused vessels that, like angiogenic sprouts, were not covered by pericytes; and (3) larger vessels, which were predominantly pericyte-covered with quiescent ECs and

few associated sprouts. Only type 1 and type 2 vessels were sensitive to anti-vascular agents[187,188].

Another classification of microvessels based on their morphological features was proposed by Nagy *et al*[130]. The researchers identified six types of microvessels, which, in their opinion, developed sequentially over time: mother vessels, glomeruloid microvascular proliferations, vascular malformations, capillaries, feeding arteries, and draining veins[99,130]. Only immature mother vessels and glomeruloid microvascular proliferations were sensitive to therapy with antiangiogenic drugs[185,186].

Furthermore, Kuczynski *et al*[184], in an investigation of vessels in hepatocellular carcinoma, identified five types of vessels: (1) tumor-embedded vessels, defined as CD31+ vessels bordered only by lamin A/C+ tumor cells; (2) connective tissue vessels, which were CD31+ vessels bordered by fibroblasts; (3) hepatocyte vessels, which were CD31+ vessels bordered by hepatocytes; (4) hepatic central veins; and (5) normal vessels of the portal triads. The authors considered the presence of vessel types 3 through 5 in the tumor as evidence for vessel co-option since these vessels were present in the structure of the normal liver and their presence was believed to be associated with resistance to sorafenib treatment.

First, it should be noted that the above classifications took into account the degree of tumor microvessel maturity and their sensitivity to antiangiogenic therapy. These classifications do not allow distinction between tumor microvessels, depending on their prognostic significance. Considering that tumor microvessels have different origins and are heterogeneous in morphology, we set the goal of classifying them according to morphology and clinical significance. For this, we studied the features of tumor microvessel morphology in 73 patients with GC and compared the data obtained with the clinical characteristics and prognosis of the disease[189]. As a result of the study, five types of microvessels and structures with endothelial lining were identified (Figure 1).

Normal capillaries

Vessels 5–40 microns in diameter lined with EC with flat, hyperchromic nuclei. The correlations between the vessels of this type and the factors of GC progression were not revealed.

Dilated capillaries

Large vessels of predominantly round or oval shape with a diameter of 40 microns or more that possessed clear, even contours and endothelial lining formed both by cells with flattened, hyperchromic nuclei and cells with large, pale nuclei with fine-netted chromatin structure. The cytoplasm of the lining cells was evenly stained by CD34. We also found no correlations between the vessels of this type and the factors of GC progression.

Atypical dilated capillaries

Vessels of an irregular shape with a diameter of 40 microns or more with indistinct, uneven contours. The endothelial lining of such vessels was formed by randomly located cells of irregular shape, unevenly accumulating the CD34 marker. In the lumen of such vessels, tumor emboli were often found.

Structures with partial endothelial linings (previously, cavitary structures of type-1)

Their characteristic feature was the chaotic arrangement of ECs with irregular shape, uneven contours, and uneven expression of CD34 markers. In GC, multiple, atypical, dilated capillaries and structures with partial endothelial linings were significantly more frequently observed at stages T3–4 (P = 0.001) and N2 (P = 0.001). With or without multiple structures with partial endothelial lining, the three-year overall survival was 52.7% and 93.9%, respectively (P = 0.0013), and the relapse-free survival was 32.4% and 87.7%, respectively (P = 0.0001).

Dilated capillaries with weak expression of CD34 (previously, cavitary structures of type-2)

Vessels located in the gastric submucosa adjacent to the tumor. The presence of these vessels was observed more often in patients with lymphatic metastases (P = 0.01) and in grade 3–4 tumors (P = 0.04) and was associated with a decrease in three-year relapse-free and overall survival (P = 0.049 and P = 0.008, respectively).

It should be noted that we changed the names of some vessels, which made it possible to more accurately characterize the features of their morphology. In particular, cavitary structures of type-1 were renamed structures with partial

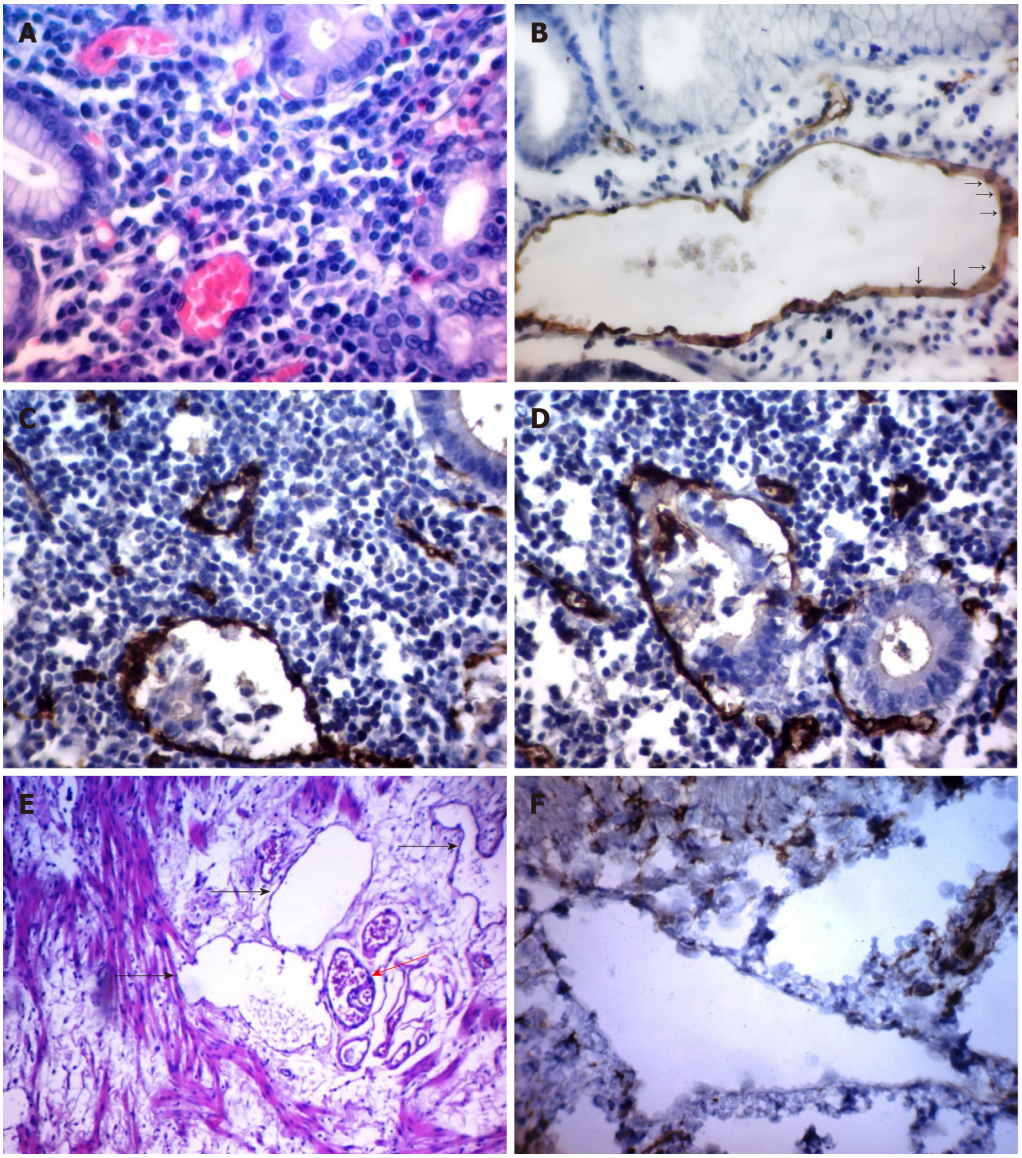

Figure 1 Different types of tumor microvessels in gastric cancer. A: Normal capillaries in the gastric mucosa adjacent to the tumor [hematoxylin and eosin (HE), 600×]; B: Dilated capillary formed by endothelial cells with large, pale nuclei with fine-netted chromatin structure (arrows) in the gastric mucosa adjacent to the tumor [immunohistochemistry (IHC) staining with antibodies to CD34, 400×]; C: Atypical dilated capillary with tumor emboli in the lumen (IHC staining with antibodies to CD34, 600×); D: Structure with partial endothelial linings (IHC staining with antibodies to CD34, 600×); E: Dilated capillaries with low expression of CD34 (black arrows) and dilated capillary (red arrow) in the gastric submucosa adjacent to the tumor (HE, 200×); F: Dilated capillaries with low expression of CD34 in the gastric submucosa adjacent to the tumor (IHC staining with antibodies to CD34, 600×).

endothelial linings, and cavitary structures of type-1 were renamed dilated capillaries with weak expression of CD34. In further studies, it was shown that the proposed classification of tumor microvessels can be used for other localizations of malignant neoplasms[190,191].

CONCLUSION

Overall, angiogenesis plays a key role in tumor progression, affecting the growth and metastasis of malignant neoplasms. At the same time, the origin, degree of maturity, morphological features, and functionality of tumor microvessels are of decisive importance for the delivery of drugs to the tumor, and in addition, they determine the sensitivity of tumor microvessels to angiogenic therapy. Most of the proposed classifications of tumor microvessels are based on assessing the degree of their maturity and do not take into account the different roles of individual types of microvessels in tumor progression. In contrast to the classifications proposed by other authors, our classification considers not only the morphology of the vessels but also their clinical

significance. We believe, however, that further studies are needed to understand angiogenesis mechanisms in GC and verify the hypotheses made regarding the role of different types of tumor vessels in the progression of GC and GC chemoresistance.

REFERENCES

1 **Baniak N**, Senger JL, Ahmed S, Kanthan SC, Kanthan R. Gastric biomarkers: a global review. *World J Surg Oncol* 2016; **14**: 212 [PMID: 27514667 DOI: 10.1186/s12957-016-0969-3]

2 **Thrift AP**, El-Serag HB. Burden of Gastric Cancer. *Clin Gastroenterol Hepatol* 2020; **18**: 534-542 [PMID: 31362118 DOI: 10.1016/j.cgh.2019.07.045]

3 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

4 **Aktaş SH**, Akbulut Yazici HO, Zengin N, Akgün HN, Üstüner Z, Içli F. A new angiogenesis prognostic index with VEGFA, PlGF, and angiopoietin1 predicts survival in patients with advanced gastric cancer. *Turk J Med Sci* 2017; **47**: 399-406 [PMID: 28425270 DOI: 10.3906/sag-1509-80]

5 **Liu X**, Guo W, Zhang W, Yin J, Zhang J, Zhu X, Liu T, Chen Z, Wang B, Chang J, Lv F, Hong X, Wang H, Wang J, Zhao X, Wu X, Li J. A multi-center phase II study and biomarker analysis of combined cetuximab and modified FOLFIRI as second-line treatment in patients with metastatic gastric cancer. *BMC Cancer* 2017; **17**: 188 [PMID: 28288572 DOI: 10.1186/s12885-017-3174-z]

6 **Chang Y**, Niu W, Lian PL, Wang XQ, Meng ZX, Liu Y, Zhao R. Endocan-expressing microvessel density as a prognostic factor for survival in human gastric cancer. *World J Gastroenterol* 2016; **22**: 5422-5429 [PMID: 27340359 DOI: 10.3748/wjg.v22.i23.5422]

7 **Nienhüser H**, Schmidt T. Angiogenesis and Anti-Angiogenic Therapy in Gastric Cancer. *Int J Mol Sci* 2017; **19** [PMID: 29295534 DOI: 10.3390/ijms19010043]

8 **Hsieh HL**, Tsai MM. Tumor progression-dependent angiogenesis in gastric cancer and its potential application. *World J Gastrointest Oncol* 2019; **11**: 686-704 [PMID: 31558974 DOI: 10.4251/wjgo.v11.i9.686]

9 **Sun Y**, Yu X, Li M, Zou Z. Expression of CD44v6 and lymphatic vessel density in early gastric cancer tissues and their clinical significance. *Pak J Med Sci* 2019; **35**: 549-554 [PMID: 31086549 DOI: 10.12669/pjms.35.2.464]

10 **Zecchin A**, Kalucka J, Dubois C, Carmeliet P. How Endothelial Cells Adapt Their Metabolism to Form Vessels in Tumors. *Front Immunol* 2017; **8**: 1750 [PMID: 29321777 DOI: 10.3389/fimmu.2017.01750]

11 **Caporarello N**, Lupo G, Olivieri M, Cristaldi M, Cambria MT, Salmeri M, Anfuso CD. Classical VEGF, Notch and Ang signalling in cancer angiogenesis, alternative approaches and future directions (Review). *Mol Med Rep* 2017; **16**: 4393-4402 [PMID: 28791360 DOI: 10.3892/mmr.2017.7179]

12 **Chen S**, Zhang X, Peng J, Zhai E, He Y, Wu H, Chen C, Ma J, Wang Z, Cai S. VEGF promotes gastric cancer development by upregulating CRMP4. *Oncotarget* 2016; **7**: 17074-17086 [PMID: 26934554 DOI: 10.18632/oncotarget.7717]

13 **Yehya AHS**, Asif M, Petersen SH, Subramaniam AV, Kono K, Majid AMSA, Oon CE. Angiogenesis: Managing the Culprits behind Tumorigenesis and Metastasis. *Medicina (Kaunas)* 2018; **54** [PMID: 30344239 DOI: 10.3390/medicina54010008]

14 **Vaahtomeri K**, Karaman S, Mäkinen T, Alitalo K. Lymphangiogenesis guidance by paracrine and pericellular factors. *Genes Dev* 2017; **31**: 1615-1634 [PMID: 28947496 DOI: 10.1101/gad.303776.117]

15 **Gutierrez-Miranda L**, Yaniv K. Cellular Origins of the Lymphatic Endothelium: Implications for Cancer Lymphangiogenesis. *Front Physiol* 2020; **11**: 577584 [PMID: 33071831 DOI: 10.3389/fphys.2020.577584]

16 **Ran S**, Volk-Draper L. Lymphatic Endothelial Cell Progenitors in the Tumor Microenvironment. *Adv Exp Med Biol* 2020; **1234**: 87-105 [PMID: 32040857 DOI: 10.1007/978-3-030-37184-5_7]

17 **Lian L**, Li XL, Xu MD, Li XM, Wu MY, Zhang Y, Tao M, Li W, Shen XM, Zhou C, Jiang M. VEGFR2 promotes tumorigenesis and metastasis in a pro-angiogenic-independent way in gastric cancer. *BMC Cancer* 2019; **19**: 183 [PMID: 30819137 DOI: 10.1186/s12885-019-5322-0]

18 **Yang J**, Yan J, Liu B. Targeting VEGF/VEGFR to Modulate Antitumor Immunity. *Front Immunol* 2018; **9**: 978 [PMID: 29774034 DOI: 10.3389/fimmu.2018.00978]

19 **Johnston PA**, Grandis JR. STAT3 signaling: anticancer strategies and challenges. *Mol Interv* 2011; **11**: 18-26 [PMID: 21441118 DOI: 10.1124/mi.11.1.4]

20 **Li H**, Huang N, Zhu W, Wu J, Yang X, Teng W, Tian J, Fang Z, Luo Y, Chen M, Li Y. Modulation the crosstalk between tumor-associated macrophages and non-small cell lung cancer to inhibit tumor migration and invasion by ginsenoside Rh2. *BMC Cancer* 2018; **18**: 579 [PMID: 29783929 DOI: 10.1186/s12885-018-4299-4]

21 **Osinsky S**, Bubnovskaya L, Ganusevich I, Kovelskaya A, Gumenyuk L, Olijnichenko G, Merentsev S. Hypoxia, tumour-associated macrophages, microvessel density, VEGF and matrix metalloproteinases in human gastric cancer: interaction and impact on survival. *Clin Transl Oncol* 2011; **13**: 133-138 [PMID: 21324802 DOI: 10.1007/s12094-011-0630-0]

22 **Zhou Y**, Li G, Wu J, Zhang Z, Wu Z, Fan P, Hao T, Zhang X, Li M, Zhang F, Li Q, Lu B, Qiao L. Clinicopathological significance of E-cadherin, VEGF, and MMPs in gastric cancer. *Tumour Biol* 2010; **31**: 549-558 [PMID: 20563765 DOI: 10.1007/s13277-010-0068-y]

23 **Beamish JA**, Juliar BA, Cleveland DS, Busch ME, Nimmagadda L, Putnam AJ. Deciphering the relative roles of matrix metalloproteinase- and plasmin-mediated matrix degradation during capillary morphogenesis using engineered hydrogels. *J Biomed Mater Res B Appl Biomater* 2019; **107**: 2507-2516 [PMID: 30784190 DOI: 10.1002/jbm.b.34341]

24 **Wen YL**, Li L. Correlation between matrix metalloproteinase-9 and vascular endothelial growth factor expression in lung adenocarcinoma. *Genet Mol Res* 2015; **14**: 19342-19348 [PMID: 26782587 DOI: 10.4238/2015.December.29.44]

25 **Yang Q**, Ye ZY, Zhang JX, Tao HQ, Li SG, Zhao ZS. Expression of matrix metalloproteinase-9 mRNA and vascular endothelial growth factor protein in gastric carcinoma and its relationship to its pathological features and prognosis. *Anat Rec (Hoboken)* 2010; **293**: 2012-2019 [PMID: 21089052 DOI: 10.1002/ar.21071]

26 **Andreuzzi E**, Capuano A, Poletto E, Pivetta E, Fejza A, Favero A, Doliana R, Cannizzaro R, Spessotto P, Mongiat M. Role of Extracellular Matrix in Gastrointestinal Cancer-Associated Angiogenesis. *Int J Mol Sci* 2020; **21** [PMID: 32456248 DOI: 10.3390/ijms21103686]

27 **Winkler J**, Abisoye-Ogunniyan A, Metcalf KJ, Werb Z. Concepts of extracellular matrix remodelling in tumour progression and metastasis. *Nat Commun* 2020; **11**: 5120 [PMID: 33037194 DOI: 10.1038/s41467-020-18794-x]

28 **Nam SY**, Ko YS, Jung J, Yoon J, Kim YH, Choi YJ, Park JW, Chang MS, Kim WH, Lee BL. A hypoxia-dependent upregulation of hypoxia-inducible factor-1 by nuclear factor-κB promotes gastric tumour growth and angiogenesis. *Br J Cancer* 2011; **104**: 166-174 [PMID: 21119667 DOI: 10.1038/sj.bjc.6606020]

29 **Li H**, Jia Y, Wang Y. Targeting HIF-1α signaling pathway for gastric cancer treatment. *Pharmazie* 2019; **74**: 3-7 [PMID: 30782242 DOI: 10.1691/ph.2019.8674]

30 **King HW**, Michael MZ, Gleadle JM. Hypoxic enhancement of exosome release by breast cancer cells. *BMC Cancer* 2012; **12**: 421 [PMID: 22998595 DOI: 10.1186/1471-2407-12-421]

31 **Kuriyama N**, Yoshioka Y, Kikuchi S, Azuma N, Ochiya T. Extracellular Vesicles Are Key Regulators of Tumor Neovasculature. *Front Cell Dev Biol* 2020; **8**: 611039 [PMID: 33363175 DOI: 10.3389/fcell.2020.611039]

32 **Kuosmanen SM**, Kansanen E, Sihvola V, Levonen AL. MicroRNA Profiling Reveals Distinct Profiles for Tissue-Derived and Cultured Endothelial Cells. *Sci Rep* 2017; **7**: 10943 [PMID: 28887500 DOI: 10.1038/s41598-017-11487-4]

33 **Voellenkle C**, Rooij Jv, Guffanti A, Brini E, Fasanaro P, Isaia E, Croft L, David M, Capogrossi MC, Moles A, Felsani A, Martelli F. Deep-sequencing of endothelial cells exposed to hypoxia reveals the complexity of known and novel microRNAs. *RNA* 2012; **18**: 472-484 [PMID: 22282338 DOI: 10.1261/rna.027615.111]

34 **Jung KO**, Youn H, Lee CH, Kang KW, Chung JK. Visualization of exosome-mediated miR-210 transfer from hypoxic tumor cells. *Oncotarget* 2017; **8**: 9899-9910 [PMID: 28038441 DOI: 10.18632/oncotarget.14247]

35 **Guduric-Fuchs J**, Pedrini E, Lechner J, Chambers SEJ, O'Neill CL, Mendes Lopes de Melo J, Pathak V, Church RH, McKeown S, Bojdo J, Mcloughlin KJ, Stitt AW, Medina RJ. miR-130a activates the VEGFR2/STAT3/HIF1α axis to potentiate the vasoregenerative capacity of endothelial colony-forming cells in hypoxia. *Mol Ther Nucleic Acids* 2021; **23**: 968-981 [PMID: 33614244 DOI: 10.1016/j.omtn.2021.01.015]

36 **Wei X**, Chen Y, Jiang X, Peng M, Liu Y, Mo Y, Ren D, Hua Y, Yu B, Zhou Y, Liao Q, Wang H, Xiang B, Zhou M, Li X, Li G, Li Y, Xiong W, Zeng Z. Mechanisms of vasculogenic mimicry in hypoxic tumor microenvironments. *Mol Cancer* 2021; **20**: 7 [PMID: 33397409 DOI: 10.1186/s12943-020-01288-1]

37 **Wang M**, Zhao X, Zhu D, Liu T, Liang X, Liu F, Zhang Y, Dong X, Sun B. HIF-1α promoted vasculogenic mimicry formation in hepatocellular carcinoma through LOXL2 up-regulation in hypoxic tumor microenvironment. *J Exp Clin Cancer Res* 2017; **36**: 60 [PMID: 28449718 DOI: 10.1186/s13046-017-0533-1]

38 **Kim D**, Dai J, Park YH, Fai LY, Wang L, Pratheeshkumar P, Son YO, Kondo K, Xu M, Luo J, Shi X, Zhang Z. Activation of Epidermal Growth Factor Receptor/p38/Hypoxia-inducible Factor-1α Is Pivotal for Angiogenesis and Tumorigenesis of Malignantly Transformed Cells Induced by Hexavalent Chromium. *J Biol Chem* 2016; **291**: 16271-16281 [PMID: 27226640 DOI: 10.1074/jbc.M116.715797]

39 **Pei YF**, Liu J, Cheng J, Wu WD, Liu XQ. Silencing of LAMC2 Reverses Epithelial-Mesenchymal Transition and Inhibits Angiogenesis in Cholangiocarcinoma *via* Inactivation of the Epidermal Growth Factor Receptor Signaling Pathway. *Am J Pathol* 2019; **189**: 1637-1653 [PMID: 31345467 DOI: 10.1016/j.ajpath.2019.03.012]

40 **Huo FC**, Zhu WT, Liu X, Zhou Y, Zhang LS, Mou J. Epidermal growth factor-like domain multiple 6 (EGFL6) promotes the migration and invasion of gastric cancer cells by inducing epithelial-mesenchymal transition. *Invest New Drugs* 2021; **39**: 304-316 [PMID: 32949323 DOI: 10.1007/s10637-020-01004-2]

41 **Martorana A**, La Monica G, Lauria A. Quinoline-Based Molecules Targeting c-Met, EGF, and VEGF Receptors and the Proteins Involved in Related Carcinogenic Pathways. *Molecules* 2020; **25**

[PMID: 32961977 DOI: 10.3390/molecules25184279]
42 **Song H**, Wang T, Tian L, Bai S, Chen L, Zuo Y, Xue Y. Macrophages on the Peritoneum are involved in Gastric Cancer Peritoneal Metastasis. *J Cancer* 2019; **10**: 5377-5387 [PMID: 31632482 DOI: 10.7150/jca.31787]
43 **Forma A**, Tyczyńska M, Kędzierawski P, Gietka K, Sitarz M. Gastric carcinogenesis: a comprehensive review of the angiogenic pathways. *Clin J Gastroenterol* 2021; **14**: 14-25 [PMID: 33206367 DOI: 10.1007/s12328-020-01295-1]
44 **Hacker UT**, Escalona-Espinosa L, Consalvo N, Goede V, Schiffmann L, Scherer SJ, Hedge P, Van Cutsem E, Coutelle O, Büning H. Evaluation of Angiopoietin-2 as a biomarker in gastric cancer: results from the randomised phase III AVAGAST trial. *Br J Cancer* 2016; **114**: 855-862 [PMID: 27031850 DOI: 10.1038/bjc.2016.30]
45 **Toiyama Y**, Tanaka K, Kitajima T, Shimura T, Imaoka H, Mori K, Okigami M, Yasuda H, Okugawa Y, Saigusa S, Ohi M, Inoue Y, Mohri Y, Goel A, Kusunoki M. Serum angiopoietin-like protein 2 as a potential biomarker for diagnosis, early recurrence and prognosis in gastric cancer patients. *Carcinogenesis* 2015; **36**: 1474-1483 [PMID: 26420253 DOI: 10.1093/carcin/bgv139]
46 **Suzuki S**, Dobashi Y, Hatakeyama Y, Tajiri R, Fujimura T, Heldin CH, Ooi A. Clinicopathological significance of platelet-derived growth factor (PDGF)-B and vascular endothelial growth factor-A expression, PDGF receptor-β phosphorylation, and microvessel density in gastric cancer. *BMC Cancer* 2010; **10**: 659 [PMID: 21118571 DOI: 10.1186/1471-2407-10-659]
47 **Zhang J**, Zhang H, Chen Y, Fu J, Lei Y, Sun J, Tang B. Plateletderived growth factor D promotes the angiogenic capacity of endothelial progenitor cells. *Mol Med Rep* 2019; **19**: 125-132 [PMID: 30483778 DOI: 10.3892/mmr.2018.9692]
48 **Cheng X**, Jin Z, Ji X, Shen X, Feng H, Morgenlander W, Ou B, Wu H, Gao H, Ye F, Zhang Y, Peng Y, Liang J, Jiang Y, Zhang T, Qiu W, Lu X, Zhao R. ETS variant 5 promotes colorectal cancer angiogenesis by targeting platelet-derived growth factor BB. *Int J Cancer* 2019; **145**: 179-191 [PMID: 30650178 DOI: 10.1002/ijc.32071]
49 **Higuchi A**, Oshima T, Yoshihara K, Sakamaki K, Aoyama T, Suganuma N, Yamamoto N, Sato T, Cho H, Shiozawa M, Yoshikawa T, Rino Y, Kunisaki C, Imada T, Masuda M. Clinical significance of platelet-derived growth factor receptor-β gene expression in stage II/III gastric cancer with S-1 adjuvant chemotherapy. *Oncol Lett* 2017; **13**: 905-911 [PMID: 28356977 DOI: 10.3892/ol.2016.5494]
50 **Xie F**, Zhang X, Luo W, Ge H, Sun D, Liu P. Notch Signaling Pathway Is Involved in bFGF-Induced Corneal Lymphangiogenesis and Hemangiogenesis. *J Ophthalmol* 2019; **2019**: 9613923 [PMID: 31531237 DOI: 10.1155/2019/9613923]
51 **Zhang YK**, Wang H, Guo YW, Yue Y. Novel role of Snail 1 in promoting tumor neoangiogenesis. *Biosci Rep* 2019; **39** [PMID: 30975732 DOI: 10.1042/BSR20182161]
52 **Yashiro M**, Matsuoka T. Fibroblast growth factor receptor signaling as therapeutic targets in gastric cancer. *World J Gastroenterol* 2016; **22**: 2415-2423 [PMID: 26937130 DOI: 10.3748/wjg.v22.i8.2415]
53 **Gao LM**, Wang F, Zheng Y, Fu ZZ, Zheng L, Chen LL. Roles of Fibroblast Activation Protein and Hepatocyte Growth Factor Expressions in Angiogenesis and Metastasis of Gastric Cancer. *Pathol Oncol Res* 2019; **25**: 369-376 [PMID: 29134462 DOI: 10.1007/s12253-017-0359-3]
54 **Li Y**, Guo XB, Wang JS, Wang HC, Li LP. Function of fibroblast growth factor 2 in gastric cancer occurrence and prognosis. *Mol Med Rep* 2020; **21**: 575-582 [PMID: 31789423 DOI: 10.3892/mmr.2019.10850]
55 **Sammarco G**, Gadaleta CD, Zuccalà V, Albayrak E, Patruno R, Milella P, Sacco R, Ammendola M, Ranieri G. Tumor-Associated Macrophages and Mast Cells Positive to Tryptase Are Correlated with Angiogenesis in Surgically-Treated Gastric Cancer Patients. *Int J Mol Sci* 2018; **19** [PMID: 29649166 DOI: 10.3390/ijms19041176]
56 **Micu GV**, Stăniceanu F, Sticlaru LC, Popp CG, Bastian AE, Gramada E, Pop G, Mateescu RB, Rimbaş M, Archip B, Bleotu C. Correlations Between the Density of Tryptase Positive Mast Cells (DMCT) and that of New Blood Vessels (CD105+) in Patients with Gastric Cancer. *Rom J Intern Med* 2016; **54**: 113-120 [PMID: 27352440 DOI: 10.1515/rjim-2016-0016]
57 **Ammendola M**, Sacco R, Zuccalà V, Luposella M, Patruno R, Gadaleta P, Zizzo N, Gadaleta CD, De Sarro G, Sammarco G, Oltean M, Ranieri G. Mast Cells Density Positive to Tryptase Correlate with Microvascular Density in both Primary Gastric Cancer Tissue and Loco-Regional Lymph Node Metastases from Patients That Have Undergone Radical Surgery. *Int J Mol Sci* 2016; **17** [PMID: 27854307 DOI: 10.3390/ijms17111905]
58 **Shi J**, Wei PK. Interleukin-8: A potent promoter of angiogenesis in gastric cancer. *Oncol Lett* 2016; **11**: 1043-1050 [PMID: 26893688 DOI: 10.3892/ol.2015.4035]
59 **Ju L**, Zhou Z, Jiang B, Lou Y, Guo X. Autocrine VEGF and IL-8 Promote Migration *via* Src/Vav2/Rac1/PAK1 Signaling in Human Umbilical Vein Endothelial Cells. *Cell Physiol Biochem* 2017; **41**: 1346-1359 [PMID: 28278510 DOI: 10.1159/000465389]
60 **Ciesielski M**, Szajewski M, Pęksa R, Lewandowska MA, Zieliński J, Walczak J, Szefel J, Kruszewski WJ. The relationship between HER2 overexpression and angiogenesis in gastric cancer. *Medicine (Baltimore)* 2018; **97**: e12854 [PMID: 30334990 DOI: 10.1097/MD.0000000000012854]
61 **Li F**, Meng G, Tan B, Chen Z, Ji Q, Wang X, Liu C, Niu S, Li Y, Liu Y. Relationship between HER2 expression and tumor interstitial angiogenesis in primary gastric cancer and its effect on prognosis. *Pathol Res Pract* 2021; **217**: 153280 [PMID: 33253925 DOI: 10.1016/j.prp.2020.153280]

62 **Wang J**, Yang L, Liang F, Chen Y, Yang G. Integrin alpha x stimulates cancer angiogenesis through PI3K/Akt signaling-mediated VEGFR2/VEGF-A overexpression in blood vessel endothelial cells. *J Cell Biochem* 2019; **120**: 1807-1818 [PMID: 30873824 DOI: 10.1002/jcb.27480]

63 **Dallinga MG**, Habani YI, Kayser RP, Van Noorden CJF, Klaassen I, Schlingemann RO. IGF-binding proteins 3 and 4 are regulators of sprouting angiogenesis. *Mol Biol Rep* 2020; **47**: 2561-2572 [PMID: 32133604 DOI: 10.1007/s11033-020-05339-0]

64 **Wang X**, Song X, Cheng G, Zhang J, Dong L, Bai J, Luo D, Xiong Y, Li S, Liu F, Sun Y, Wang X, Li Y, Huang Y. The Regulatory Mechanism and Biological Significance of Mitochondrial Calcium Uniporter in the Migration, Invasion, Angiogenesis and Growth of Gastric Cancer. *Onco Targets Ther* 2020; **13**: 11781-11794 [PMID: 33235465 DOI: 10.2147/OTT.S262049]

65 **Liu N**, Zhou N, Chai N, Liu X, Jiang H, Wu Q, Li Q. Helicobacter pylori promotes angiogenesis depending on Wnt/beta-catenin-mediated vascular endothelial growth factor *via* the cyclooxygenase-2 pathway in gastric cancer. *BMC Cancer* 2016; **16**: 321 [PMID: 27198692 DOI: 10.1186/s12885-016-2351-9]

66 **Xiang T**, Lin YX, Ma W, Zhang HJ, Chen KM, He GP, Zhang X, Xu M, Feng QS, Chen MY, Zeng MS, Zeng YX, Feng L. Vasculogenic mimicry formation in EBV-associated epithelial malignancies. *Nat Commun* 2018; **9**: 5009 [PMID: 30479336 DOI: 10.1038/s41467-018-07308-5]

67 **Kim HS**, Won YJ, Shim JH, Kim HJ, Kim J, Hong HN, Kim BS. Morphological characteristics of vasculogenic mimicry and its correlation with EphA2 expression in gastric adenocarcinoma. *Sci Rep* 2019; **9**: 3414 [PMID: 30833656 DOI: 10.1038/s41598-019-40265-7]

68 **Olejarz W**, Kubiak-Tomaszewska G, Chrzanowska A, Lorenc T. Exosomes in Angiogenesis and Anti-angiogenic Therapy in Cancers. *Int J Mol Sci* 2020; **21** [PMID: 32823989 DOI: 10.3390/ijms21165840]

69 **Rosano S**, Corà D, Parab S, Zaffuto S, Isella C, Porporato R, Hoza RM, Calogero RA, Riganti C, Bussolino F, Noghero A. A regulatory microRNA network controls endothelial cell phenotypic switch during sprouting angiogenesis. *Elife* 2020; **9** [PMID: 31976858 DOI: 10.7554/eLife.48095]

70 **Bai M**, Li J, Yang H, Zhang H, Zhou Z, Deng T, Zhu K, Ning T, Fan Q, Ying G, Ba Y. miR-135b Delivered by Gastric Tumor Exosomes Inhibits FOXO1 Expression in Endothelial Cells and Promotes Angiogenesis. *Mol Ther* 2019; **27**: 1772-1783 [PMID: 31416776 DOI: 10.1016/j.ymthe.2019.06.018]

71 **Deng T**, Zhang H, Yang H, Wang H, Bai M, Sun W, Wang X, Si Y, Ning T, Zhang L, Li H, Ge S, Liu R, Lin D, Li S, Ying G, Ba Y. Exosome miR-155 Derived from Gastric Carcinoma Promotes Angiogenesis by Targeting the c-MYB/VEGF Axis of Endothelial Cells. *Mol Ther Nucleic Acids* 2020; **19**: 1449-1459 [PMID: 32160713 DOI: 10.1016/j.omtn.2020.01.024]

72 **Li Y**, Wu Z, Yuan J, Sun L, Lin L, Huang N, Bin J, Liao Y, Liao W. Long non-coding RNA MALAT1 promotes gastric cancer tumorigenicity and metastasis by regulating vasculogenic mimicry and angiogenesis. *Cancer Lett* 2017; **395**: 31-44 [PMID: 28268166 DOI: 10.1016/j.canlet.2017.02.035]

73 **Gong Z**, Ma J, Su H, Guo T, Cai H, Chen Q, Zhao X, Qi J, Du J. Interleukin-1 receptor antagonist inhibits angiogenesis in gastric cancer. *Int J Clin Oncol* 2018; **23**: 659-670 [PMID: 29344744 DOI: 10.1007/s10147-018-1242-2]

74 **Zhang C**, Liang Y, Ma MH, Wu KZ, Zhang CD, Dai DQ. Downregulation of microRNA-376a in Gastric Cancer and Association with Poor Prognosis. *Cell Physiol Biochem* 2018; **51**: 2010-2018 [PMID: 30522118 DOI: 10.1159/000495820]

75 **Mei B**, Chen J, Yang N, Peng Y. The regulatory mechanism and biological significance of the Snail-miR590-VEGFR-NRP1 axis in the angiogenesis, growth and metastasis of gastric cancer. *Cell Death Dis* 2020; **11**: 241 [PMID: 32303680 DOI: 10.1038/s41419-020-2428-x]

76 **Xie M**, Dart DA, Guo T, Xing XF, Cheng XJ, Du H, Jiang WG, Wen XZ, Ji JF. MicroRNA-1 acts as a tumor suppressor microRNA by inhibiting angiogenesis-related growth factors in human gastric cancer. *Gastric Cancer* 2018; **21**: 41-54 [PMID: 28493075 DOI: 10.1007/s10120-017-0721-x]

77 **Zhu F**, Wang KB, Rui L. STAT3 Activation and Oncogenesis in Lymphoma. *Cancers (Basel)* 2019; **12** [PMID: 31861597 DOI: 10.3390/cancers12010019]

78 **Wu X**, Yang T, Liu X, Guo JN, Xie T, Ding Y, Lin M, Yang H. IL-17 promotes tumor angiogenesis through Stat3 pathway mediated upregulation of VEGF in gastric cancer. *Tumour Biol* 2016; **37**: 5493-5501 [PMID: 26566627 DOI: 10.1007/s13277-015-4372-4]

79 **Zhao G**, Zhu G, Huang Y, Zheng W, Hua J, Yang S, Zhuang J, Ye J. IL-6 mediates the signal pathway of JAK-STAT3-VEGF-C promoting growth, invasion and lymphangiogenesis in gastric cancer. *Oncol Rep* 2016; **35**: 1787-1795 [PMID: 26750536 DOI: 10.3892/or.2016.4544]

80 **Zhou Y**, Xia L, Liu Q, Wang H, Lin J, Oyang L, Chen X, Luo X, Tan S, Tian Y, Su M, Wang Y, Chen P, Wu Y, Liao Q. Induction of Pro-Inflammatory Response *via* Activated Macrophage-Mediated NF-κB and STAT3 Pathways in Gastric Cancer Cells. *Cell Physiol Biochem* 2018; **47**: 1399-1410 [PMID: 29929193 DOI: 10.1159/000490829]

81 **Zhang X**, Tang J, Zhi X, Xie K, Wang W, Li Z, Zhu Y, Yang L, Xu H, Xu Z. miR-874 functions as a tumor suppressor by inhibiting angiogenesis through STAT3/VEGF-A pathway in gastric cancer. *Oncotarget* 2015; **6**: 1605-1617 [PMID: 25596740 DOI: 10.18632/oncotarget.2748]

82 **Krstić M**, Stojanović NM, Stojnev S, Radenković G, Čukuranović Kokoris J, Mladenović B, Janković Veličković L. Interplay between STAT3, Cell Adhesion Molecules and Angiogenesis-Related Parameters in Gastric Carcinoma. Does STAT3 Really Have a Prognostic Value? *Medicina (Kaunas)* 2019; **55** [PMID: 31234597 DOI: 10.3390/medicina55060300]

83 **Sokolova O**, Naumann M. NF-κB Signaling in Gastric Cancer. *Toxins (Basel)* 2017; **9** [PMID: 28350359 DOI: 10.3390/toxins9040119]

84 **Wang N**, Chang LL. Maspin suppresses cell invasion and migration in gastric cancer through inhibiting EMT and angiogenesis *via* ITGB1/FAK pathway. *Hum Cell* 2020; **33**: 663-675 [PMID: 32409959 DOI: 10.1007/s13577-020-00345-7]

85 **Tang L**, Wen JB, Wen P, Li X, Gong M, Li Q. Long non-coding RNA LINC01314 represses cell migration, invasion, and angiogenesis in gastric cancer *via* the Wnt/β-catenin signaling pathway by down-regulating KLK4. *Cancer Cell Int* 2019; **19**: 94 [PMID: 31007611 DOI: 10.1186/s12935-019-0799-9]

86 **Chen P**, Zhao D, Wang W, Zhang Y, Yuan Y, Wang L, Wu Y. High expression of RELM-α correlates with poor prognosis and promotes angiogenesis in gastric cancer. *Oncol Rep* 2015; **34**: 77-86 [PMID: 25937206 DOI: 10.3892/or.2015.3943]

87 **Qian CN**, Pezzella F. Tumor vasculature: a sally port for inhibiting cancer cell spreading. *Cancer Commun (Lond)* 2018; **38**: 52 [PMID: 30075743 DOI: 10.1186/s40880-018-0322-z]

88 **Lugano R**, Ramachandran M, Dimberg A. Tumor angiogenesis: causes, consequences, challenges and opportunities. *Cell Mol Life Sci* 2020; **77**: 1745-1770 [PMID: 31690961 DOI: 10.1007/s00018-019-03351-7]

89 **Zuazo-Gaztelu I**, Casanovas O. Unraveling the Role of Angiogenesis in Cancer Ecosystems. *Front Oncol* 2018; **8**: 248 [PMID: 30013950 DOI: 10.3389/fonc.2018.00248]

90 **Zhang Y**, Qu H. Expression and clinical significance of aquaporin-1, vascular endothelial growth factor and microvessel density in gastric cancer. *Medicine (Baltimore)* 2020; **99**: e21883 [PMID: 32899018 DOI: 10.1097/MD.0000000000021883]

91 **Li B**, Nie Z, Zhang D, Wu J, Peng B, Guo X, Shi Y, Cai X, Xu L, Cao F. Roles of circulating endothelial progenitor cells and endothelial cells in gastric carcinoma. *Oncol Lett* 2018; **15**: 324-330 [PMID: 29391882 DOI: 10.3892/ol.2017.7272]

92 **Hafez NH**, Tahoun NS. Expression of cyclooxygenase 2 and vascular endothelial growth factor in gastric carcinoma: Relationship with clinicopathological parameters. *J Egypt Natl Canc Inst* 2016; **28**: 149-156 [PMID: 27342370 DOI: 10.1016/j.jnci.2016.05.005]

93 **Hong WG**, Ko YS, Pyo JS. Clinicopathological significance and prognostic role of microvessel density in gastric cancer: A meta-analysis. *Pathol Res Pract* 2017; **213**: 1459-1463 [PMID: 29129495 DOI: 10.1016/j.prp.2017.11.001]

94 **Zhou L**, Yu L, Feng ZZ, Gong XM, Cheng ZN, Yao N, Wang DN, Wu SW. Aberrant Expression of Markers of Cancer Stem Cells in Gastric Adenocarcinoma and their Relationship to Vasculogenic Mimicry. *Asian Pac J Cancer Prev* 2015; **16**: 4177-4183 [PMID: 26028069 DOI: 10.7314/apjcp.2015.16.10.4177]

95 **Hosseini F**, Naghavi N. Modelling Tumor-induced Angiogenesis: Combination of Stochastic Sprout Spacing and Sprout Progression. *J Biomed Phys Eng* 2017; **7**: 233-256 [PMID: 29082215]

96 **Palm MM**, Dallinga MG, van Dijk E, Klaassen I, Schlingemann RO, Merks RM. Computational Screening of Tip and Stalk Cell Behavior Proposes a Role for Apelin Signaling in Sprout Progression. *PLoS One* 2016; **11**: e0159478 [PMID: 27828952 DOI: 10.1371/journal.pone.0159478]

97 **Shamloo A**, Mohammadaliha N, Heilshorn SC, Bauer AL. A Comparative Study of Collagen Matrix Density Effect on Endothelial Sprout Formation Using Experimental and Computational Approaches. *Ann Biomed Eng* 2016; **44**: 929-941 [PMID: 26271521 DOI: 10.1007/s10439-015-1416-2]

98 **Feng X**, Tonnesen MG, Mousa SA, Clark RA. Fibrin and collagen differentially but synergistically regulate sprout angiogenesis of human dermal microvascular endothelial cells in 3-dimensional matrix. *Int J Cell Biol* 2013; **2013**: 231279 [PMID: 23737792 DOI: 10.1155/2013/231279]

99 **Dvorak HF**. Tumor Stroma, Tumor Blood Vessels, and Antiangiogenesis Therapy. *Cancer J* 2015; **21**: 237-243 [PMID: 26222073 DOI: 10.1097/PPO.0000000000000124]

100 **Burri PH**, Hlushchuk R, Djonov V. Intussusceptive angiogenesis: its emergence, its characteristics, and its significance. *Dev Dyn* 2004; **231**: 474-488 [PMID: 15376313 DOI: 10.1002/dvdy.20184]

101 **Díaz-Flores L**, Gutiérrez R, Gayoso S, García MP, González-Gómez M, Díaz-Flores L Jr, Sánchez R, Carrasco JL, Madrid JF. Intussusceptive angiogenesis and its counterpart intussusceptive lymphangiogenesis. *Histol Histopathol* 2020; **35**: 1083-1103 [PMID: 32329808 DOI: 10.14670/HH-18-222]

102 **Ali Z**, Mukwaya A, Biesemeier A, Ntzouni M, Ramsköld D, Giatrellis S, Mammadzada P, Cao R, Lennikov A, Marass M, Gerri C, Hildesjö C, Taylor M, Deng Q, Peebo B, Del Peso L, Kvanta A, Sandberg R, Schraermeyer U, Andre H, Steffensen JF, Lagali N, Cao Y, Kele J, Jensen LD. Intussusceptive Vascular Remodeling Precedes Pathological Neovascularization. *Arterioscler Thromb Vasc Biol* 2019; **39**: 1402-1418 [PMID: 31242036 DOI: 10.1161/ATVBAHA.118.312190]

103 **Hlushchuk R**, Riesterer O, Baum O, Wood J, Gruber G, Pruschy M, Djonov V. Tumor recovery by angiogenic switch from sprouting to intussusceptive angiogenesis after treatment with PTK787/ZK222584 or ionizing radiation. *Am J Pathol* 2008; **173**: 1173-1185 [PMID: 18787105 DOI: 10.2353/ajpath.2008.071131]

104 **Ribatti D**. Tumor refractoriness to anti-VEGF therapy. *Oncotarget* 2016; **7**: 46668-46677 [PMID: 27081695 DOI: 10.18632/oncotarget.8694]

105 **Asahara T**, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997; **275**: 964-967 [PMID: 9020076 DOI: 10.1126/science.275.5302.964]

106 **Moschetta M**, Mishima Y, Sahin I, Manier S, Glavey S, Vacca A, Roccaro AM, Ghobrial IM. Role of endothelial progenitor cells in cancer progression. *Biochim Biophys Acta* 2014; **1846**: 26-39 [PMID: 24709008 DOI: 10.1016/j.bbcan.2014.03.005]

107 **Paprocka M**, Kieda C, Kantor A, Bielawska-Pohl A, Dus D, Czekanski A, Heimrath J. Increased Endothelial Progenitor Cell Number in Early Stage of Endometrial Cancer. *Int J Gynecol Cancer* 2017; **27**: 947-952 [PMID: 28498245 DOI: 10.1097/IGC.0000000000000961]

108 **Yu M**, Men HT, Niu ZM, Zhu YX, Tan BX, Li LH, Jiang J. Meta-Analysis of Circulating Endothelial Cells and Circulating Endothelial Progenitor Cells as Prognostic Factors in Lung Cancer. *Asian Pac J Cancer Prev* 2015; **16**: 6123-6128 [PMID: 26320506 DOI: 10.7314/apjcp.2015.16.14.6123]

109 **Ha XQ**, Zhao M, Li XY, Peng JH, Dong JZ, Deng ZY, Zhao HB, Zhao Y, Zhang YY. Distribution of endothelial progenitor cells in tissues from patients with gastric cancer. *Oncol Lett* 2014; **7**: 1695-1700 [PMID: 24765203 DOI: 10.3892/ol.2014.1944]

110 **Pezzella F**, Gatter KC. Evidence Showing That Tumors Can Grow Without Angiogenesis and Can Switch Between Angiogenic and Nonangiogenic Phenotypes. *J Natl Cancer Inst* 2016; **108** [PMID: 27059375 DOI: 10.1093/jnci/djw032]

111 **Lugassy C**, Kleinman HK, Vermeulen PB, Barnhill RL. Angiotropism, pericytic mimicry and extravascular migratory metastasis: an embryogenesis-derived program of tumor spread. *Angiogenesis* 2020; **23**: 27-41 [PMID: 31720876 DOI: 10.1007/s10456-019-09695-9]

112 **Kuczynski EA**, Reynolds AR. Vessel co-option and resistance to anti-angiogenic therapy. *Angiogenesis* 2020; **23**: 55-74 [PMID: 31865479 DOI: 10.1007/s10456-019-09698-6]

113 **Hong SA**, Hwang HW, Kim MK, Lee TJ, Yim K, Won HS, Sun S, Kim EY, Ko YH. High Endothelial Venule with Concomitant High CD8+ Tumor-Infiltrating Lymphocytes Is Associated with a Favorable Prognosis in Resected Gastric Cancer. *J Clin Med* 2020; **9** [PMID: 32823631 DOI: 10.3390/jcm9082628]

114 **Martinet L**, Garrido I, Filleron T, Le Guellec S, Bellard E, Fournie JJ, Rochaix P, Girard JP. Human solid tumors contain high endothelial venules: association with T- and B-lymphocyte infiltration and favorable prognosis in breast cancer. *Cancer Res* 2011; **71**: 5678-5687 [PMID: 21846823 DOI: 10.1158/0008-5472.CAN-11-0431]

115 **Martinet L**, Le Guellec S, Filleron T, Lamant L, Meyer N, Rochaix P, Garrido I, Girard JP. High endothelial venules (HEVs) in human melanoma lesions: Major gateways for tumor-infiltrating lymphocytes. *Oncoimmunology* 2012; **1**: 829-839 [PMID: 23162750 DOI: 10.4161/onci.20492]

116 **Valdivia A**, Mingo G, Aldana V, Pinto MP, Ramirez M, Retamal C, Gonzalez A, Nualart F, Corvalan AH, Owen GI. Fact or Fiction, It Is Time for a Verdict on Vasculogenic Mimicry? *Front Oncol* 2019; **9**: 680 [PMID: 31428573 DOI: 10.3389/fonc.2019.00680]

117 **Nowak-Sliwinska P**, Alitalo K, Allen E, Anisimov A, Aplin AC, Auerbach R, Augustin HG, Bates DO, van Beijnum JR, Bender RHF, Bergers G, Bikfalvi A, Bischoff J, Böck BC, Brooks PC, Bussolino F, Cakir B, Carmeliet P, Castranova D, Cimpean AM, Cleaver O, Coukos G, Davis GE, De Palma M, Dimberg A, Dings RPM, Djonov V, Dudley AC, Dufton NP, Fendt SM, Ferrara N, Fruttiger M, Fukumura D, Ghesquière B, Gong Y, Griffin RJ, Harris AL, Hughes CCW, Hultgren NW, Iruela-Arispe ML, Irving M, Jain RK, Kalluri R, Kalucka J, Kerbel RS, Kitajewski J, Klaassen I, Kleinmann HK, Koolwijk P, Kuczynski E, Kwak BR, Marien K, Melero-Martin JM, Munn LL, Nicosia RF, Noel A, Nurro J, Olsson AK, Petrova TV, Pietras K, Pili R, Pollard JW, Post MJ, Quax PHA, Rabinovich GA, Raica M, Randi AM, Ribatti D, Ruegg C, Schlingemann RO, Schulte-Merker S, Smith LEH, Song JW, Stacker SA, Stalin J, Stratman AN, Van de Velde M, van Hinsbergh VWM, Vermeulen PB, Waltenberger J, Weinstein BM, Xin H, Yetkin-Arik B, Yla-Herttuala S, Yoder MC, Griffioen AW. Consensus guidelines for the use and interpretation of angiogenesis assays. *Angiogenesis* 2018; **21**: 425-532 [PMID: 29766399 DOI: 10.1007/s10456-018-9613-x]

118 **Tauchi Y**, Tanaka H, Kumamoto K, Tokumoto M, Sakimura C, Sakurai K, Kimura K, Toyokawa T, Amano R, Kubo N, Muguruma K, Yashiro M, Maeda K, Ohira M, Hirakawa K. Tumor-associated macrophages induce capillary morphogenesis of lymphatic endothelial cells derived from human gastric cancer. *Cancer Sci* 2016; **107**: 1101-1109 [PMID: 27227358 DOI: 10.1111/cas.12977]

119 **Li M**, Gu Y, Zhang Z, Zhang S, Zhang D, Saleem AF, Zhao X, Sun B. Vasculogenic mimicry: a new prognostic sign of gastric adenocarcinoma. *Pathol Oncol Res* 2010; **16**: 259-266 [PMID: 20016961 DOI: 10.1007/s12253-009-9220-7]

120 **Guo Q**, Yuan Y, Jin Z, Xu T, Gao Y, Wei H, Li C, Hou W, Hua B. Association between Tumor Vasculogenic Mimicry and the Poor Prognosis of Gastric Cancer in China: An Updated Systematic Review and Meta-Analysis. *Biomed Res Int* 2016; **2016**: 2408645 [PMID: 27812528 DOI: 10.1155/2016/2408645]

121 **Ren HY**, Shen JX, Mao XM, Zhang XY, Zhou P, Li SY, Zheng ZW, Shen DY, Meng JR. Correlation Between Tumor Vasculogenic Mimicry and Poor Prognosis of Human Digestive Cancer Patients: A Systematic Review and Meta-Analysis. *Pathol Oncol Res* 2019; **25**: 849-858 [PMID: 30361906 DOI: 10.1007/s12253-018-0496-3]

122 **Seftor RE**, Seftor EA, Koshikawa N, Meltzer PS, Gardner LM, Bilban M, Stetler-Stevenson WG, Quaranta V, Hendrix MJ. Cooperative interactions of laminin 5 gamma2 chain, matrix metalloproteinase-2, and membrane type-1-matrix/metalloproteinase are required for mimicry of embryonic vasculogenesis by aggressive melanoma. *Cancer Res* 2001; **61**: 6322-6327 [PMID: 11522618]

123 **Lv J**, Sun B, Sun H, Zhang Y, Sun J, Zhao X, Gu Q, Dong X, Che N. Significance of Vasculogenic

Mimicry Formation in Gastric Carcinoma. *Oncol Res Treat* 2017; **40**: 35-41 [PMID: 28118629 DOI: 10.1159/000455144]

124 **Said AH**, Raufman JP, Xie G. The role of matrix metalloproteinases in colorectal cancer. *Cancers (Basel)* 2014; **6**: 366-375 [PMID: 24518611 DOI: 10.3390/cancers6010366]

125 **McDonald DM**, Munn L, Jain RK. Vasculogenic mimicry: how convincing, how novel, and how significant? *Am J Pathol* 2000; **156**: 383-388 [PMID: 10666365 DOI: 10.1016/S0002-9440(10)64740-2]

126 **Sood AK**, Seftor EA, Fletcher MS, Gardner LM, Heidger PM, Buller RE, Seftor RE, Hendrix MJ. Molecular determinants of ovarian cancer plasticity. *Am J Pathol* 2001; **158**: 1279-1288 [PMID: 11290546 DOI: 10.1016/S0002-9440(10)64079-5]

127 **Less JR**, Skalak TC, Sevick EM, Jain RK. Microvascular architecture in a mammary carcinoma: branching patterns and vessel dimensions. *Cancer Res* 1991; **51**: 265-273 [PMID: 1988088]

128 **Mărgăritescu C**, Simionescu C, Pirici D, Mogoantă L, Ciurea R, Stepan A. Immunohistochemical characterization of tumoral vessels in oral squamous cell carcinoma. *Rom J Morphol Embryol* 2008; **49**: 447-458 [PMID: 19050792]

129 **Fukumura D**, Duda DG, Munn LL, Jain RK. Tumor microvasculature and microenvironment: novel insights through intravital imaging in pre-clinical models. *Microcirculation* 2010; **17**: 206-225 [PMID: 20374484 DOI: 10.1111/j.1549-8719.2010.00029.x]

130 **Nagy JA**, Chang SH, Shih SC, Dvorak AM, Dvorak HF. Heterogeneity of the tumor vasculature. *Semin Thromb Hemost* 2010; **36**: 321-331 [PMID: 20490982 DOI: 10.1055/s-0030-1253454]

131 **Baluk P**, Hashizume H, McDonald DM. Cellular abnormalities of blood vessels as targets in cancer. *Curr Opin Genet Dev* 2005; **15**: 102-111 [PMID: 15661540 DOI: 10.1016/j.gde.2004.12.005]

132 **Morikawa S**, Baluk P, Kaidoh T, Haskell A, Jain RK, McDonald DM. Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *Am J Pathol* 2002; **160**: 985-1000 [PMID: 11891196 DOI: 10.1016/S0002-9440(10)64920-6]

133 **Birau A**, Ceausu RA, Cimpean AM, Gaje P, Raica M, Olariu T. Assessement of angiogenesis reveals blood vessel heterogeneity in lung carcinoma. *Oncol Lett* 2012; **4**: 1183-1186 [PMID: 23205116 DOI: 10.3892/ol.2012.893]

134 **Ribatti D**, Nico B, Crivellato E, Vacca A. The structure of the vascular network of tumors. *Cancer Lett* 2007; **248**: 18-23 [PMID: 16879908 DOI: 10.1016/j.canlet.2006.06.007]

135 **Jiménez-Torres JA**, Virumbrales-Muñoz M, Sung KE, Lee MH, Abel EJ, Beebe DJ. Patient-specific organotypic blood vessels as an in vitro model for anti-angiogenic drug response testing in renal cell carcinoma. *EBioMedicine* 2019; **42**: 408-419 [PMID: 30902740 DOI: 10.1016/j.ebiom.2019.03.026]

136 **Nagy JA**, Dvorak AM, Dvorak HF. Vascular hyperpermeability, angiogenesis, and stroma generation. *Cold Spring Harb Perspect Med* 2012; **2**: a006544 [PMID: 22355795 DOI: 10.1101/cshperspect.a006544]

137 **Tekesin K**, Emin Gunes M, Tural D, Akar E, Zirtiloglu A, Karaca M, Selcukbiricik F, Bayrak S, Ozet A. Clinicopathological characteristics, prognosis and survival outcome of gastric cancer in young patients: A large cohort retrospective study. *J BUON* 2019; **24**: 672-678 [PMID: 31128022]

138 **Zhai Z**, Zhu ZY, Zhang Y, Yin X, Han BL, Gao JL, Lou SH, Fang TY, Wang YM, Li CF, Yu XF, Ma Y, Xue YW. Prognostic significance of Borrmann type combined with vessel invasion status in advanced gastric cancer. *World J Gastrointest Oncol* 2020; **12**: 992-1004 [PMID: 33005293 DOI: 10.4251/wjgo.v12.i9.992]

139 **De Franco L**, Marrelli D, Voglino C, Vindigni C, Ferrara F, Di Mare G, Iudici L, Marini M, Roviello F. Prognostic Value of Perineural Invasion in Resected Gastric Cancer Patients According to Lauren Histotype. *Pathol Oncol Res* 2018; **24**: 393-400 [PMID: 28555306 DOI: 10.1007/s12253-017-0257-8]

140 **Gao S**, Cao GH, Ding P, Zhao YY, Deng P, Hou B, Li K, Liu XF. Retrospective evaluation of lymphatic and blood vessel invasion and Borrmann types in advanced proximal gastric cancer. *World J Gastrointest Oncol* 2019; **11**: 642-651 [PMID: 31435465 DOI: 10.4251/wjgo.v11.i8.642]

141 **Du CY**, Chen JG, Zhou Y, Zhao GF, Fu H, Zhou XK, Shi YQ. Impact of lymphatic and/or blood vessel invasion in stage II gastric cancer. *World J Gastroenterol* 2012; **18**: 3610-3616 [PMID: 22826628 DOI: 10.3748/wjg.v18.i27.3610]

142 **Zhao LY**, Chen XL, Wang YG, Xin Y, Zhang WH, Wang YS, Chen XZ, Yang K, Liu K, Xue L, Zhang B, Chen ZX, Chen JP, Zhou ZG, Hu JK. A new predictive model combined of tumor size, lymph nodes count and lymphovascular invasion for survival prognosis in patients with lymph node-negative gastric cancer. *Oncotarget* 2016; **7**: 72300-72310 [PMID: 27509175 DOI: 10.18632/oncotarget.11035]

143 **Bernabeu MO**, Köry J, Grogan JA, Markelc B, Beardo A, d'Avezac M, Enjalbert R, Kaeppler J, Daly N, Hetherington J, Krüger T, Maini PK, Pitt-Francis JM, Muschel RJ, Alarcón T, Byrne HM. Abnormal morphology biases hematocrit distribution in tumor vasculature and contributes to heterogeneity in tissue oxygenation. *Proc Natl Acad Sci U S A* 2020; **117**: 27811-27819 [PMID: 33109723 DOI: 10.1073/pnas.2007770117]

144 **Hughes VS**, Wiggins JM, Siemann DW. Tumor oxygenation and cancer therapy-then and now. *Br J Radiol* 2019; **92**: 20170955 [PMID: 29513032 DOI: 10.1259/bjr.20170955]

145 **Liu M**, Xie S, Zhou J. Use of animal models for the imaging and quantification of angiogenesis. *Exp Anim* 2018; **67**: 1-6 [PMID: 28757511 DOI: 10.1538/expanim.17-0054]

146 **Shimo T**, Takigawa M. Cell Biological Assays for Measuring Angiogenic Activities of CCN

Proteins. *Methods Mol Biol* 2017; **1489**: 239-249 [PMID: 27734381 DOI: 10.1007/978-1-4939-6430-7_22]

147 **Macedo F**, Ladeira K, Longatto-Filho A, Martins SF. Gastric Cancer and Angiogenesis: Is VEGF a Useful Biomarker to Assess Progression and Remission? *J Gastric Cancer* 2017; **17**: 1-10 [PMID: 28337358 DOI: 10.5230/jgc.2017.17.e1]

148 **Wang TB**, Deng MH, Qiu WS, Dong WG. Association of serum vascular endothelial growth factor-C and lymphatic vessel density with lymph node metastasis and prognosis of patients with gastric cancer. *World J Gastroenterol* 2007; **13**: 1794-7; discussion 1797 [PMID: 17465468 DOI: 10.3748/wjg.v13.i12.1794]

149 **Wang TB**, Wang J, Wei XQ, Wei B, Dong WG. Serum vascular endothelial growth factor-C combined with multi-detector CT in the preoperative diagnosis of lymph node metastasis of gastric cancer. *Asia Pac J Clin Oncol* 2012; **8**: 180-186 [PMID: 22524577 DOI: 10.1111/j.1743-7563.2011.01490.x]

150 **Zhao WX**, Liu ZF, Li XL, Li Z. Correlations of serum homocysteine, VEGF and gastrin 17 with gastric cancer and precancerous lesions. *Eur Rev Med Pharmacol Sci* 2019; **23**: 4192-4198 [PMID: 31173290 DOI: 10.26355/eurrev_201905_17922]

151 **Park DJ**, Seo AN, Yoon C, Ku GY, Coit DG, Strong VE, Suh YS, Lee HS, Yang HK, Kim HH, Yoon SS. Serum VEGF-A and Tumor Vessel VEGFR-2 Levels Predict Survival in Caucasian but Not Asian Patients Undergoing Resection for Gastric Adenocarcinoma. *Ann Surg Oncol* 2015; **22** Suppl 3: S1508-S1515 [PMID: 26259755 DOI: 10.1245/s10434-015-4790-y]

152 **Tsirlis TD**, Kostakis A, Papastratis G, Masselou K, Vlachos I, Papachristodoulou A, Nikiteas NI. Predictive significance of preoperative serum VEGF-C and VEGF-D, independently and combined with Ca19-9, for the presence of malignancy and lymph node metastasis in patients with gastric cancer. *J Surg Oncol* 2010; **102**: 699-703 [PMID: 20672317 DOI: 10.1002/jso.21677]

153 **Cheng R**, Yong H, Xia Y, Xie Q, Gao G, Zhou X. Chemotherapy regimen based on sorafenib combined with 5-FU on HIF-1α and VEGF expression and survival in advanced gastric cancer patients. *Oncol Lett* 2017; **13**: 2703-2707 [PMID: 28454454 DOI: 10.3892/ol.2017.5769]

154 **Han K**, Claret L, Piao Y, Hegde P, Joshi A, Powell JR, Jin J, Bruno R. Simulations to Predict Clinical Trial Outcome of Bevacizumab Plus Chemotherapy vs. Chemotherapy Alone in Patients With First-Line Gastric Cancer and Elevated Plasma VEGF-A. *CPT Pharmacometrics Syst Pharmacol* 2016; **5**: 352-358 [PMID: 27404946 DOI: 10.1002/psp4.12064]

155 **Wei B**, Tai Y, Tong H, Wen SL, Tang SH, Huan H, Huang ZY, Liu R, Tang YM, Yang JH, Tang CW, Gao JH. Correlations between VEGF-A expression and prognosis in patients with gastric adenocarcinoma. *Int J Clin Exp Pathol* 2017; **10**: 8461-8469 [PMID: 31966698]

156 **Dai Y**, Jiang J, Wang Y, Jin Z, Hu S. The correlation and clinical implication of VEGF-C expression in microvascular density and lymph node metastasis of gastric carcinoma. *Am J Transl Res* 2016; **8**: 5741-5747 [PMID: 28078045]

157 **Li X**, Zhu X, Wang Y, Wang R, Wang L, Zhu ML, Zheng L. Prognostic value and association of Lauren classification with VEGF and VEGFR-2 expression in gastric cancer. *Oncol Lett* 2019; **18**: 4891-4899 [PMID: 31611999 DOI: 10.3892/ol.2019.10820]

158 **Weidner N**, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. *N Engl J Med* 1991; **324**: 1-8 [PMID: 1701519 DOI: 10.1056/NEJM199101033240101]

159 **Vermeulen PB**, Gasparini G, Fox SB, Colpaert C, Marson LP, Gion M, Beliën JA, de Waal RM, Van Marck E, Magnani E, Weidner N, Harris AL, Dirix LY. Second international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumours. *Eur J Cancer* 2002; **38**: 1564-1579 [PMID: 12142044 DOI: 10.1016/s0959-8049(02)00094-1]

160 **Rada M**, Lazaris A, Kapelanski-Lamoureux A, Mayer TZ, Metrakos P. Tumor microenvironment conditions that favor vessel co-option in colorectal cancer liver metastases: A theoretical model. *Semin Cancer Biol* 2021; **71**: 52-64 [PMID: 32920126 DOI: 10.1016/j.semcancer.2020.09.001]

161 **Marien KM**, Croons V, Waumans Y, Sluydts E, De Schepper S, Andries L, Waelput W, Fransen E, Vermeulen PB, Kockx MM, De Meyer GR. Development and Validation of a Histological Method to Measure Microvessel Density in Whole-Slide Images of Cancer Tissue. *PLoS One* 2016; **11**: e0161496 [PMID: 27583442 DOI: 10.1371/journal.pone.0161496]

162 **Pavlovic M**, Gajovic N, Jurisevic M, Mitrovic S, Radosavljevic G, Pantic J, Arsenijevic N, Jovanovic I. Diverse Expression of IL-32 in Diffuse and Intestinal Types of Gastric Cancer. *Gastroenterol Res Pract* 2018; **2018**: 6578273 [PMID: 30402092 DOI: 10.1155/2018/6578273]

163 **Oliver G**, Kipnis J, Randolph GJ, Harvey NL. The Lymphatic Vasculature in the 21st Century: Novel Functional Roles in Homeostasis and Disease. *Cell* 2020; **182**: 270-296 [PMID: 32707093 DOI: 10.1016/j.cell.2020.06.039]

164 **Petrova TV**, Koh GY. Organ-specific lymphatic vasculature: From development to pathophysiology. *J Exp Med* 2018; **215**: 35-49 [PMID: 29242199 DOI: 10.1084/jem.20171868]

165 **Stacker SA**, Williams SP, Karnezis T, Shayan R, Fox SB, Achen MG. Lymphangiogenesis and lymphatic vessel remodelling in cancer. *Nat Rev Cancer* 2014; **14**: 159-172 [PMID: 24561443 DOI: 10.1038/nrc3677]

166 **Wilczak W**, Wittmer C, Clauditz T, Minner S, Steurer S, Büscheck F, Krech T, Lennartz M, Harms L, Leleu D, Ahrens M, Ingwerth S, Günther CT, Koop C, Simon R, Jacobsen F, Tsourlakis MC, Chirico V, Höflmayer D, Vettorazzi E, Haese A, Steuber T, Salomon G, Michl U, Budäus L, Tilki D, Thederan I, Fraune C, Göbel C, Henrich MC, Juhnke M, Möller K, Bawahab AA, Uhlig R, Adam

M, Weidemann S, Beyer B, Huland H, Graefen M, Sauter G, Schlomm T. Marked Prognostic Impact of Minimal Lymphatic Tumor Spread in Prostate Cancer. *Eur Urol* 2018; **74**: 376-386 [PMID: 29908878 DOI: 10.1016/j.eururo.2018.05.034]

167 **Lund AW**, Wagner M, Fankhauser M, Steinskog ES, Broggi MA, Spranger S, Gajewski TF, Alitalo K, Eikesdal HP, Wiig H, Swartz MA. Lymphatic vessels regulate immune microenvironments in human and murine melanoma. *J Clin Invest* 2016; **126**: 3389-3402 [PMID: 27525437 DOI: 10.1172/JCI79434]

168 **Song E**, Mao T, Dong H, Boisserand LSB, Antila S, Bosenberg M, Alitalo K, Thomas JL, Iwasaki A. VEGF-C-driven lymphatic drainage enables immunosurveillance of brain tumours. *Nature* 2020; **577**: 689-694 [PMID: 31942068 DOI: 10.1038/s41586-019-1912-x]

169 **Sasaki H**, Morohashi S, Toba T, Seino H, Yoshizawa T, Hirai H, Haga T, Wu Y, Kijima H. Neoangiogenesis of gastric submucosa-invasive adenocarcinoma. *Oncol Lett* 2018; **16**: 3895-3900 [PMID: 30128004 DOI: 10.3892/ol.2018.9116]

170 **Lu L**, Wu M, Sun L, Li W, Fu W, Zhang X, Liu T. Clinicopathological and prognostic significance of cancer stem cell markers CD44 and CD133 in patients with gastric cancer: A comprehensive meta-analysis with 4729 patients involved. *Medicine (Baltimore)* 2016; **95**: e5163 [PMID: 27759647 DOI: 10.1097/MD.0000000000005163]

171 **Gresta LT**, Rodrigues-Júnior IA, de Castro LP, Cassali GD, Cabral MM. Assessment of vascular invasion in gastric cancer: a comparative study. *World J Gastroenterol* 2013; **19**: 3761-3769 [PMID: 23840114 DOI: 10.3748/wjg.v19.i24.3761]

172 **Woodham BL**, Chmelo J, Donohoe CL, Madhavan A, Phillips AW. Prognostic Significance of Lymphatic, Venous and Perineural Invasion After Neoadjuvant Chemotherapy in Patients with Gastric Adenocarcinoma. *Ann Surg Oncol* 2020; **27**: 3296-3304 [PMID: 32219726 DOI: 10.1245/s10434-020-08389-7]

173 **Tao Q**, Zhu W, Zhao X, Li M, Shu Y, Wang D, Li X. Perineural Invasion and Postoperative Adjuvant Chemotherapy Efficacy in Patients With Gastric Cancer. *Front Oncol* 2020; **10**: 530 [PMID: 32373527 DOI: 10.3389/fonc.2020.00530]

174 **Bentolila LA**, Prakash R, Mihic-Probst D, Wadehra M, Kleinman HK, Carmichael TS, Péault B, Barnhill RL, Lugassy C. Imaging of Angiotropism/Vascular Co-Option in a Murine Model of Brain Melanoma: Implications for Melanoma Progression along Extravascular Pathways. *Sci Rep* 2016; **6**: 23834 [PMID: 27048955 DOI: 10.1038/srep23834]

175 **Prieto-Vila M**, Yan T, Calle AS, Nair N, Hurley L, Kasai T, Kakuta H, Masuda J, Murakami H, Mizutani A, Seno M. iPSC-derived cancer stem cells provide a model of tumor vasculature. *Am J Cancer Res* 2016; **6**: 1906-1921 [PMID: 27725898]

176 **Cojoc M**, Mäbert K, Muders MH, Dubrovska A. A role for cancer stem cells in therapy resistance: cellular and molecular mechanisms. *Semin Cancer Biol* 2015; **31**: 16-27 [PMID: 24956577 DOI: 10.1016/j.semcancer.2014.06.004]

177 **Lizárraga-Verdugo E**, Avendaño-Félix M, Bermúdez M, Ramos-Payán R, Pérez-Plasencia C, Aguilar-Medina M. Cancer Stem Cells and Its Role in Angiogenesis and Vasculogenic Mimicry in Gastrointestinal Cancers. *Front Oncol* 2020; **10**: 413 [PMID: 32296643 DOI: 10.3389/fonc.2020.00413]

178 **Costa G**, Harrington KI, Lovegrove HE, Page DJ, Chakravartula S, Bentley K, Herbert SP. Asymmetric division coordinates collective cell migration in angiogenesis. *Nat Cell Biol* 2016; **18**: 1292-1301 [PMID: 27870831 DOI: 10.1038/ncb3443]

179 **Hamm MJ**, Kirchmaier BC, Herzog W. Sema3d controls collective endothelial cell migration by distinct mechanisms *via* Nrp1 and PlxnD1. *J Cell Biol* 2016; **215**: 415-430 [PMID: 27799363 DOI: 10.1083/jcb.201603100]

180 **Williams SP**, Gould CM, Nowell CJ, Karnezis T, Achen MG, Simpson KJ, Stacker SA. Systematic high-content genome-wide RNAi screens of endothelial cell migration and morphology. *Sci Data* 2017; **4**: 170009 [PMID: 28248931 DOI: 10.1038/sdata.2017.9]

181 **Brassard-Jollive N**, Monnot C, Muller L, Germain S. *In vitro* 3D Systems to Model Tumor Angiogenesis and Interactions With Stromal Cells. *Front Cell Dev Biol* 2020; **8**: 594903 [PMID: 33224956 DOI: 10.3389/fcell.2020.594903]

182 **Zhang L**, Zheng F, Peng Z, Hu Z, Yang Z. A Feasible Method of Angiogenesis Assessment in Gastric Cancer Using 3D Microvessel Density. *Stem Cells Int* 2018; **2018**: 7813729 [PMID: 29765420 DOI: 10.1155/2018/7813729]

183 **Angelucci A**, Delle Monache S, Cortellini A, Di Padova M, Ficorella C. "Vessels in the Storm": Searching for Prognostic and Predictive Angiogenic Factors in Colorectal Cancer. *Int J Mol Sci* 2018; **19** [PMID: 29351242 DOI: 10.3390/ijms19010299]

184 **Kuczynski EA**, Yin M, Bar-Zion A, Lee CR, Butz H, Man S, Daley F, Vermeulen PB, Yousef GM, Foster FS, Reynolds AR, Kerbel RS. Co-option of Liver Vessels and Not Sprouting Angiogenesis Drives Acquired Sorafenib Resistance in Hepatocellular Carcinoma. *J Natl Cancer Inst* 2016; **108** [PMID: 27059374 DOI: 10.1093/jnci/djw030]

185 **Sitohy B**, Chang S, Sciuto TE, Masse E, Shen M, Kang PM, Jaminet SC, Benjamin LE, Bhatt RS, Dvorak AM, Nagy JA, Dvorak HF. Early Actions of Anti-Vascular Endothelial Growth Factor/Vascular Endothelial Growth Factor Receptor Drugs on Angiogenic Blood Vessels. *Am J Pathol* 2017; **187**: 2337-2347 [PMID: 28736316 DOI: 10.1016/j.ajpath.2017.06.010]

186 **Sitohy B**, Nagy JA, Jaminet SC, Dvorak HF. Tumor-surrogate blood vessel subtypes exhibit differential susceptibility to anti-VEGF therapy. *Cancer Res* 2011; **71**: 7021-7028 [PMID: 21937680

DOI: 10.1158/0008-5472.CAN-11-1693]
187 **Gee MS**, Procopio WN, Makonnen S, Feldman MD, Yeilding NM, Lee WM. Tumor vessel development and maturation impose limits on the effectiveness of anti-vascular therapy. *Am J Pathol* 2003; **162**: 183-193 [PMID: 12507901 DOI: 10.1016/S0002-9440(10)63809-6]
188 **Cascone T**, Herynk MH, Xu L, Du Z, Kadara H, Nilsson MB, Oborn CJ, Park YY, Erez B, Jacoby JJ, Lee JS, Lin HY, Ciardiello F, Herbst RS, Langley RR, Heymach JV. Upregulated stromal EGFR and vascular remodeling in mouse xenograft models of angiogenesis inhibitor-resistant human lung adenocarcinoma. *J Clin Invest* 2011; **121**: 1313-1328 [PMID: 21436589 DOI: 10.1172/JCI42405]
189 **Senchukova M**, Kiselevsky MV. The "cavitary" type of angiogenesis by gastric cancer. Morphological characteristics and prognostic value. *J Cancer* 2014; **5**: 311-319 [PMID: 24723973 DOI: 10.7150/jca.8716]
190 **Senchukova MA**, Nikitenko NV, Tomchuk ON, Zaitsev NV, Stadnikov AA. Different types of tumor vessels in breast cancer: morphology and clinical value. *Springerplus* 2015; **4**: 512 [PMID: 26405632 DOI: 10.1186/s40064-015-1293-z]
191 **Senchukova MA**, Makarova EV, Shurygina EI, Volchenko NN. Morphological Characteristics and Clinical Significance of Different Types of Tumor Vessels in Patients with Stages I-IIA of Squamous Cervical Cancer. *J Oncol* 2020; **2020**: 3818051 [PMID: 32849870 DOI: 10.1155/2020/3818051]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 December 28; 27(48): 8283-8301

DOI: 10.3748/wjg.v27.i48.8283

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

REVIEW

Reciprocal interactions between gut microbiota and autophagy

Pierre Lapaquette, Jean-Baptiste Bizeau, Niyazi Acar, Marie-Agnès Bringer

ORCID number: Pierre Lapaquette 0000-0002-2680-351X; Jean-Baptiste Bizeau 0000-0003-3986-794X; Niyazi Acar 0000-0002-3401-8079; Marie-Agnès Bringer 0000-0002-3746-4998.

Author contributions: Lapaquette P and Bringer MA wrote the paper; Bizeau JB and Acar N revised the manuscript.

Conflict-of-interest statement: The authors declare no conflict of interest for this article.

Supported by Agence Nationale de la Recherche, No. ANR-11-LABX-0021-01;
French "Investissements d'Avenir" program, project ISITE-BFC, No. ANR-15-IDEX-0003; Conseil Régional de Bourgogne, Franche-Comté, No. RECH-E1INV-000131-0-EarlEAT; Institut Carnot Qualiment, INPROBIAUS grant; and FEDER (European Funding for Regional Economic Development), EARLEAT, PO FEDER-FSE Bourgogne 2014-2020 BG 0027905 BG 0027810.

Country/Territory of origin: France

Specialty type: Microbiology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Pierre Lapaquette, UMR PAM A 02.102, University Bourgogne Franche-Comté, Agrosup Dijon, Dijon 21000, France

Jean-Baptiste Bizeau, Niyazi Acar, Marie-Agnès Bringer, Eye and Nutrition Research Group, Centre des Sciences du Goût et de l'Alimentation, AgroSup Dijon, CNRS, INRAE, Université Bourgogne Franche-Comté, Dijon 21000, France

Corresponding author: Marie-Agnès Bringer, PhD, Academic Research, Eye and Nutrition Research Group, Centre des Sciences du Goût et de l'Alimentation, AgroSup Dijon, CNRS, INRAE, Université Bourgogne Franche-Comté, Centre INRAE, 17 rue Sully, Dijon 21000, France. marie-agnes.bringer@inrae.fr

Abstract

A symbiotic relationship has set up between the gut microbiota and its host in the course of evolution, forming an interkingdom consortium. The gut offers a favorable ecological niche for microbial communities, with the whole body and external factors (*e.g.*, diet or medications) contributing to modulating this microenvironment. Reciprocally, the gut microbiota is important for maintaining health by acting not only on the gut mucosa but also on other organs. However, failure in one or another of these two partners can lead to the breakdown in their symbiotic equilibrium and contribute to disease onset and/or progression. Several microbial and host processes are devoted to facing up the stress that could alter the symbiosis, ensuring the resilience of the ecosystem. Among these processes, autophagy is a host catabolic process integrating a wide range of stress in order to maintain cell survival and homeostasis. This cytoprotective mechanism, which is ubiquitous and operates at basal level in all tissues, can be rapidly down- or up-regulated at the transcriptional, post-transcriptional, or post-translational levels, to respond to various stress conditions. Because of its sensitivity to all, metabolic-, immune-, and microbial-derived stimuli, autophagy is at the crossroad of the dialogue between changes occurring in the gut microbiota and the host responses. In this review, we first delineate the modulation of host autophagy by the gut microbiota locally in the gut and in peripheral organs. Then, we describe the autophagy-related mechanisms affecting the gut microbiota. We conclude this review with the current challenges and an outlook toward the future interventions aiming at modulating host autophagy by targeting the gut microbiota.

Key Words: Gut microbiota; Autophagy; Probiotic; Brain; Liver; Muscle

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: June 18, 2021
Peer-review started: June 18, 2021
First decision: August 19, 2021
Revised: September 9, 2021
Accepted: December 2, 2021
Article in press: December 7, 2021
Published online: December 28, 2021

P-Reviewer: Shi JL, Zhai Q
S-Editor: Liu M
L-Editor: Wang TQ
P-Editor: Liu M

Core Tip: We are now aware that maintaining a fine equilibrium between the host and its gut microbiota is a prerequisite to maintain host homeostasis and promote long-term health. Several host and microbial processes interact dynamically to respond to external stresses. Among these processes, host autophagy acts as a cytoprotective mechanism responsive to a wide range of stress conditions, including metabolic, immune, and microbial stimuli. Autophagy was initially described as a degradative process active upon nutrient starvation. However, this process fulfils a wide range of other functions that are essential to host homeostasis. We discuss herein reciprocal interactions of autophagy with the gut microbiota in health and disease conditions.

Citation: Lapaquette P, Bizeau JB, Acar N, Bringer MA. Reciprocal interactions between gut microbiota and autophagy. *World J Gastroenterol* 2021; 27(48): 8283-8301
URL: https://www.wjgnet.com/1007-9327/full/v27/i48/8283.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i48.8283

INTRODUCTION

The commensal microbiota living in the human gut is a unique ecosystem that has co-evolved with human to establish a symbiotic relationship. This microbial community is estimated to encompass about 10^{14} resident microorganisms, dominated by bacteria, but containing also populations of archaea, fungi, protozoa, and viruses[1]. The host provides nutrients and a favorable environment (*i.e.*, ecological niches) for its microbial inhabitants. In return, the gut microbiota plays multiple roles that contribute to the host whole-body homeostasis, in particular by metabolizing dietary nutrients, by preventing colonization by enteric pathogens, and by regulating the host immune system and metabolism. The gut microbiota is, for instance, essential for the synthesis of vitamins (*e.g.*, K and B-group vitamins) and the fermentation of dietary fibers and carbohydrates, which generate short-chain fatty acids (SCFAs). These fermentation products are used as energy source by organs and are also involved in the regulation of various cellular processes (*e.g.*, intestinal barrier integrity, mucus production, and inflammation)[2,3].

Through their interactions with the host, gut microbes and their derived products are involved not only in the physiological regulation of the gut mucosa but also in that of organs located at distance from the gut mucosa, as illustrated by the studies detailing molecular features of the gut-microbiota-brain axis[4-6]. Keeping the mutualistic relationship between the gut microbiota and the host throughout host's life is thus essential to maintain the health status of the host[7]. Deleterious shifts in the composition of the gut microbiota, called dysbiosis, can unbalance its functions, leading to the disruption of host homeostasis. This is particularly well illustrated by the ability of fecal microbiota transplantation (FMT) to transmit detrimental metabolic and/or pro-inflammatory traits from a sick donor to healthy recipient mice[8-10]. In addition to environmental stresses, the symbiotic equilibrium of the gut microbiota and the host can also be broken by dysfunctions/alterations in the host metabolism and immune system, which are conditions that can contribute to dysbiosis[8,11,12]. In this context, the roles of autophagy in strengthening the intestinal barrier and in maintaining host metabolic and inflammatory balance position it as the cornerstone of the symbiotic relationship between the gut microbiota and the host[4,13].

Macroautophagy/autophagy is an intracellular and multistep process starting with the formation of a membranous cup-shaped structure, called phagophore, which engulfs portions of the cytoplasm. The phagophore elongates and finally closes to form a sealed double-membraned vacuole, called autophagosome, whose maturation ends by its fusion with lysosomes[14-16]. Autophagy was initially described as a lysosomal catabolic process occurring under starvation that degrades and recycles cytoplasmic macromolecules (*e.g.*, proteins, lipids, and carbohydrates) for the biosynthesis of essential cellular components and to restore energy balance[17]. Nowadays, autophagy process and autophagy-related proteins are recognized as key cellular components whose roles are not restricted to the regulation of energy balance[18,19]. These roles include, but are not limited to, the regulation of the inflammatory response, the cytoprotection by preventing the accumulation of intracellular waste (*e.g.*, damaged organelles and misfolded or aggregated proteins), the protection against

intracellular pathogens (*e.g.*, bacteria, fungi, or viruses), the membrane dynamic (*e.g.*, transport or secretion), and the regulation of cell differentiation and survival. Autophagy also regulates specific functions related to the features of organs. For example, at the gut mucosa - the first tissue at the interface between the gut microbiota and the host - autophagy is involved in the regulation of the functions of the secretory cells and of the intestinal stem cell[4]. In the central nervous system, autophagy plays roles in neuronal development and survival and other various functions[20]. The central role of autophagy in maintaining homeostasis, and thus the health status, is supported by the observed embryonic or neonatal lethality of mice deficient for most autophagy-related (*Atg*) core genes (*Becn1, Vps34, Atg9a, Ulk1/2, Atg3, Atg5, Atg7,* and *Atg16l1*) as well as association of numerous diseases and disorders with autophagy defects[19,21].

Of note, a growing number of recent studies highlight that most of the proteins of the autophagy machinery also mediate autophagy-independent functions, including phagocytosis, exocytosis, cytokinesis, DNA repair, or innate and adaptive immune signaling[22]. To exert their numerous functions, the machineries involving autophagy proteins are intricated with molecular sensors specialized in the detection of various stimuli such as microbial sensors [*e.g.*, Toll-like receptors (TLR) and Nod-like receptors (NLR)], stress sensors (*e.g.*, HMGB1, Sestrins, ER-stress sensor proteins, P2XR, and cGAS-STING pathway), or energy status sensors (*e.g.*, AMPK and mTOR pathways) [23-29].

In this review, we summarize the current knowledge on how the gut microbiota influences host autophagy locally in the gut mucosa or remotely in peripheral organs (brain, heart, liver, or muscles), and how autophagy or autophagy-related proteins can reciprocally shape the gut microbiota composition and modify its functions (Figure 1). We finally discuss the potential of targeting the gut microbiota as a strategy to modulate autophagy or restore its functionality in pathological context.

INFLUENCE OF THE MICROBIOTA ON GUT AUTOPHAGY

A first clue that points out a direct implication of the gut microbiota in the regulation of host autophagy has been provided by analyzing autophagy in germ-free mice (*i.e.*, mice lacking microorganisms and bred in isolators without any microbial exposure). Basal autophagy was decreased in the colonic epithelium of germ-free mice compared to conventionally raised mice, suggesting that the gut microbiota influences intestinal autophagy in physiological condition[30]. The increase in basal activity of autophagy in germ-free mice was attributed to an energy-deprived status of colonocytes. Treatment of these cells with butyrate, a SCFA generated by some gut bacteria and serving as main energy source for colonocytes, was sufficient to reverse the phenotype. *In vivo*, colonization of germ-free mice with the butyrate-producing bacterial strain *Butyrivibrio fibrisolvens* was sufficient to restore autophagy steady state. In addition to butyrate, other bacteria-derived metabolites may have the ability to reduce basal autophagy in the colon. They include indole-3-lactate, which is a tryptophan metabolite produced notably by the bacteria belonging to the *Lacticaseibacillus*, *Lactobacillus*, *Bifidobacterium*, *Megamonas*, *Roseburia*, or *Ruminococcus* genus[31,32].

Pathogen-associated molecular patterns (PAMPs), which are conserved microbial molecules, are also able to modulate autophagy usually by stimulating the process [23]. These effects have been particularly well described for pathogens. PAMPs mainly act by interacting with specific host cell receptors that belong to the TLR and NLR families. This has been illustrated by the ability of the lipopolysaccharide (LPS) from Gram negative bacteria to stimulate autophagy through its binding to TLR4[33], or the peptidoglycan (PGN) from Gram positive bacteria through NOD1-, NOD2-, and TLR2-associated signaling[34,35]. Besides those of bacteria, fungal PAMPs can also mobilize components of the autophagy machinery. This is true for β-glucans that are found in fungal cell walls and stimulate autophagy-related processes though their binding to the host receptor Dectin-1[36,37]. Trehalose, a non-reducing disaccharide produced by bacteria and fungi, is also a potent autophagy inducer, for which the ability to stimulate colonic autophagy during colitis in mice has been described[38,39]. In addition, in-depth studies of the infectious cycle of some pathogenic bacteria have shed the light on the existence of secreted bacterial effectors able to activate (*e.g.*, Ats-1 protein from *Anaplasma phagocytophilum*) or inhibit (*e.g.*, RavZ protein from *Legionella pneumophila*) autophagy at various stages of the process[40,41]. It is not excluded that some commensal microorganisms in the gut express such proteins that influence host autophagy.

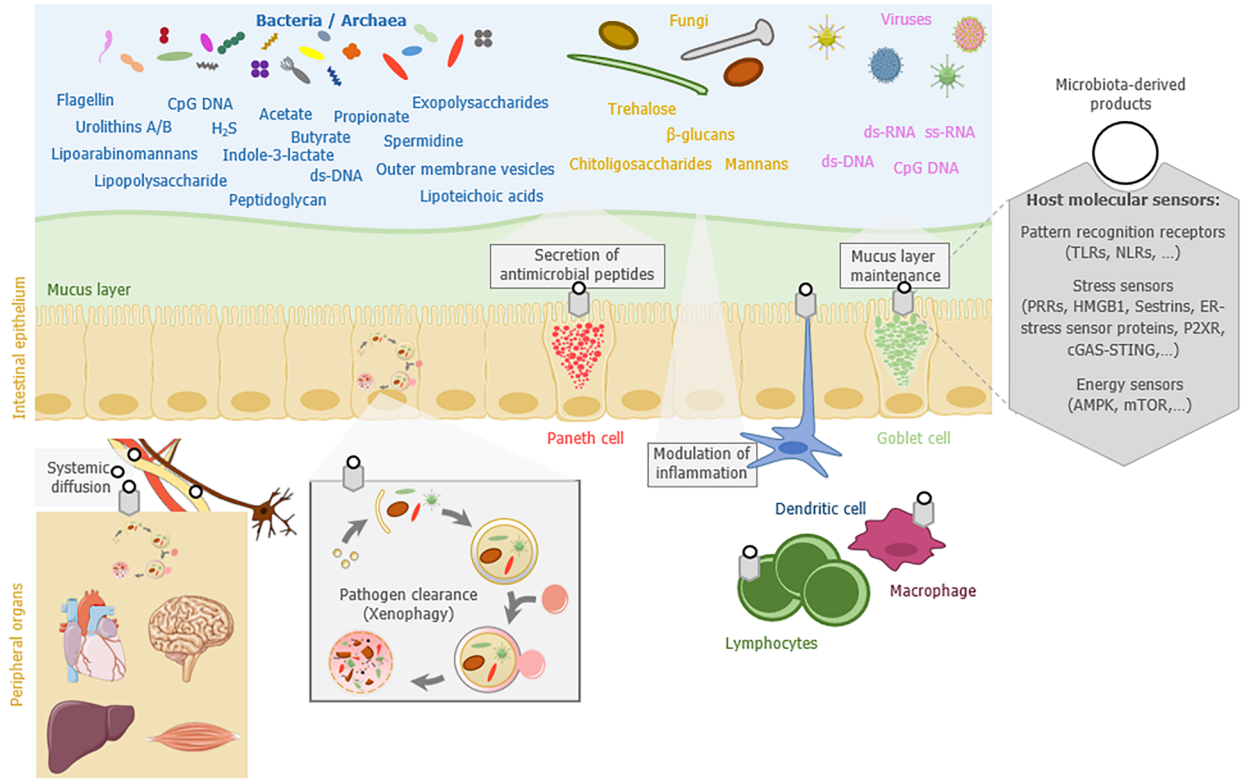

Figure 1 Complex interplay between gut microbiota and autophagy. The interactions between the gut microbiota and autophagy are bidirectional. Autophagy is involved in the regulation of several mechanisms (grey boxes) that shape the gut microbiota. Reciprocally, some bacterial- (blue), fungal- (orange), or viral-derived (pink) compounds are able to modulate autophagy in the gut mucosa as well as in distant organs through systemic pathways (circulatory system, nervous system ...). Modulation of autophagy by the gut microbiota involves microbiota-derived products such as microbial compounds (lipopolysaccharide, peptidoglycan ...), microbial derived-compounds (short chain fatty acids, secondary biliary acids ...), or signaling molecules (cytokines, hormones ...). They stimulate a wide range of host molecular sensors (pattern recognition receptors, stress sensors, and energy sensors; grey hexagons) located in the gut or peripheral organs. PRR: Pattern recognition receptor; TLR: Toll-like receptor; NLR: Nod-like receptor.

Given the influence of gut microbiota-related factors on autophagy, one could expect that alterations in the composition of the gut microbiota would affect autophagy in the gut mucosa. Indeed, an increase in the expression of some autophagy-related proteins (FoxO1, FoxO3, GABARAP, and ATG7) and LC3-II/LC3-I ratio and a decrease in AKT activation have been reported in newborn piglets receiving FMT[42]. In addition, alteration of the gut microbiota resulting from the administration of a cocktail of broad-spectrum antibiotics increased the basal activity of autophagy as well as the expression of some autophagy-related proteins (ATG16L1, ATG5, and IRGM1) in the ileal mucosa of mice[43,44]. Interestingly, oral administration of a single bacterial species (*e.g.*, *Desulfovibrio* spp., *Fusobacterium nucleatum*, or *Escherichia coli*) in conventional mice can also be sufficient to modulate gut autophagy [42,44,45]. Altogether, these studies suggest that autophagy regulatory network is sensitive to changes in the gut microbiota.

SYSTEMIC EFFECTS OF THE GUT MICROBIOTA ON HOST AUTOPHAGY

Microbial-derived metabolites (*e.g.*, PAMPs), compounds that are issued from the gut microbiota metabolism (*e.g.*, neuroactive compounds and SCFAs) and host bioactive molecules that are produced in response to its interaction with the gut microbiota (*e.g.*, cytokines), can have large systemic effects and modulate the physiology of organs that are distant from the gut. Influence of the gut microbiota on the brain is a well-documented example of such effects[6]. Several communication routes (immune system, autonomic nervous system, neuroendocrine system, hypothalamic – pituitary – adrenal axis, and other metabolic pathways) between the microbiota and the brain have been identified[6]. It is very likely that similar pathways and microbiota-derived players, or at least some of them, modulate as well the physiology of other organs in the body. Evidence is accumulating on the modulation of autophagy by the gut

microbiota in distant organs and several of these are presented below (Table 1).

Modulation of autophagy in nervous tissues

Although few studies are available on this emerging topic, they suggest that the gut microbiota could influence autophagy in the brain throughout life in both physiological and pathological conditions.

Diet is a key environmental factor that drives the composition and metabolic functions of the gut microbiota[46,47]. In particular, maternal diet can influence post-natal gut microbiota and neurological development of the offspring[48]. In a recent study, Wang and colleagues reported that feeding mothers with a high sugar and high fat (HSHF) diet, a condition that modifies the gut microbiota of the offspring, modulates also the expression of neuronal and autophagy markers in the brain during early life stage[49]. Particularly, they observed that the LC3A and LC3B levels were modified in the brain of the offspring in the HSHF group compared to controls before 28 d of age, and then decreased, meaning that autophagy may be differentially regulated in HSHF offspring[49].

Aging is associated with a decline of host autophagy including in the brain[50]. Influence of the gut microbiota on brain autophagy in aging has been evidenced in *in vivo* models. Alteration of autophagy has been reported in the brain of D-gal-treated mice, a model of accelerated aging[51,52]. These alterations were characterized by decreases in the LC3-II/LC3-I ratio and in the expression of ATG7 and SIRT1, as well as by increased phosphorylation of the master negative regulator of autophagy mTOR (S2448) and expression of p62 in the hippocampus tissue of D-gal-induced aging mice [52]. Interestingly, the administration of urolithin A (UA), a bioactive metabolite generated by the gut microbiota, was efficient in rescuing these autophagy-related defects. To note, UA administration also allowed to reverse increases in the LC3-II/LC3-I ratio, the expression of p62, and the phosphorylation of mTOR (S2448), as well as the decreased expression of Sirt-1 and ATG7 observed in the hippocampus of 12-mo-old mice[52].

Autophagy defect is thought to play a role in neurodegenerative processes associated with numerous diseases, including Alzheimer's disease (AD)[53]. Interestingly, although a causal relationship remains to be demonstrated, a few studies suggest that dysbiosis associated with AD could influence brain autophagy[54]. Decreased Beclin-1 expression and increased expression of p62 have been observed in the brain of old 3xTg-AD mice (a transgenic mouse model of AD) compared to young control mice, indicating alterations in autophagy[55]. Interestingly, in addition to modifying the composition and predicted function of the gut microbiota, oral supplementation of old 3xTg-AD mice with a combination of nine probiotic strains (*Streptococcus thermophilus*, *Bifidobacterium longum*, *B. breve*, *B. infantis*, *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, *Lactobacillus delbrueckii* subsp. bulgaricus, and *Levilactobacillus brevis*; SLAB51 formulation) also partially restored defects in autophagy[55]. Moreover, SLAB51 was also effective in restoring the impaired expression level and activity of SIRT1, a positive regulator of autophagy, in the brain of 3xTg-AD mice[56,57].

In another context, changes in the composition of the fecal microbiota have been reported in patients with acute ischemic stroke (AIS), a common cerebrovascular disease caused by sudden loss of blood circulation in a specific brain area[58,59]. Interestingly, anal administration of the fecal supernatant obtained from an AIS patient to antibiotics-treated mice resulted in increased expression of genes encoding Beclin-1, ATG12, and LC3 as well as increased expression of Beclin-1 at the protein level and an increased level of LC3-II in brain tissue compared to antibiotics-treated mice that received the fecal supernatant of healthy controls[59].

The retina, which is the light sensitive neural tissue that lines the back of the eyes, displays numerous similarities with the brain either anatomically or functionally[60]. Neurodegenerative conditions that affect the brain seem to compromise the retina, and *vice versa*[60-62]. Similarly to the brain, the retina is also highly sensitive to nutritional variations[63]. Retina autophagy[64,65] as well as modifications in the gut microbiota [66-69] is suspected to contribute to retinal diseases such as diabetic retinopathy, age-related macular degeneration, and glaucoma. Although no causal relationship has been yet established, one can assume that, as in the brain, the gut microbiota might influence retinal autophagy and that changes in its composition might alter retinal autophagy and contribute to the development of retinopathies.

Modulation of liver autophagy

Evidence of the influence of the gut microbiota on liver autophagy came from studies in gut microbiota-deprived mouse models. Comparison of germ-free mice and altered

Table 1 Data supporting the existence of a systemic regulation of autophagy by the gut microbiota

Ref.		Impact on autophagy		
		Brain	Liver	Muscles
[49,74-76]	Diet-induced changes in the gut microbiota	Feeding of mother mice with an HSHF diet: Changes in the expression levels of LC3A-I/LC3A-II/ LC3B-I/LC3B-II in the offspring.	Feeding mice or rats with an HF diet: Changes in the expression levels of *LC3*, p62, mTOR, and p-AKT and modulation of the LC3-II amount.	
[55,56,59,70]	Mice with specific gut microbiota	AD mice[1]: Modulation of the lysosomal activity (Cathepsin L) and SIRT1 activity and changes in the expression levels of Beclin-1, p62, and SIRT1. FMT from patients with AIS to mice: Changes in the expression levels of *Becn1*, *ATG12*, and *LC3* expression and in the amount of LC3-II.	ASF colonized mice: Changes in the expression of a set of genes related to autophagy/membrane trafficking (*Uvrag*, *Atg14*, *Becn1*, *Bcl2l1*, and *Pik3c3*) and lysosomal functions (*Chmp4c* and *Chmp2a*) compared to germ-free mice.	
[71,79]	Germ free or antibiotic-treated animals		Antibiotic treatment of mice fed a normal diet: Alteration of the basal expression of LC3 compared to controls.	Germ free piglets: Changes in the expression levels of *LC3A*, *LC3B*, and *Becn1* and of mTOR, p-mTOR, AKT, and p-AKT levels compared to normal and/or FMT piglets.
[55,56,75,76,78]	Probiotics	SLAB51[2]: Modulation of SIRT1 activity and changes in the expression levels of Beclin-1, p62, and SIRT-1 as well as in the LC3-II amount in AD mice[1].	*Limosilactobacillus reuteri*: Modulation of the expression levels of mTOR and p-AKT in HFD-fed rats.	*Lacticaseibacillus rhamnosus*, *Pediococcus acidilactici*, *Bifidobacterium adolescentis*: Changes in the expression levels of LC3 and ATG7 in rats fed a high-calorie diet.
[52,71,74,77,80]	Gut microbiota-derived products	UA: Modulation of LC3-II/LC3-I and p-mTOR/mTOR ratio and changes in the expression levels of ATG7 and p62 in mouse models of aging[3].	SCFAs: Activation of the PPARγ-UCP2-AMPK pathway, and induction of autophagy flux and lysosomal activity in mouse hepatocyte AML-12 cells. FXR and TGR5[4]: Involved in autophagy modulation.	UA: Induction of mitophagy in *Caenorhabditis elegans* and in rodents. UB: Modulation of LC3-II/LC3-I, p-mTOR/mTOR and p-ULK1/ULK1 ratio and change in the expression level of p62 in a rat model of ischemia/reperfusion injury.

[1]AD mice: Mouse model of Alzheimer's disease (3xTg-AD mice).

[2]SLAB51: A combination of nine probiotic strains (*Streptococcus thermophilus*, *Bifidobacterium longum*, *B. breve*, *B. infantis*, *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, *Lactobacillus delbrueckii* subsp. bulgaricus, and *Levilactobacillus brevis*).

[3]D-gal-treated mice and 12-mo-old mice.

[4]FXR and TGR5: Bile acid receptors.

HSHF diet: High sugar and high fat diet; HF diet: High fat diet; FMT: Fecal microbiota transplantation; SCFAs: Short chain fatty acids (propionate and butyrate); AIS: Acute ischemic stroke; ASF: Altered Schaedler's flora; UA: Urolithin A; UB: urolithin B.

Schaedler's flora (a community of eight bacterial species) colonized mice revealed that absence of the gut microbiota altered hepatic expression of genes involved in autophagy and lysosomal functions[70]. In another study, a decrease in the expression of LC3 at the protein level has been reported in the liver of mice deprived from gut microbiota as a consequence of chronic treatment with antibiotics (ampicillin and neomycin) compared to control mice[71]. In addition, those authors showed that microbial-derived SCFAs (propionate and butyrate) activated autophagy, induced lysosomal activity, and increased autophagy flux *in vitro* in mouse hepatocyte AML-12

cells[71]. The mechanism involves the activation of the PPARγ-UCP2-AMPK pathway [71].

Primary bile acids are synthesized from cholesterol in the liver and are converted into secondary bile acids by the gut microbiota[72]. Bile acids are signaling molecules that can activate nuclear hormone receptors including FXR and TGR5 (also known as GPBAR1), which is a cell-surface receptor of the G protein-coupled receptor family [73]. These two bile acid receptors have been described to modulate autophagy in the liver and adipose tissue in fed and fasted states[74].

Several alterations of autophagy, including a decreased amount of *LC3* mRNA and LC3-II and an increased amount of p62, have been observed in the liver of mice fed a high-fat diet (HFD), a potent inducer of dysbiosis[74]. Chronic exposure of rats to an HFD can lead to NASH (non-alcoholic fatty steatohepatitis). Development of this liver disease has been associated with dysbiosis and alterations in autophagy, particularly increased expression of hepatic mTOR and p-AKT[75,76]. Interestingly, supplementation of an HFD with a probiotic strain (*Limosilactobacillus reuteri*) and/or treatment of NASH mice with antibiotics (metronidazole) tended to normalize the hepatic content of these two autophagy-related proteins, as well as SCFAs and *Firmicutes* and *Bacteroidetes* fecal contents, thus suggesting a role of the gut microbiota in the modulation of hepatic autophagy[75,76]. To note, some data suggest a role for TGR5 in the regulation of autophagy in response to HFD[74].

Modulation of autophagy in muscle tissues

An induction of autophagy, characterized by decreased phosphorylation of mTOR (S2448) and ULK1 (S757), an increased amount of LC3-II, and decreased expression of p62, has been reported in a rat model of ischemia/reperfusion injury[77]. Interestingly, intraperitoneal injection of urolithin B (UB), a gut microbiota-derived metabolite, was able to reverse this phenotype[77]. The inhibitory effect of UB on autophagy is thought to activate the Nrf2-related antioxidant response by increasing p62 accumulation and favoring p62-Keap1interaction[77]. Another argument that suggests the influence of the gut microbiota on heart autophagy has been provided by changes in the expression levels of LC3 and ATG7 observed in heart tissue of rats fed a high-calorie diet supplemented with probiotics (*Lacticaseibacillus rhamnosus*, *Pediococcus acidilactici*, and *Bifidobacterium adolescentis*)[78].

In addition to the heart, autophagy might be regulated by the gut microbiota in other muscles. Recently, high-throughput RNA-seq analysis revealed that the expression levels of autophagy-related genes (*LC3A*, *LC3B*, and *Beclin-1*) were modulated in the skeletal muscles of germ-free piglets compared to control piglets [79]. Moreover, germ-free piglets harbored decreased expression of mTOR and AKT and their phosphorylated forms, phospho-mTOR (S2448) and phospho-AKT (S473), respectively, compared to control piglets[79]. FMT of germ-free piglets with stools collected on healthy donors pigs was effective in restoring the amounts of phospho-AKT and mTOR to a level similar to that of controls[79]. Some microbial-derived metabolites able to influence the muscle autophagy have been identified. For example, a role of UA as a mitophagy (selective degradation of mitochondria by autophagy) inducer in the muscle tissue has been described in the model organism *Caenorhabditis elegans* and in rodents[80].

SHAPING OF THE GUT MICROBIOTA BY AUTOPHAGY

As developed in the first part of this review, the gut microbiota is able to influence host autophagy by several pathways and through complex regulatory networks governing the autophagy machinery. Reciprocally, autophagy and autophagy-related proteins can shape the gut microbiota (Figure 1). This is particularly well illustrated by changes in the gut microbiota composition observed in mice conditionally deficient for autophagy (*Atg5-/-*, *Atg7-/-*, and *ATG16L1* T300A knock-in) in the gut[81-83]. Interestingly, alterations of autophagy in peripheral organs such as the liver have been shown to influence the composition of the gut microbiota[84].

A first overall reason that would explain why autophagy activity in the gut mucosa can modulate the abundance of gut microorganisms is that this process is essential to maintain homeostasis of their ecological niche. Indeed, basal autophagy is crucial to maintain the integrity of Lgr5-positive intestinal stem cells that give rise to all differentiated lineages of the intestinal epithelium throughout life[85]. In addition, autophagy contributes to the maintaining of intestinal barrier integrity, particularly by regulating proteins involved in tight junctions (*e.g.*, Claudin-2 and Occludin) on the apical side of

intestinal epithelial cells and by promoting cell survival upon various stress (*e.g.*, bacterial or viral infection, inflammation, or chemical stress)[4,86-88].

The main cellular mechanisms by which host autophagy shapes the gut microbiota (including pathosymbionts) are described below.

Clearance of pathogens

Autophagy mediates the bulk or selective lysosomal degradation of cellular components. In selective autophagy, selective autophagy receptors (SARs) recognize and bind specific cargoes to promote phagophore formation around them, ultimately leading to their degradation into a mature autolysosome. These specific cargoes can be for instance mitochondria (mitophagy), lipid droplets (lipophagy), protein aggregates (aggrephagy), or peroxysomes (pexophagy)[89]. A selective form of autophagy termed xenophagy is dedicated to the elimination of intracellular pathogens (*e.g.*, bacteria, viruses, fungi, or protozoa) and is supported by the expression of several SARs including NDP52, Optineurin, p62, TAX1BP1, Galectin 8, and TECPR1[90]. Xenophagy has been shown to restrict or avoid the intracellular persistence and the replication of various human pathogenic or pathosymbiotic bacteria, residing either in damaged vacuoles [*e.g.*, *Salmonella* Typhimurium or adherent-invasive *Escherichia coli* (AIEC)] or free in the host cytosol (Group A *Streptococcus*)[91-93]. Thus, by limiting the dissemination of invasive pathogens from the gut lumen to extra-intestinal sites, autophagy also restrains their persistency in the gut microbiota[94,95]. Defects in xenophagy are thought to contribute to the etiology of Crohn's disease (CD) an inflammatory bowel disease (IBD) characterized by chronic and severe intestinal inflammation associated with dysbiosis[96]. In particular, a coding polymorphism (Thr300Ala) in the autophagy-related gene *ATG16L1* that confers an increased risk for the development of CD has been shown *in vitro* and *in vivo* to alter the xenophagy process, thus favoring persistency of the CD-associated AIEC bacteria[92,97,98]. CD risk polymorphisms have also been identified in other autophagy-related genes, including core autophagy genes (*IRGM*, *ULK1*, *ATG4a*, and *ATG4d*) and genes involved more specifically in xenophagy (*NOD2* and *NDP52*)[99-101].

One important point is that, besides xenophagy, non-canonical autophagy such as LC3-associated phagocytosis (LAP) can also contribute to the clearance of intracellular pathogens. This specific form of phagocytosis requires an important set of core autophagy proteins (UVRAG, BECN1, VPS34, LC3, ATG3, ATG4, ATG5, ATG7, ATG12, and ATG16L1), but some other proteins involved in canonical autophagy remain dispensable (ATG14, ULK1, FIP200, and AMBRA1). LAP also distinguishes from canonical autophagy by the formation of single-membrane vacuoles called LAPosomes[102]. Efficiency of LAP to increase clearance of pathogens such as *Listeria monocytogenes* or *Aspergillus fumigatus* has been shown[103,104].

Mucus layer maintenance

A mucus layer composed of highly glycosylated proteins (mucins) overlays the gut epithelium and represents an important physical barrier limiting the contact of luminal microbes with the epithelium, thus avoiding their potential translocation into underlying tissues[105]. The mucus layer differs between the small and large intestine in terms of physicochemical properties (*e.g.*, thickness, density, and composition) and it is under the influence of numerous factors, including the gut microbiota and the diet [106-108]. Whereas in the small intestine the mucus is non-attached and constitutes a discontinuous layer, it is organized in two layers - the inner and outer mucus layers - in the large intestine. Compared to the intestinal lumen, only few bacterial species are able to live and to persist in the mucus layer. This is partly due to the important amount of various antimicrobial compounds (*e.g.*, IgA, lysozyme, defensins, REG3γ, and phospholipase A2-IIA) found in the mucus layer, particularly in the small intestine. However, some commensal bacteria are molecularly equipped to bind, degrade the mucus glycans, and/or harvest the oligosaccharides, giving them a selective advantage in colonizing this particular ecological niche[109]. Among others, mucin-degrading specialists include species belonging to the genera *Bacteroides* (*e.g.*, *B. thetaiotaomicron* and *B. fragilis*), *Ruminococcus* (*e.g.*, *R. gnavus* and *R. torques*), and *Akkermansia* (*e.g.*, *A. muciniphila*). Interestingly, *A. muciniphila*, a bacterial species belonging to the phylum Verrucomicrobia, is considered as a healthy marker of the intestine since its presence in high abundance is associated with a healthy mucosa whereas reduction of its abundance is associated with intestinal disorders (*e.g.*, obesity and IBD)[110,111]. Studies suggest that the composition of mucus-associated microbiota differs depending on the intestinal segment or the mucus layer (outer or inner layer) that is considered[105]. Bacteria belonging to the phylum Firmicutes have been found in higher abundance in the mucus layer than Bacteroidetes, both in

humans and in rodents[105].

Mucus plays a critical role in the maintenance of the symbiotic relationship between the host and the gut microbiota[112]. Deletion of the *Muc2* gene in mice results in changes in the gut microbiota composition characterized in particular by an increase in the abundance of potential pathobionts (*e.g.*, *Desulfovibrio*, *Escherichia*, and *Erysipelotrichaceae*), and the reduction of beneficial bacteria (*e.g.*, *Lactobacilli*) and *Lachnospiraceae* [112]. In addition to ensuring an habitat and energy sources for a specific part of the gut microbiota, the mucus constitutes a protective layer against pathogen invasion and infection, although some pathogenic bacteria have developed efficient strategies to colonize this special environment and reach the intestinal epithelium (*e.g.*, *Shigella flexneri* and AIEC)[113,114]. Thus, modifications in mucus layer structure or composition by genetic and environmental factors, such as diet, can modify the gut microbiota[105]. These changes can be beneficial when they strengthen the mucus barrier properties, but they can also be deleterious by favoring emergence of pathobionts, by bringing harmful bacteria closer to the epithelial barrier and by destabilizing the symbiotic relationship between the gut microbiota and the host, at the gut mucosa as well as at systemic levels[107].

Mucus secretion into the gut lumen is achieved by specialized secretory cells, the goblet cells. Mucins, the proteins forming the mucus, are packed into secretory granules that are localized on the apical side of the goblet cells and constitutively secreted by fusion of the granules with the plasma membrane. Proteins belonging to the core autophagy machinery (ATG5, ATG7, and LC3B) are critical in mice for the release of these secretory granules by supporting the generation of reactive oxygen species[115].

The NLRP6 inflammasome has been identified, among others roles, as a key factor involved in autophagy-induced regulation of goblet cell secretory functions[116,117]. NLRP6-deficient mice exhibit defective autophagy in intestinal cells including in goblet cells, a phenotype that is associated with impaired mucus layer formation. This mucus alteration may contribute, together with the other NLRP6-related defects, to modulating the composition of the gut microbiota and abnormally bring microbes closer to the epithelial barrier in NLRP6-deficient mice. Analyses of the gut microbiota in NLRP6-deficient mice revealed an abnormal representation of the bacterial phyla Bacteroidetes (*Prevotellaceae*) and Saccharibacteria (formerly known as TM7)[116]. In addition, alteration of the mucus layer in NLRP6-deficient mice enables *Citrobacter rodentium*, a mouse-specific pathogen, to penetrate deeper into the crypts and be more invasive[117]. The role of autophagy in shaping the gut microbiota through the regulation of mucus layer maintenance is also supported by observations made in *Atg7*-deficient mice. Secretion of mucins from goblet cells was diminished in colonic-epithelial cell-specific *Atg7* knock-out mice[82]. This phenotype was associated with an abnormal composition of the gut microbiota characterized in particular by an increased abundance of *Clostridia* and *Prevotellaceae* in *Atg7*-deficient mice. In addition, those authors observed an increased bacterial burden in the colon, a phenotype that could contribute to the exacerbated sensitivity to experimental colitis observed in *Atg7* knock-out mice. Interestingly, stimulation of the autophagy-related process, either by a beneficial bacterial strain (*Bifidobacterium dentium*) or by a polyphenol (oxyresveratrol), has been shown to enhance mucin production by goblet cells in *in vivo* and *in vitro* models[118,119].

Secretion of antimicrobial compounds in the gut lumen

Autophagy and autophagy-related proteins can also affect the composition of the gut microbiota by regulating the secretion of some antimicrobial compounds released into the gut lumen by enterocytes, Paneth cells, or immune cells. Among them, immunoglobulins of the A class (IgAs) are daily released in huge amount (several grams per day) into the gut lumen and shape the composition of the gut microbiota. Alterations of the gut microbial ecosystem have been reported in the absence of hypermutated intestinal IgA in mice with deficiency of activation-induced cytidine deaminase[120-122]. Changes in the gut microbiota were particularly characterized by expansion of anaerobic bacteria in the small intestine, with a domination by segmented filamentous bacteria[121]. Several other studies in mouse models support the role of IgAs in regulating the diversity and composition of microbiota[123,124]. Data obtained in humans showed that selective IgA-deficiency (sIgAd) is associated with a mild intestinal dysbiosis, characterized by expansion of pro-inflammatory bacteria (*e.g.*, *E. coli, Prevotella*), reduction of anti-inflammatory commensals (e.g., *Faecalibacterium*), and perturbation of bacterial dependency association network[125]. In addition, Catanzaro and colleagues reported also a trend toward a decreased alpha diversity and shifts in the relative abundance of some taxa (*e.g.*, increase in *Eubacterium dolichum* and *Rumino-*

coccus bromii and decrease in *Paraprevotellaceae*) in human sIgAd subjects compared to controls[126]. IgAs are produced by gut-resident antibody-secreting plasma cells (PCs) that display important metabolic adaptations and endoplasmic reticulum expansion to cope with the stress of producing very large amounts of IgAs[127]. Some studies suggest that autophagy is required for sustainable production of immunoglobulins by PCs since mice with conditional deficiency of *Atg5* in B cells had defective antibody responses, with an increased sensitivity of PCs to cell death[128]. In addition, mice deficient for *Atg5* in B cells harbored a decreased number of IgA-secreting PCs isolated from the gut-associated lamina propria, Peyer's patches, and mesenteric lymph nodes in comparison to control mice[129].

Another important antimicrobial compound to which commensal bacteria are directly exposed in the gut lumen is the lysozyme secreted by Paneth cells, which are secretory epithelial cells located at the bottom of the crypts in the small intestine. This antimicrobial protein is also produced by macrophages and neutrophils in the lamina propria. Three types of lysozyme have been described so far across the animal kingdom[130]. Lysozyme causes bacterial lysis by hydrolyzing bacterial cell wall PGN, but it can also induce cationic killing of bacteria by inserting into and forming pores into the lipid bilayer of the bacterial cell membrane. This is the case with c-type lysozyme expressed in human[130]. Not all bacteria are equally sensitive to lysozyme and some pathogenic bacteria have developed strategies to escape its antimicrobial activity[130]. The contribution of lysozyme in shaping the gut microbiota is illustrated by the dysbiosis observed in lysozyme-deficient mice (*Lyz1*$^{-/-}$ mice) that is characterized by the expansion of some mucolytic bacteria such as *Blautia gnavus* (formerly known as *Ruminococcus gnavus*)[130,131]. No change in luminal bacterial load and alpha-diversity was observed in the cecum- and mucosal-associated bacteria in the ileum and the colon of *Lyz1*$^{-/-}$ mice[131]. However, changes occurred in the composition of the fecal microbiota (expansion of *Dorea formicigenerans* and reduction of *Candidatus Arthromitus*) as well as the ileal microbiota (expansion of *B. gnavus* and *D. formicigenerans* and reduction of *C. Arthromitus*) in *Lyz1*$^{-/-}$ mice[131].

Alpha-defensins (also called crypt defensins or cryptdins) are another example of antimicrobial factors that are produced by Paneth cells, whose roles in host defense against enteric pathogens and regulation of the composition of the gut indigenous microbiota have been described[132]. Interestingly, abnormal packaging and secretion of antimicrobial compounds by Paneth cells have been reported in mice harboring Paneth cells deficient for the autophagy-related genes *Atg5*, *Atg7*, and *Atg16l1* and in patients with CD-associated *NOD2* and *ATG16L1* variants[133-135]. Of note, this defect in lysozyme packaging in autophagy-deficient mice required an infectious (viral or bacterial) trigger[136,137].

Even if canonical autophagy is considered as a degradative process, some infectious agents such as *Salmonella* Typhimurium can trigger a secretory autophagy resulting in the formation of LC3-positive, double-membraned lysozyme granules[136]. These autophagosome-like vacuoles are not directed for the fusion with the lysosomes but instead reach the plasma membrane for the release of their content into the gut lumen. Thus, the autophagy machinery participates in the unconventional protein secretion of lysozyme, thereby affecting the composition of the gut microbiota by counter-selecting the lysozyme-sensitive bacteria. In this context, it has been suggested that vitamin D, *via* binding to the vitamin D receptor expressed by Paneth cells, can sustain autophagy activities in these cells[138]. To note, several studies suggest that expression and secretion of other antimicrobial peptides than lyzozyme, such as the defensins and cathelicidins, would be regulated by autophagy. However, the exact molecular mechanisms remain to be determined[82,139].

Modulation of inflammation

Cell stimulation by microorganisms (*e.g.*, invasive pathogens) or danger signals (*e.g.*, extracellular ATP, uric acid, or HMGB1) are usually associated with the triggering of inflammatory processes through the release of cytokines and chemokines. Inflammation is a protective response that results in tissue repair. However, this response needs to be tightly regulated in order to avoid excessive and/or chronic inflammation that could be detrimental for host tissues. In the gut mucosa, immune tolerance toward the resident gut microbiota should be maintained to avoid chronic gut inflammation and sustain homeostasis[140]. Unbalanced inflammatory responses can also alter the gut microbiota as shown in mouse models of colitis that mimic human IBD, in which inflammation induces microbial dysbiosis[141,142]. Chronic inflammatory state was also suggested to contribute to dysbiosis in IBD patients[143]. This inflammation-driven bacterial dysbiosis is commonly characterized by an overall decrease in bacterial diversity, especially in Firmicutes (*Clostridium* groups) and an overgrowth of

species belonging to *Enterobacteriaceae*[143,144].

Autophagy machinery and autophagy-related proteins are key contributors to the regulation of the inflammatory processes. Thus, one could assume that modulation of inflammation by autophagy could influence the composition of the gut microbiota. Autophagy is usually considered as an anti-inflammatory process, particularly since it controls activation of inflammasomes that are multimeric protein complexes involved in the maturation of pro-inflammatory cytokines[145]. Mice deficient for *Atg16l1* in haematopoietic cells have been shown to be highly sensitive to chemically-induced colitis and produce increased levels of IL-1β and IL-18, two cytokines processed by inflammasomes[146]. *Atg16l1*-deficient macrophages that were stimulated by LPS also produced higher amounts of these cytokines compared to wild-type macrophages. Autophagy can alleviate activation of inflammasomes, at least by removing stimuli that induced them (*e.g.*, intracellular infectious agents) and by degrading some inflammasome components (*e.g.*, NLRP1, NLRP3, AIM2, or pro-CASP1)[147]. Interestingly, alterations of the gut microbiota (*e.g.*, increased abundance of Bacteroidetes) as well as enhancement of the local Th1 and Th17 immune responses have been reported in mice with dextran sodium sulfate (DSS) colitis that express the CD risk allele *ATG16L1* T300A - a genetic context known to impair some autophagy-related functions - compared to DSS-treated wild-type mice[81]. Similar observations have been made in gnotobiotic mice expressing the CD risk allele *ATG16L1* T300A and inoculated with human stools from active CD patients[81]. These data illustrated how a subtle polymorphism in an autophagy-related gene could deeply impact the equilibrium between immune responses and the gut microbiota.

Autophagy is also able to modulate signaling of interferons, notably by degrading key players of type-I interferon responses (*e.g.*, RIG-I, STING, MDA5, IRF3, MAVS, and cGAS)[148]. Abnormal regulation of interferon signaling can lead to alterations of the gut microbiota as described in knock-out mice and viral infection models[149]. Interestingly, the gut microbiota has been described to stimulate intestinal autophagy *via* the induction of the type-II interferon, and this microbiota-mediated activation of autophagy has been shown to protect the host against infection by the protozoan parasite *Toxoplasma gondii* by limiting the deleterious production of the pro-inflammatory cytokine TNF-α[150]. Autophagy has also been described to limit the production and the secretion of various cytokines including TNF-α, IL-1β, IL-23, IL-6, TGF-β, and MIF[151,152]. However, the molecular mechanisms by which autophagy regulates their expression remain elusive. In many cases, autophagy reduces secretion of cytokines by simply alleviating cellular stress that triggers the inflammatory responses.

CONCLUSION

Given its crucial role in regulating homeostasis at both cell and tissue levels, it is not surprising that alterations of autophagy are connected to a large number of disorders (*e.g.*, IBD, cancers, and neurodegenerative diseases). To assume its various functions, autophagy activation is tightly regulated and the gut microbiota has recently emerged as a contributor in its regulatory networks in both the gut mucosa and other tissues. This advance in the understanding of the molecular mechanisms supporting this highly integrated cellular process that tip the balance between health and disease offers new opportunities to develop preventive or therapeutic tools. Indeed, the gut microbiota appears as a promising target to restore functional autophagy or to prevent its alterations in various disease conditions. The growing interest that was aroused from the discovery of such a hub position occupied by the gut microbiota in maintaining physical and mental health status has led to the conceptualization, development, and/or examination of various tools to manipulate the gut microbiota (probiotics, prebiotics, synbiotics, postbiotics, FMT, Crispr/Cas9, diet…). In the era of personalized medicine, such a toolbox could constitute a key element that could be integrated in the therapeutic strategies. However, further explorations of the interplay between the gut microbiota and autophagy are needed. Important advances have been made in understanding the local dialogue between the gut microbiota and autophagy at the level of the gut mucosa, but less is known about how and in which extent they communicate at the systemic level. Bi-directionality of the interactions between the gut microbiota and the autophagy network, plasticity and complexity of the gut microbiota and its multiple effects on host, as well as pleiotropy of the functions of autophagy are all factors that increase the level of complexity of the system. Better characterization of the cellular and molecular actors from both sides - the gut

microbiota and autophagy - that contribute and regulate the framework of their interactions to maintain homeostasis constitutes a prerequisite to propose new preventive and therapeutic tools in pathological conditions associated with dysbiosis and/or autophagy dysfunction.

REFERENCES

1 **Matijašić M**, Meštrović T, Paljetak HČ, Perić M, Barešić A, Verbanac D. Gut Microbiota beyond Bacteria-Mycobiome, Virome, Archaeome, and Eukaryotic Parasites in IBD. *Int J Mol Sci* 2020; **21** [PMID: 32290414 DOI: 10.3390/ijms21082668]

2 **Blaak EE**, Canfora EE, Theis S, Frost G, Groen AK, Mithieux G, Nauta A, Scott K, Stahl B, van Harsselaar J, van Tol R, Vaughan EE, Verbeke K. Short chain fatty acids in human gut and metabolic health. *Benef Microbes* 2020; **11**: 411-455 [PMID: 32865024 DOI: 10.3920/BM2020.0057]

3 **LeBlanc JG**, Milani C, de Giori GS, Sesma F, van Sinderen D, Ventura M. Bacteria as vitamin suppliers to their host: a gut microbiota perspective. *Curr Opin Biotechnol* 2013; **24**: 160-168 [PMID: 22940212 DOI: 10.1016/j.copbio.2012.08.005]

4 **Foerster EG**, Mukherjee T, Cabral-Fernandes L, Rocha JDB, Girardin SE, Philpott DJ. How autophagy controls the intestinal epithelial barrier. *Autophagy* 2021; 1-18 [PMID: 33906557 DOI: 10.1080/15548627.2021.1909406]

5 **Schroeder BO**, Bäckhed F. Signals from the gut microbiota to distant organs in physiology and disease. *Nat Med* 2016; **22**: 1079-1089 [PMID: 27711063 DOI: 10.1038/nm.4185]

6 **Morais LH**, Schreiber HL 4th, Mazmanian SK. The gut microbiota-brain axis in behaviour and brain disorders. *Nat Rev Microbiol* 2021; **19**: 241-255 [PMID: 33093662 DOI: 10.1038/s41579-020-00460-0]

7 **Lynch SV**, Pedersen O. The Human Intestinal Microbiome in Health and Disease. *N Engl J Med* 2016; **375**: 2369-2379 [PMID: 27974040 DOI: 10.1056/NEJMra1600266]

8 **Schaubeck M**, Clavel T, Calasan J, Lagkouvardos I, Haange SB, Jehmlich N, Basic M, Dupont A, Hornef M, von Bergen M, Bleich A, Haller D. Dysbiotic gut microbiota causes transmissible Crohn's disease-like ileitis independent of failure in antimicrobial defence. *Gut* 2016; **65**: 225-237 [PMID: 25887379 DOI: 10.1136/gutjnl-2015-309333]

9 **Turnbaugh PJ**, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 2006; **444**: 1027-1031 [PMID: 17183312 DOI: 10.1038/nature05414]

10 **Zenewicz LA**, Yin X, Wang G, Elinav E, Hao L, Zhao L, Flavell RA. IL-22 deficiency alters colonic microbiota to be transmissible and colitogenic. *J Immunol* 2013; **190**: 5306-5312 [PMID: 23585682 DOI: 10.4049/jimmunol.1300016]

11 **Al Nabhani Z**, Lepage P, Mauny P, Montcuquet N, Roy M, Le Roux K, Dussaillant M, Berrebi D, Hugot JP, Barreau F. Nod2 Deficiency Leads to a Specific and Transmissible Mucosa-associated Microbial Dysbiosis Which Is Independent of the Mucosal Barrier Defect. *J Crohns Colitis* 2016; **10**: 1428-1436 [PMID: 27147452 DOI: 10.1093/ecco-jcc/jjw095]

12 **Yang M**, Liu Y, Xie H, Wen Z, Zhang Y, Wu C, Huang L, Wu J, Xie C, Wang T, Peng W, Liu S, Chen L, Liu X. Gut Microbiota Composition and Structure of the Ob/Ob and Db/Db Mice. *Int J Endocrinol* 2019; **2019**: 1394097 [PMID: 30984260 DOI: 10.1155/2019/1394097]

13 **Lahiri V**, Hawkins WD, Klionsky DJ. Watch What You (Self-) Eat: Autophagic Mechanisms that Modulate Metabolism. *Cell Metab* 2019; **29**: 803-826 [PMID: 30943392 DOI: 10.1016/j.cmet.2019.03.003]

14 **Galluzzi L**, Baehrecke EH, Ballabio A, Boya P, Bravo-San Pedro JM, Cecconi F, Choi AM, Chu CT, Codogno P, Colombo MI, Cuervo AM, Debnath J, Deretic V, Dikic I, Eskelinen EL, Fimia GM, Fulda S, Gewirtz DA, Green DR, Hansen M, Harper JW, Jäättelä M, Johansen T, Juhasz G, Kimmelman AC, Kraft C, Ktistakis NT, Kumar S, Levine B, Lopez-Otin C, Madeo F, Martens S, Martinez J, Melendez A, Mizushima N, Münz C, Murphy LO, Penninger JM, Piacentini M, Reggiori F, Rubinsztein DC, Ryan KM, Santambrogio L, Scorrano L, Simon AK, Simon HU, Simonsen A, Tavernarakis N, Tooze SA, Yoshimori T, Yuan J, Yue Z, Zhong Q, Kroemer G. Molecular definitions of autophagy and related processes. *EMBO J* 2017; **36**: 1811-1836 [PMID: 28596378 DOI: 10.15252/embj.201796697]

15 **Zhao YG**, Codogno P, Zhang H. Machinery, regulation and pathophysiological implications of autophagosome maturation. *Nat Rev Mol Cell Biol* 2021 [PMID: 34302147 DOI: 10.1038/s41580-021-00392-4]

16 **Boya P**, Reggiori F, Codogno P. Emerging regulation and functions of autophagy. *Nat Cell Biol* 2013; **15**: 713-720 [PMID: 23817233 DOI: 10.1038/ncb2788]

17 **Ohsumi Y**. Historical landmarks of autophagy research. *Cell Res* 2014; **24**: 9-23 [PMID: 24366340 DOI: 10.1038/cr.2013.169]

18 **Khandia R**, Dadar M, Munjal A, Dhama K, Karthik K, Tiwari R, Yatoo MI, Iqbal HMN, Singh KP, Joshi SK, Chaicumpa W. A Comprehensive Review of Autophagy and Its Various Roles in Infectious, Non-Infectious, and Lifestyle Diseases: Current Knowledge and Prospects for Disease Prevention, Novel Drug Design, and Therapy. *Cells* 2019; **8** [PMID: 31277291 DOI:

10.3390/cells8070674]
19 **Levine B**, Kroemer G. Biological Functions of Autophagy Genes: A Disease Perspective. *Cell* 2019; **176**: 11-42 [PMID: 30633901 DOI: 10.1016/j.cell.2018.09.048]
20 **Nikoletopoulou V**, Papandreou ME, Tavernarakis N. Autophagy in the physiology and pathology of the central nervous system. *Cell Death Differ* 2015; **22**: 398-407 [PMID: 25526091 DOI: 10.1038/cdd.2014.204]
21 **Kuma A**, Komatsu M, Mizushima N. Autophagy-monitoring and autophagy-deficient mice. *Autophagy* 2017; **13**: 1619-1628 [PMID: 28820286 DOI: 10.1080/15548627.2017.1343770]
22 **Galluzzi L**, Green DR. Autophagy-Independent Functions of the Autophagy Machinery. *Cell* 2019; **177**: 1682-1699 [PMID: 31199916 DOI: 10.1016/j.cell.2019.05.026]
23 **Oh JE**, Lee HK. Pattern recognition receptors and autophagy. *Front Immunol* 2014; **5**: 300 [PMID: 25009542 DOI: 10.3389/fimmu.2014.00300]
24 **Rashid HO**, Yadav RK, Kim HR, Chae HJ. ER stress: Autophagy induction, inhibition and selection. *Autophagy* 2015; **11**: 1956-1977 [PMID: 26389781 DOI: 10.1080/15548627.2015.1091141]
25 **Ro SH**, Fay J, Cyuzuzo CI, Jang Y, Lee N, Song HS, Harris EN. SESTRINs: Emerging Dynamic Stress-Sensors in Metabolic and Environmental Health. *Front Cell Dev Biol* 2020; **8**: 603421 [PMID: 33425907 DOI: 10.3389/fcell.2020.603421]
26 **Kang R**, Livesey KM, Zeh HJ 3rd, Lotze MT, Tang D. HMGB1 as an autophagy sensor in oxidative stress. *Autophagy* 2011; **7**: 904-906 [PMID: 21487246 DOI: 10.4161/auto.7.8.15704]
27 **Kroemer G**, Mariño G, Levine B. Autophagy and the integrated stress response. *Mol Cell* 2010; **40**: 280-293 [PMID: 20965422 DOI: 10.1016/j.molcel.2010.09.023]
28 **Young CN**, Sinadinos A, Lefebvre A, Chan P, Arkle S, Vaudry D, Gorecki DC. A novel mechanism of autophagic cell death in dystrophic muscle regulated by P2RX7 receptor large-pore formation and HSP90. *Autophagy* 2015; **11**: 113-130 [PMID: 25700737 DOI: 10.4161/15548627.2014.994402]
29 **Zierhut C**, Funabiki H. Regulation and Consequences of cGAS Activation by Self-DNA. *Trends Cell Biol* 2020; **30**: 594-605 [PMID: 32546434 DOI: 10.1016/j.tcb.2020.05.006]
30 **Donohoe DR**, Garge N, Zhang X, Sun W, O'Connell TM, Bunger MK, Bultman SJ. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. *Cell Metab* 2011; **13**: 517-526 [PMID: 21531334 DOI: 10.1016/j.cmet.2011.02.018]
31 **Fan Q**, Guan X, Hou Y, Liu Y, Wei W, Cai X, Zhang Y, Wang G, Zheng X, Hao H. Paeoniflorin modulates gut microbial production of indole-3-lactate and epithelial autophagy to alleviate colitis in mice. *Phytomedicine* 2020; **79**: 153345 [PMID: 33002829 DOI: 10.1016/j.phymed.2020.153345]
32 **Meng D**, Sommella E, Salviati E, Campiglia P, Ganguli K, Djebali K, Zhu W, Walker WA. Indole-3-lactic acid, a metabolite of tryptophan, secreted by Bifidobacterium longum subspecies infantis is anti-inflammatory in the immature intestine. *Pediatr Res* 2020; **88**: 209-217 [PMID: 31945773 DOI: 10.1038/s41390-019-0740-x]
33 **Xu Y**, Jagannath C, Liu XD, Sharafkhaneh A, Kolodziejska KE, Eissa NT. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. *Immunity* 2007; **27**: 135-144 [PMID: 17658277 DOI: 10.1016/j.immuni.2007.05.022]
34 **Arroyo DS**, Soria JA, Gaviglio EA, Garcia-Keller C, Cancela LM, Rodriguez-Galan MC, Wang JM, Iribarren P. Toll-like receptor 2 Ligands promote microglial cell death by inducing autophagy. *FASEB J* 2013; **27**: 299-312 [PMID: 23073832 DOI: 10.1096/fj.12-214312]
35 **Travassos LH**, Carneiro LA, Ramjeet M, Hussey S, Kim YG, Magalhães JG, Yuan L, Soares F, Chea E, Le Bourhis L, Boneca IG, Allaoui A, Jones NL, Nuñez G, Girardin SE, Philpott DJ. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol* 2010; **11**: 55-62 [PMID: 19898471 DOI: 10.1038/ni.1823]
36 **Ma J**, Becker C, Lowell CA, Underhill DM. Dectin-1-triggered recruitment of light chain 3 protein to phagosomes facilitates major histocompatibility complex class II presentation of fungal-derived antigens. *J Biol Chem* 2012; **287**: 34149-34156 [PMID: 22902620 DOI: 10.1074/jbc.M112.382812]
37 **Öhman T**, Teirilä L, Lahesmaa-Korpinen AM, Cypryk W, Veckman V, Saijo S, Wolff H, Hautaniemi S, Nyman TA, Matikainen S. Dectin-1 pathway activates robust autophagy-dependent unconventional protein secretion in human macrophages. *J Immunol* 2014; **192**: 5952-5962 [PMID: 24808366 DOI: 10.4049/jimmunol.1303213]
38 **Macias-Ceja DC**, Cosín-Roger J, Ortiz-Masiá D, Salvador P, Hernández C, Esplugues JV, Calatayud S, Barrachina MD. Stimulation of autophagy prevents intestinal mucosal inflammation and ameliorates murine colitis. *Br J Pharmacol* 2017; **174**: 2501-2511 [PMID: 28500644 DOI: 10.1111/bph.13860]
39 **Sarkar S**, Davies JE, Huang Z, Tunnacliffe A, Rubinsztein DC. Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. *J Biol Chem* 2007; **282**: 5641-5652 [PMID: 17182613 DOI: 10.1074/jbc.M609532200]
40 **Choy A**, Dancourt J, Mugo B, O'Connor TJ, Isberg RR, Melia TJ, Roy CR. The Legionella effector RavZ inhibits host autophagy through irreversible Atg8 deconjugation. *Science* 2012; **338**: 1072-1076 [PMID: 23112293 DOI: 10.1126/science.1227026]
41 **Niu H**, Xiong Q, Yamamoto A, Hayashi-Nishino M, Rikihisa Y. Autophagosomes induced by a bacterial Beclin 1 binding protein facilitate obligatory intracellular infection. *Proc Natl Acad Sci U S A* 2012; **109**: 20800-20807 [PMID: 23197835 DOI: 10.1073/pnas.1218674109]
42 **Cheng S**, Ma X, Geng S, Jiang X, Li Y, Hu L, Li J, Wang Y, Han X. Fecal Microbiota Transplantation Beneficially Regulates Intestinal Mucosal Autophagy and Alleviates Gut Barrier

Injury. *mSystems* 2018; **3** [PMID: 30320222 DOI: 10.1128/mSystems.00137-18]
43 **Feng Y**, Huang Y, Wang Y, Wang P, Song H, Wang F. Antibiotics induced intestinal tight junction barrier dysfunction is associated with microbiota dysbiosis, activated NLRP3 inflammasome and autophagy. *PLoS One* 2019; **14**: e0218384 [PMID: 31211803 DOI: 10.1371/journal.pone.0218384]
44 **Singh SB**, Wilson M, Ritz N, Lin HC. Autophagy Genes of Host Responds to Disruption of Gut Microbial Community by Antibiotics. *Dig Dis Sci* 2017; **62**: 1486-1497 [PMID: 28466260 DOI: 10.1007/s10620-017-4589-8]
45 **Yu T**, Guo F, Yu Y, Sun T, Ma D, Han J, Qian Y, Kryczek I, Sun D, Nagarsheth N, Chen Y, Chen H, Hong J, Zou W, Fang JY. Fusobacterium nucleatum Promotes Chemoresistance to Colorectal Cancer by Modulating Autophagy. *Cell* 2017; **170**: 548-563.e16 [PMID: 28753429 DOI: 10.1016/j.cell.2017.07.008]
46 **Singh RK**, Chang HW, Yan D, Lee KM, Ucmak D, Wong K, Abrouk M, Farahnik B, Nakamura M, Zhu TH, Bhutani T, Liao W. Influence of diet on the gut microbiome and implications for human health. *J Transl Med* 2017; **15**: 73 [PMID: 28388917 DOI: 10.1186/s12967-017-1175-y]
47 **Sonnenburg JL**, Bäckhed F. Diet-microbiota interactions as moderators of human metabolism. *Nature* 2016; **535**: 56-64 [PMID: 27383980 DOI: 10.1038/nature18846]
48 **Al Rubaye H**, Adamson CC, Jadavji NM. The role of maternal diet on offspring gut microbiota development: A review. *J Neurosci Res* 2021; **99**: 284-293 [PMID: 32112450 DOI: 10.1002/jnr.24605]
49 **Wang D**, Zhang H, Zeng M, Tang X, Zhu X, Guo Y, Qi L, Xie Y, Zhang M, Chen D. Maternal high sugar and fat diet benefits offspring brain function *via* targeting on the gut-brain axis. *Aging (Albany NY)* 2021; **13**: 10240-10274 [PMID: 33819195 DOI: 10.18632/aging.202787]
50 **Barbosa MC**, Grosso RA, Fader CM. Hallmarks of Aging: An Autophagic Perspective. *Front Endocrinol (Lausanne)* 2018; **9**: 790 [PMID: 30687233 DOI: 10.3389/fendo.2018.00790]
51 **Azman KF**, Zakaria R. D-Galactose-induced accelerated aging model: an overview. *Biogerontology* 2019; **20**: 763-782 [PMID: 31538262 DOI: 10.1007/s10522-019-09837-y]
52 **Chen P**, Chen F, Lei J, Li Q, Zhou B. Activation of the miR-34a-Mediated SIRT1/mTOR Signaling Pathway by Urolithin A Attenuates D-Galactose-Induced Brain Aging in Mice. *Neurotherapeutics* 2019; **16**: 1269-1282 [PMID: 31420820 DOI: 10.1007/s13311-019-00753-0]
53 **Chen S**, Zhou Q, Ni Y, Le W. Autophagy and Alzheimer's Disease. *Adv Exp Med Biol* 2020; **1207**: 3-19 [PMID: 32671736 DOI: 10.1007/978-981-15-4272-5_1]
54 **Bostancikliođlu M**. The role of gut microbiota in pathogenesis of Alzheimer's disease. *J Appl Microbiol* 2019; **127**: 954-967 [PMID: 30920075 DOI: 10.1111/jam.14264]
55 **Bonfili L**, Cecarini V, Berardi S, Scarpona S, Suchodolski JS, Nasuti C, Fiorini D, Boarelli MC, Rossi G, Eleuteri AM. Microbiota modulation counteracts Alzheimer's disease progression influencing neuronal proteolysis and gut hormones plasma levels. *Sci Rep* 2017; **7**: 2426 [PMID: 28546539 DOI: 10.1038/s41598-017-02587-2]
56 **Bonfili L**, Cecarini V, Cuccioloni M, Angeletti M, Berardi S, Scarpona S, Rossi G, Eleuteri AM. SLAB51 Probiotic Formulation Activates SIRT1 Pathway Promoting Antioxidant and Neuroprotective Effects in an AD Mouse Model. *Mol Neurobiol* 2018; **55**: 7987-8000 [PMID: 29492848 DOI: 10.1007/s12035-018-0973-4]
57 **Lee IH**, Cao L, Mostoslavsky R, Lombard DB, Liu J, Bruns NE, Tsokos M, Alt FW, Finkel T. A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy. *Proc Natl Acad Sci U S A* 2008; **105**: 3374-3379 [PMID: 18296641 DOI: 10.1073/pnas.0712145105]
58 **Battaglini D**, Pimentel-Coelho PM, Robba C, Dos Santos CC, Cruz FF, Pelosi P, Rocco PRM. Gut Microbiota in Acute Ischemic Stroke: From Pathophysiology to Therapeutic Implications. *Front Neurol* 2020; **11**: 598 [PMID: 32670191 DOI: 10.3389/fneur.2020.00598]
59 **Xu N**, Kan P, Yao X, Yang P, Wang J, Xiang L, Zhu Y. Astragaloside IV reversed the autophagy and oxidative stress induced by the intestinal microbiota of AIS in mice. *J Microbiol* 2018; **56**: 838-846 [PMID: 30353470 DOI: 10.1007/s12275-018-8327-5]
60 **London A**, Benhar I, Schwartz M. The retina as a window to the brain-from eye research to CNS disorders. *Nat Rev Neurol* 2013; **9**: 44-53 [PMID: 23165340 DOI: 10.1038/nrneurol.2012.227]
61 **Byun MS**, Park SW, Lee JH, Yi D, Jeon SY, Choi HJ, Joung H, Ghim UH, Park UC, Kim YK, Shin SA, Yu HG, Lee DY; KBASE Research Group. Association of Retinal Changes With Alzheimer Disease Neuroimaging Biomarkers in Cognitively Normal Individuals. *JAMA Ophthalmol* 2021; **139**: 548-556 [PMID: 33764406 DOI: 10.1001/jamaophthalmol.2021.0320]
62 **Nucci C**, Martucci A, Cesareo M, Garaci F, Morrone LA, Russo R, Corasaniti MT, Bagetta G, Mancino R. Links among glaucoma, neurodegenerative, and vascular diseases of the central nervous system. *Prog Brain Res* 2015; **221**: 49-65 [PMID: 26518072 DOI: 10.1016/bs.pbr.2015.04.010]
63 **Lien EL**, Hammond BR. Nutritional influences on visual development and function. *Prog Retin Eye Res* 2011; **30**: 188-203 [PMID: 21296184 DOI: 10.1016/j.preteyeres.2011.01.001]
64 **Boya P**, Esteban-Martínez L, Serrano-Puebla A, Gómez-Sintes R, Villarejo-Zori B. Autophagy in the eye: Development, degeneration, and aging. *Prog Retin Eye Res* 2016; **55**: 206-245 [PMID: 27566190 DOI: 10.1016/j.preteyeres.2016.08.001]
65 **Rosa MD**, Distefano G, Gagliano C, Rusciano D, Malaguarnera L. Autophagy in Diabetic Retinopathy. *Curr Neuropharmacol* 2016; **14**: 810-825 [PMID: 26997506 DOI: 10.2174/1570159x14666160321122900]
66 **Das T**, Jayasudha R, Chakravarthy S, Prashanthi GS, Bhargava A, Tyagi M, Rani PK, Pappuru RR, Sharma S, Shivaji S. Alterations in the gut bacterial microbiome in people with type 2 diabetes

mellitus and diabetic retinopathy. *Sci Rep* 2021; **11**: 2738 [PMID: 33531650 DOI: 10.1038/s41598-021-82538-0]

67 **Gong H**, Zhang S, Li Q, Zuo C, Gao X, Zheng B, Lin M. Gut microbiota compositional profile and serum metabolic phenotype in patients with primary open-angle glaucoma. *Exp Eye Res* 2020; **191**: 107921 [PMID: 31917963 DOI: 10.1016/j.exer.2020.107921]

68 **Huang Y**, Wang Z, Ma H, Ji S, Chen Z, Cui Z, Chen J, Tang S. Dysbiosis and Implication of the Gut Microbiota in Diabetic Retinopathy. *Front Cell Infect Microbiol* 2021; **11**: 646348 [PMID: 33816351 DOI: 10.3389/fcimb.2021.646348]

69 **Lin P**, McClintic SM, Nadeem U, Skondra D. A Review of the Role of the Intestinal Microbiota in Age-Related Macular Degeneration. *J Clin Med* 2021; **10** [PMID: 34065988 DOI: 10.3390/jcm10102072]

70 **Juanola O**, Hassan M, Kumar P, Yilmaz B, Keller I, Simillion C, Engelmann C, Tacke F, Dufour JF, De Gottardi A, Moghadamrad S. Intestinal microbiota drives cholestasis-induced specific hepatic gene expression patterns. *Gut Microbes* 2021; **13**: 1-20 [PMID: 33847205 DOI: 10.1080/19490976.2021.1911534]

71 **Iannucci LF**, Sun J, Singh BK, Zhou J, Kaddai VA, Lanni A, Yen PM, Sinha RA. Short chain fatty acids induce UCP2-mediated autophagy in hepatic cells. *Biochem Biophys Res Commun* 2016; **480**: 461-467 [PMID: 27773823 DOI: 10.1016/j.bbrc.2016.10.072]

72 **Dawson PA**, Karpen SJ. Intestinal transport and metabolism of bile acids. *J Lipid Res* 2015; **56**: 1085-1099 [PMID: 25210150 DOI: 10.1194/jlr.R054114]

73 **Schaap FG**, Trauner M, Jansen PL. Bile acid receptors as targets for drug development. *Nat Rev Gastroenterol Hepatol* 2014; **11**: 55-67 [PMID: 23982684 DOI: 10.1038/nrgastro.2013.151]

74 **Carino A**, Marchianò S, Biagioli M, Scarpelli P, Bordoni M, Di Giorgio C, Roselli R, Fiorucci C, Monti MC, Distrutti E, Zampella A, Fiorucci S. The bile acid activated receptors GPBAR1 and FXR exert antagonistic effects on autophagy. *FASEB J* 2021; **35**: e21271 [PMID: 33368684 DOI: 10.1096/fj.202001386R]

75 **Ahmed LA**, Salem MB, Seif El-Din SH, El-Lakkany NM, Ahmed HO, Nasr SM, Hammam OA, Botros SS, Saleh S. Gut microbiota modulation as a promising therapy with metformin in rats with non-alcoholic steatohepatitis: Role of LPS/TLR4 and autophagy pathways. *Eur J Pharmacol* 2020; **887**: 173461 [PMID: 32758573 DOI: 10.1016/j.ejphar.2020.173461]

76 **Seif El-Din SH**, Salem MB, El-Lakkany NM, Hammam OA, Nasr SM, Okasha H, Ahmed LA, Saleh S, Botros SS. Early intervention with probiotics and metformin alleviates liver injury in NAFLD rats *via* targeting gut microbiota dysbiosis and p-AKT/mTOR/LC-3II pathways. *Hum Exp Toxicol* 2021; **40**: 1496-1509 [PMID: 33678036 DOI: 10.1177/0960327121999445]

77 **Zheng D**, Liu Z, Zhou Y, Hou N, Yan W, Qin Y, Ye Q, Cheng X, Xiao Q, Bao Y, Luo J, Wu X. Urolithin B, a gut microbiota metabolite, protects against myocardial ischemia/reperfusion injury *via* p62/Keap1/Nrf2 signaling pathway. *Pharmacol Res* 2020; **153**: 104655 [PMID: 31996327 DOI: 10.1016/j.phrs.2020.104655]

78 **Lai CH**, Tsai CC, Kuo WW, Ho TJ, Day CH, Pai PY, Chung LC, Huang CC, Wang HF, Liao PH, Huang CY. Multi-Strain Probiotics Inhibit Cardiac Myopathies and Autophagy to Prevent Heart Injury in High-Fat Diet-Fed Rats. *Int J Med Sci* 2016; **13**: 277-285 [PMID: 27076784 DOI: 10.7150/ijms.14769]

79 **Qi R**, Sun J, Qiu X, Zhang Y, Wang J, Wang Q, Huang J, Ge L, Liu Z. The intestinal microbiota contributes to the growth and physiological state of muscle tissue in piglets. *Sci Rep* 2021; **11**: 11237 [PMID: 34045661 DOI: 10.1038/s41598-021-90881-5]

80 **Ryu D**, Mouchiroud L, Andreux PA, Katsyuba E, Moullan N, Nicolet-Dit-Félix AA, Williams EG, Jha P, Lo Sasso G, Huzard D, Aebischer P, Sandi C, Rinsch C, Auwerx J. Urolithin A induces mitophagy and prolongs lifespan in C. elegans and increases muscle function in rodents. *Nat Med* 2016; **22**: 879-888 [PMID: 27400265 DOI: 10.1038/nm.4132]

81 **Lavoie S**, Conway KL, Lassen KG, Jijon HB, Pan H, Chun E, Michaud M, Lang JK, Gallini Comeau CA, Dreyfuss JM, Glickman JN, Vlamakis H, Ananthakrishnan A, Kostic A, Garrett WS, Xavier RJ. The Crohn's disease polymorphism, *ATG16L1* T300A, alters the gut microbiota and enhances the local Th1/Th17 response. *Elife* 2019; **8** [PMID: 30666959 DOI: 10.7554/eLife.39982]

82 **Tsuboi K**, Nishitani M, Takakura A, Imai Y, Komatsu M, Kawashima H. Autophagy Protects against Colitis by the Maintenance of Normal Gut Microflora and Secretion of Mucus. *J Biol Chem* 2015; **290**: 20511-20526 [PMID: 26149685 DOI: 10.1074/jbc.M114.632257]

83 **Yang L**, Liu C, Zhao W, He C, Ding J, Dai R, Xu K, Xiao L, Luo L, Liu S, Li W, Meng H. Impaired Autophagy in Intestinal Epithelial Cells Alters Gut Microbiota and Host Immune Responses. *Appl Environ Microbiol* 2018; **84** [PMID: 30006408 DOI: 10.1128/AEM.00880-18]

84 **Yan S**, Khambu B, Chen X, Dong Z, Guo G, Yin XM. Hepatic Autophagy Deficiency Remodels Gut Microbiota for Adaptive Protection *via* FGF15-FGFR4 Signaling. *Cell Mol Gastroenterol Hepatol* 2021; **11**: 973-997 [PMID: 33127558 DOI: 10.1016/j.jcmgh.2020.10.011]

85 **Trentesaux C**, Fraudeau M, Pitasi CL, Lemarchand J, Jacques S, Duche A, Letourneur F, Naser E, Bailly K, Schmitt A, Perret C, Romagnolo B. Essential role for autophagy protein ATG7 in the maintenance of intestinal stem cell integrity. *Proc Natl Acad Sci U S A* 2020; **117**: 11136-11146 [PMID: 32371487 DOI: 10.1073/pnas.1917174117]

86 **Chang SY**, Lee SN, Yang JY, Kim DW, Yoon JH, Ko HJ, Ogawa M, Sasakawa C, Kweon MN. Autophagy controls an intrinsic host defense to bacteria by promoting epithelial cell survival: a murine model. *PLoS One* 2013; **8**: e81095 [PMID: 24260541 DOI: 10.1371/journal.pone.0081095]

87 **Liu TC**, Kern JT, VanDussen KL, Xiong S, Kaiko GE, Wilen CB, Rajala MW, Caruso R, Holtzman MJ, Gao F, McGovern DP, Nunez G, Head RD, Stappenbeck TS. Interaction between smoking and ATG16L1T300A triggers Paneth cell defects in Crohn's disease. *J Clin Invest* 2018; **128**: 5110-5122 [PMID: 30137026 DOI: 10.1172/JCI120453]

88 **Matsuzawa-Ishimoto Y**, Shono Y, Gomez LE, Hubbard-Lucey VM, Cammer M, Neil J, Dewan MZ, Lieberman SR, Lazrak A, Marinis JM, Beal A, Harris PA, Bertin J, Liu C, Ding Y, van den Brink MRM, Cadwell K. Autophagy protein ATG16L1 prevents necroptosis in the intestinal epithelium. *J Exp Med* 2017; **214**: 3687-3705 [PMID: 29089374 DOI: 10.1084/jem.20170558]

89 **Gubas A**, Dikic I. A guide to the regulation of selective autophagy receptors. *FEBS J* 2021 [PMID: 33730405 DOI: 10.1111/febs.15824]

90 **Viret C**, Duclaux-Loras R, Nancey S, Rozières A, Faure M. Selective Autophagy Receptors in Antiviral Defense. *Trends Microbiol* 2021; **29**: 798-810 [PMID: 33678557 DOI: 10.1016/j.tim.2021.02.006]

91 **Birmingham CL**, Smith AC, Bakowski MA, Yoshimori T, Brumell JH. Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. *J Biol Chem* 2006; **281**: 11374-11383 [PMID: 16495224 DOI: 10.1074/jbc.M509157200]

92 **Lapaquette P**, Glasser AL, Huett A, Xavier RJ, Darfeuille-Michaud A. Crohn's disease-associated adherent-invasive E. coli are selectively favoured by impaired autophagy to replicate intracellularly. *Cell Microbiol* 2010; **12**: 99-113 [PMID: 19747213 DOI: 10.1111/j.1462-5822.2009.01381.x]

93 **Nakagawa I**, Amano A, Mizushima N, Yamamoto A, Yamaguchi H, Kamimoto T, Nara A, Funao J, Nakata M, Tsuda K, Hamada S, Yoshimori T. Autophagy defends cells against invading group A Streptococcus. *Science* 2004; **306**: 1037-1040 [PMID: 15528445 DOI: 10.1126/science.1103966]

94 **Benjamin JL**, Sumpter R Jr, Levine B, Hooper LV. Intestinal epithelial autophagy is essential for host defense against invasive bacteria. *Cell Host Microbe* 2013; **13**: 723-734 [PMID: 23768496 DOI: 10.1016/j.chom.2013.05.004]

95 **Bretin A**, Carrière J, Dalmasso G, Bergougnoux A, B'chir W, Maurin AC, Müller S, Seibold F, Barnich N, Bruhat A, Darfeuille-Michaud A, Nguyen HT. Activation of the EIF2AK4-EIF2A/eIF2α-ATF4 pathway triggers autophagy response to Crohn disease-associated adherent-invasive Escherichia coli infection. *Autophagy* 2016; **12**: 770-783 [PMID: 26986695 DOI: 10.1080/15548627.2016.1156823]

96 **Larabi A**, Barnich N, Nguyen HTT. New insights into the interplay between autophagy, gut microbiota and inflammatory responses in IBD. *Autophagy* 2020; **16**: 38-51 [PMID: 31286804 DOI: 10.1080/15548627.2019.1635384]

97 **Lassen KG**, Kuballa P, Conway KL, Patel KK, Becker CE, Peloquin JM, Villablanca EJ, Norman JM, Liu TC, Heath RJ, Becker ML, Fagbami L, Horn H, Mercer J, Yilmaz OH, Jaffe JD, Shamji AF, Bhan AK, Carr SA, Daly MJ, Virgin HW, Schreiber SL, Stappenbeck TS, Xavier RJ. Atg16L1 T300A variant decreases selective autophagy resulting in altered cytokine signaling and decreased antibacterial defense. *Proc Natl Acad Sci U S A* 2014; **111**: 7741-7746 [PMID: 24821797 DOI: 10.1073/pnas.1407001111]

98 **Sadaghian Sadabad M**, Regeling A, de Goffau MC, Blokzijl T, Weersma RK, Penders J, Faber KN, Harmsen HJ, Dijkstra G. The ATG16L1-T300A allele impairs clearance of pathosymbionts in the inflamed ileal mucosa of Crohn's disease patients. *Gut* 2015; **64**: 1546-1552 [PMID: 25253126 DOI: 10.1136/gutjnl-2014-307289]

99 **Ellinghaus D**, Zhang H, Zeissig S, Lipinski S, Till A, Jiang T, Stade B, Bromberg Y, Ellinghaus E, Keller A, Rivas MA, Skieceviciene J, Doncheva NT, Liu X, Liu Q, Jiang F, Forster M, Mayr G, Albrecht M, Häsler R, Boehm BO, Goodall J, Berzuini CR, Lee J, Andersen V, Vogel U, Kupcinskas L, Kayser M, Krawczak M, Nikolaus S, Weersma RK, Ponsioen CY, Sans M, Wijmenga C, Strachan DP, McArdle WL, Vermeire S, Rutgeerts P, Sanderson JD, Mathew CG, Vatn MH, Wang J, Nöthen MM, Duerr RH, Büning C, Brand S, Glas J, Winkelmann J, Illig T, Latiano A, Annese V, Halfvarson J, D'Amato M, Daly MJ, Nothnagel M, Karlsen TH, Subramani S, Rosenstiel P, Schreiber S, Parkes M, Franke A. Association between variants of PRDM1 and NDP52 and Crohn's disease, based on exome sequencing and functional studies. *Gastroenterology* 2013; **145**: 339-347 [PMID: 23624108 DOI: 10.1053/j.gastro.2013.04.040]

100 **Girardelli M**, Basaldella F, Paolera SD, Vuch J, Tommasini A, Martelossi S, Crovella S, Bianco AM. Genetic profile of patients with early onset inflammatory bowel disease. *Gene* 2018; **645**: 18-29 [PMID: 29248579 DOI: 10.1016/j.gene.2017.12.029]

101 **Hoefkens E**, Nys K, John JM, Van Steen K, Arijs I, Van der Goten J, Van Assche G, Agostinis P, Rutgeerts P, Vermeire S, Cleynen I. Genetic association and functional role of Crohn disease risk alleles involved in microbial sensing, autophagy, and endoplasmic reticulum (ER) stress. *Autophagy* 2013; **9**: 2046-2055 [PMID: 24247223 DOI: 10.4161/auto.26337]

102 **Birgisdottir ÅB**, Johansen T. Autophagy and endocytosis - interconnections and interdependencies. *J Cell Sci* 2020; **133** [PMID: 32501285 DOI: 10.1242/jcs.228114]

103 **Gluschko A**, Herb M, Wiegmann K, Krut O, Neiss WF, Utermöhlen O, Krönke M, Schramm M. The β_2 Integrin Mac-1 Induces Protective LC3-Associated Phagocytosis of Listeria monocytogenes. *Cell Host Microbe* 2018; **23**: 324-337.e5 [PMID: 29544096 DOI: 10.1016/j.chom.2018.01.018]

104 **Sprenkeler EG**, Gresnigt MS, van de Veerdonk FL. LC3-associated phagocytosis: a crucial mechanism for antifungal host defence against Aspergillus fumigatus. *Cell Microbiol* 2016; **18**: 1208-1216 [PMID: 27185357 DOI: 10.1111/cmi.12616]

105 **Paone P**, Cani PD. Mucus barrier, mucins and gut microbiota: the expected slimy partners? *Gut*

2020; **69**: 2232-2243 [PMID: 32917747 DOI: 10.1136/gutjnl-2020-322260]

106 **Arike L**, Holmén-Larsson J, Hansson GC. Intestinal Muc2 mucin O-glycosylation is affected by microbiota and regulated by differential expression of glycosyltranferases. *Glycobiology* 2017; **27**: 318-328 [PMID: 28122822 DOI: 10.1093/glycob/cww134]

107 **Desai MS**, Seekatz AM, Koropatkin NM, Kamada N, Hickey CA, Wolter M, Pudlo NA, Kitamoto S, Terrapon N, Muller A, Young VB, Henrissat B, Wilmes P, Stappenbeck TS, Núñez G, Martens EC. A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility. *Cell* 2016; **167**: 1339-1353.e21 [PMID: 27863247 DOI: 10.1016/j.cell.2016.10.043]

108 **Jakobsson HE**, Rodríguez-Piñeiro AM, Schütte A, Ermund A, Boysen P, Bemark M, Sommer F, Bäckhed F, Hansson GC, Johansson ME. The composition of the gut microbiota shapes the colon mucus barrier. *EMBO Rep* 2015; **16**: 164-177 [PMID: 25525071 DOI: 10.15252/embr.201439263]

109 **Schroeder BO**. Fight them or feed them: how the intestinal mucus layer manages the gut microbiota. *Gastroenterol Rep (Oxf)* 2019; **7**: 3-12 [PMID: 30792861 DOI: 10.1093/gastro/goy052]

110 **Pittayanon R**, Lau JT, Leontiadis GI, Tse F, Yuan Y, Surette M, Moayyedi P. Differences in Gut Microbiota in Patients With *vs* Without Inflammatory Bowel Diseases: A Systematic Review. *Gastroenterology* 2020; **158**: 930-946.e1 [PMID: 31812509 DOI: 10.1053/j.gastro.2019.11.294]

111 **Xu Y**, Wang N, Tan HY, Li S, Zhang C, Feng Y. Function of *Akkermansia muciniphila* in Obesity: Interactions With Lipid Metabolism, Immune Response and Gut Systems. *Front Microbiol* 2020; **11**: 219 [PMID: 32153527 DOI: 10.3389/fmicb.2020.00219]

112 **Wu M**, Wu Y, Li J, Bao Y, Guo Y, Yang W. The Dynamic Changes of Gut Microbiota in *Muc2* Deficient Mice. *Int J Mol Sci* 2018; **19** [PMID: 30231491 DOI: 10.3390/ijms19092809]

113 **Gibold L**, Garenaux E, Dalmasso G, Gallucci C, Cia D, Mottet-Auselo B, Faïs T, Darfeuille-Michaud A, Nguyen HT, Barnich N, Bonnet R, Delmas J. The Vat-AIEC protease promotes crossing of the intestinal mucus layer by Crohn's disease-associated Escherichia coli. *Cell Microbiol* 2016; **18**: 617-631 [PMID: 26499863 DOI: 10.1111/cmi.12539]

114 **Sperandio B**, Fischer N, Joncquel Chevalier-Curt M, Rossez Y, Roux P, Robbe Masselot C, Sansonetti PJ. Virulent Shigella flexneri affects secretion, expression, and glycosylation of gel-forming mucins in mucus-producing cells. *Infect Immun* 2013; **81**: 3632-3643 [PMID: 23876800 DOI: 10.1128/IAI.00551-13]

115 **Patel KK**, Miyoshi H, Beatty WL, Head RD, Malvin NP, Cadwell K, Guan JL, Saitoh T, Akira S, Seglen PO, Dinauer MC, Virgin HW, Stappenbeck TS. Autophagy proteins control goblet cell function by potentiating reactive oxygen species production. *EMBO J* 2013; **32**: 3130-3144 [PMID: 24185898 DOI: 10.1038/emboj.2013.233]

116 **Elinav E**, Strowig T, Kau AL, Henao-Mejia J, Thaiss CA, Booth CJ, Peaper DR, Bertin J, Eisenbarth SC, Gordon JI, Flavell RA. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* 2011; **145**: 745-757 [PMID: 21565393 DOI: 10.1016/j.cell.2011.04.022]

117 **Wlodarska M**, Thaiss CA, Nowarski R, Henao-Mejia J, Zhang JP, Brown EM, Frankel G, Levy M, Katz MN, Philbrick WM, Elinav E, Finlay BB, Flavell RA. NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating goblet cell mucus secretion. *Cell* 2014; **156**: 1045-1059 [PMID: 24581500 DOI: 10.1016/j.cell.2014.01.026]

118 **Engevik MA**, Luk B, Chang-Graham AL, Hall A, Herrmann B, Ruan W, Endres BT, Shi Z, Garey KW, Hyser JM, Versalovic J. Bifidobacterium dentium Fortifies the Intestinal Mucus Layer *via* Autophagy and Calcium Signaling Pathways. *mBio* 2019; **10** [PMID: 31213556 DOI: 10.1128/mBio.01087-19]

119 **Yeom J**, Ma S, Lim YH. Oxyresveratrol Induces Autophagy *via* the ER Stress Signaling Pathway, and Oxyresveratrol-Induced Autophagy Stimulates MUC2 Synthesis in Human Goblet Cells. *Antioxidants (Basel)* 2020; **9** [PMID: 32150901 DOI: 10.3390/antiox9030214]

120 **Fagarasan S**, Muramatsu M, Suzuki K, Nagaoka H, Hiai H, Honjo T. Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora. *Science* 2002; **298**: 1424-1427 [PMID: 12434060 DOI: 10.1126/science.1077336]

121 **Suzuki K**, Meek B, Doi Y, Muramatsu M, Chiba T, Honjo T, Fagarasan S. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. *Proc Natl Acad Sci U S A* 2004; **101**: 1981-1986 [PMID: 14766966 DOI: 10.1073/pnas.0307317101]

122 **Wei M**, Shinkura R, Doi Y, Maruya M, Fagarasan S, Honjo T. Mice carrying a knock-in mutation of Aicda resulting in a defect in somatic hypermutation have impaired gut homeostasis and compromised mucosal defense. *Nat Immunol* 2011; **12**: 264-270 [PMID: 21258321 DOI: 10.1038/ni.1991]

123 **Kawamoto S**, Maruya M, Kato LM, Suda W, Atarashi K, Doi Y, Tsutsui Y, Qin H, Honda K, Okada T, Hattori M, Fagarasan S. Foxp3(+) T cells regulate immunoglobulin a selection and facilitate diversification of bacterial species responsible for immune homeostasis. *Immunity* 2014; **41**: 152-165 [PMID: 25017466 DOI: 10.1016/j.immuni.2014.05.016]

124 **Kawamoto S**, Tran TH, Maruya M, Suzuki K, Doi Y, Tsutsui Y, Kato LM, Fagarasan S. The inhibitory receptor PD-1 regulates IgA selection and bacterial composition in the gut. *Science* 2012; **336**: 485-489 [PMID: 22539724 DOI: 10.1126/science.1217718]

125 **Fadlallah J**, El Kafsi H, Sterlin D, Juste C, Parizot C, Dorgham K, Autaa G, Gouas D, Almeida M, Lepage P, Pons N, Le Chatelier E, Levenez F, Kennedy S, Galleron N, de Barros JP, Malphettes M, Galicier L, Boutboul D, Mathian A, Miyara M, Oksenhendler E, Amoura Z, Doré J, Fieschi C, Ehrlich SD, Larsen M, Gorochov G. Microbial ecology perturbation in human IgA deficiency. *Sci*

Transl Med 2018; **10** [PMID: 29720448 DOI: 10.1126/scitranslmed.aan1217]

126 **Catanzaro JR**, Strauss JD, Bielecka A, Porto AF, Lobo FM, Urban A, Schofield WB, Palm NW. IgA-deficient humans exhibit gut microbiota dysbiosis despite secretion of compensatory IgM. *Sci Rep* 2019; **9**: 13574 [PMID: 31537840 DOI: 10.1038/s41598-019-49923-2]

127 **Ma Y**, Hendershot LM. The stressful road to antibody secretion. *Nat Immunol* 2003; **4**: 310-311 [PMID: 12660729 DOI: 10.1038/ni0403-310]

128 **Pengo N**, Scolari M, Oliva L, Milan E, Mainoldi F, Raimondi A, Fagioli C, Merlini A, Mariani E, Pasqualetto E, Orfanelli U, Ponzoni M, Sitia R, Casola S, Cenci S. Plasma cells require autophagy for sustainable immunoglobulin production. *Nat Immunol* 2013; **14**: 298-305 [PMID: 23354484 DOI: 10.1038/ni.2524]

129 **Conway KL**, Kuballa P, Khor B, Zhang M, Shi HN, Virgin HW, Xavier RJ. ATG5 regulates plasma cell differentiation. *Autophagy* 2013; **9**: 528-537 [PMID: 23327930 DOI: 10.4161/auto.23484]

130 **Ragland SA**, Criss AK. From bacterial killing to immune modulation: Recent insights into the functions of lysozyme. *PLoS Pathog* 2017; **13**: e1006512 [PMID: 28934357 DOI: 10.1371/journal.ppat.1006512]

131 **Yu S**, Balasubramanian I, Laubitz D, Tong K, Bandyopadhyay S, Lin X, Flores J, Singh R, Liu Y, Macazana C, Zhao Y, Béguet-Crespel F, Patil K, Midura-Kiela MT, Wang D, Yap GS, Ferraris RP, Wei Z, Bonder EM, Häggblom MM, Zhang L, Douard V, Verzi MP, Cadwell K, Kiela PR, Gao N. Paneth Cell-Derived Lysozyme Defines the Composition of Mucolytic Microbiota and the Inflammatory Tone of the Intestine. *Immunity* 2020; **53**: 398-416.e8 [PMID: 32814028 DOI: 10.1016/j.immuni.2020.07.010]

132 **Sankaran-Walters S**, Hart R, Dills C. Guardians of the Gut: Enteric Defensins. *Front Microbiol* 2017; **8**: 647 [PMID: 28469609 DOI: 10.3389/fmicb.2017.00647]

133 **Cadwell K**, Liu JY, Brown SL, Miyoshi H, Loh J, Lennerz JK, Kishi C, Kc W, Carrero JA, Hunt S, Stone CD, Brunt EM, Xavier RJ, Sleckman BP, Li E, Mizushima N, Stappenbeck TS, Virgin HW 4th. A key role for autophagy and the autophagy gene Atg16 L1 in mouse and human intestinal Paneth cells. *Nature* 2008; **456**: 259-263 [PMID: 18849966 DOI: 10.1038/nature07416]

134 **Cadwell K**, Patel KK, Komatsu M, Virgin HW 4th, Stappenbeck TS. A common role for Atg16L1, Atg5 and Atg7 in small intestinal Paneth cells and Crohn disease. *Autophagy* 2009; **5**: 250-252 [PMID: 19139628 DOI: 10.4161/auto.5.2.7560]

135 **VanDussen KL**, Liu TC, Li D, Towfic F, Modiano N, Winter R, Haritunians T, Taylor KD, Dhall D, Targan SR, Xavier RJ, McGovern DP, Stappenbeck TS. Genetic variants synthesize to produce paneth cell phenotypes that define subtypes of Crohn's disease. *Gastroenterology* 2014; **146**: 200-209 [PMID: 24076061 DOI: 10.1053/j.gastro.2013.09.048]

136 **Bel S**, Pendse M, Wang Y, Li Y, Ruhn KA, Hassell B, Leal T, Winter SE, Xavier RJ, Hooper LV. Paneth cells secrete lysozyme *via* secretory autophagy during bacterial infection of the intestine. *Science* 2017; **357**: 1047-1052 [PMID: 28751470 DOI: 10.1126/science.aal4677]

137 **Cadwell K**, Patel KK, Maloney NS, Liu TC, Ng AC, Storer CE, Head RD, Xavier R, Stappenbeck TS, Virgin HW. Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine. *Cell* 2010; **141**: 1135-1145 [PMID: 20602997 DOI: 10.1016/j.cell.2010.05.009]

138 **Lu R**, Zhang YG, Xia Y, Zhang J, Kaser A, Blumberg R, Sun J. Paneth Cell Alertness to Pathogens Maintained by Vitamin D Receptors. *Gastroenterology* 2021; **160**: 1269-1283 [PMID: 33217447 DOI: 10.1053/j.gastro.2020.11.015]

139 **Muniz LR**, Knosp C, Yeretssian G. Intestinal antimicrobial peptides during homeostasis, infection, and disease. *Front Immunol* 2012; **3**: 310 [PMID: 23087688 DOI: 10.3389/fimmu.2012.00310]

140 **Sun M**, He C, Cong Y, Liu Z. Regulatory immune cells in regulation of intestinal inflammatory response to microbiota. *Mucosal Immunol* 2015; **8**: 969-978 [PMID: 26080708 DOI: 10.1038/mi.2015.49]

141 **Kozik AJ**, Nakatsu CH, Chun H, Jones-Hall YL. Comparison of the fecal, cecal, and mucus microbiome in male and female mice after TNBS-induced colitis. *PLoS One* 2019; **14**: e0225079 [PMID: 31703107 DOI: 10.1371/journal.pone.0225079]

142 **Park H**, Yeo S, Kang S, Huh CS. Longitudinal Microbiome Analysis in a Dextran Sulfate Sodium-Induced Colitis Mouse Model. *Microorganisms* 2021; **9** [PMID: 33673349 DOI: 10.3390/microorganisms9020370]

143 **Zeng MY**, Inohara N, Nuñez G. Mechanisms of inflammation-driven bacterial dysbiosis in the gut. *Mucosal Immunol* 2017; **10**: 18-26 [PMID: 27554295 DOI: 10.1038/mi.2016.75]

144 **Qin J**, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR, Li J, Xu J, Li S, Li D, Cao J, Wang B, Liang H, Zheng H, Xie Y, Tap J, Lepage P, Bertalan M, Batto JM, Hansen T, Le Paslier D, Linneberg A, Nielsen HB, Pelletier E, Renault P, Sicheritz-Ponten T, Turner K, Zhu H, Yu C, Jian M, Zhou Y, Li Y, Zhang X, Qin N, Yang H, Wang J, Brunak S, Doré J, Guarner F, Kristiansen K, Pedersen O, Parkhill J, Weissenbach J; MetaHIT Consortium, Bork P, Ehrlich SD, Wang J. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 2010; **464**: 59-65 [PMID: 20203603 DOI: 10.1038/nature08821]

145 **Malik A**, Kanneganti TD. Inflammasome activation and assembly at a glance. *J Cell Sci* 2017; **130**: 3955-3963 [PMID: 29196474 DOI: 10.1242/jcs.207365]

146 **Saitoh T**, Fujita N, Jang MH, Uematsu S, Yang BG, Satoh T, Omori H, Noda T, Yamamoto N, Komatsu M, Tanaka K, Kawai T, Tsujimura T, Takeuchi O, Yoshimori T, Akira S. Loss of the

autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature* 2008; **456**: 264-268 [PMID: 18849965 DOI: 10.1038/nature07383]

147 **Deretic V**, Levine B. Autophagy balances inflammation in innate immunity. *Autophagy* 2018; **14**: 243-251 [PMID: 29165043 DOI: 10.1080/15548627.2017.1402992]

148 **Hou P**, Yang K, Jia P, Liu L, Lin Y, Li Z, Li J, Chen S, Guo S, Pan J, Wu J, Peng H, Zeng W, Li C, Liu Y, Guo D. A novel selective autophagy receptor, CCDC50, delivers K63 polyubiquitination-activated RIG-I/MDA5 for degradation during viral infection. *Cell Res* 2021; **31**: 62-79 [PMID: 32612200 DOI: 10.1038/s41422-020-0362-1]

149 **Pott J**, Stockinger S. Type I and III Interferon in the Gut: Tight Balance between Host Protection and Immunopathology. *Front Immunol* 2017; **8**: 258 [PMID: 28352268 DOI: 10.3389/fimmu.2017.00258]

150 **Burger E**, Araujo A, López-Yglesias A, Rajala MW, Geng L, Levine B, Hooper LV, Burstein E, Yarovinsky F. Loss of Paneth Cell Autophagy Causes Acute Susceptibility to Toxoplasma gondii-Mediated Inflammation. *Cell Host Microbe* 2018; **23**: 177-190.e4 [PMID: 29358083 DOI: 10.1016/j.chom.2018.01.001]

151 **Bussi C**, Peralta Ramos JM, Arroyo DS, Gaviglio EA, Gallea JI, Wang JM, Celej MS, Iribarren P. Autophagy down regulates pro-inflammatory mediators in BV2 microglial cells and rescues both LPS and alpha-synuclein induced neuronal cell death. *Sci Rep* 2017; **7**: 43153 [PMID: 28256519 DOI: 10.1038/srep43153]

152 **Cadwell K**. Crosstalk between autophagy and inflammatory signalling pathways: balancing defence and homeostasis. *Nat Rev Immunol* 2016; **16**: 661-675 [PMID: 27694913 DOI: 10.1038/nri.2016.100]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 December 28; 27(48): 8302-8322

DOI: 10.3748/wjg.v27.i48.8302

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

ORIGINAL ARTICLE

Basic Study

Hepatitis B core antigen modulates exosomal miR-135a to target vesicle-associated membrane protein 2 promoting chemoresistance in hepatocellular carcinoma

Xiao-Cui Wei, Ya-Ru Xia, Ping Zhou, Xing Xue, Shuang Ding, Li-Juan Liu, Fan Zhu

ORCID number: Xiao-Cui Wei 0000-0003-4606-2066; Ya-Ru Xia 0000-0001-7933-6532; Ping Zhou 0000-0003-4717-3390; Xing Xue 0000-0003-4441-4347; Li-Juan Liu 0000-0002-2549-5463; Shuang Ding 0000-0002-9810-8428; Fan Zhu 0000-0001-7031-2956.

Author contributions: Zhu F conceived the study, was in charge of overall direction and planning, and revised the manuscript; Wei XC designed and performed the experiments, and wrote the paper; Wei XC and Liu LJ revised the manuscript; Xia YR and Zhou P participated in data collection; Xue X was responsible for bioinformatics data; Ding S, and Liu LJ analyzed data; all authors read and approved the final manuscript and agreed to be accountable for all aspects of the report.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Wuhan University, School of Basic Medical Sciences, and the study was carried out following The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments

Xiao-Cui Wei, Ya-Ru Xia, Ping Zhou, Xing Xue, Shuang Ding, Li-Juan Liu, Fan Zhu, State Key Laboratory of Virology, Hubei Province Key Laboratory of Allergy and Immunology, Department of Medical Microbiology, School of Medicine, Wuhan University, Wuhan 430071, Hubei Province, China

Corresponding author: Fan Zhu, PhD, Professor, State Key Laboratory of Virology, Hubei Province Key Laboratory of Allergy and Immunology, Department of Medical Microbiology, School of Medicine, Wuhan University, No. 185 Donghu Road, Wuhan 430071, Hubei Province, China. fanzhu@whu.edu.cn

Abstract

BACKGROUND

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors. The association of hepatitis B virus (HBV) infection with HCC is hitherto documented. Exosomal miRNAs contribute to cancer progression and chemoresistance. HBV X protein has been known to modulate miRNAs that facilitate cell proliferation and the process of hepatocarcinogenesis. However, there has been no report on hepatitis B core antigen (HBc) regulating exosomal miRNAs to induce drug resistance of HCC cells.

AIM

To elucidate the mechanism by which HBc promotes Doxorubicin hydrochloride (Dox) resistance in HCC.

METHODS

Exosomes were isolated by ultracentrifugation. The morphology and size of exosomes were evaluated by Dynamic Light Scattering (DLS) and transmission electron microscopy (TEM). The miRNAs differentially expressed in HCC were identified using The Cancer Genome Atlas (TCGA) database. The level of miR-135a-5p in patient tissue samples was detected by quantitative polymerase chain reaction. TargetScan and luciferase assay were used to predict and prove the target gene of miR-135a-5p. Finally, we identified the effects of miR-135a-5p on anti-apoptosis and the proliferation of HCC in the presence or absence of Dox using flow cytometry, Cell counting kit 8 (CCK-8) assay and western blot.

RESULTS

involving humans. Informed consent was obtained for experimentation with human subjects, and their privacy rights were consistently observed.

Conflict-of-interest statement: The authors hereby declare that no conflict of interest exists.

Data sharing statement: Datasets available from the corresponding author upon request.

Supported by National Natural Science Foundation of China, No. 81971943 and 81772196; and the Medical Science Advancement Program (Basic Medical Sciences) of Wuhan University, No. TFJC 2018002.

Country/Territory of origin: China

Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: April 27, 2021
Peer-review started: April 27, 2021
First decision: June 13, 2021
Revised: June 22, 2021
Accepted: November 11, 2021

We found that HBc increased the expression of exosomal miR-135a-5p. Integrated analysis of bioinformatics and patient samples found that miR-135a-5p was increased in HCC tissues in comparison with paracancerous tissues. Bioinformatic analysis and *in vitro* validation identified vesicle-associated membrane protein 2 (VAMP2) as a novel target gene of miR-135a-5p. Functional assays showed that exosomal miR-135a-5p induced apoptosis protection, cell proliferation, and chemotherapy resistance in HCC. In addition, the rescue experiment demonstrated that VAMP2 reversed apoptosis protection, cell growth, and drug resistance by miR-135a-5p. Finally, HBc promoted HCC anti-apoptosis, proliferation, and drug resistance and prevented Dox-induced apoptosis *via* the miR-135a-5p/VAMP2 axis.

CONCLUSION

These data suggested that HBc upregulated the expression of exosomal miR-135a-5p and promoted anti-apoptosis, cell proliferation, and chemical resistance through miR-135a-5p/VAMP2. Thus, our work indicated an essential role of the miR-135a-5p/VAMP2 regulatory axis in chemotherapy resistance of HCC and a potential molecular therapeutic target for HCC.

Key Words: Hepatocellular carcinoma; Exosomes; miR-135a-5p; Anti-apoptosis; Proliferation; Chemoresistance

Core Tip: Hepatitis B virus infection is the most common cause of hepatocellular carcinoma (HCC). Drug resistance is the primary reason for the high mortality of HCC patients. We demonstrated that hepatitis B core antigen (HBc) increased exosomal miR-135a-5p. Tissue samples showed that the level of miR-135a-5p was significantly elevated in HCC tissues. Vesicle-associated membrane protein 2 (VAMP2) was demonstrated to be a target gene of miR-135a-5p. Further investigation recommended that HBc enhanced the anti-apoptosis, cell proliferation, and chemotherapy resistance of HCC cells through exosomal miR-135a-5p by targeting VAMP2. Our findings reveal that HBc can cause anti-cancer drug resistance in HCC and provide us with a novel mechanism underlying drug resistance in cancer chemotherapy.

Citation: Wei XC, Xia YR, Zhou P, Xue X, Ding S, Liu LJ, Zhu F. Hepatitis B core antigen modulates exosomal miR-135a to target vesicle-associated membrane protein 2 promoting chemoresistance in hepatocellular carcinoma. *World J Gastroenterol* 2021; 27(48): 8302-8322
URL: https://www.wjgnet.com/1007-9327/full/v27/i48/8302.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i48.8302

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer-related death worldwide, accounting for 90% of primary liver cancer[1]. Approximately 383000 individuals die from liver cancer every year in China, accounting for 51% of liver cancer deaths worldwide[2]. Surgical resection is the cornerstone of treatment for HCC patients with early stages. However, most patients with HCC are diagnosed at an advanced stage, which prevents surgical management. Chemotherapy is the primary treatment for patients with advanced HCC. Nevertheless, drug resistance has become more and more prominent in HCC[3]. Therefore, it is essential to understand the mechanism of pathology and drug resistance in HCC.

Hepatitis B virus (HBV) is one of the major causes of HCC development in Asia, including China[4]. Studies have shown that exosomes are critical mediators of cell-to-cell communication in HBV infection[5]. Exosomes are a class of lipid bilayer vesicles 30-150 nm in size and are secreted from cells into the extracellular environment[6]. Almost all cells can secrete exosomes. The number of circulating exosomes is elevated in various diseases, including cancers. However, exosomes from different cells contain several marker proteins (CD9, CD63, and CD81)[7]. Additionally, exosomes carry

Article in press: December 11, 2021
Published online: December 28, 2021

P-Reviewer: Sahin TT, Yu JS
S-Editor: Wu YXJ
L-Editor: Webster JR
P-Editor: Wu YXJ

some signaling molecules, such as proteins, lipids, nucleic acids, and non-coding RNAs, to the recipient cell to perform their functions[8]. Among these cargos carried by exosomes, miRNAs receive sufficient attention due to their high conservation across species and extensive regulatory roles in gene expression[9].

MicroRNAs (miRNAs) belong to small non-coding RNAs, about 19-25 nucleotides in length. MiRNAs regulate posttranscriptional gene expression by binding to the 3′ untranslated regions (3′ UTRs) of messenger RNA to induce gene silencing or degradation[10]. In cancer, exosomal miRNAs play an essential role in cell apoptosis, proliferation, and chemical resistance[11,12]. Studies have shown that abnormal expression of miRNA is closely related to HBV-associated HCC[13]. The abnormal expression of miRNAs can affect the apoptosis, proliferation, and drug resistance in HCC[14,15]. In recent years, miR-135a has emerged as a critical miRNA in several cancers[16]. Several data suggest a markedly downregulated expression of miR-135a in some diseases and cancers[17,18]. Nonetheless, a high level of miR-135a-5p is associated with postoperative recurrence of HCC[19]. Hepatitis C virus (HCV) can drive the occurrence of HCV-associated hepatocarcinogenesis by upregulating miR-135a-5p[20]. Nevertheless, there is no existing literature on the roles and molecular mechanisms of miR-135a-5p in HCC chemotherapy resistance and the relationship between miR-135a-5p and HBV.

In this study, we discovered that Hepatitis B core antigen (HBc) changed the exosomes release and enhanced the expression of exosomal miR-135a-5p. Tissues and bioinformatics analysis revealed that the level of miR-135a-5p in HCC was higher than that in normal tissues. Vesicle-associated membrane protein 2 (VAMP2) was identified as the target gene of miR-135a-5p *via* the online prediction website TargetScan (http://www.targetscan.org) and luciferase assay. *In vitro* studies indicated that miR-135a-5p promoted anti-apoptosis, proliferation, and chemoresistance in HCC by targeting VAMP2. Additional experiments revealed that HBc enhanced anti-apoptosis, cell proliferation, and chemotherapy resistance in HCC *via* miR-135a-5p/VAMP2. In general, this study revealed a novel mechanism of HBV which counteracted apoptosis, enhanced cell proliferation, and developed chemotherapy resistance in HCC. Our findings also suggested that miR-135a-5p might be a potential therapeutic target in the treatment of HCC chemoresistance.

MATERIALS AND METHODS

The Cancer Genome Atlas dataset

The Cancer Genome Atlas (TCGA) database (http://cancergenome.nih.gov/) was used to analyze the differentially expressed miRNAs in HCC. We analyzed the data obtained from TCGA through the R package (ggplot2, rjson, ggpubr, dplyr, limma, stringr) and determined the expression of miR-135a in HCC tissues and normal tissues.

Tissue samples

Eighteen paired HCC and adjacent tissues were collected during surgical procedures at Ren-Min Hospital of Wuhan University in China. Samples were obtained under a consensus agreement approved by the Institutional Review Committee of the School of Medicine of Wuhan University. The samples were stored at -80°C until experiments were carried out. Table 1 shows the patients' information.

Cell culture and Doxorubicin treatment

The HepG2 cell line was purchased from American Type Culture Collection (Manassas, VA, United States). The HBV-transfected HepG2.2.15 cell line was obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB, Osaka, Japan). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco, United States) with fetal bovine serum (10%, Biological Industries, China), streptomycin (0.1 mg/mL, Gibco, United States) and penicillin (100 units/mL, Gibco, United States).

Doxorubicin hydrochloride (Dox) for injection was from Shenzhen Main Luck Pharmaceutical Company (10 mg, China). Cells were treated with Dox at a concentration of 1.2 μmol/L.

Plasmid construction, synthesis of mimic and inhibitor, and transfection

The HBV (strain ayw) genome (NC_003977.2; c1903-2454) was amplified using pUC18-

Table 1 Clinical sample information

Characteristics	Total	miR-135a-5p		*P* value
		Negative	Positive	
Age (yr)				
< 55	12	3	9	0.82
≥ 55	6	2	4	
Gender				
Male	8	1	7	0.291
Female	10	3	7	
Hepatitis B s antigen				
Negative	-			
Positive	18	5	13	-

HBV1.3 according to sequences in NCBI and cloned into the pcDNA3.1 (-) vector. Human VAMP2 (NM_001330125.1) gene was amplified from HepG2 cDNA and cloned into the pcDNA3.1 (-) vector. Wild-type (WT) VAMP2 3'UTR (NM_001330125.1) and mutant (MUT) VAMP2 3'UTR (70-76: AGCCATA to ACGTGCA) luciferase reporter vectors were constructed and subcloned into the pmiRGLO dual-luciferase miRNA target expression vector (Promega, Wisconsin, United States). All synthesized plasmids were sequenced at Sangon Biotech, Shanghai, China, and the sequences are completely consistent.

MiR-135a-5p mimic, miR-135a-5p inhibitor, and the negative controls were synthesized at Sangon Biotech, Shanghai, China (the specific sequence is listed in Table 2). Cells with 80%-90% confluency were transfected using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions.

Isolation of exosomes

Exosomes were separated from the supernatant of cell cultures *via* ultracentrifugation, slightly modified, as reported[21]. Ultracentrifugation was performed using a fixed angle 70 Ti rotor (Beckman optimal L-100XP, CA, United States) with a speed of 110000 × g at 4°C for 70 min. The precipitate was refrigerated at -80°C until it was used in the experiment.

Exosome detection and characterization

For transmission electron microscopy (TEM), 10 μL of exosome suspension was absorbed onto carbon-coated copper grids (200 mesh) for 5 min. Samples were stained with 2% uranyl acetate for 2 min. After air drying, the sample was visualized under a microscope at 80 kV in TEM (HT7700, Tokyo, Japan).

Particle size distribution of purified exosomes was evaluated using dynamic light scattering (DLS). Briefly, about 200 μL of exosome sample was diluted in 1.5 mL PBS. DLS measurement was conducted using a Zetasizer Nano ZSP (Malvern Instruments Ltd., United Kingdom).

Cellular uptake of PKH67-labeled exosomes

Exosomes isolated from HepG2 cells transfected with miR-135a-5p mimic were stained with PKH67 membrane dye (UR52303, Umibio, Shanghai, China) according to the manufacturer's instructions. HepG2 cells were cultured in confocal Petri dishes 20 mm in diameter (801001, Nest Scientific USA Inc.). When confluency of 70%-80% was reached, 2.5 μg of PKH67-labeled exosomes was added to each well. After incubation for 4 h, the cells were washed with PBS and then stained with 0.5 μg of 4', 6-diamidino-2-phenylindole (DAPI, Solarbio, Beijing, China) at 37°C. Cellular uptake of PKH67-labeled exosomes was visualized using confocal laser scanning microscopy LCS SP8 (Leica, Wetzlar, Germany).

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted from HCC tissues, HCC cell lines and exosomes using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) and complementary DNA

Table 2 Sequences and primers for vector construction

Category	Sequence (5'-3')
miR-135a-5p mimic	F-UAUGGCUUUUUAUUCCUAUGUGA
	R-UCACAUAGGAAUAAAAAGCCAUA
miR-135a-5p inhibitor	UCACAUAGGAAUAAAAAGCCAUA
VAMP2 (NM_001330125.1)	F-CTAGCTAGCATGGACAGGTCTGCTAC
	R-CGCGGATCCTTAAGTGCTGAAGT
VAMP2 3′-UTR-WT	F-CTAGCTAGCATCCCCGAGGAGTCT
	R-ACGCGTCGACAGAGAGGGGTGAAG
VAMP2 3′-UTR-MUT	F-GTTCCTCCACCTCTCACGTGCATCTTTCAGCC CC
	R-GGGGCTGAAAGATGCACGTGAGAGGTGGAGGAAC
Hepatitis B virus-1903/2454	F-CTAGCTAGCGCCACCATGGACATCGACCCTT
	R-CCGCTCGAGCTAACATTGAGATTCCCGAGAT

VAMP2: Vesicle-associated membrane protein 2; WT: Wild-type; MUT: Mutant.

(cDNA) was synthesized using ReverTra Ace quantitative real-time polymerase chain reaction (qPCR) RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). qPCR was carried out using SYBR Green I dye master mix (Invitrogen, Carlsbad, CA, United States). The primer sequences are listed in Table 2. The mRNA expression levels of genes were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or U6.

Primer Premier 5.0 software (Premier, Delaware, Canada) was used to design the primers (primers for vector construction are listed in Table 2; qPCR Primers are listed in Table 3).

Western blotting

Cells were collected for protein extraction using M-PER reagents (Pierce Chemical, Rockford, IL, United States) after 48 h transfection. Total protein content was quantified using the BCA Protein Quantification kit (Thermo Fisher Scientific, Waltham, MA, United States). Protein samples were separated on 12% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membranes (Millipore, United States). After blocking, the membranes were incubated with primary antibodies overnight at 4°C and then with secondary antibodies for 1 h at room temperature. Using ECL chemiluminescence solution (Biosharp, Hefei, China), the band signal was visualized in an automatic chemiluminescence system (Tanon5200, Shanghai, China). The antibodies used in this article were all purchased from ABclonal (Wuhan, China), including anti-GAPDH (AC002), anti-VAMP2 (A1249), anti-CD63 (A5271), anti-CD9 (A1703), anti- Calnexin (CANX, A15631), anti-proliferating cell nuclear antigen (PCNA, A0264), anti-mini-chromosome maintenance protein-2 (MCM2, A1056), anti-B-cell lymphoma-2 (Bcl-2, A0208).

Luciferase reporter assay

The luciferase reporter vectors containing the 3'UTR-WT or 3'UTR-MUT of VAMP2, along with miR-135a-5p mimics or negative control (NC), respectively, were co-transfected into HepG2 cells. Luciferase activities were assessed using the Dual Glo Luciferase Assay System (Promega, Madison, WI, United States) according to the manufacturer's instructions.

Flow cytometry

After washing and collecting, cells were treated with the Annexin V-FITC/PI Apoptosis Assay Kit (Zomanbio, Beijing, China) according to the manufacturer's instructions. The apoptosis rate of cells was analyzed by flow cytometry (FACS Aria III, BD, United States) with FlowJo v10 software (Leonard Herzenberg, United States).

Cell proliferation assay

Cell counting kit 8 (CCK-8) (Zomanbio, Beijing, China) was used to assess cell prolif-

Table 3 Primers for quantitative polymerase chain reaction

Primer	Product size	Sequence (5'-3')
VAMP2 (NM_001330125.1)	182 bp	F-GGTCTCTCCTGCGTTCCC
		R-TCGACCCGAAAAGACAGGC
GAPDH (NM_002046.7)	197 bp	F-GGAGCGAGATCCCTCCAAAAT
		R-GGCTGTTGTCATACTTCTCATGG
U6 (NR_004394.1)	94 bp	F-CTCGCTTCGGCAGCACA
		R-AACGCTTCACGAATTTGCGT
miR135a (NR_029677.1)	68 bp	F-ACACTCCAGCTGGGTATGGCTTTTTATTCCT
		R-TGGTGTCGTGGAGTCG

VAMP2: Vesicle-associated membrane protein 2; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

eration according to the manufacturer's instructions. Generally, 5 × 10^3 cells were allowed to grow in 96-well plates. After incubation with Dox or tumor-derived exosomes for 0 h, 24 h, and 48 h, 10 μL CCK-8 solution was added to each sample and incubated for a further 30 min. The absorbance value was measured at 450 nm using the micro-plate reader.

Statistical analysis

Each experiment was carried out using at least three replicates. Clinical data analysis was performed using SPSS25.0. R software for bioinformatics analysis. Other data analysis was carried out with GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, United States), and data were mentioned as mean ± standard error of the mean (SEM). The *t*-test was implemented to compare the data between 2 groups. $P < 0.05$ was considered to represent a statistically significant difference ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$).

RESULTS

HBV may upregulate the expression levels of miR-135a-5p in exosomes

HBV infection changes the release of extracellular vesicles (EVs) from hepatocytes[22]. In this study, we extracted EVs from HepG2 cells and HepG2.2.15 cells. The TEM image showed that the EVs had a classic "cup" or "dish" morphology[23] (Figure 1A). EVs secreted from HepG2.2.15 cells with HBV replication contained exosomes, subviral particles, and virions[24]. Therefore, western blotting was utilized to verify the marker proteins of exosomes. The results revealed that CD63 and CD9, which commonly serve as specific marker proteins of exosomes, were present in purified EVs (Figure 1B). The negative control Calnexin was detected only in the cell lysate. Furthermore, DLS results demonstrated that the distribution of isolated EVs ranged from 30 nm to 150 nm (Figure 1C). These results suggested that we successfully isolated exosomes from HepG2 cells and HepG2.2.15 cells.

The miRNA content in exosomes is likely responsible for cancer progression, including anti-apoptosis, cell proliferation, and chemoresistance[12]. Notably, we detected the expression of several miRNAs in exosomes purified from HepG2 cells and HepG2.2.15 cells. The qPCR results indicated that the expression level of miR-135a-5p in exosomes isolated from HepG2.2.15 cells was significantly higher than that of HepG2 cells (Figure 1D). The same results were derived in cells (Figure 1E).

HCV promotes the expression of miR-135a-5p in HCC[25]. To our knowledge, there is no report on the effect of HBV on miR-135a-5p. Here, we found that high expression of HBc (Figure 1F) could significantly upregulate the level of miR-135a-5p (Figure 1G) in HCC cells and exosomes (Figure 1H). Moreover, patient tissue samples showed increased expression of miR-135a-5p in HCC tissues compared to paracancerous tissues (Figure 1I). TCGA data analysis identified high expression of miR-135a-5p in HCC tissues (Figure 1J). These results indicated that HBc might upregulate the expression of miR-135a-5p in HCC cell-derived exosomes.

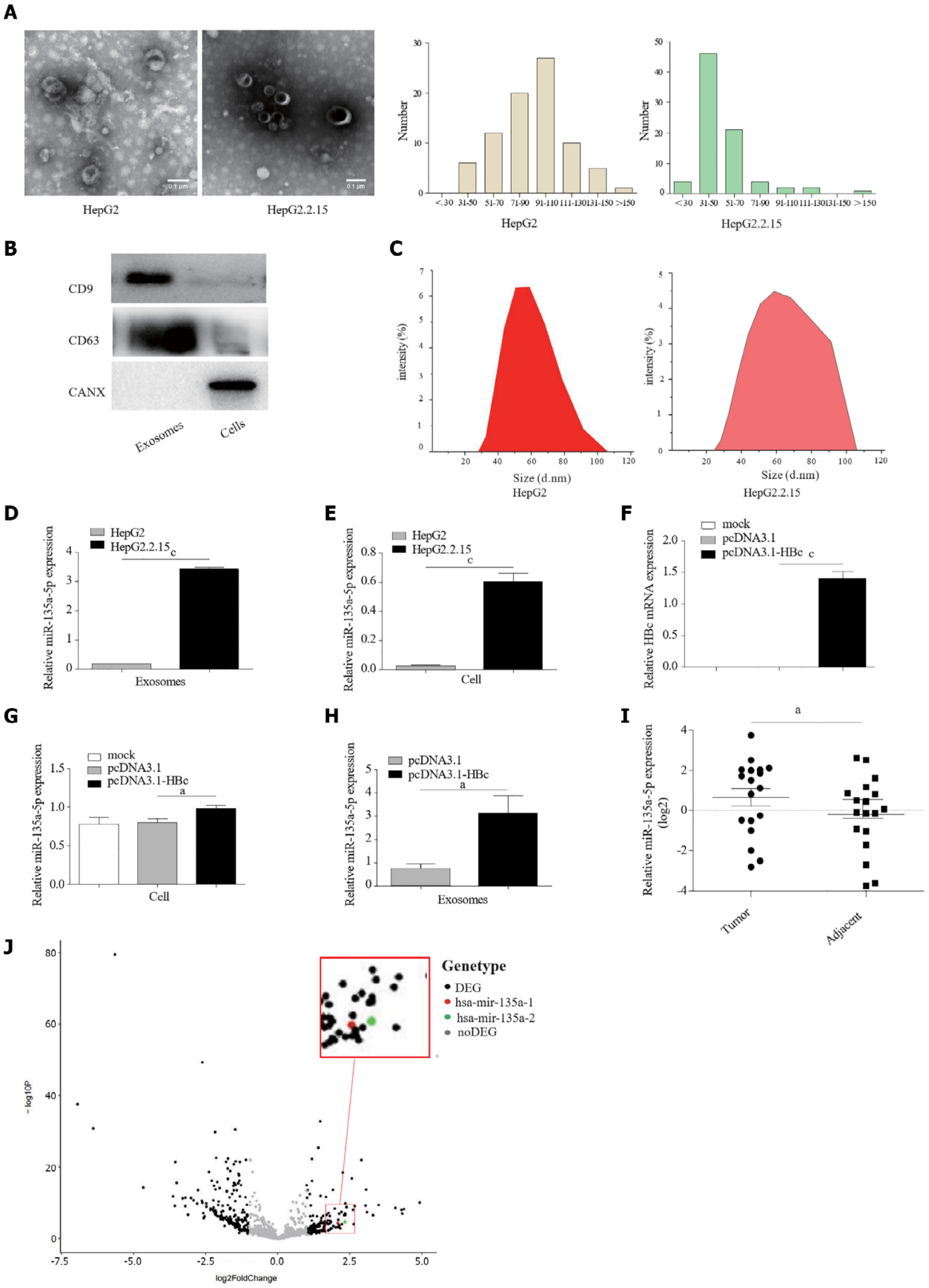

Figure 1 Hepatitis B virus upregulated the expression levels of miR-135a-5p in exosomes. A: Transmission electron microscopy image of exosomes; B: Western blotting indicated proteins in exosomes; C: Analysis of particle size distribution of exosomes; D and E: Quantitative polymerase chain reaction (qPCR) assay examined the expression of miR-135a-5p in exosomes derived from cancer cells and in HCC cell lines; F: Overexpression of Hepatitis B core antigen (HBc) in HepG2 cells was detected by qPCR; G and H: The qRCP assay identified the level of miR-135a-5p in HepG2 cells overexpressed HBc and exosomes

isolated from HepG2 cells after transfected with pcDNA3.1-HBc plasmids; I: Detection of miR-135a-5p in adjacent and tumor tissues from 18 patients; J: Expression of miR-135a-5p obtained from TCGA in HCC. $^{a}P < 0.05$; $^{c}P < 0.001$. HBc: Hepatitis B core antigen.

VAMP2 is one of the potential target genes of miR-135a-5p in HCC cells

Generally, miRNAs exert their functions by inhibiting downstream target genes[26]. Thus, it is important to identify the biological targets of miR-135a-5p. Subsequently, TargetScan[27] and DIANA[28] predicted a potential binding site of miR-135a-5p on the 3′-UTR of VAMP2 (Figure 2A). To validate this bioinformatic prediction, HepG2 cells were transfected with miR-135a-5p mimics (mimic-HepG2). A high level of miR-135a-5p was found in HepG2 cells by qPCR assays (Figure 2B) and a down-regulation was seen when miR-135a-5p inhibitor was involved. The results of qPCR and western blot indicated miR-135a-5p inhibited the expression of VAMP2 (Figure 2C and D) and elevated VAMP2 mRNA and protein were observed (Figure 2F and G) when miR-135a-5p was knocked down (Figure 2E). Moreover, the fluorescence intensity in the cells co-transfected with miR-135a-5p and EGFP-VAMP2-3′UTR was significantly decreased as compared with that in the controls (Figure 2F), indicating that miR-135a-5p interacted with VAMP2. These results suggested that VAMP2 might be a target gene of miR-135a-5p.

MiR-135a-5p exerts anti-apoptotic and proliferative effects by targeting VAMP2 in HCC cells

Our molecular analysis of patient tissue samples found that miR-135a-5p increased in HCC. Apoptosis can eliminate cancer cells. Apoptosis resistance commonly occurs in HCC[29]. Our experiment demonstrated reduced apoptosis in mimic-HepG2 cells when compared to the control group (Figure 3A). Moreover, miR-135a-5p inhibitor effectively increased apoptosis compared to control (Figure 3B). Western blot showed that miR-135a-5p enhanced the expression of Bcl-2 protein, one of the most common anti-apoptotic proteins[30] (Figure 3C), while the level of Bcl-2 protein was decreased in HepG2 cells transfected with miR-135a-5p inhibitor (inhibitor-HepG2) (Figure 3D).

Suppression of apoptosis can lead to cell proliferation[31], one of the prerequisites for cancer progression or carcinogenesis[32]. We found that miR-135a-5p promoted HCC cell proliferation as compared with the control group (Figure 3E). Subsequently, miR-135a-5p inhibitor suppressed cell proliferation in HepG2 cells (Figure 3F). PCNA [33] and MCM2[34] are the traditional proliferating protein molecules. MiR-135a-5p upregulated the expression levels of PCNA and MCM2 (Figure 3G), while miR-135a-5p inhibitor downregulated the levels of these two genes in HepG2 cells (Figure 3H).

Our previous study suggested increased miR-135a-5p in exosomes from HepG2.2.15 cells. Here, we found that these purified exosomes from HepG2 cells transfected with miR-135a-5p mimic (mimic-loaded EXO) could be absorbed by HepG2 cells (Figure 3I). QPCR showed an increased level of miR-135a-5p and a decreased expression of VAMP2 in the recipient cells (Supplementary Figure 1). It is worth mentioning that after absorbing exosomes, the recipient cells exerted anti-apoptotic (Figure 3J) and proliferative effects (Figure 3K). Interestingly, the protein expression levels of Bcl-2, PCNA, and MCM2 increased in the recipient cells, while target gene VAMP2 decreased (Figure 3L).

As the target gene of miR-135a-5p, increased VAMP2 (Supplementary Figure 2) induced the apoptosis in HepG2 cells (Figure 4A). As miR-135a-5p induced anti-apoptosis, we also measured the apoptosis rates in mimic-HepG2 cells co-transfected with pcDNA3.1-VAMP2 or pcDNA3.1 and found that VAMP2 led to excessive apoptosis (Figure 4B). Western blot demonstrated that VAMP2 markedly down-regulated the Bcl-2 protein (Figure 4C). The CCK-8 assay further demonstrated that VAMP2 restrained the proliferation of HCC cells (Figure 4D). In addition, VAMP2 suppressed the protein levels of PCNA and MCM2 in HepG2 cells (Figure 4E).

HBc protects HCC cells against apoptosis and promotes proliferation by miR-135a-5p/VAMP2

HBc has been reported to inhibit apoptosis[35] and promote HCC proliferation[36]. Our data also confirmed this (Supplementary Figure 3). Combined with our data that suggested that HBc upregulated miR-135a-5p, we attempted to determine the functions of miR-135a-5p and its target VAMP2 in the process of anti-apoptosis and proliferation induced by HBc. HBc restrained the expression of VAMP2 in HCC (Figure 5A). Noticeably, we found that miR-135a-5p inhibitors recovered the level of VAMP2 (Figure 5B). To further investigate the role of the miR-135a-5p/VAMP2 axis in

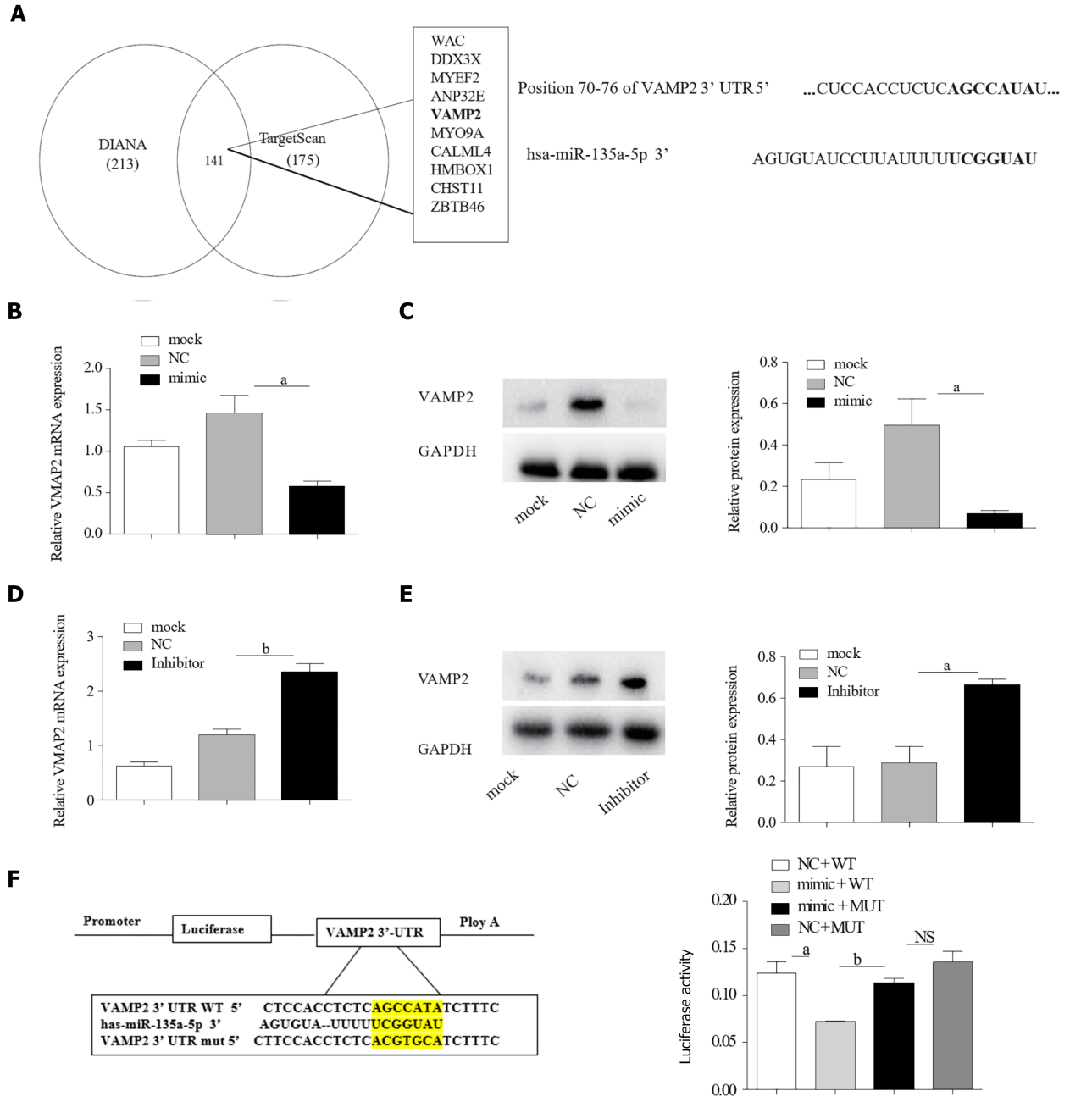

Figure 2 miR-135a-5p targeted vesicle-associated membrane protein 2 in hepatocellular carcinoma cells. A: Prediction results of target gene of miR-135a-5p; B: The expression of miR-135a-5p was measured by quantitative polymerase chain reaction (qPCR) in HepG2 cells transfected with miR-135a-5p mimics; C and D: The mRNA and protein levels of vesicle-associated membrane protein 2 (VAMP2) were detected in the overexpressed miR-135a-5p cells; E: Quantification of miR-135a-5p in HepG2 cells transfected with miR-135a-5p inhibitors; F and G: qPCR and western blot analyses of VAMP2 level in HepG2 cells transfected with miR-135a-5p inhibitors; H: Luciferase assay in HepG2 cells. $^{a}P < 0.05$; $^{b}P < 0.01$; NS: Not Statistically Significant. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; VAMP2: Vesicle-associated membrane protein 2; WT: Wild-type; MUT: Mutant.

the effect of HBc on anti-apoptosis, we co-transfected HBc and miR-135a-5p inhibitors or VAMP2 into HepG2 cells. The data showed that the expression of miR-135a-5p was decreased (Supplementary Figure 4A), and VAMP2 was upregulated (Supplementary Figure 5). As expected, both miR-135a-5p inhibitors (Figure 5C) and VAMP2 (Figure 5D) reversed the effect of HBc against apoptosis. Western blotting showed that anti-apoptotic protein decreased (Figure 5E and F). Subsequently, both miR-135a-5p inhibitors (Figure 5G) and VAMP2 (Figure 5H) impaired the enhancement of HCC cell proliferation by HBc. In addition, MCM2 and PCNA decreased (Figure 5I and J). These results suggested that HBc protects HCC cells against apoptosis and promotes proliferation by miR-135a-5p/VAMP2.

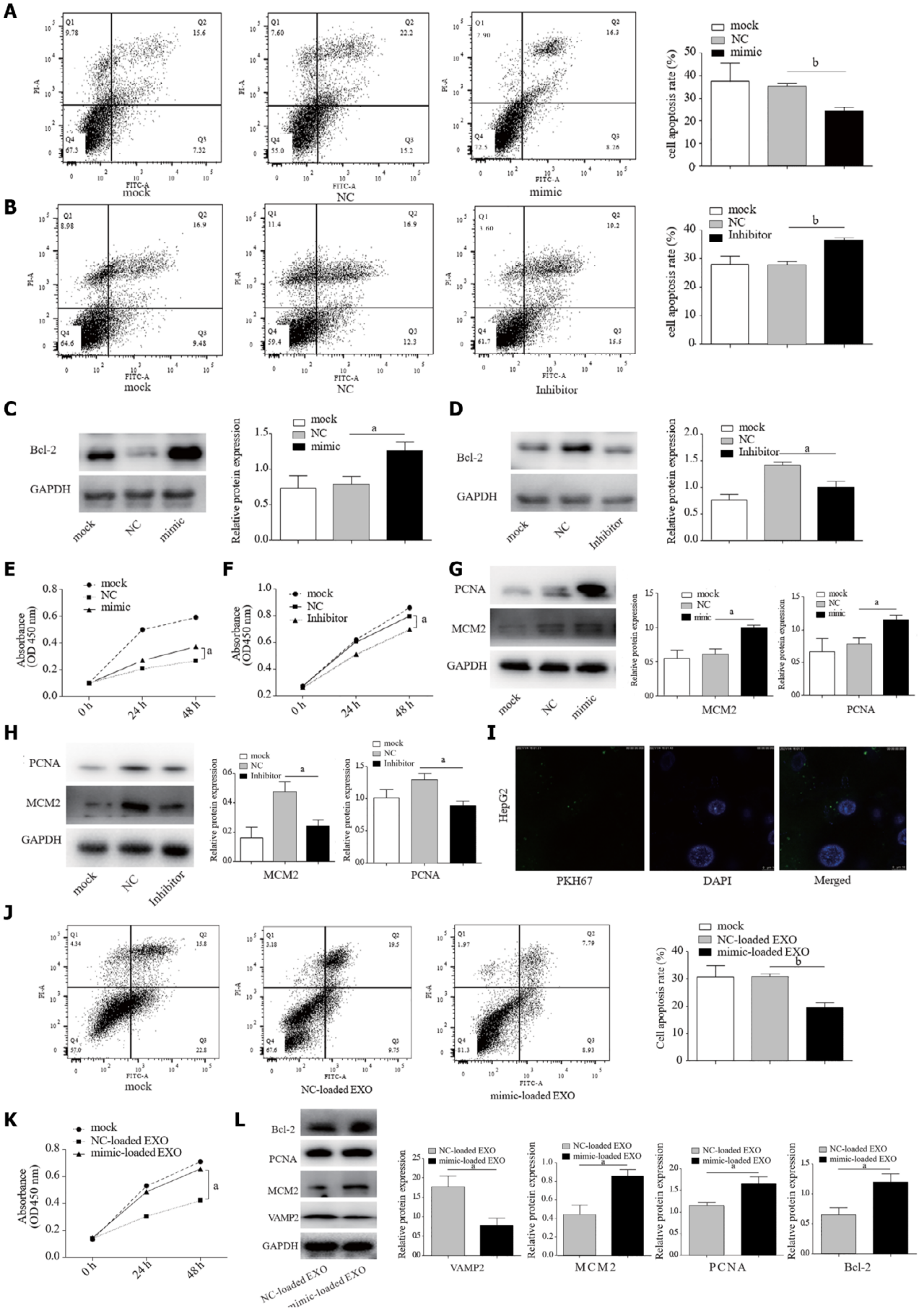

Figure 3 miR-135a-5p suppressed apoptosis and promoted proliferation. A and B: Annexin V-FITC/PI assay for the effect of overexpression or

knockdown of miR-135a-5p on apoptosis of HepG2 cells; C and D: The protein expression of B-cell lymphoma-2 (Bcl-2) in the group with overexpression of miR-135a-5p and the miR-135a-5p inhibited group; E and F: Cell counting kit 8 assays were used to determine the proliferation of HepG2 cells transfected with miR-135a-5p mimics and miR-135a-5p inhibitors; G and H: Western blot analyses of the level of mini-chromosome maintenance protein-2 (MCM2) and proliferating cell nuclear antigen (PCNA) in the group with overexpression of miR-135a-5p and the miR-135a-5p inhibited group; I: Confocal image showing that HepG2 cells were treated with exosomes rich in miR-135a-5p; J: Flow cytometry analysis of the effect of exosomal miR-135a-5p on cell apoptosis; K: Cell counting assay was performed to determine the proliferation of HepG2 cells treated with exosomes with overexpressed miR-135a-5p; L: Western blot analyses of Bcl-2, MCM2, PCNA and vesicle-associated membrane protein 2 in HepG2 cells incubated with mimic-loaded EXO or NC-loaded EXO. $^{a}P < 0.05$; $^{b}P < 0.01$. Bcl-2: B-cell lymphoma-2; MCM2: Mini-chromosome maintenance protein-2; PCNA: Proliferating cell nuclear antigen.

MiR-135a-5p blocks Dox-induced apoptosis by downregulating VAMP2 in HCC

Cell survival and proliferation usually counter the chemotherapy drug effect[37]. Herein, we tried to demonstrate whether miR-135a-5p/VAMP2 is involved in the resistance to anti-cancer drugs. Intriguingly, miR-135a-5p reversed the apoptosis caused by Dox (Figure 6A). Mimic-loaded EXO confirmed this result (Figure 6B). On the contrary, VAMP2 enhanced the effect of Dox-induced apoptosis in HepG2 cells (Figure 6C). The results from co-transfected miR-135a-5p mimics and pcDNA3.1-VAMP2 suggested that VAMP2 reversed Dox resistance induced by miR-135a-5p (Figure 6D).

Similarly, miR-135a-5p recovered cell proliferation in HepG2 cells treated with Dox (Figure 6E and F). Moreover, VAMP2 played a critical role in the Dox resistance triggered by miR-135a-5p (Figure 6G and H). Taken together, these results suggest that miR-135a-5p could be transported to other cells by exosomes and lead to Dox resistance of recipient cells by down-regulating VAMP2.

HBc mediates resistance of HCC cells to Dox via miR-135a-5p/VAMP2

Dox can directly promote HBV replication[38]. However, there are no publicly available data on the effect of HBV or HBV proteins on the chemotherapy resistance of HCC. We noted that HBc protects HCC cells against apoptosis in the Dox treatment groups (Figure 7A). Since HBc increased miR-135a-5p and decreased VAMP2, we co-transfected HBc and miR-135a-5p inhibitors or VAMP2 in HepG2 cells. Flow cytometry revealed that the apoptosis rate was higher in HepG2 cells co-transfected with pcDNA3.1-HBc plasmid and miR-135a-5p inhibitors than in the control after treatment with Dox (Figure 7B). Similarly, VAMP2 also recovered the apoptosis rate (Figure 7C), suggesting that miR-135a-5p/VAMP2 participated in the HBc-mediated chemotherapy resistance of HCC.

The cell proliferation assay further demonstrated that HBc mediated resistance of HCC cells to Dox (Figure 7D) and miR-135a-5p/VAMP2 played an essential role in this (Figure 7E and F). In summary, HBc mediated Dox resistance in HCC cells *via* miR-135a-5p/VAMP2.

DISCUSSION

Chronic HBV infection is still a significant risk factor for HCC. Various studies have underlined the usefulness of exosomal miRNAs as potential biomarkers to detect early stages of HBV-related HCC[39]. Hepatitis B virus X protein (HBx) has been reported to modulate several exosomal miRNAs that facilitate the process of hepatocarcinogenesis [22]. A recent finding revealed that HBc promotes liver cancer metastasis through the miR-382-5p/DLC-1 axis[40]. However, it is less clear on the effect of HBc on drug resistance in HCC. Here, we reported that HBc reduced apoptosis, induced cell proliferation, and mediated resistance of HCC to chemotherapeutic drugs by increasing and modulating exosomal miR-135a-5p to target VAMP2.

Viral infections can induce exosomal cargos, including miRNAs, to change them profoundly[41]. This study successfully isolated exosomes from HepG2 cells and HepG2.2.15 cells and found that HBc could induce the overexpression of miR-135a-5p in exosomes. HBV-associated miRNAs can distinguish HBV-related HCC from healthy controls[39]. Our clinical data revealed that miR-135a was upregulated in liver cancer tissues, consistent with other studies.

As a small non-coding RNA, miRNA mainly inhibits the expression of downstream target genes. Most miRNAs may regulate more than one target gene[42]. Forkhead box O1 (FOXO1)[43], protein tyrosine phosphatase receptor delta (PTPRD)[20], Kruppel-like factor-4 (KLF4)[44], signal transducer and activator of transcription 6 (STAT6)[45], ELK1 and ELK3[46] have been proven to be direct target genes of miR-135a-5p. We

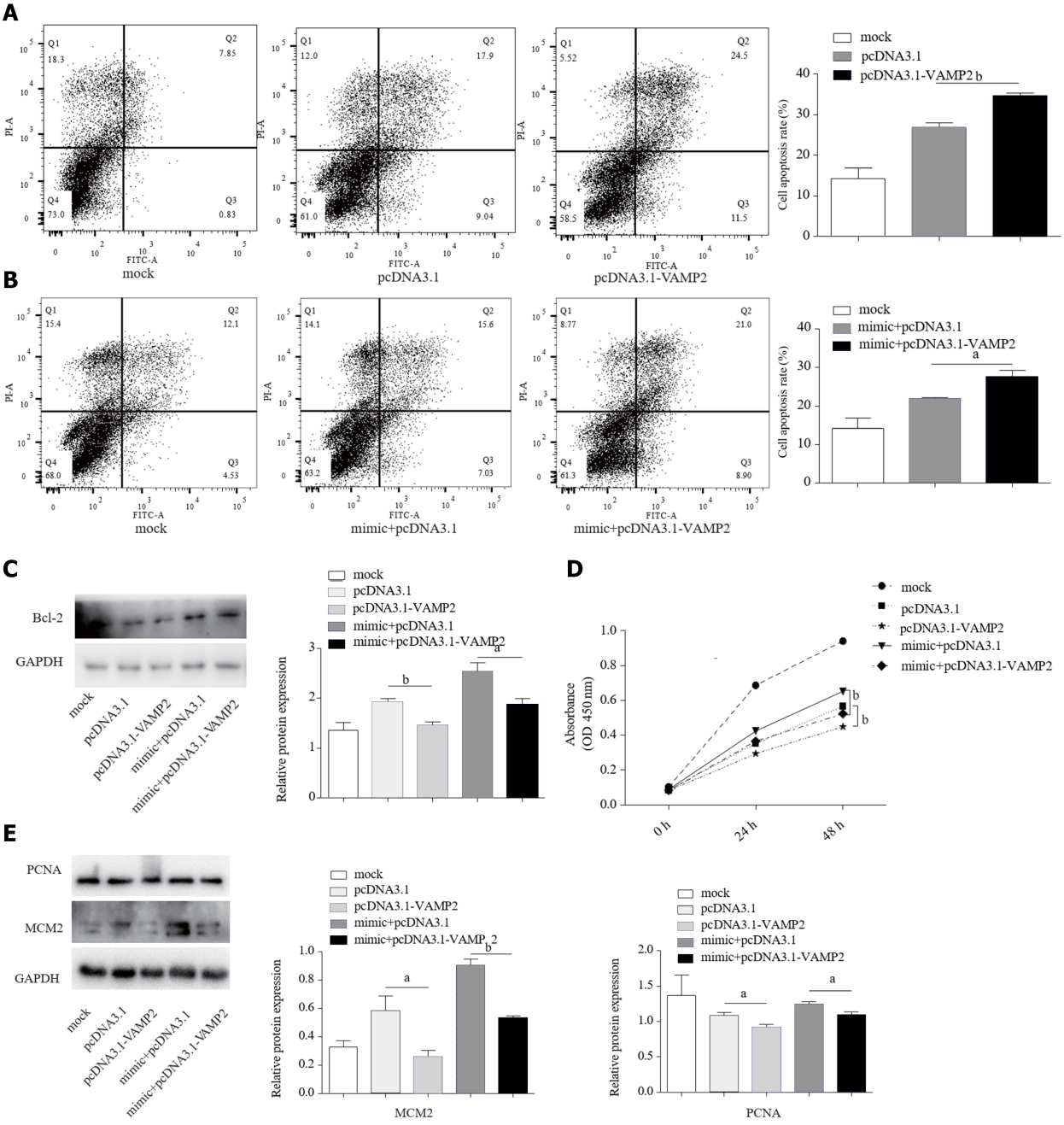

Figure 4 Vesicle-associated membrane protein 2 contributed to anti-apoptosis and proliferation induced by miR-135a-5p. A and B: Cell apoptosis was examined by flow cytometry in HepG2 cells transfected with the specific plasmid combinations; C: B-cell lymphoma-2 expression was detected by Western blot in HepG2 cells after transfection with the indicated plasmids; D: Cell counting kit 8 assay showed the proliferation of HepG2 cells after transfection with the plasmid combination shown above; E: The protein level of mini-chromosome maintenance protein-2 and proliferating cell nuclear antigen was measured by Western blot in HepG2 cells transfected with the plasmid group shown in the figure above. $^{a}P < 0.05$; $^{b}P < 0.01$. Bcl-2: B-cell lymphoma-2; MCM2: Mini-chromosome maintenance protein-2; PCNA: Proliferating cell nuclear antigen; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; VAMP2: Vesicle-associated membrane protein 2.

tried to identify a novel target gene of miR-135a-5p in HCC. Both TargetScan and DIANA predicted VAMP2 as a candidate target gene of miR-135a-5p. The present study verified the prediction and added VAMP2 as one more target gene of miR-135a-5p.

Exosomal miRNAs have a significant function in the regulation of tumor progression[47]. Numerous studies have suggested that miR-135a has shown protective effects under some conditions[46,48,49]. Zhou and his collaborators showed that apoptosis was induced by miR-135a through the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling pathway in human renal cancer cells[50]. Moreover, miR-135a-5p also induces the apoptosis of glioma[48] and cardiomyocyte cells[51], whereas miR-135a-5p inhibitor significantly protects nerve

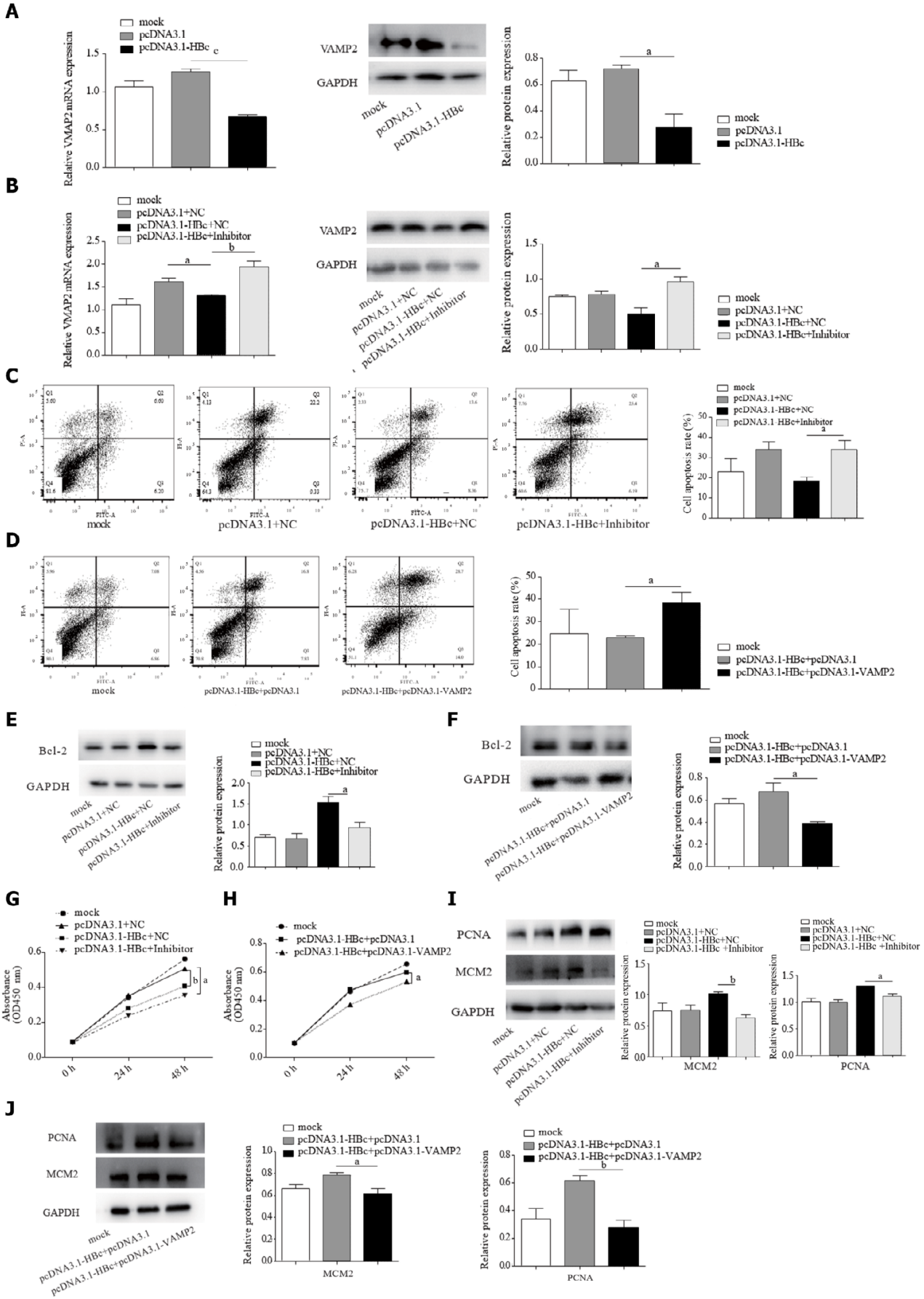

Figure 5 Hepatitis B core antigen induced anti-apoptosis and proliferation *via* miR-135a-5p and its target gene vesicle-associated membrane protein 2. A and B: Quantitative polymerase chain reaction and western blot analyses of the level of vesicle-associated membrane protein 2 in HepG2

cells after transfection with the specific plasmid combinations; C and D: Annexin V-FITC/PI assay was performed to assess cell apoptosis in HepG2 cells after transfected with the indicated plasmids; E and F: Western blot was performed to analyze the level of B-cell lymphoma-2 in HepG2 cells after transfection with the plasmid combination shown above; G and H: Cell counting kit 8 assay was performed to assess cell proliferation in HepG2 cells transfected with the specific plasmid combinations; I and J: Western blot analyses of mini-chromosome maintenance protein-2 and proliferating cell nuclear antigen in HepG2 cells transfected with the plasmid group shown in the figure above. $^{a}P < 0.05$; $^{b}P < 0.01$. Bcl-2: B-cell lymphoma-2; MCM2: Mini-chromosome maintenance protein-2; PCNA: Proliferating cell nuclear antigen; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; VAMP2: Vesicle-associated membrane protein 2.

cells against epilepsy-induced apoptosis[52]. However, our findings suggested an opposite role of miR-135a-5p in mediating cell apoptosis, indicating that miR-135a-5p might serve a dual role as a regulator of cancer progression. In gastric cancer, miR-135a has been reported to have an anti-apoptotic effect consistent with our results[53].

Abnormal cell apoptosis is one of the causes of excessive proliferation and oncogenesis[31]. It is interesting to note that miR-135a-5p also exerts different functions in cell proliferation. It is clear that miR-135a-5p acts as a tumor suppressor miRNA in some cancers, including prostate cancer[45], renal carcinoma cells[50], nasopharyngeal carcinoma[54], and as an oncogenic miRNA in bladder cancer[55] and HCC[19,44]. Our experimental results also demonstrated that miR-135a-5p acts as an onco-miRNA to promote HCC proliferation *via* inhibition of VAMP2. Many recent studies showed that the same individual miRNA has different purposes in different diseases[56]. This study also showed that miR-135a has a distinct purpose in HCC, implying that miR-135a might also play diverse roles in different cancers. Therefore, the effects of miR-135a on diseases depend on its target genes.

There are two different conclusions regarding the effect of HBc on apoptosis in HCC [57,58]. Several studies report that HBc, involved in HBV self-regulation, can inhibit apoptosis or enhance anti-apoptosis in HCC[35,57]. Liu and his partners reported that HBc inhibits Fas-mediated hepatocyte apoptosis[35]. Du *et al*[57] found that HBc enhances anti-apoptosis of hepatocytes by blocking death receptor 5 (DR5) expression. On the contrary, researchers in the Institut Pasteur of Shanghai revealed that HBc increases tumor necrosis factor alpha (TNF-α) -induced apoptosis in HCC cells[58]. Our experimental results showed that HBc prevented cell apoptosis and promoted cell proliferation through the miR-135a-5p/VAMP2 axis in HCC cells, which is similar to the report that HBc fosters the proliferation of HCC by upregulating the expression of c-Ets2[36].

Chemotherapy is the primary treatment for patients with advanced cancer. Exosomes secreted by drug-resistant cell lines can deliver miRNAs to sensitive cells and induce drug-resistant characteristics[59]. A few articles describe that miR-135a increases chemical resistance in some cancers[60-62]. Upregulation of miR-135a contributes to paclitaxel resistance in human non-small cell lung cancer cells[60]. High levels of miR-135b-5p promote resistance to cisplatin treatment in endometrial cancer cells[62] and gastric cancer cells[63]. MiR-135a also seems to have different effects on drug resistance, as well as cell apoptosis. A report from Nanjing Medical University shows that enforced miR-135a/b expression sensitizes A549/Cisplatin (CDDP) cells to CDDP-induced apoptosis[64]. Our results suggested that miR-135a-5p could resist Dox-induced apoptosis by targeting VAMP2 in HCC.

Our research group and other groups have published several articles on HBx protein promoted chemotherapeutic resistance in HCC[65-67]. A recently published paper concludes that HBx protein leads to resistance to the chemotherapy drug 5-Fluorouracil in HCC by downregulating SHIP2 through SKP2[65]. We also reported that HBx protein can promote Dox chemoresistance in HCC through overexpression of Variant 1 of KIAA0101[66] and Transcript variant 2 of the chemokine-like factor (CKLF1)[67]. However, there is no relevant study to assess the effect of HBc on HCC drug resistance. Herein, we found that HBc protected HCC from Dox-induced apoptosis through the miR-135a-5p/VAMP2 axis.

CONCLUSION

HBc could upregulate the expression of miR-135a-5p in HBV-infected hepatocytes. Then, miR-135a-5p was packaged into exosomes. After adjacent or distant recipient cells absorbed these exosomes, miR-135a-5p was delivered into recipient cells and led to a decrease in VAMP2 transcription, a novel target gene. The decreased VAMP2 facilitated tumor anti-apoptosis, cell proliferation, and drug resistance in HCC (Figure 8).

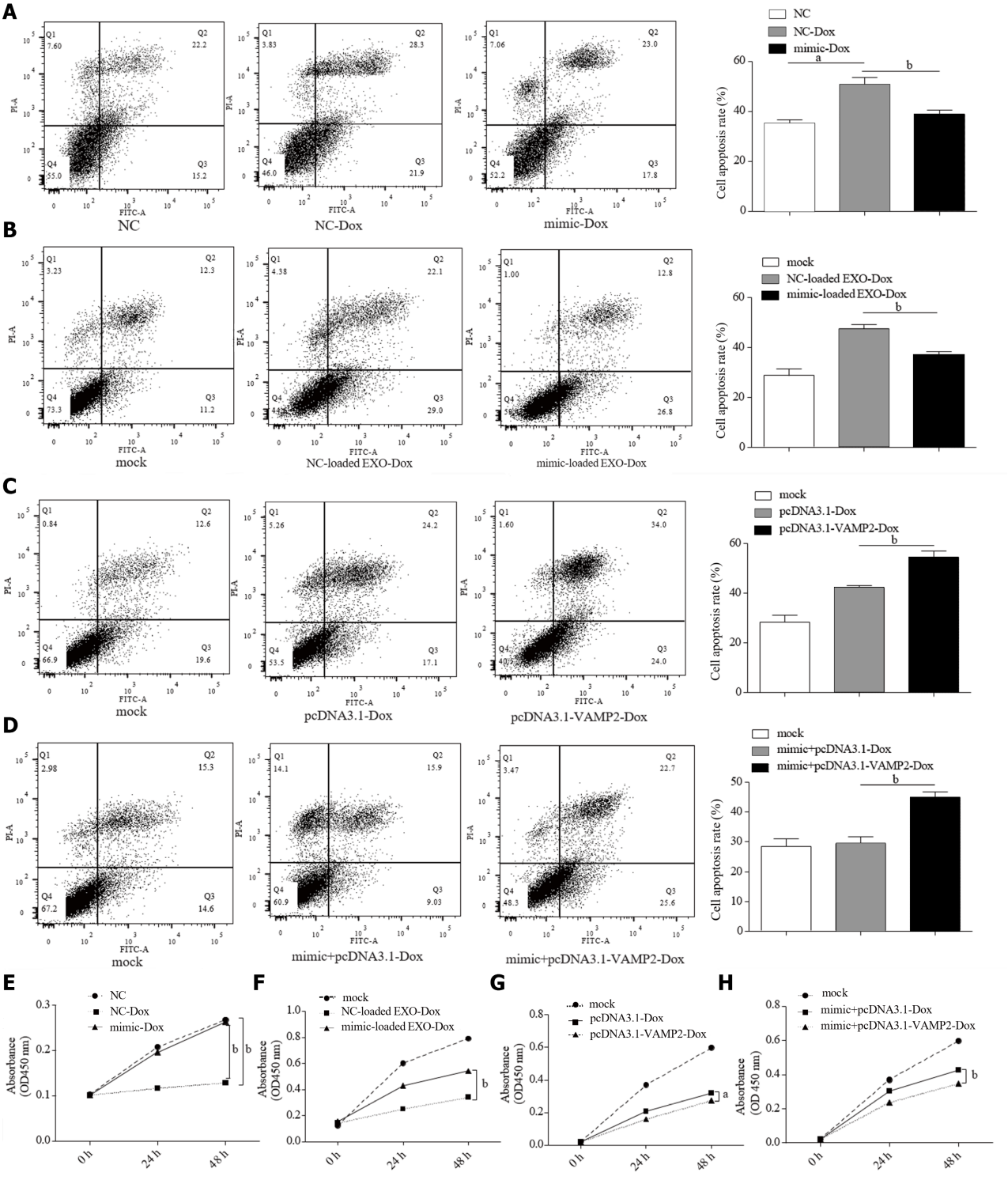

Figure 6 miR-135a-5p enhanced Dox-resistance and reduced cell apoptosis of hepatocellular carcinoma cells by down-regulating vesicle-associated membrane protein 2. A: The apoptosis rate of HepG2 cells after treatment with Doxorubicin hydrochloride (Dox). Flow cytometry was used to detected the effect of HepG2 cells with overexpressed miR-135a-5p after treatment with Dox; B: Annexin V-FITC/PI assay was used to discover the rate of apoptosis in HepG2 cells cultured with mimic-loaded exosomes; C and D: Flow cytometry was used to detect the rate of Dox-induced apoptosis in HepG2 cells after transfected with the plasmid group shown in the figure above; E and F: Cell counting kit 8 assay was used to determine the proliferation rate of HepG2 cells transfected with miR-135a-5p mimics after treated with Dox; G and H: Cell counting assay was performed to determine the proliferation of HepG2 cells transfected with pcDNA3.1-vesicle-associated membrane protein 2. $^{a}P < 0.05$; $^{b}P < 0.01$. Dox: Doxorubicin hydrochloride; VAMP2: Vesicle-associated membrane protein 2.

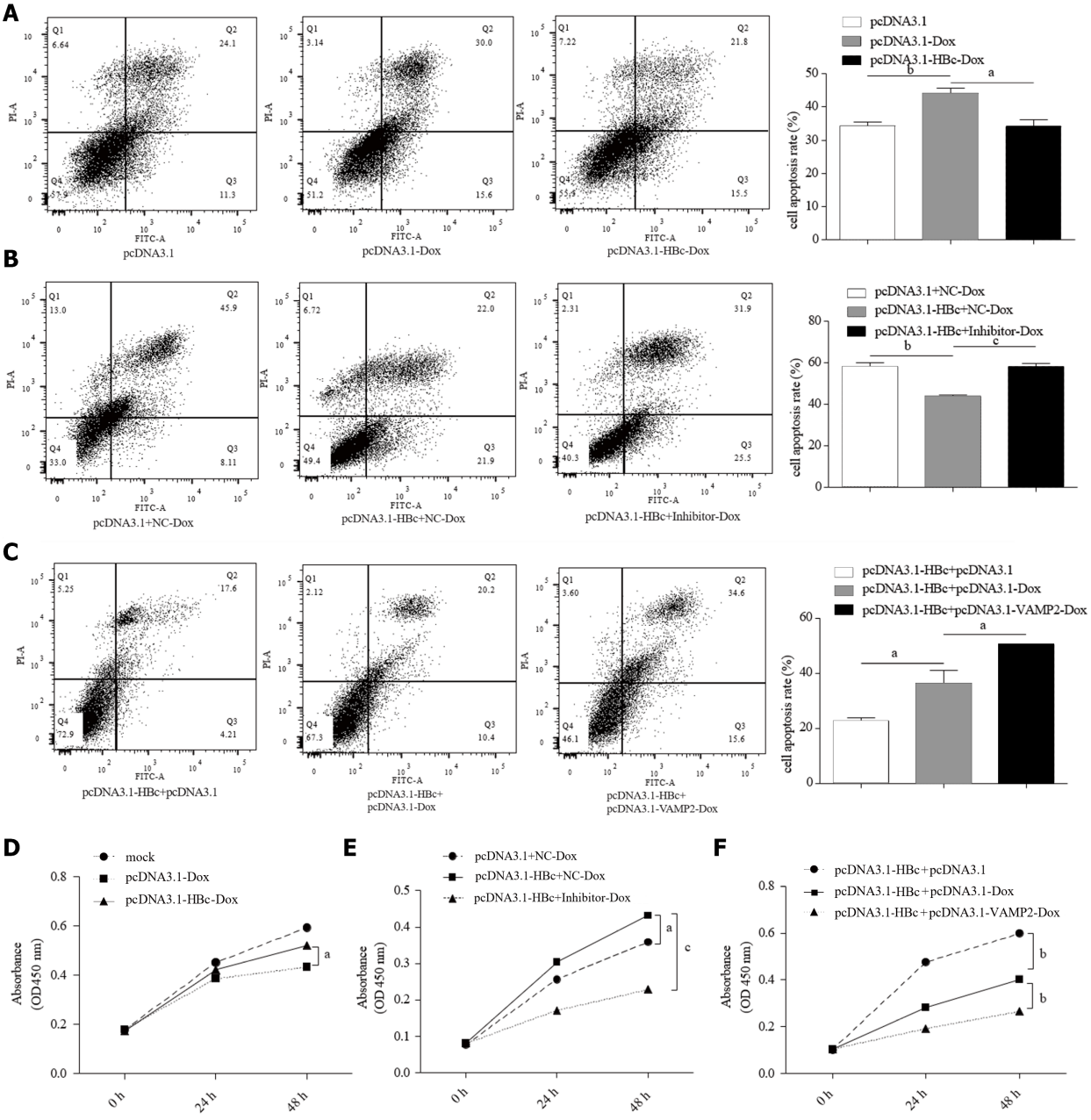

Figure 7 Hepatitis B core antigen mediated resistance of hepatocellular carcinoma cells to Doxorubicin hydrochloride *via* miR-135a-5p/vesicle-associated membrane protein 2. A: HepG2 cells were transfected with pcDNA3.1-hepatitis B core antigen (HBc) plasmids, flow cytometry was used to determine the rate of Doxorubicin hydrochloride (Dox)-induced apoptosis; B and C: Cell apoptosis rate was measured in HepG2 cells treated with Dox by flow cytometry after transfection with the indicated plasmid; D: Cell counting assay was performed to determine the proliferation of HepG2 cells transfected with pcDNA3.1-HBc plasmids after treatment with Dox; E: Cell proliferation in HepG2 cells co-transfected with pcDNA3.1-HBc plasmids and miR-135a-5p inhibitors assessed by the cell counting kit 8 assay; F: Cell counting assay used to determine the proliferation of HepG2 cells co-transfected with pcDNA3.1-HBc and pcDNA3.1-vesicle-associated membrane protein 2 plasmids after treatment with Dox. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. HBc: Hepatitis B core antigen; VAMP2: Vesicle-associated membrane protein 2; Dox: Doxorubicin hydrochloride.

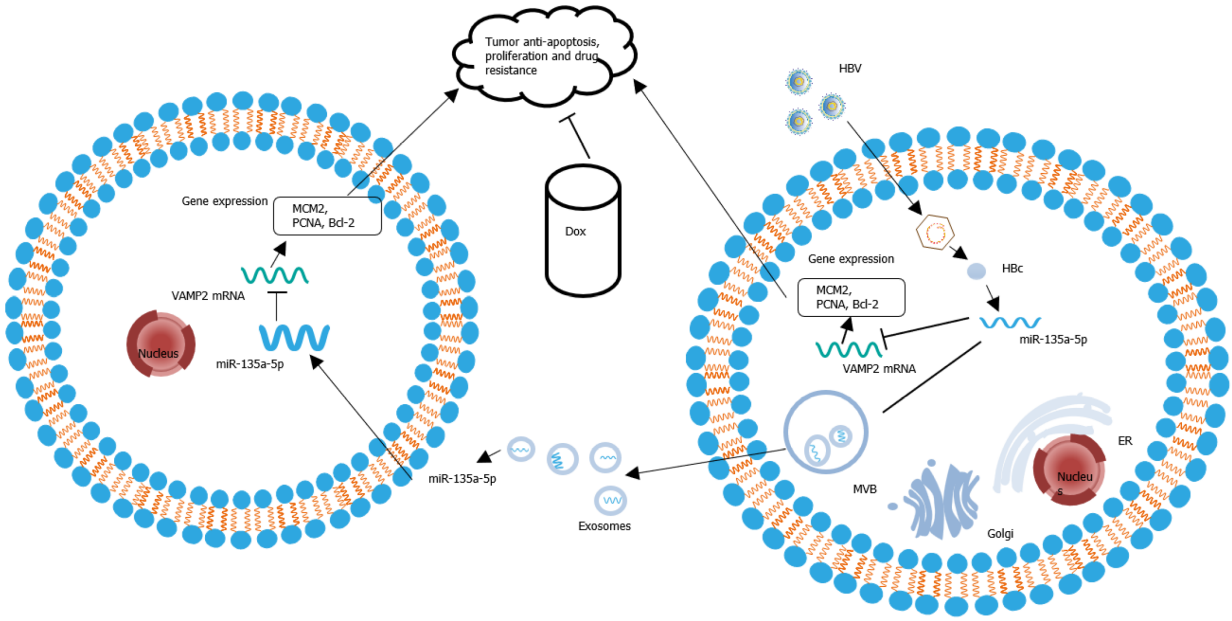

Figure 8 Hepatitis B core antigen promoted tumor anti-apoptosis, proliferation and chemoresistance in hepatocellular carcinoma cells by the miR-135a-5p/vesicle-associated membrane protein 2 axis. HBV: Hepatitis B virus; HBc: Hepatitis B core antigen; Bcl-2: B-cell lymphoma-2; MCM2: Mini-chromosome maintenance protein-2; PCNA: Proliferating cell nuclear antigen; VAMP2: Vesicle-associated membrane protein 2; Dox: Doxorubicin hydrochloride; MVB: Multivesicular body; ER: Endoplasmic reticulum.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is a frequently diagnosed malignant tumor caused by its main risk factor, hepatitis B virus (HBV) infection. HBV infection alters the level of miRNA in cells, which can be delivered to surrounding cells by exosomes to affect disease progression.

Research motivation

HCC is a common malignant tumor with relatively insipid early symptoms, rapid disease progression, burdensome treatment, and poor prognosis. Since HBV infection is still one of the major causes of HCC in China, the mechanism of HBV in HCC resistance remains unclear.

Research objectives

To explore the role of hepatitis B core antigen (HBc) on Dox-induced HCC resistance and the underlying mechanism.

Research methods

Exosomes were isolated by ultracentrifugation. The miRNAs differentially expressed in HCC were identified using the Cancer Genome Atlas (TCGA) database. The level of miR-135a-5p in patient tissues and exosomes was detected by quantitative polymerase chain reaction. After transfection with the indicated plasmids, cell functions affected by the HBV-regulated miR-135a/vesicle-associated membrane protein 2 (VAMP2) axis were assessed by flow cytometry and cell counting kit 8 assay.

Research results

miR-135a-5p expression was upregulated in HCC tissues and cells. HBc increased the expression of exosomal miR-135a-5p. VAMP2 is one of the potential target genes of miR-135a-5p, and functional assays showed that HBc mediated the miR-135a/VAMP2 axis to induce apoptosis protection, cell proliferation, and chemotherapy resistance in HCC.

Research conclusions

HBc elevated the expression of exosomal miR-135a-5p and promoted anti-apoptosis,

cell proliferation, and chemical resistance through miR-135a-5p/VAMP2 in HCC.

Research perspectives

The role of the miR-135a-5p/VAMP2 regulatory axis in chemotherapy resistance of HCC may serve as a potential molecular therapeutic target for HCC.

ACKNOWLEDGEMENTS

We are very grateful to Wang Y for her skillful statistical analysis guidance. Many thanks to Medical Research Center for Structural biology of Wuhan University for providing Ultracentrifuge- Beckman optimal L-100XP; Transmission electron microscopy- HT7700; Confocal microscopy- Leica-LCS-SP8-STED; Flow cytometry- FACS AriaIII and Wuhan University Testing Center for providing Dynamic Light Scatterer-Zetasizer Nano ZSP. We appreciate the contributors of TCGA databases for providing open access resources for exploring cancer genomics data.

REFERENCES

1 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

2 **Wang FS**, Fan JG, Zhang Z, Gao B, Wang HY. The global burden of liver disease: the major impact of China. *Hepatology* 2014; **60**: 2099-2108 [PMID: 25164003 DOI: 10.1002/hep.27406]

3 **Llovet JM**, Montal R, Sia D, Finn RS. Molecular therapies and precision medicine for hepatocellular carcinoma. *Nat Rev Clin Oncol* 2018; **15**: 599-616 [PMID: 30061739 DOI: 10.1038/s41571-018-0073-4]

4 **El-Serag HB**, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007; **132**: 2557-2576 [PMID: 17570226 DOI: 10.1053/j.gastro.2007.04.061]

5 **Yang Y**, Han Q, Hou Z, Zhang C, Tian Z, Zhang J. Exosomes mediate hepatitis B virus (HBV) transmission and NK-cell dysfunction. *Cell Mol Immunol* 2017; **14**: 465-475 [PMID: 27238466 DOI: 10.1038/cmi.2016.24]

6 **Kalluri R**. The biology and function of exosomes in cancer. *J Clin Invest* 2016; **126**: 1208-1215 [PMID: 27035812 DOI: 10.1172/JCI81135]

7 **Kowal J**, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, Dingli F, Loew D, Tkach M, Théry C. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc Natl Acad Sci U S A* 2016; **113**: E968-E977 [PMID: 26858453 DOI: 10.1073/pnas.1521230113]

8 **Wu Q**, Zhou L, Lv D, Zhu X, Tang H. Exosome-mediated communication in the tumor microenvironment contributes to hepatocellular carcinoma development and progression. *J Hematol Oncol* 2019; **12**: 53 [PMID: 31142326 DOI: 10.1186/s13045-019-0739-0]

9 **Zhang J**, Li S, Li L, Li M, Guo C, Yao J, Mi S. Exosome and exosomal microRNA: trafficking, sorting, and function. *Genomics Proteomics Bioinformatics* 2015; **13**: 17-24 [PMID: 25724326 DOI: 10.1016/j.gpb.2015.02.001]

10 **Bracken CP**, Scott HS, Goodall GJ. A network-biology perspective of microRNA function and dysfunction in cancer. *Nat Rev Genet* 2016; **17**: 719-732 [PMID: 27795564 DOI: 10.1038/nrg.2016.134]

11 **Zheng P**, Chen L, Yuan X, Luo Q, Liu Y, Xie G, Ma Y, Shen L. Exosomal transfer of tumor-associated macrophage-derived miR-21 confers cisplatin resistance in gastric cancer cells. *J Exp Clin Cancer Res* 2017; **36**: 53 [PMID: 28407783 DOI: 10.1186/s13046-017-0528-y]

12 **Sun Z**, Shi K, Yang S, Liu J, Zhou Q, Wang G, Song J, Li Z, Zhang Z, Yuan W. Effect of exosomal miRNA on cancer biology and clinical applications. *Mol Cancer* 2018; **17**: 147 [PMID: 30309355 DOI: 10.1186/s12943-018-0897-7]

13 **Sartorius K**, Makarova J, Sartorius B, An P, Winkler C, Chuturgoon A, Kramvis A. The Regulatory Role of MicroRNA in Hepatitis-B Virus-Associated Hepatocellular Carcinoma (HBV-HCC) Pathogenesis. *Cells* 2019; **8** [PMID: 31771261 DOI: 10.3390/cells8121504]

14 **Lu C**, Jia S, Zhao S, Shao X. MiR-342 regulates cell proliferation and apoptosis in hepatocellular carcinoma through Wnt/β-catenin signaling pathway. *Cancer Biomark* 2019; **25**: 115-126 [PMID: 31006667 DOI: 10.3233/CBM-192399]

15 **Zhou Y**, Chen E, Tang Y, Mao J, Shen J, Zheng X, Xie S, Zhang S, Wu Y, Liu H, Zhi X, Ma T, Ni H, Chen J, Chai K, Chen W. miR-223 overexpression inhibits doxorubicin-induced autophagy by targeting FOXO3a and reverses chemoresistance in hepatocellular carcinoma cells. *Cell Death Dis* 2019; **10**: 843 [PMID: 31695022 DOI: 10.1038/s41419-019-2053-8]

16 **Cao Z**, Qiu J, Yang G, Liu Y, Luo W, You L, Zheng L, Zhang T. MiR-135a biogenesis and regulation in malignancy: a new hope for cancer research and therapy. *Cancer Biol Med* 2020; **17**: 569-582 [PMID: 32944391 DOI: 10.20892/j.issn.2095-3941.2020.0033]

17 **Yang C**, Zheng X, Ye K, Sun Y, Lu Y, Fan Q, Ge H. miR-135a Inhibits the Invasion and Migration of Esophageal Cancer Stem Cells through the Hedgehog Signaling Pathway by Targeting Smo. *Mol Ther Nucleic Acids* 2020; **19**: 841-852 [PMID: 31981861 DOI: 10.1016/j.omtn.2019.10.037]

18 **Xie Y**, Li F, Li Z, Shi Z. miR-135a suppresses migration of gastric cancer cells by targeting TRAF5-mediated NF-κB activation. *Onco Targets Ther* 2019; **12**: 975-984 [PMID: 30774383 DOI: 10.2147/OTT.S189976]

19 **von Felden J**, Heim D, Schulze K, Krech T, Ewald F, Nashan B, Lohse AW, Wege H. High expression of micro RNA-135A in hepatocellular carcinoma is associated with recurrence within 12 months after resection. *BMC Cancer* 2017; **17**: 60 [PMID: 28100188 DOI: 10.1186/s12885-017-3053-7]

20 **Van Renne N**, Roca Suarez AA, Duong FHT, Gondeau C, Calabrese D, Fontaine N, Ababsa A, Bandiera S, Croonenborghs T, Pochet N, De Blasi V, Pessaux P, Piardi T, Sommacale D, Ono A, Chayama K, Fujita M, Nakagawa H, Hoshida Y, Zeisel MB, Heim MH, Baumert TF, Lupberger J. miR-135a-5p-mediated downregulation of protein tyrosine phosphatase receptor delta is a candidate driver of HCV-associated hepatocarcinogenesis. *Gut* 2018; **67**: 953-962 [PMID: 28159835 DOI: 10.1136/gutjnl-2016-312270]

21 **Théry C**, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* 2006; **Chapter** 3: Unit 3.22 [PMID: 18228490 DOI: 10.1002/0471143030.cb0322s30]

22 **Kouwaki T**, Fukushima Y, Daito T, Sanada T, Yamamoto N, Mifsud EJ, Leong CR, Tsukiyama-Kohara K, Kohara M, Matsumoto M, Seya T, Oshiumi H. Extracellular Vesicles Including Exosomes Regulate Innate Immune Responses to Hepatitis B Virus Infection. *Front Immunol* 2016; **7**: 335 [PMID: 27630638 DOI: 10.3389/fimmu.2016.00335]

23 **Zhao L**, Liu W, Xiao J, Cao B. The role of exosomes and "exosomal shuttle microRNA" in tumorigenesis and drug resistance. *Cancer Lett* 2015; **356**: 339-346 [PMID: 25449429 DOI: 10.1016/j.canlet.2014.10.027]

24 **Hu X**, Jiang J, Ni C, Xu Q, Ye S, Wu J, Ge F, Han Y, Mo Y, Huang D, Yang L. HBV Integration-mediated Cell Apoptosis in HepG2.2.15. *J Cancer* 2019; **10**: 4142-4150 [PMID: 31417659 DOI: 10.7150/jca.30493]

25 **Sodroski C**, Lowey B, Hertz L, Jake Liang T, Li Q. MicroRNA-135a Modulates Hepatitis C Virus Genome Replication through Downregulation of Host Antiviral Factors. *Virol Sin* 2019; **34**: 197-210 [PMID: 30456659 DOI: 10.1007/s12250-018-0055-9]

26 **Bartel DP**. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; **116**: 281-297 [PMID: 14744438 DOI: 10.1016/s0092-8674(04)00045-5]

27 **Agarwal V**, Bell GW, Nam JW, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. *Elife* 2015; **4** [PMID: 26267216 DOI: 10.7554/eLife.05005]

28 **Karagkouni D**, Paraskevopoulou MD, Chatzopoulos S, Vlachos IS, Tastsoglou S, Kanellos I, Papadimitriou D, Kavakiotis I, Maniou S, Skoufos G, Vergoulis T, Dalamagas T, Hatzigeorgiou AG. DIANA-TarBase v8: a decade-long collection of experimentally supported miRNA-gene interactions. *Nucleic Acids Res* 2018; **46**: D239-D245 [PMID: 29156006 DOI: 10.1093/nar/gkx1141]

29 **Liu M**, Jiang L, Guan XY. The genetic and epigenetic alterations in human hepatocellular carcinoma: a recent update. *Protein Cell* 2014; **5**: 673-691 [PMID: 24916440 DOI: 10.1007/s13238-014-0065-9]

30 **Tsujimoto Y**. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? *Genes Cells* 1998; **3**: 697-707 [PMID: 9990505 DOI: 10.1046/j.1365-2443.1998.00223.x]

31 **Goyal L**. Cell death inhibition: keeping caspases in check. *Cell* 2001; **104**: 805-808 [PMID: 11290317 DOI: 10.1016/s0092-8674(01)00276-8]

32 **Sarig R**, Tzahor E. The cancer paradigms of mammalian regeneration: can mammals regenerate as amphibians? *Carcinogenesis* 2017; **38**: 359-366 [PMID: 28334384 DOI: 10.1093/carcin/bgw103]

33 **Gramantieri L**, Trerè D, Chieco P, Lacchini M, Giovannini C, Piscaglia F, Cavallari A, Bolondi L. In human hepatocellular carcinoma in cirrhosis proliferating cell nuclear antigen (PCNA) is involved in cell proliferation and cooperates with P21 in DNA repair. *J Hepatol* 2003; **39**: 997-1003 [PMID: 14642618 DOI: 10.1016/s0168-8278(03)00458-6]

34 **Maiorano D**, Lutzmann M, Méchali M. MCM proteins and DNA replication. *Curr Opin Cell Biol* 2006; **18**: 130-136 [PMID: 16495042 DOI: 10.1016/j.ceb.2006.02.006]

35 **Liu W**, Lin YT, Yan XL, Ding YL, Wu YL, Chen WN, Lin X. Hepatitis B virus core protein inhibits Fas-mediated apoptosis of hepatoma cells *via* regulation of mFas/FasL and sFas expression. *FASEB J* 2015; **29**: 1113-1123 [PMID: 25466893 DOI: 10.1096/fj.14-263822]

36 **Gai X**, Zhao P, Pan Y, Shan H, Yue X, Du J, Zhang Z, Liu P, Ma H, Guo M, Yang X, Sun W, Gao L, Ma C, Liang X. Hepatitis B virus core protein enhances human telomerase reverse transcriptase expression and hepatocellular carcinoma cell proliferation in a c-Ets2-dependent manner. *Int J Biochem Cell Biol* 2013; **45**: 1174-1185 [PMID: 23542016 DOI: 10.1016/j.biocel.2013.03.015]

37 **Jones VS**, Huang RY, Chen LP, Chen ZS, Fu L, Huang RP. Cytokines in cancer drug resistance: Cues to new therapeutic strategies. *Biochim Biophys Acta* 2016; **1865**: 255-265 [PMID: 26993403 DOI: 10.1016/j.bbcan.2016.03.005]

38 **Wang W**, Peng H, Li J, Zhao X, Zhao F, Hu K. Controllable inhibition of hepatitis B virus replication by a DR1-targeting short hairpin RNA (shRNA) expressed from a DOX-inducible lentiviral vector. *Virus Genes* 2013; **46**: 393-403 [PMID: 23397077 DOI: 10.1007/s11262-013-0886-2]

39 **Bandopadhyay M**, Bharadwaj M. Exosomal miRNAs in hepatitis B virus related liver disease: a new hope for biomarker. *Gut Pathog* 2020; **12**: 23 [PMID: 32346400 DOI: 10.1186/s13099-020-00353-w]

40 **Du J**, Bai F, Zhao P, Li X, Gao L, Ma C, Liang X. Hepatitis B core protein promotes liver cancer metastasis through miR-382-5p/DLC-1 axis. *Biochim Biophys Acta Mol Cell Res* 2018; **1865**: 1-11 [PMID: 28982593 DOI: 10.1016/j.bbamcr.2017.09.020]

41 **Kouwaki T**, Okamoto M, Tsukamoto H, Fukushima Y, Oshiumi H. Extracellular Vesicles Deliver Host and Virus RNA and Regulate Innate Immune Response. *Int J Mol Sci* 2017; **18** [PMID: 28335522 DOI: 10.3390/ijms18030666]

42 **Lu TX**, Rothenberg ME. MicroRNA. *J Allergy Clin Immunol* 2018; **141**: 1202-1207 [PMID: 29074454 DOI: 10.1016/j.jaci.2017.08.034]

43 **Zeng YB**, Liang XH, Zhang GX, Jiang N, Zhang T, Huang JY, Zhang L, Zeng XC. miRNA-135a promotes hepatocellular carcinoma cell migration and invasion by targeting forkhead box O1. *Cancer Cell Int* 2016; **16**: 63 [PMID: 27486383 DOI: 10.1186/s12935-016-0328-z]

44 **Yao S**, Tian C, Ding Y, Ye Q, Gao Y, Yang N, Li Q. Down-regulation of Krüppel-like factor-4 by microRNA-135a-5p promotes proliferation and metastasis in hepatocellular carcinoma by transforming growth factor-β1. *Oncotarget* 2016; **7**: 42566-42578 [PMID: 27302923 DOI: 10.18632/oncotarget.9934]

45 **Xu B**, Lu X, Zhao Y, Liu C, Huang X, Chen S, Zhu W, Zhang L, Chen M. MicroRNA-135a induces prostate cancer cell apoptosis *via* inhibition of STAT6. *Oncol Lett* 2019; **17**: 1889-1895 [PMID: 30675252 DOI: 10.3892/ol.2018.9791]

46 **Ahmad A**, Zhang W, Wu M, Tan S, Zhu T. Tumor-suppressive miRNA-135a inhibits breast cancer cell proliferation by targeting ELK1 and ELK3 oncogenes. *Genes Genomics* 2018; **40**: 243-251 [PMID: 29892795 DOI: 10.1007/s13258-017-0624-6]

47 **Bach DH**, Hong JY, Park HJ, Lee SK. The role of exosomes and miRNAs in drug-resistance of cancer cells. *Int J Cancer* 2017; **141**: 220-230 [PMID: 28240776 DOI: 10.1002/ijc.30669]

48 **Wu S**, Lin Y, Xu D, Chen J, Shu M, Zhou Y, Zhu W, Su X, Qiu P, Yan G. MiR-135a functions as a selective killer of malignant glioma. *Oncogene* 2012; **31**: 3866-3874 [PMID: 22139076 DOI: 10.1038/onc.2011.551]

49 **Zhang T**, Shao Y, Chu TY, Huang HS, Liou YL, Li Q, Zhou H. MiR-135a and MRP1 play pivotal roles in the selective lethality of phenethyl isothiocyanate to malignant glioma cells. *Am J Cancer Res* 2016; **6**: 957-972 [PMID: 27293991]

50 **Zhou W**, Bi X, Gao G, Sun L. miRNA-133b and miRNA-135a induce apoptosis *via* the JAK2/STAT3 signaling pathway in human renal carcinoma cells. *Biomed Pharmacother* 2016; **84**: 722-729 [PMID: 27710896 DOI: 10.1016/j.biopha.2016.09.074]

51 **Liu N**, Shi YF, Diao HY, Li YX, Cui Y, Song XJ, Tian X, Li TY, Liu B. MicroRNA-135a Regulates Apoptosis Induced by Hydrogen Peroxide in Rat Cardiomyoblast Cells. *Int J Biol Sci* 2017; **13**: 13-21 [PMID: 28123342 DOI: 10.7150/ijbs.16769]

52 **Wang Y**, Yang Z, Zhang K, Wan Y, Zhou Y. miR-135a-5p inhibitor protects glial cells against apoptosis *via* targeting SIRT1 in epilepsy. *Exp Ther Med* 2021; **21**: 431 [PMID: 33747170 DOI: 10.3892/etm.2021.9848]

53 **Pan Y**, Ren F, Zhang W, Liu G, Yang D, Hu J, Feng K, Feng Y. Regulation of BGC-823 cell sensitivity to adriamycin *via* miRNA-135a-5p. *Oncol Rep* 2014; **32**: 2549-2556 [PMID: 25322930 DOI: 10.3892/or.2014.3546]

54 **Wang LX**, Kang ZP, Yang ZC, Ma RX, Tan Y, Peng XB, Dai RZ, Li J, Yu Y, Xu M. MicroRNA-135a Inhibits Nasopharyngeal Carcinoma Cell Proliferation Through Targeting Interleukin-17. *Cell Physiol Biochem* 2018; **46**: 2232-2238 [PMID: 29734196 DOI: 10.1159/000489591]

55 **Mao XP**, Zhang LS, Huang B, Zhou SY, Liao J, Chen LW, Qiu SP, Chen JX. Mir-135a enhances cellular proliferation through post-transcriptionally regulating PHLPP2 and FOXO1 in human bladder cancer. *J Transl Med* 2015; **13**: 86 [PMID: 25888950 DOI: 10.1186/s12967-015-0438-8]

56 **Iacona JR**, Lutz CS. miR-146a-5p: Expression, regulation, and functions in cancer. *Wiley Interdiscip Rev RNA* 2019; **10**: e1533 [PMID: 30895717 DOI: 10.1002/wrna.1533]

57 **Du J**, Liang X, Liu Y, Qu Z, Gao L, Han L, Liu S, Cui M, Shi Y, Zhang Z, Yu L, Cao L, Ma C, Zhang L, Chen Y, Sun W. Hepatitis B virus core protein inhibits TRAIL-induced apoptosis of hepatocytes by blocking DR5 expression. *Cell Death Differ* 2009; **16**: 219-229 [PMID: 18927587 DOI: 10.1038/cdd.2008.144]

58 **Jia B**, Guo M, Li G, Yu D, Zhang X, Lan K, Deng Q. Hepatitis B virus core protein sensitizes hepatocytes to tumor necrosis factor-induced apoptosis by suppression of the phosphorylation of mitogen-activated protein kinase kinase 7. *J Virol* 2015; **89**: 2041-2051 [PMID: 25428880 DOI: 10.1128/JVI.03106-14]

59 **Fu X**, Liu M, Qu S, Ma J, Zhang Y, Shi T, Wen H, Yang Y, Wang S, Wang J, Nan K, Yao Y, Tian T. Exosomal microRNA-32-5p induces multidrug resistance in hepatocellular carcinoma *via* the PI3K/Akt pathway. *J Exp Clin Cancer Res* 2018; **37**: 52 [PMID: 29530052 DOI: 10.1186/s13046-018-0677-7]

60 **Holleman A**, Chung I, Olsen RR, Kwak B, Mizokami A, Saijo N, Parissenti A, Duan Z, Voest EE, Zetter BR. miR-135a contributes to paclitaxel resistance in tumor cells both *in vitro* and in vivo. *Oncogene* 2011; **30**: 4386-4398 [PMID: 21552288 DOI: 10.1038/onc.2011.148]

61 **Yan LH**, Chen ZN, Li-Li, Chen J, Wei WE, Mo XW, Qin YZ, Lin Y, Chen JS. miR-135a promotes gastric cancer progression and resistance to oxaliplatin. *Oncotarget* 2016; **7**: 70699-70714 [PMID: 27683111 DOI: 10.18632/oncotarget.12208]

62 **Wang J**, Zhang L, Jiang W, Zhang R, Zhang B, Silayiding A, Duan X. MicroRNA-135a promotes proliferation, migration, invasion and induces chemoresistance of endometrial cancer cells. *Eur J*

Obstet Gynecol Reprod Biol X 2020; **5**: 100103 [PMID: 32021975 DOI: 10.1016/j.eurox.2019.100103]

63 **Shao L**, Chen Z, Soutto M, Zhu S, Lu H, Romero-Gallo J, Peek R, Zhang S, El-Rifai W. Helicobacter pylori-induced miR-135b-5p promotes cisplatin resistance in gastric cancer. *FASEB J* 2019; **33**: 264-274 [PMID: 29985646 DOI: 10.1096/fj.201701456RR]

64 **Zhou L**, Qiu T, Xu J, Wang T, Wang J, Zhou X, Huang Z, Zhu W, Shu Y, Liu P. miR-135a/b modulate cisplatin resistance of human lung cancer cell line by targeting MCL1. *Pathol Oncol Res* 2013; **19**: 677-683 [PMID: 23640248 DOI: 10.1007/s12253-013-9630-4]

65 **Su KJ**, Yu YL. Downregulation of SHIP2 by Hepatitis B Virus X Promotes the Metastasis and Chemoresistance of Hepatocellular Carcinoma through SKP2. *Cancers (Basel)* 2019; **11** [PMID: 31357665 DOI: 10.3390/cancers11081065]

66 **Liu L**, Chen X, Xie S, Zhang C, Qiu Z, Zhu F. Variant 1 of KIAA0101, overexpressed in hepatocellular carcinoma, prevents doxorubicin-induced apoptosis by inhibiting p53 activation. *Hepatology* 2012; **56**: 1760-1769 [PMID: 22576474 DOI: 10.1002/hep.25834]

67 **Liu Y**, Liu L, Zhou Y, Zhou P, Yan Q, Chen X, Ding S, Zhu F. CKLF1 Enhances Inflammation-Mediated Carcinogenesis and Prevents Doxorubicin-Induced Apoptosis *via* IL6/STAT3 Signaling in HCC. *Clin Cancer Res* 2019; **25**: 4141-4154 [PMID: 30918019 DOI: 10.1158/1078-0432.CCR-18-3510]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 December 28; 27(48): 8323-8342

DOI: 10.3748/wjg.v27.i48.8323

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

ORIGINAL ARTICLE

Basic Study

Dual therapy with zinc acetate and rifaximin prevents from ethanol-induced liver fibrosis by maintaining intestinal barrier integrity

Yuki Fujimoto, Kosuke Kaji, Norihisa Nishimura, Masahide Enomoto, Koji Murata, Soichi Takeda, Hiroaki Takaya, Hideto Kawaratani, Kei Moriya, Tadashi Namisaki, Takemi Akahane, Hitoshi Yoshiji

ORCID number: Yuki Fujimoto 0000-0002-5046-0588; Kosuke Kaji 0000-0002-1822-6759; Norihisa Nishimura 0000-0002-6295-3283; Masahide Enomoto 0000-0001-8980-4758; Koji Murata 0000-0001-5149-2437; Soichi Takeda 0000-0001-6457-2954; Hiroaki Takaya 0000-0002-4990-7573; Hideto Kawaratani 0000-0002-4361-0592; Kei Moriya 0000-0002-2878-8296; Tadashi Namisaki 0000-0002-3158-5318; Takemi Akahane 0000-0002-6675-0475; Hitoshi Yoshiji 0000-0002-5243-8544.

Author contributions: Fujimoto Y and Kaji K designed the research study; Fujimoto Y, Kaji K, Nishimura N, Enomoto M, Murata K, Takeda S, Takaya H and Kawaratani H performed the research; Moriya K, Namisaki T and Akahane T offered new reagents, analytic tools and material support; Fujimoto Y and Kaji K analyzed the data and wrote the manuscript; Yoshiji H supervised this study; All authors have read and approve the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Institutional review board of Nara Medical University, Kashihara, Japan (authorization numbers: 12734).

Yuki Fujimoto, Kosuke Kaji, Norihisa Nishimura, Masahide Enomoto, Koji Murata, Soichi Takeda, Hiroaki Takaya, Hideto Kawaratani, Kei Moriya, Tadashi Namisaki, Takemi Akahane, Hitoshi Yoshiji, Department of Gastroenterology, Nara Medical University, Kashihara 6348521, Nara, Japan

Corresponding author: Kosuke Kaji, MD, PhD, Doctor, Lecturer, Senior Researcher, Department of Gastroenterology, Nara Medical University, 840, Shijo-cho, Kashihara 6348521, Nara, Japan. kajik@naramed-u.ac.jp

Abstract

BACKGROUND

Hepatic overload of gut-derived lipopolysaccharide dictates the progression of alcoholic liver disease (ALD) by inducing oxidative stress and activating Kupffer cells and hepatic stellate cells through toll-like receptor 4 signaling. Therefore, targeting the maintenance of intestinal barrier integrity has attracted attention for the treatment of ALD. Zinc acetate and rifaximin, which is a nonabsorbable antibiotic, had been clinically used for patients with cirrhosis, particularly those with hepatic encephalopathy, and had been known to improve intestinal barrier dysfunction. However, only few studies focused on their efficacies in preventing the ALD-related fibrosis development.

AIM

To investigate the effects of a combined zinc acetate with rifaximin on liver fibrosis in a mouse ALD model.

METHODS

To induce ALD-related liver fibrosis, female C57BL/6J mice were fed a 2.5% (v/v) ethanol-containing Lieber-DeCarli liquid diet and received intraperitoneal carbon tetrachloride (CCl_4) injection twice weekly (1 mL/kg) for 8 wk. Zinc acetate (100 mg/L) and/or rifaximin (100 mg/L) were orally administered during experimental period. Hepatic steatosis, inflammation and fibrosis as well as intestinal barrier function were evaluated by histological and molecular analyses. Moreover, the direct effects of both agents on Caco-2 barrier function were assessed by *in vitro* assays.

RESULTS

Conflict-of-interest statement: All authors have nothing to disclose.

Data sharing statement: No additional data are available.

Country/Territory of origin: Japan

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: July 6, 2021
Peer-review started: July 6, 2021
First decision: July 26, 2021
Revised: July 6, 2021
Accepted: December 10, 2021
Article in press: December 22, 2021
Published online: December 28, 2021

P-Reviewer: Wang QY
S-Editor: Liu M
L-Editor: A
P-Editor: Liu M

In the ethanol plus CCl_4-treated mice, combination of zinc acetate and rifaximin attenuated oxidative lipid peroxidation with downregulation of *Nox2* and *Nox4*. This combination significantly inhibited the Kupffer cells expansion and the proinflammatory response with blunted hepatic exposure of lipopolysaccharide and the toll-like receptor 4/nuclear factor kB pathway. Consequently, liver fibrosis and hepatic stellate cells activation were efficiently suppressed with downregulation of *Mmp-2, -9, -13, and Timp1*. Both agents improved the atrophic changes and permeability in the ileum, with restoration of tight junction proteins (TJPs) by decreasing the expressions of tumor necrosis factor α and myosin light chain kinase. In the *in vitro* assay, both agents directly reinforced ethanol or lipopolysaccharide-stimulated paracellular permeability and upregulated TJPs in Caco-2 cells.

CONCLUSION
Dual therapy with zinc acetate and rifaximin may serve as a strategy to prevent ALD-related fibrosis by maintaining intestinal barrier integrity.

Key Words: Liver fibrosis; Intestinal permeability; Alcoholic liver disease; Lipopolysaccharide; Toll-like receptor; Tight junction protein

Core Tip: Gut-derived lipopolysaccharide dictates the progression of alcoholic liver disease (ALD) hence the maintenance of intestinal barrier integrity has attracted attention for the treatment of ALD. This study elucidates the preventive effect of combined zinc supplementation and rifaximin from ALD-related liver fibrosis induced by ethanol plus carbon tetrachloride in mice. This effect is involved in the multifaceted regulatory functions that maintain intestinal barrier integrity and reduce hepatic lipopolysaccharide exposure, thereby, leading to Kupffer cell expansion and hepatic stellate cell activation by inhibiting the toll-like receptor 4 pathway, highlighting that this regimen may represent a potential novel strategy against ALD-related liver fibrosis.

Citation: Fujimoto Y, Kaji K, Nishimura N, Enomoto M, Murata K, Takeda S, Takaya H, Kawaratani H, Moriya K, Namisaki T, Akahane T, Yoshiji H. Dual therapy with zinc acetate and rifaximin prevents from ethanol-induced liver fibrosis by maintaining intestinal barrier integrity. *World J Gastroenterol* 2021; 27(48): 8323-8342
URL: https://www.wjgnet.com/1007-9327/full/v27/i48/8323.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i48.8323

INTRODUCTION

Alcoholic liver disease (ALD), which is the most common and serious complication of excessive alcohol consumption, includes a spectrum of disorders, such as acute or chronic hepatitis, fatty liver disease, cirrhosis, and hepatocellular carcinoma[1,2]. The increasing mortality from ALD has become a major health problem in both Western and Asian countries[3]. Currently, limiting alcohol intake remains the most effective therapy for patients in all stages of ALD, although only few individuals succeed in substantially abstaining from alcohol consumption. Therefore, novel efficacious medications are urgently required to prevent the development of ALD.

ALD is known to progress through several communications between the liver and several physiologic systems in other organs[4,5]. Among various factors that mediate these cross-talks, the gut-derived endotoxin lipopolysaccharide (LPS), which is produced by gram-negative bacteria, particularly plays a pivotal role in inflammation and fibrosis in ALD[6]. Accumulation of LPS in response to alcohol consumption may be attributed to the functional impairment of intestinal barrier integrity, including intestinal hyperpermeability secondary to disrupted tight junction[7]. LPS is transported into the liver and activates Kupffer cells and macrophages that had been recruited in the liver through toll-like receptor 4 (TLR4) and its coreceptor CD14,

which in turn force these cells to produce inflammatory cytokines[8]. Moreover, gut-derived LPS triggers hepatic stellate cell (HSC) activation by increasing its susceptibility to acetaldehyde and transforming growth factor (TGF)-b and leading to extracellular matrix (ECM) deposition, intrahepatic inflammation, and fibrosis[9]. Therefore, maintenance of intestinal barrier integrity and blockage of the transfer of LPS from the intestine to the liver may be a therapeutic strategy to prevent alcohol-induced liver fibrosis.

Zinc is the second most abundant trace metal in humans after iron and is the only metal that appears in all enzyme classes[10]. Zinc deficiency is often observed in patients with ALD and usually becomes evident with increasing severity and with the progression of ALD from steatosis to cirrhosis[11,12]. Zinc supplementation in patients with cirrhosis provides metabolic effects that assist in the improvement of liver function, hepatic encephalopathy, and nutritional status[13-15]. Notably, Zhong *et al*[16] have documented that zinc deficiency induced by chronic alcohol exposure augmented epithelial barrier dysfunction with subsequent increase in gut permeability and development of endotoxemia in alcoholic liver injury. Meanwhile, several animal studies have shown that zinc supplementation could ameliorate intestinal barrier dysfunction[17,18]. However, supplementation with zinc alone was considered to only partially improve the outcome of patients with chronic liver diseases, including ALD [19]. Therefore, we postulated that a combination of zinc and another agent with antifibrotic effects would add benefits in the treatment of alcohol-induced liver fibrosis.

Rifaximin is an antibiotic that is minimally absorbed, has broad-spectrum activity against gram-positive and gram-negative aerobic and anaerobic enteric bacteria, and is clinically available for hepatic encephalopathy or travelers' diarrhea[20,21]. Our recent clinical studies have elucidated that rifaximin significantly decreased serum endotoxin activity and potentially improved intestinal permeability without modifying the gut microbiome in patients with cirrhosis[22]. Moreover, a recent study demonstrated that rifaximin inhibited toxin-induced apoptosis and deprivation of tight junction proteins (TJPs) in human intestinal cells through pregnane X receptor (PXR)-dependent inhibition of the TLR4/MyD88/nuclear factor kB (NF-kB) pathway[23]. However, the therapeutic potential of rifaximin against alcohol-induced liver fibrosis had been obscure.

This study aimed to investigate the combined effects of zinc supplementation and rifaximin on liver fibrosis induced by ethanol plus carbon tetrachloride (CCl_4) in connection with their protective properties against intestinal barrier disruption.

MATERIALS AND METHODS

Animals and experimental protocol

Ten-week-old female C57BL/6J mice (CLEA Japan, Osaka, Japan) were housed under 23 °C ± 3 °C with 50% ± 20% humidity and a 12-h light/12-h dark cycle. All experiments were performed over an 8-wk period, since our previous report has shown that administration of ethanol plus CCl_4 for this period definitely developed ALD-related liver fibrosis[24].

The mice were divided into five treatment groups (Figure 1). The control group (C/V; n = 10) were fed non-ethanol liquid diet (Research Diets, New Brunswick, NJ, United States). The E/V group (n = 10) were fed a 2.5% (v/v) ethanol-containing Lieber–DeCarli liquid diet (research diets) and received intraperitoneal injection of CCl_4 (FUJIFILM, Wako Pure Chemical Corporation, Osaka, Japan) twice a week (1 mL/kg body weight)[25]. The E/Zn (n = 10) and E/RFX (n = 10) groups were fed ethanol diet with 100 mg/L of zinc acetate (FUJIFILM, Wako Pure Chemical Corporation) and 100 mg/L of rifaximin (ASKA Pharmaceutical Co. Ltd., Tokyo, Japan), respectively[26,27], and received intraperitoneal CCl_4 injection twice weekly. The E/both group (n = 10) were fed ethanol diet that contained a combination of zinc acetate and rifaximin and received intraperitoneal CCl_4 injection. The same amount of lactose hydrate (FUJIFILM, Wako Pure Chemical Corporation) was used as vehicle for the C/V and E/V groups. Another set of mice groups were used to measure intestinal permeability, as described in Measurement of *in vivo* intestinal permeability. For sample collection, all mice underwent the following procedures: anesthesia with barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium), blood collection from the cervical artery and harvesting of the liver and ileum immediately after sacrifice. Serum biologic markers were measured by SRL, Inc. (Tokyo, Japan). The animal care and experimental procedures were approved by the ethics committee of

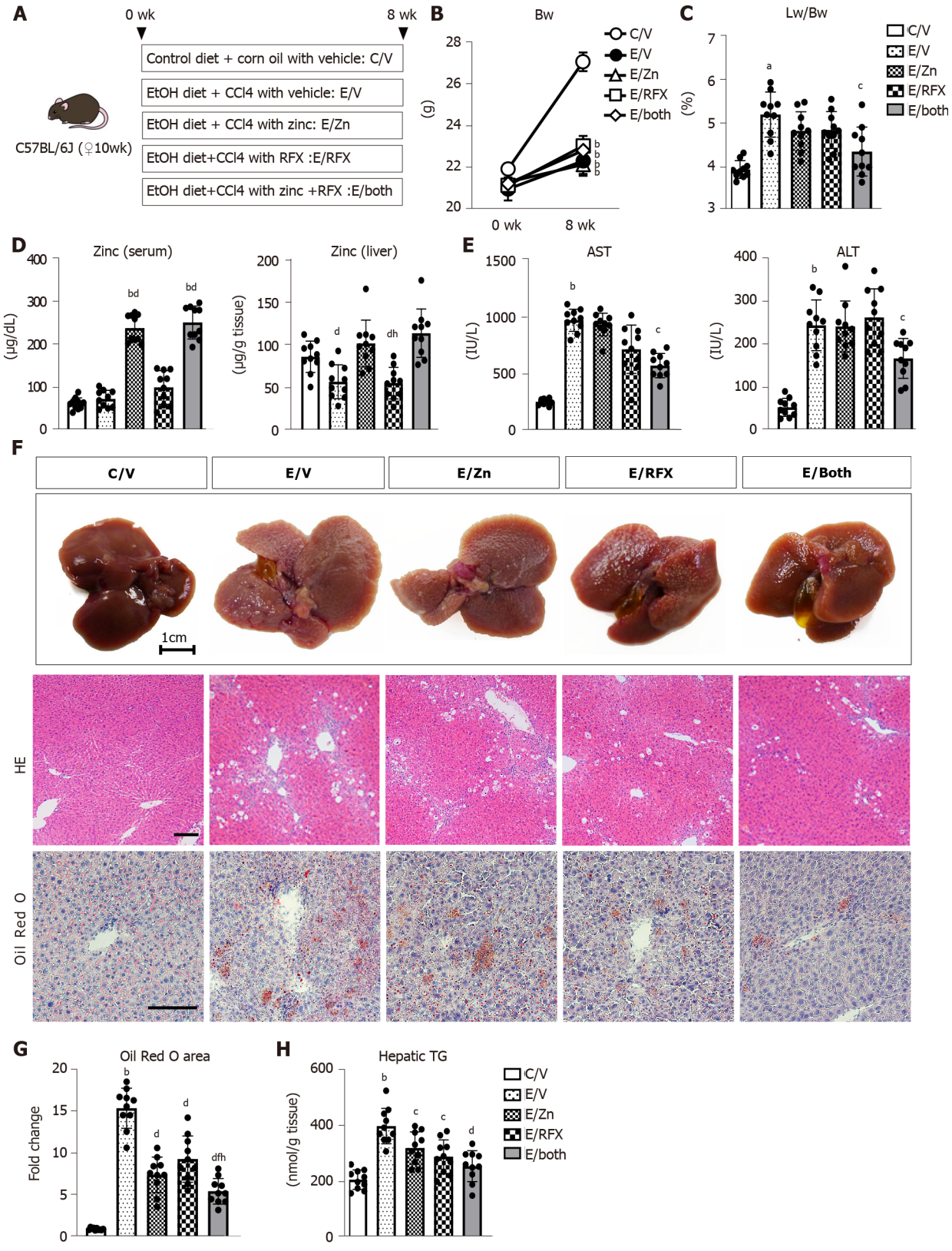

Figure 1 Zinc acetate and rifaximin against hepatic steatosis in alcoholic liver disease mice. A: Experimental protocols; B: Changes in body weights during experimental period; C: Ratio of liver weight to body weight at the end of experiment; D: Zinc concentrations of the serum (left) and the liver (right); E: Serum levels of aspartate aminotransferase (left) and alanine aminotransferase (right); F: Representative macroscopic appearances (upper), microphotographs of hematoxylin and eosin (middle) and Oil Red O staining (lower) of the livers in the experimental mice. Scale bar: 25 μm; G: Semi-quantification of lipid accumulation stained by Oil Red O in high-power field by NIH imageJ software. Histochemical quantitative analyses included five fields per section. Quantitative values are indicated as fold changes to the values of C/V group; H: Hepatic concentrations of triglyceride. Data are mean ± SD (n = 10), $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* C/V group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* E/V group; $^{e}P < 0.05$ and $^{f}P < 0.01$ *vs* E/Zn group; $^{g}P < 0.05$ and $^{h}P < 0.01$ *vs* E/RFX group. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HE: Hematoxylin and eosin.

Experimental Animal Care of Nara Medical University, Kashihara, Japan (authorization numbers: 12734).

Histologic and immunohistochemical analyses

The liver and ileum specimens were fixed in 10% formalin and embedded in paraffin, and other liver specimens were fixed with 4% paraformaldehyde for 24 h, then frozen in a Cryomold with Tissue-Tek OCT compound (Sakura Finetek Japan, Tokyo, Japan) for frozen sections. Paraffin-embedded sections of 5-μm thickness were stained with hematoxylin and eosin (HE) for the liver and ileum and with Sirius Red for the liver and frozen liver sections were stained with Oil Red O at Narabyouri Research Co. (Nara, Japan). To evaluate the morphologic changes in the ileum, 10 well-oriented crypt–villus units were examined per slide under a microscope. Immunohistochemistry was performed as described previously and α-smooth muscle actin (SMA) (#ab5694; 1:200, Abcam, Cambridge, United Kingdom), F4/80 (#ab100790; 1:100, Abcam) and COL-1 (#14695-1-AP; 1:500, Proteintech, Rosemont, IL, United States) were used as primary antibodies[28,29]. Immunofluorescence test for zonula occludens-1 (ZO-1) (#61-7300; 1:250, Invitrogen, Carlsbad, CA, United States) and Occludin (#ab216327; 1:200, Abcam) was performed on the paraffin-embedded ileum sections. Primary antibodies were detected using Alexa Fluor-conjugated secondary antibodies (Invitrogen). Images were captured using a BX53 (Olympus, Tokyo, Japan) for histology and immunohistochemistry and a BZ-X700 (Keyence, Osaka, Japan) for immunofluorescence. Semiquantitative analysis was performed using Image J software version 64 (National Institutes of Health, Bethesda, MD, United States).

Intrahepatic zinc and triglyceride concentration

Intrahepatic zinc and triglyceride concentrations were measured in 100 mg of frozen liver tissue per mouse using the Metalloassay Kit (Metallogenics, Chiba, Japan) and Triglyceride-Glo™ Assay (Promega, Madison, WI, United States), respectively, according to the manufacturer's instructions.

Intrahepatic alcohol dehydrogenase 1, aldehyde dehydrogenase 2 and cytochrome P450 2E1 (CYP2E1) activity

Intrahepatic alcohol dehydrogenase 1 (ADH1) and aldehyde dehydrogenase 2 (ALDH2) activities were measured by using Alcohol Dehydrogenase Activity Colorimetric Assay Kit (BioVision, Milpitas, CA, United States) and ALDH2 activity assay kit (Abcam), respectively, according to the manufacturer's instructions. Intrahepatic CYP2E1 activity was determined by measuring the hydroxylation of *p*-nitrophenol in whole liver extract as described[30].

Intrahepatic catalase, superoxide dismutase, and malondialdehyde concentration

Intrahepatic levels of catalase (CAT), superoxide dismutase (SOD) and malondialdehyde (MDA) were measured in 25 mg frozen liver tissue in each mouse using Mouse catalase ELISA Kit (CUnited StatesBIO, Houston, TX, United States), Mouse Super Oxidase Dimutase, SOD ELISA Kit (CUnited StatesBIO) and OxiSelect™ TBARS Assay Kit (Cell Biolabs, Inc., San Diego, CA, United States), according to the manufacturer's protocol.

Mouse matrix metalloproteinase-9 activity assay

Intrahepatic matrix metalloproteinase (MMP)-9 activities were evaluated in frozen liver tissue per mouse by the Mouse MMP-9 Activity Assay Kit (QuickZyme Biosciences, Leiden, Netherlands), according to the manufacturers protocol.

Measurement of in vivo intestinal permeability

In vivo intestinal permeability was determined as previously described with brief modifications[31]. Six hours after initiating fasting conditions, the mice (n = 5) were orally given 600 mg/kg body weight of fluorescein isothiocyanate (FITC)-dextran (4 kDa) (TdB Labs, Uppsala, Sweden). Blood was collected from the portal vein 4 h after FITC-dextran administration. To evaluate the degree of gut permeability, plasma was analyzed by fluorescence measurement of the concentration of FITC-labeled dextran at an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

Cell culture

To explore *in vitro* effects of zinc acetate and rifaximin on enterocytes, we used the human colorectal adenocarcinoma line Caco-2. Caco-2 cells were obtained from Riken

BRC Cell Bank (Ibaraki, Japan) and were cultured, as described previously[32]. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 100 U/mL of penicillin, 100 g/mL of streptomycin, 0.1-mM nonessential amino acids, 10-mM HEPES, and 10% fetal bovine serum at 37 °C in an environment with 5% carbon dioxide. Culture medium was replaced every 2 d. Caco-2 cells were subcultured after partial digestion with 0.25% trypsin-EDTA, and passages 19–30 were used. For alcohol intoxication, 5% ethanol was added to the culture medium for 3 h, with or without the addition of different concentrations of zinc acetate (1−100 μM) and/or rifaximin (0.1−10 μM) 30 min before alcohol intoxication. A previous report has shown that 5% ethanol significantly affected the Caco-2 monolayer barrier function[33]. For tumor necrosis factor (TNF)α stimulation, recombinant human TNFα (100 ng/mL, Abcam) was added to the Caco-2 cell monolayers for 6 h, with or without zinc acetate (100 μM) or rifaximin (10 μM) 30 minutes prior. For LPS stimulation, LPS (O55:B5) (2 μg/mL; Sigma-Aldrich, St. Louis, MO, United States) was added to the Caco-2 cell monolayers with and without zinc acetate (100 μM) or rifaximin (10 μM) for 24 h. The phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (10 μM, ChemScene, Monmouth Junction, NJ, United States) or the human PXR antagonist, SPA70 (510 μM, Axon Medchem, Groningen, Netherlands) was added to the culture media that had been treated with zinc acetate or rifaximin, respectively[34,35].

Measurement of transepithelial electrical resistance

To assess the *in vitro* Caco-2 monolayer barrier function, we measured the transepithelial electrical resistance (TEER) using an electrical resistance system (Millicell-ERS®; Millipore Corporation, Bedford, MA, United States), as reported previously[36]. The electrical resistance was expressed in units of Ω/cm^2 using the surface area of the Trans-well insert.

Cell viability assay

In vitro cell viability was determined using the Premix WST-1 Cell Proliferation Assay system (Takara Bio, Kusatsu, Japan), according to the manufacturer's protocol. Cell viability was calculated as the relative value to the start of exposure to each agent.

Quantitative real-time polymerase chain reaction assay

Total RNA was extracted from the liver and ileum tissues and cultured Caco-2 cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). After assessing the quality and concentration, 2 μg of total RNA was subjected to cDNA synthesis using the High-Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, United States). Quantitative real-time polymerase chain reaction (qRT-PCR) with gene-specific primer pairs (Supplementary Table 1) was performed using the StepOnePlus Real-time PCR system and SYBR Green (Applied Biosystems). The levels of mRNA expression were normalized according to the internal control of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase. All reactions were performed using 1:10 diluted cDNA; mRNA expression levels were estimated using the $2^{-\Delta\Delta CT}$ method and were presented as fold changes relative to the controls for each experiment.

Protein extraction and western blotting

Proteins were extracted from frozen liver tissues and Caco-2 cells using T-PER Tissue Protein Extraction Reagent supplemented with proteinase and phosphatase inhibitors (Thermo Scientific, Rockford, IL, United States). Western blot was performed, as described previously[37]. The membranes were incubated overnight with antibodies against phospho-IKKα/β (Ser176/180) (#2697; Cell Signaling Technology, Danvers, MA, United States), IKKβ (#2370; CST), IkBα (#4812; CST), NF-kB p65 (#8242; CST), phospho-NF-kB p65 (Ser536) (#3033; CST), COL-1 (#14695-1-AP; Proteintech), ZO-1 (#61-7300; Invitrogen), Occludin (#ab216327; Abcam), AKT (#9272; CST), phospho-AKT (Ser473) (#9271; CST), and β actin (#4967). Densitometric analysis was performed using ImageJ software version 64.

Statistical analyses

Continuous variables are presented as mean ± SD. Statistical significance was analyzed with a 2-sided Student's *t*-test or one-way analysis of variance, followed by Bonferroni's multiple comparison test, as appropriate. Statistical analyses were performed using Prism, version 9.1.2 (GraphPad Software, La Jolla, CA, United States). *P* values of < 0.05 were considered to indicate statistical significance.

RESULTS

Combination of zinc acetate and rifaximin improved liver dysfunction and suppressed hepatic steatosis in ethanol plus CCl_4-treated mice

Figure 1A shows our initial examination of the effects of zinc acetate and rifaximin on ALD-related fibrosis induced *via* combined ethanol and CCl_4 administration in mice. After 8 wk, the administration of ethanol plus CCl_4 group had remarkable delay in body weight gain, compared with that in the control group, and this delay in body weight gain could not be prevented by treatments with zinc acetate and rifaximin (Figure 1B). Conversely, the relative liver weights increased in the ethanol plus CCl_4-treated mice, and combined treatment with zinc acetate and rifaximin efficiently attenuated hepatomegaly (Figure 1C). To confirm the effect of zinc supplementation, we measured the serum and hepatic levels of zinc in the experimental groups. As shown in Figure 1D, the ethanol plus CCl_4-treated mice, compared with the control group, showed almost equivalent levels of serum zinc but lower levels of hepatic zinc, and treatment with zinc acetate significantly increased both the serum and hepatic zinc levels in the ethanol plus CCl_4-treated mice. Administration of zinc acetate (100 mg/L) and rifaximin (100 mg/L) at the present doses did not cause hypocupremia and renal dysfunction, respectively (Supplementary Figure 1A and B). The serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were increased by chronic ethanol exposure and CCl_4 administration (Figure 1E). Interestingly, combined treatment with zinc acetate and rifaximin lowered the AST and ALT levels in the ethanol plus CCl_4-treated mice (Figure 1E). Serum γ-glutamyl transpeptidase levels were elevated in the ethanol plus CCl_4-treated mice and remained unchanged by treatments with both agents (Supplementary Figure 1C). Meanwhile, serum alkaline phosphatase and albumin levels were not affected by chronic ethanol exposure and CCl_4 administration (Supplementary Figure 1C). In serum lipid test, serum triglyceride levels were elevated in the ethanol plus CCl_4-treated mice that were attenuated by treatments with zinc acetate and rifaximin. However, there were no significant differences in serum total-, high density lipoprotein (HDL)-, and low density lipoprotein (LDL)-cholesterol levels among the experimental groups (Supplementary Figure 1D and E).

Histologic assessment on HE and Oil Red O staining revealed hepatic steatosis in the ethanol plus CCl_4-treated mice (Figure 1F and G). Notably, treatment with zinc acetate and rifaximin remarkably attenuated hepatic fat accumulation, and consistently combined treatment with both agents attenuated the hepatic level of triglyceride (Figure 1F-H).

Zinc acetate and rifaximin prevented the accumulation of oxidative stress in ethanol plus CCl_4-treated mice

Next, we evaluated the changes in the activities of metabolic enzymes related to alcohol, acetaldehyde, and cytochrome CYP2E1 in the liver tissues of experimental group. As shown in Figure 2A and B, ethanol and CCl_4 administration significantly decreased both ADH1 and ALDH2 activities. Treatment with zinc acetate significantly suppressed the decline of ADH1 activity but did not affect ALDH2 in the ethanol plus CCl_4-treated mice. However, neither ADH1 nor ALDH2 activities changed after treatment with rifaximin. CYP2E1 activity was increased in the ethanol plus CCl_4-treated mice, and zinc acetate significantly suppressed the increase of CYP2E1 activity but rifaximin did not affected (Figure 2C). These findings indicate that zinc acetate would attenuate CYP2E1-mediated accumulation of oxidative stress.

In the ethanol plus CCl_4-treated mice, hepatic levels of antioxidant enzymes CAT and SOD were decreased as compared to control mice, and treatments with zinc acetate and rifaximin significantly prevented the decreases in CAT and SOD levels (Figure 2D and E). The chronic ethanol exposure and CCl_4 administration also induced the increase in hepatic levels of MDA, one of the final products of polyunsaturated fatty acids peroxidation (Figure 2F). It was noteworthy that treatments with zinc acetate and rifaximin suppressed the alteration in the levels of MDA (Figure 2F).

Moreover, compared with the control mice, the ethanol plus CCl_4-treated mice exhibited higher mRNA levels of the hepatic nicotinamide adenine dinucleotide phosphate oxidase (*Nox*) gene family members (*i.e.*, *Nox1*, *Nox2*, and *Nox4*); treatment with zinc acetate and rifaximin reduced the observed increase in the mRNA levels of *Nox2* and *Nox4* (Figure 2G).

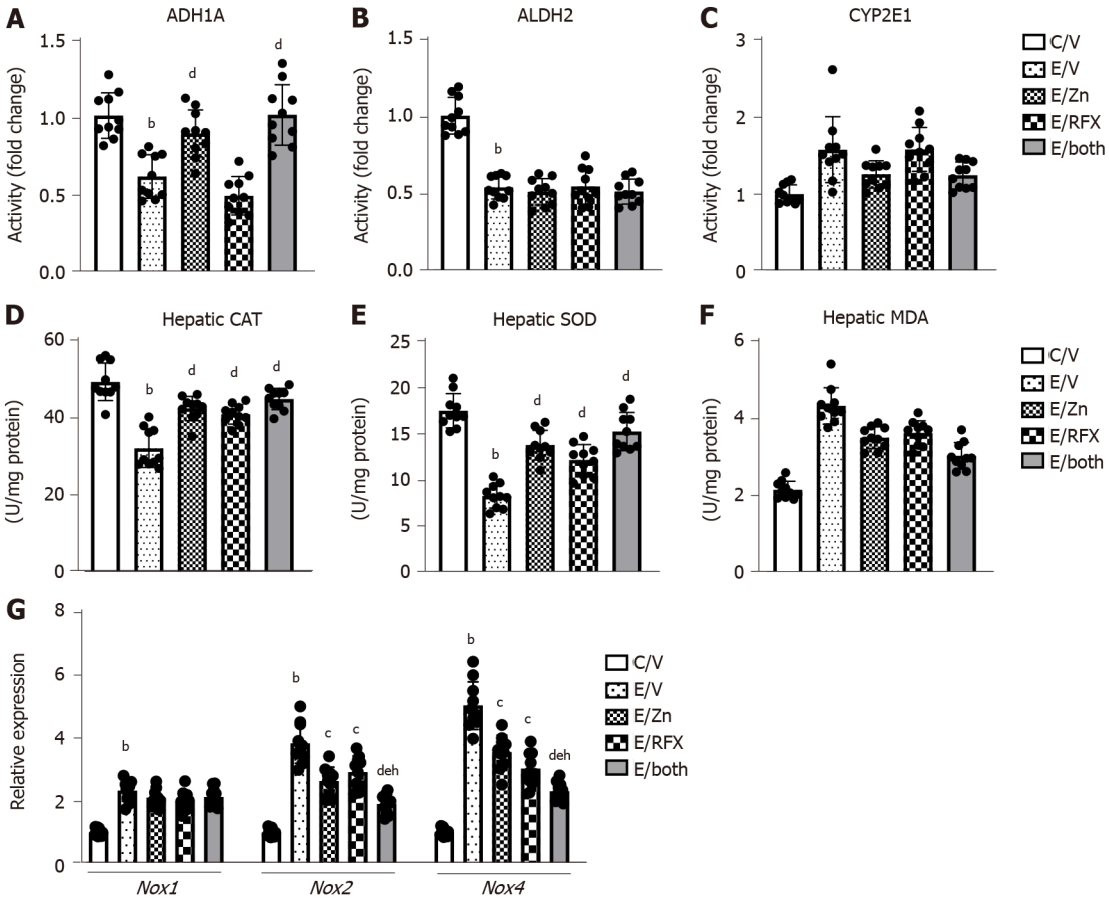

Figure 2 Zinc acetate and rifaximin on ethanol metabolism and accumulation of oxidative stress in alcoholic liver disease mice. A-C: Hepatic activity of alcohol dehydrogenase 1 (A), aldehyde dehydrogenase 2 (B) and cytochrome P450 2E1 (CYP2E1) (C). Quantitative values are indicated as fold changes to the values of C/V group; D-F: Hepatic levels of of catalase (D), superoxide dismutase (E) and malondialdehyde (F); G: Relative mRNA expression levels of *Nox1*, *Nox2* and *Nox4* in the liver of experimental mice. The mRNA expression levels were measured by RT-qPCR, and *Gapdh* was used as internal control. Quantitative values are indicated as fold changes to the values of C/V group. Data are mean ± SD (n = 10), ^{a}P < 0.05 and ^{b}P < 0.01 *vs* C/V group; ^{c}P < 0.05 and ^{d}P < 0.01 *vs* E/V group; ^{e}P < 0.05 and ^{f}P < 0.01 *vs* E/Zn group; ^{g}P < 0.05 and ^{h}P < 0.01 *vs* E/RFX group. ADH1: Alcohol dehydrogenase 1; ALDH2: Aldehyde dehydrogenase 2; CAT: Catalase; SOD: Superoxide dismutase; MDA: Malondialdehyde.

Zinc acetate and rifaximin attenuated Kupffer cell expansion and the lipopolysaccharide/TLR4 signaling activation in ethanol plus CCl_4-treated mice

On the basis of the suppressions in ethanol plus CCl_4-induced steatosis and inflammation following zinc acetate and rifaximin treatment, we next evaluated the proinflammatory status of the liver in the experimental mice. We observed extensive infiltration of F4/80-positive Kupffer cells and an increased mRNA levels of *Cd68* in the liver of ethanol plus CCl_4-treated mice (Figure 3A–C). Treatment with zinc acetate and rifaximin attenuated the expanded Kupffer cell infiltration and reduced the mRNA expression of *Cd68* which were robustly boosted by combination of the two agents (Figure 3A-C). We also observed that the combination treatment significantly suppressed the increases of M1-polarized macrophages while it had little effect on M2-polarized macrophages in the liver of ethanol plus CCl_4-treated mice (Figure 3D and E).

We further assessed to the effect of zinc acetate and rifaximin on the hepatic LPS/TLR4 signaling. Administration of ethanol plus CCl_4 caused an upregulation of hepatic LPS-binding protein (LBP), which forms a complex with LPS to interact with the macrophage receptor and initiate a proinflammatory host response (Figure 3F). In accordance with the upregulated hepatic *Lbp* expression, the mRNA levels of *Tlr4* and its coreceptor *Cd14*, which function to detect LPS, were increased in the ethanol plus CCl_4-treated mice (Figure 3G). Notably, treatment with zinc acetate and rifaximin ameliorated these increases, suggesting that both agents could reduce the load of LPS to the liver (Figure 3F and G). In the ethanol plus CCl_4-treaed mice, the hepatic overload of LPS induced the IKKα/β phosphorylation and in turn promoted the IkBα degradation; NF-kB p65 Levels were consequently increased as a sequence of the LBP/CD14/TLR4 pathway (Figure 3H). The combination of both agents efficiently

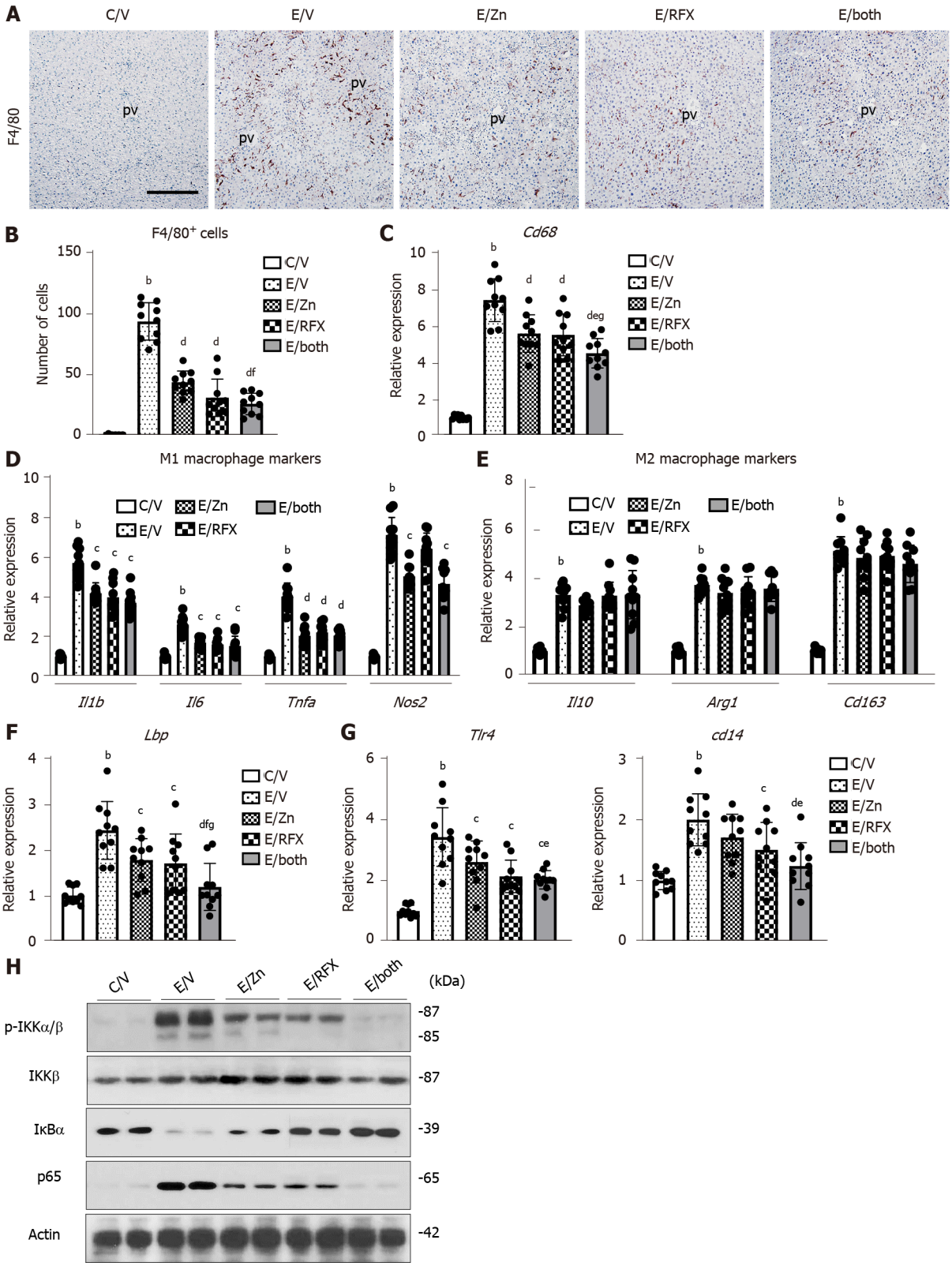

Figure 3 Zinc acetate and rifaximin against toll-like receptor 4-mediated pro-inflammatory response in alcoholic liver disease mice. A: Representative microphotographs of liver sections stained with F4/80. Scale bar: 50 μm. B: Semi-quantitation of F4/80 immuno-positive Kupffer cells in high-power field by NIH imageJ software. Histochemical quantitative analyses included five fields per section; C-G: Relative mRNA expression level of *Cd68* (C), M1-polarized macrophage-related genes (*Il1b*, *Il6*, *Tnfa* and *Nos2*) (D), M2-polarized macrophage-related genes (*Il10*, *Arg1* and *Cd163*) (E), *Lbp* (F), *Tlr4* and *Cd14* (G) in the liver of experimental mice. The mRNA expression levels were measured by RT-qPCR, and *Gapdh* was used as internal control. Quantitative values are indicated as fold changes to the values of C/V group; H: Western blots for p-IKKα/β, IKKβ, IkBα and NF-kB p65 in the liver of experimental mice. Actin was used as internal control. Data are mean ± SD (B-G; $n = 10$), $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* C/V group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* E/V group; $^{e}P < 0.05$ and $^{f}P < 0.01$ *vs* E/Zn group; $^{g}P < 0.05$ and $^{h}P < 0.01$ *vs* E/RFX group. pv: Portal vein.

inhibited these LPS-triggered accumulation of NF-kB in the ethanol plus CCl_4-treaed mice (Figure 3H).

Zinc acetate and rifaximin inhibited liver fibrosis development in ethanol plus CCl_4-treated mice

Given the antiinflammatory properties of rifaximin and zinc acetate, we evaluated their effects on the development of liver fibrosis. The ethanol plus CCl_4-treated mice showed extensive development of fibrous septa on Sirius Red staining (Figure 4A). Treatment with either zinc acetate or rifaximin alone significantly attenuated the ethanol plus CCl_4-induced fibrosis, and the antifibrotic effect was augmented by parallel use of both agents combined (Figure 4A). Correspondingly, there was a remarkable reduction in the α-SMA-immunopositive areas, which represented activation of HSCs, after treatment with zinc acetate and rifaximin (Figure 4A). Semiquantitative analysis demonstrated that the combination treatment caused more than 50% reduction in the areas of fibrotic septa and α-SMA-positive activated HSCs in the ethanol plus CCl_4-treated mice (Figure 4B and 4C). We also found that COL-1-immunopositive ECM deposition was decreased in parallel with the attenuation of liver fibrosis after treatment with both agents in the ethanol plus CCl_4-treated mice (Figure 4A and D). The western blot results substantiated that the hepatic expression of COL-1 protein was reduced *via* treatment with both agents (Figure 4E). Consistently, the hepatic gene expressions of profibrotic markers (*i.e.*, *Acta2*, *Col1a1*, and *Tgfb1*) were decreased after treatment with zinc acetate and rifaximin (Figure 4F). We further assessed the hepatic expressions of MMPs and TIMPs in the experimental groups. The ethanol plus CCl_4-treated mice showed increase in the hepatic mRNA levels of *Mmp2*, *Mmp9*, and *Mmp13* as liver fibrosis developed (Figure 4G). In line with the improvement of liver fibrosis, these MMP expressions were reduced after treatment with zinc acetate and rifaximin (Figure 4G). In response to this, the hepatic mRNA level of *Timp1* also varied according to liver fibrosis development (Figure 4H).

Based on the fact that zinc is essential as a component of the catalytic domain in MMPs[38], we investigated the effect of zinc supplementation on MMP activity. Interestingly, MMP-9 activity, which was indicated by active/pro MMP-9, was increased in the liver of the zinc acetate-treated groups, compared with that in the liver of the vehicle-treated group (Figure 4I).

Zinc acetate and rifaximin recovered the intestinal barrier function in ethanol plus CCl_4-treated mice

Both zinc acetate and rifaximin efficiently prevented the accumulation of LPS in the liver, as indicated by the reduced hepatic mRNA level of *Lbp* (Figure 3D). To uncover the mechanism of these effects, we next evaluated intestinal barrier integrity in the experimental groups. In the ethanol plus CCl_4-treated mice, the intestinal mucosal architecture was not significantly different from that of the controls, and epithelial shedding was absent. However, there was a decrease in the villus height of the ileum mucosa in the ethanol plus CCl_4-treated mice (Figure 5A and B). Conversely, we found an increase in the crypt depth of the ileum in the ethanol plus CCl_4-treated mice (Figure 5A and C). Notably, these atrophic changes were suppressed by treatment with zinc acetate and rifaximin (Figure 5A and B). Immunofluorescent analysis showed that in the ethanol plus CCl_4-treated mice, the intestinal expressions of ZO-1 and Occludin, which are the markers of TJP, were markedly decreased but were effectively restored by treatment with zinc acetate and rifaximin (Figure 5A and C). The western blot results confirmed the restoration of intestinal ZO-1 and Occludin protein expressions through treatment with both agents (Figure 5D). Along with these findings, RT-qPCR analysis revealed that combination treatment with both agents increased the intestinal mRNA expressions of the other TJP markers *Cldn1*, and *Cldn4*, which encode for Claudin1, and Claudin4, respectively, as well as *Zo1* and *Ocln* (Figure 5E). To examine the functional consequence of altered cellular junctions, we determined the flux through the leak pathway, which is responsible for the paracellular movement of larger molecules, including LPS. Inversely proportional to the loss of TJPs, leakage of plasma FITC-dextran (4 kDa) increased by more than two-fold in the ethanol plus CCl_4-treated mice, compared with that in the control mice (Figure 5F). In correspondence with the improvement of TJP expression, leakage of FITC-dextran was significantly alleviated by treatment with both agents (Figure 5F). Moreover, we measured the intestinal mRNA levels of *Tnfα* as a downstream cytokine of TLR4, which plays a key role in ethanol-mediated disruption of the intestinal barrier function in ALD[39]. As shown in Figure 5G, intestinal *Tnfα* mRNA levels increased by three-fold in the ethanol plus CCl_4-treated mice, compared with those in the control

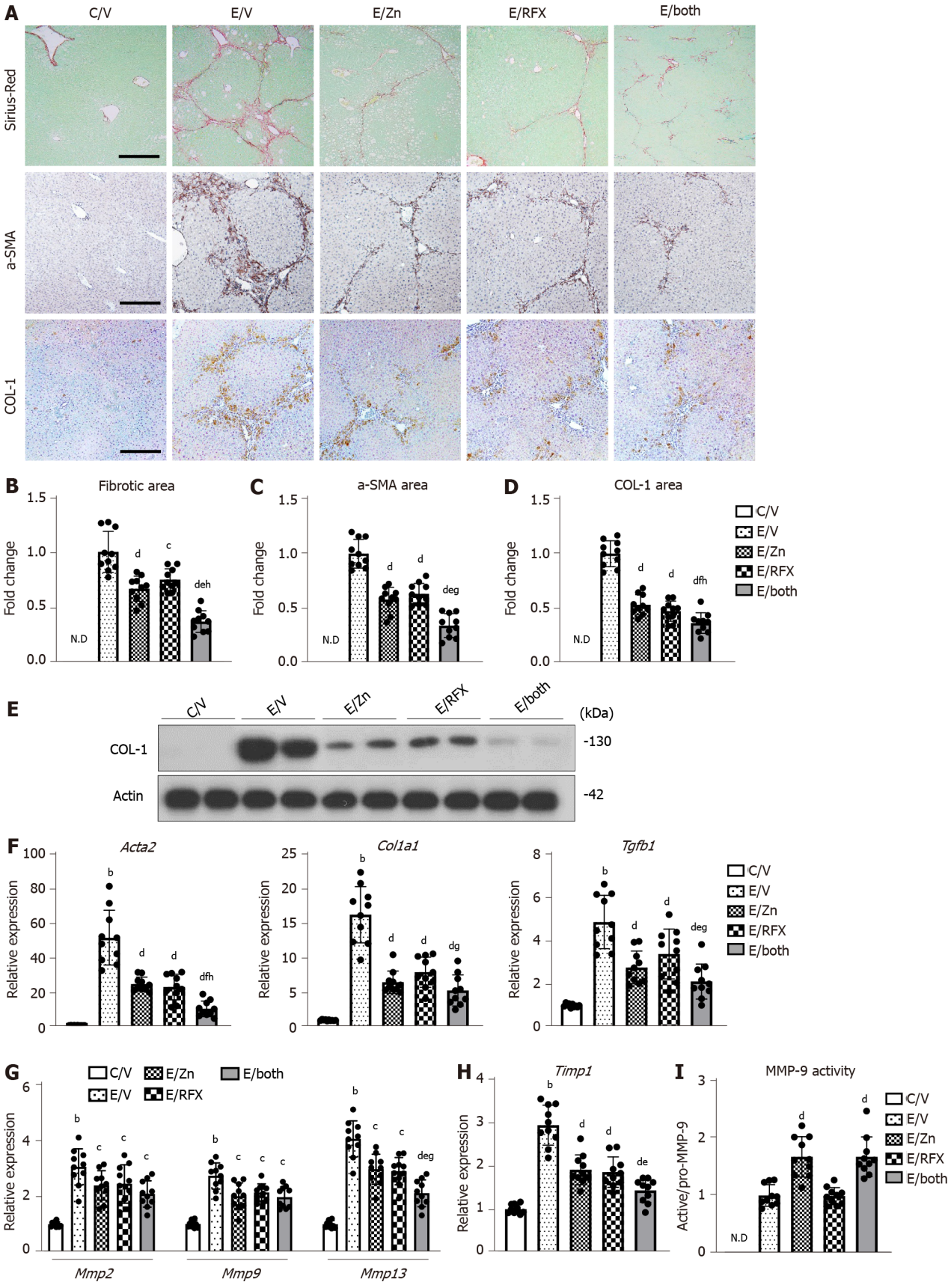

Figure 4 Zinc acetate and rifaximin against liver fibrosis development in alcoholic liver disease mice. A: Representative microphotographs of liver sections stained with Sirius-Red, α-smooth muscle actin (αSMA) and COL-1. Scale bar: 50 μm; B-D: Semi-quantitation of Sirius-Red-stained fibrotic area (B), α-SMA (C) and COL-1 (D) immuno-positive areas in high-power field (HPF) by NIH imageJ software. Histochemical quantitative analyses included five fields per section; E: Western blots for COL-1 in the liver of experimental mice. Actin was used as internal control; F-H: Relative mRNA expression levels of *Acta2*, *Col1a1* and *Tgfb1* (F), *Mmp-2*, *-9* and *-13* (G), and *Timp1* (H) in the liver of experimental mice. The mRNA expression levels were measured by RT-qPCR, and *Gapdh* was used

as internal control; I: Intrahepatic MMP-9 activity determined by ELISA. Quantitative values are indicated as fold changes to the values of E/V (B-D and I) or C/V group (F-H). Data are mean ± SD (n = 10), [a]P < 0.05 and [b]P < 0.01 *vs* C/V group; [c]P < 0.05 and [d]P < 0.01 *vs* E/V group; [e]P < 0.05 and [f]P < 0.01 *vs* E/Zn group; [g]P < 0.05 and [h]P < 0.01 *vs* E/RFX group. ND: Not detected; αSMA: α-smooth muscle actin.

mice; moreover, combination of zinc acetate and rifaximin reduced these mRNA levels by approximately 50% of the levels after vehicle treatment (Figure 5G).

Myosin light chain kinase (MLCK) is known to play a key role in intestinal barrier disruption as a downstream target of TNFα following alcohol stimulation[40]. Therefore, we further investigated the intestinal *Mylk* mRNA levels in the experimental groups. The ethanol plus CCl_4-treated mice showed marked increase in the intestinal *Mylk* mRNA levels; interestingly, both zinc acetate and rifaximin reduced these mRNA levels in parallel with downregulation of TNFα (Figure 5H).

Direct effects of zinc acetate and rifaximin on ethanol-induced barrier dysfunction in human enterocytes

Next, we assessed the effects of zinc acetate and rifaximin on enterocytes by *in vitro* assays using Caco-2 cells. The stimulation of 5% ethanol reduced the TEER values in the Caco-2 cells, but it did not affect cell viability (Figure 6A, B and Supplementary Figure 2A); this result indicated that this 5% ethanol-induced barrier dysfunction without cell death. The ethanol-induced reduction of TEER values was efficiently attenuated by treatment with zinc acetate, and the PI3K inhibitor LY294002 was shown to negate the zinc-mediated recovery of electrical resistance in the ethanol-stimulated Caco-2 cells (Figure 6A). It was noteworthy that rifaximin likewise dose-dependently improved the ethanol-stimulated decrease in the TEER values of the Caco-2 cells, which was sufficiently offset by treatment with a known as a PXR inhibitor SPA70 (Figure 6B). Moreover, zinc acetate or rifaximin also attenuated the LPS-stimulated decrease in the TEER values, and these attenuations were negated by treatments with LY294002 or SPA70, respectively (Figure 6C and D). At the concentrations used in the present assays, both zinc acetate and rifaximin did not affect Caco-2 cell viability (Supplementary Figure 2B). In parallel with the increase in TEER values, both zinc acetate and rifaximin restored the intestinal protein expressions of TJPs, including ZO-1 and Occludin in either ethanol- or LPS-stimulated Caco-2 cells (Figure 6E and F). Interestingly, the abovementioned methods of restoring TJPs *via* zinc acetate administration was accompanied by the augmentation of AKT phosphorylation and negated by treatments with LY294002 in either ethanol- or LPS-stimulated Caco-2 cells (Figure 6E). Notably, we found that rifaximin-mediated TJPs restoration involved the amelioration of p65 phosphorylation and negated by treatments with SPA70 in either ethanol- or LPS-stimulated Caco-2 cells (Figure 6F). These findings suggest that zinc acetate and rifaximin reintegrate the gut barrier function *via* the activation of PI3K/AKT signaling and the PXR-mediated inhibition of TLR4/NF-kB, respectively.

Additionally, the TNFα-stimulated *MYLK* expressions were not altered by treatment with zinc acetate but reduced by that with rifaximin (Figure 6G and H). Since this effect of rifaximin was also canceled by SPA-mediated PXR inhibition, rifaximin could be suggested to protect the intestinal barrier function against ethanol and LPS through PXR activation (Figure 6H).

DISCUSSION

The gut–liver axis is an operative unit that works to protect the human body against potentially harmful substances and microorganisms, thereby, maintaining the homeostasis of the immune system[41,42]. In patients with cirrhosis, the intestine often becomes a leaky gut, which is characterized by increased permeability with defects in the intestinal TJPs[43]. Leaky gut allows the translocation of bacteria, bacterial products, and fragments, including LPS, into the portal circulation and can trigger hepatic inflammation and fibrosis[6,7,41,42]. In the present study, we elucidated that combination of zinc acetate with rifaximin additively attenuated steatosis, inflammation, and fibrosis and reduced oxidative stress in the liver of ethanol plus CCl_4-treated mice. As an underlying mechanism of these hepatoprotective effects mediated by both agents, we focused on the maintenance of intestinal barrier integrity, which resulted in reduced hepatic exposure of LPS.

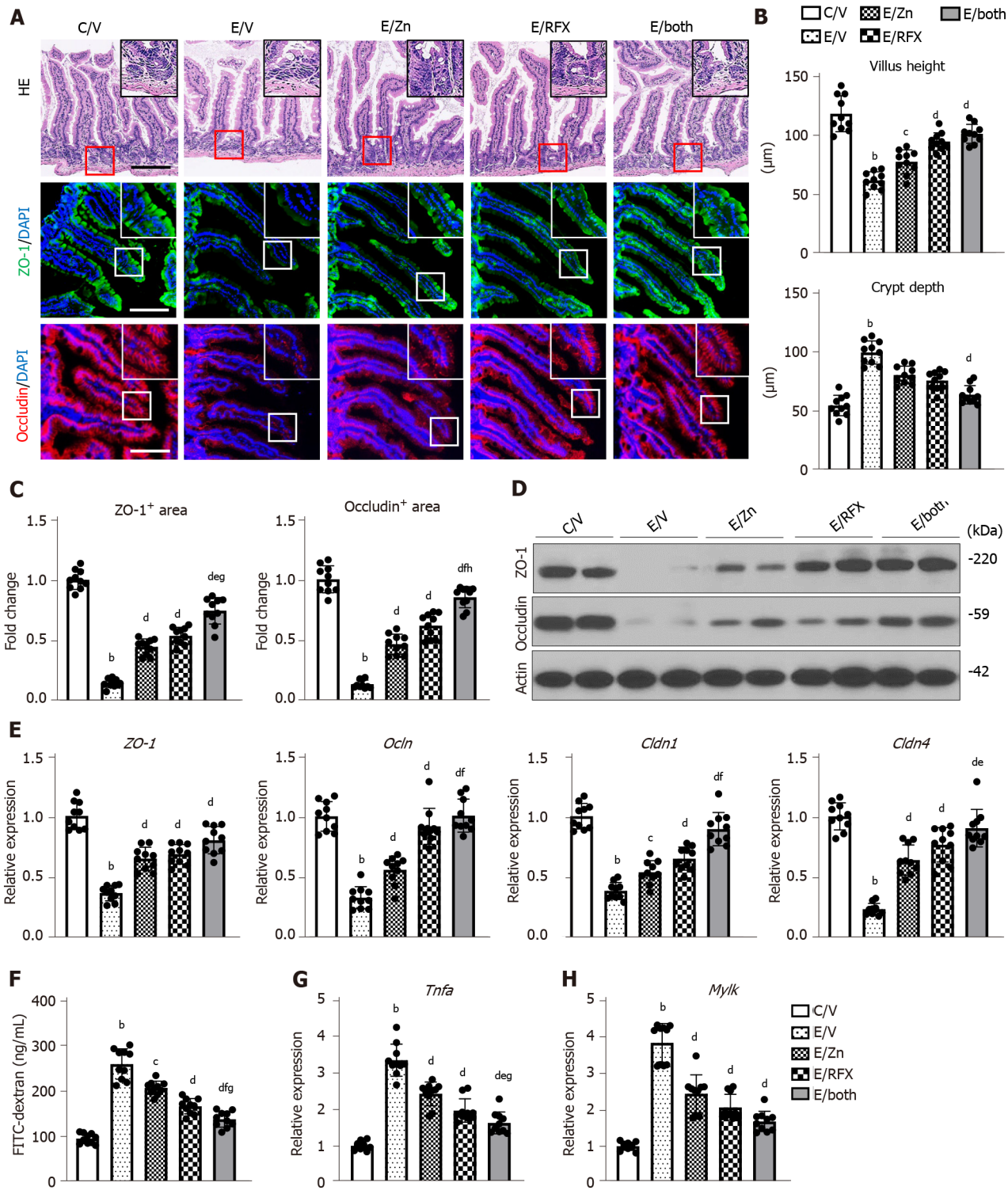

Figure 5 Zinc acetate and rifaximin on intestinal barrier function in alcoholic liver disease mice. A: Representative microphotographs of ileum sections stained with hematoxylin-eosin (upper), zonula occludens-1 (ZO-1) (middle) and Occludin (lower) in the experimental groups. Boxes are selected regions for magnified. Nuclei counterstained with 4',6-diamidino-2-phenylindole. Scale Bar: 50 μm; B: Villus height (upper) and crypt depth (lower) of the ileum in the experimental mice; C: Semi-quantitation of ZO-1 and Occludin immuno-positive areas in high-power field by NIH imageJ software; D: Western blots for ZO-1 and Occludin in the liver of experimental mice. Actin was used as internal control; E: Relative mRNA expression levels of *Zo1*, *Ocln*, *Cldn1* and *Cldn4* in the ileum of experimental mice; F: Blood levels of fluorescein isothiocyanate (FITC)-dextran (4kDa) 4 h after oral administration; G and H): Relative mRNA expression levels of *Tnfa* (G) and *Mylk* (H) in the ileum of experimental mice. Histochemical quantitative analyses included five fields per section (B and C). The mRNA expression levels were measured by RT-qPCR, and *Gapdh* was used as internal control (E, G and H). Quantitative values are indicated as fold changes to the values of C/V group (C, E, G and H). Data are mean ± SD (B, C, E, G and H; $n = 10$, F; $n = 5$), $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* C/V group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* E/V group; $^{e}P < 0.05$ and $^{f}P < 0.01$ *vs* E/Zn group; $^{g}P < 0.05$ and $^{h}P < 0.01$ *vs* E/RFX group. DAPI: 4',6-diamidino-2-phenylindole; ZO-1: Zonula occludens; HE: Hematoxylin and eosin.

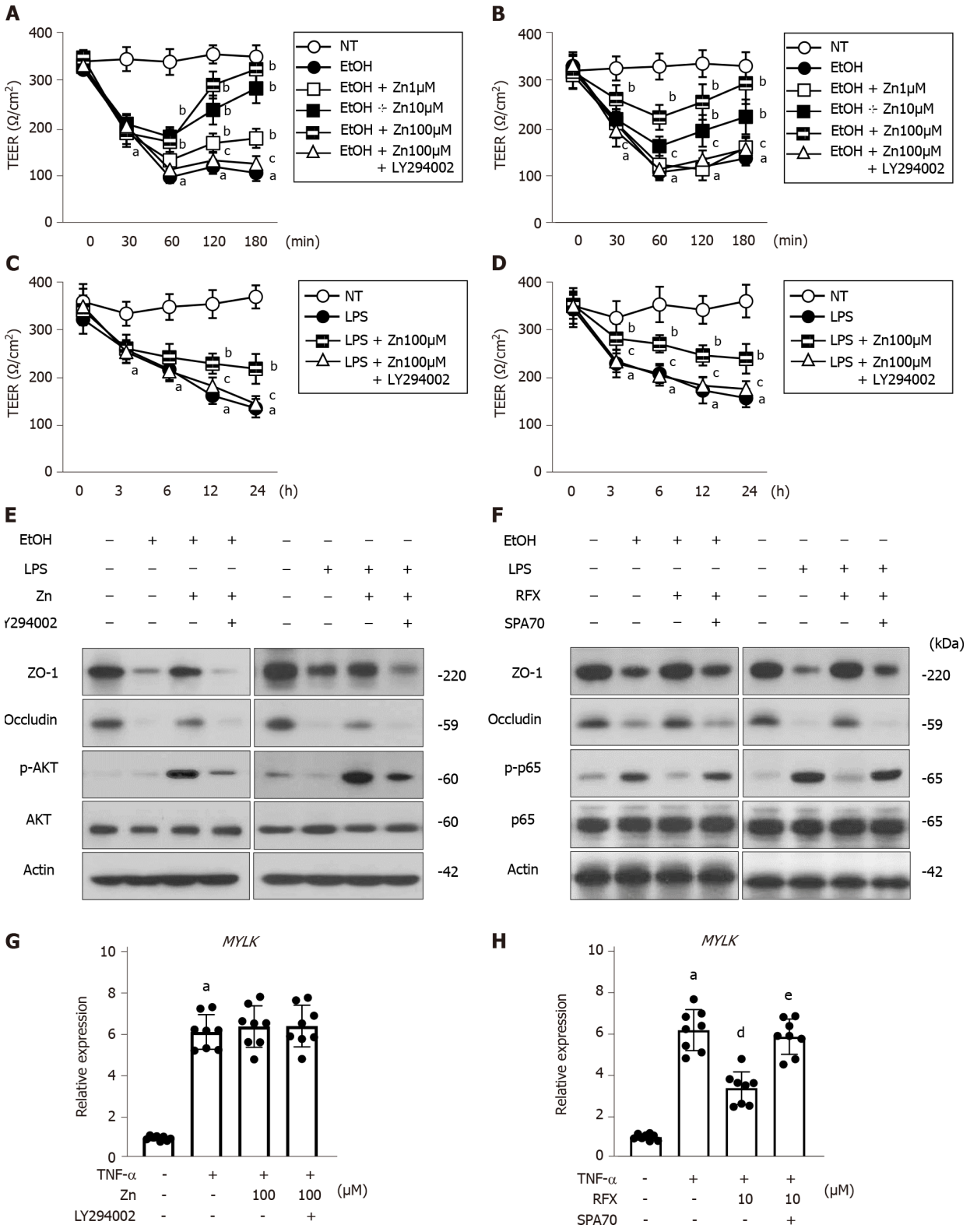

Figure 6 Effects of zinc acetate and rifaximin on *in vitro* EtOH/LPS/TNF-α-stimulated Caco-2 cells. A-D: *In vitro* paracellular permeability in ethanol (EtOH) (A and B)- or lipopolysaccharide (LPS) (C and D)-stimulated Caco-2 cells determined as transepithelial electrical resistance; E and F: Western blots for the effects of zinc acetate (100 μM) on ZO-1, Occludin, p-AKT and AKT expressions (E) and rifaximin (10 μM) on ZO-1, Occludin, p-p65 and p65 expressions (F) in the whole cell lysate of Caco-2 cells. Actin was used as internal control; G and H: Relative mRNA expression levels of *MYLK* in TNF-α-stimulated Caco-2 cells. The mRNA expression levels were measured by RT-qPCR, and *GAPDH* was used as internal control. Quantitative values are indicated as fold changes to the values of non-treatment group. Caco-2 were treated with each agent as following; (A, C, E and G) zinc acetate (Zn) and/or PI3K inhibitor, LY294002, (B, D, F and H) rifaximin (RFX) and/or human PXR inhibitor, SPA70. Data are mean ± SD (A-D; $n = 6$, G and H; $n = 8$), $^{a}P < 0.01$ *vs* non-treated groups (A-D, G and H), $^{b}P < 0.01$ *vs* EtOH (A and B) or LPS-treated groups (C and D), $^{c}P < 0.01$ *vs* EtOH with Zn (100 μM) (A), EtOH with RFX (10 μM) (B), LPS with Zn (100 μM) (C) or LPS with RFX (10 μM) (D)-treated groups, $^{d}P < 0.01$ *vs* TNF-α-treated group (H), $^{e}P < 0.01$ *vs* TNF-α with RFX (10 μM)-treated group (H). LPS: Lipopolysaccharide; TEER: Transepithelial electrical resistance; EtOH: Ethanol; ZO-1: Zonula occludens.

The presence of alcohol and its metabolites, such as acetaldehyde, in the bloodstream is known to injure intestinal epithelial cells directly and indirectly[44]. Alcohol binge at high concentrations causes intestinal cellular damage, and chronic exposure to ethanol decreases the expressions of TJPs in between colon epithelial cells [44]. In this context, recent clinical evidences have shown that acute alcohol binge drinking significantly increased serum endotoxin levels in healthy human volunteers and that serum endotoxin was elevated in patients with chronic alcohol consumption and ALD[45,46]. A previous study on rodents showed that exposure of ethanol and CCl_4 reduced the diversity of gut microbiota which resulted in bacterial translocation [47]. Similarly, our current model was observed to have remarkable increase in the hepatic *Lbp* expression, in accordance with decreased intestinal TJP expression and increased leakage of plasma FITC-dextran, which indicated augmentation of LPS exposure to the liver along with intestinal hyperpermeability. These features were supported by our results on the *in vitro* assay, which showed that the ethanol-stimulus profoundly weakened epithelial resistance and reduced TJP expressions in Caco-2 cells, in agreement with previous reports.

Our therapeutic models showed that both zinc acetate and rifaximin reinforced the tight junctions in the intestine of ethanol plus CCl_4-treated mice. We assumed the involvement of multifunctional pathways in these effects of both agents (Figure 7). First, both drugs suppressed the intestinal TNFα and MLCK expressions in mice. Chen *et al*[40] demonstrated that dysbiosis triggered by chronic alcohol administration induced TNFα production in the inflammatory cells of the intestinal lamina propria and that the TNFα/TNF receptor I axis potentially regulated tight junction disruption through activation of MLCK. Thus, the decrease of intestinal TNFα mediated by both agents participates in the improved intestinal barrier function. Moreover, Garg *et al*[48] documented that rifaximin attenuated TNFα-induced MLCK expression through PXR activation in human enterocytes. Accordingly, our *in vitro* assay in Caco-2 cells validated the inhibitory effect of rifaximin on TNFα-stimulated upregulation of MLCK through PXR activation. These results indicated that suppression of TNFα/MLCK pathway was partially associated with the reinforced tight junctions in the ethanol plus CCl_4-treated mice. Second, both zinc acetate and rifaximin also improved the LPS-stimulated intestinal barrier dysfunction. Zinc has been reported to enhance intestinal epithelial barrier function by directly affecting enterocytes through activation of PI3K/AKT/mTOR signaling[34]. He *et al*[49] demonstrated that the pharmacological activation of PI3K/AKT could inhibit the LPS-induced downregulation of TJP expressions in Caco-2 cells. Meanwhile, rifaximin-mediated PXR activation has been suggested to attenuate the LPS-stimulated barrier dysfunction in intestinal epithelial cells through the inhibition of TLR4/NF-kB p65 pathway as well as the abovementioned TNFα/MLCK pathway[50]. Consistently, our *in vitro* study found that zinc acetate or rifaximin suppressed the LPS-stimulated disruption of intestinal barrier function, which was mitigated by inhibition of PI3K or PXR, respectively in the Caco-2 cells. These findings support that both agents protect the intestinal barrier breakdown triggered by LPS. Other than the above, a variety of molecular mechanisms have been supposed to be relevant to the zinc-mediated alteration of intestinal barrier permeability and TJP expression. Zinc-induced activation of different signaling pathways such as PKCd or MAPK/ERK has been reported to improve epithelial integrity[51,52]. Moreover, dietary zinc supplementation could promote the metabolism of acetaldehyde in the gut by enhancing ALDH1B1 activity[53]. To explore the possible involvement of these molecular mechanisms in the present model, further investigations are required.

In addition to intestinal barrier maintenance, several pharmacologic actions have been suggested to be associated with the antifibrotic properties of zinc. Szuster-Ciesielska *et al*[54] demonstrated that zinc supplementation could silence ethanol- or acetaldehyde-mediated HSC activation by acting as an antioxidant and inhibitor of MAPK, TGFβ, and NF-kB transduction signaling. In our models, the increased hepatic zinc levels and hepatic MMP-9 activity after zinc acetate treatment implied that the antifibrotic effect was at least partially associated with a direct effect on the profibrogenic activity of HSCs. However, detailed consideration by analyzing the molecular mechanisms in HSCs isolated from the liver of the experimental groups would be needed.

When considering the results of this study, several important limitations should be acknowledged. First, although our study addressed the effects of zinc acetate and rifaximin on intestinal barrier integrity in the ethanol plus CCl_4-treated mice, their effects on microbial profiles were not clarified. Several studies have indicated the impacts of both agents on the gut microbiota. Zhang *et al*[55] showed that zinc modified the cecal microbial community in broilers by making abundant in the

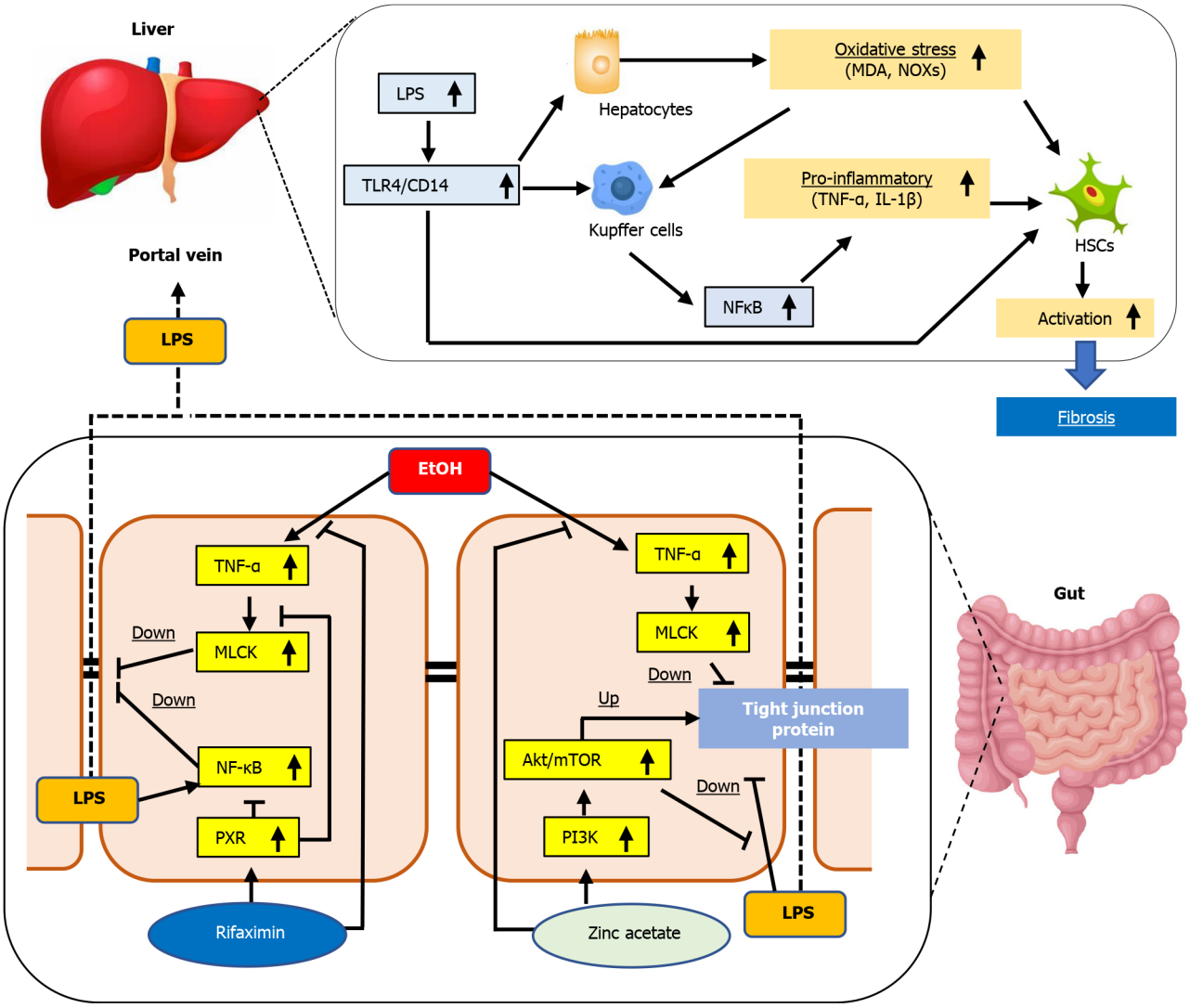

Figure 7 Graphic summary of the effect of zinc acetate and rifaximin on the alcoholic liver disease-related liver fibrosis. ALD: Alcoholic liver disease; TLR4: Toll-like receptor 4; MDA: Malondialdehyde; LPS: Lipopolysaccharide; HSC: Hepatic stellate cell; TNFα: Tumor necrosis factor α; MLCK: Myosin light chain kinase; NF-kB: Nuclear factor kB; PXR: Pregnane X receptor.

populations of total bacteria, including Lactobacillus, and reducing the populations of Salmonella. Foligné *et al*[56] suggested that zinc supplementation provided a significant increase in endogenous Clostridiaceae in mice. Meanwhile, in a mouse steatohepatitis model, Kitagawa *et al*[27] have recently demonstrated that rifaximin improved ethanol-induced liver injury with drastic modification of the small intestine microbiota; they elucidated that rifaximin decreased the relative abundance of Erysipelotrichales and increased Bacteroidales. Given these evidences, additional analyses are necessary to determine the interaction between microbial alterations by both agents and the therapeutic effects in our model. Second, this study elucidated the preventive effects of zinc acetate and rifaximin on the progression of ethanol plus CCl_4 -induced liver fibrosis; however, the pharmacologic properties of fibrinolysis and liver regeneration in an established model of liver fibrosis remain obscure. Future studies should address whether both drugs could induce fibrinolysis and efficient liver regeneration in other models of cirrhosis.

CONCLUSION

Taken together, our results indicated that combination of zinc acetate and rifaximin exerted a preventive effect on the ALD-related liver fibrosis in a mouse model treated with ethanol plus CCl_4. We believed that this antifibrotic effect is involved in the multifaceted regulatory functions that maintain intestinal barrier integrity and reduce

hepatic LPS exposure, thereby, leading to Kupffer cell expansion and HSC activation by inhibition of the TLR4 signaling pathway. We emphasize that both drugs are clinically available for patients with chronic liver diseases and that the abovementioned effects on alcohol-related liver fibrosis were achieved using the pharmacologic doses, without adverse effects, such as hypocupremia or renal dysfunction. Therefore, the results of this study demonstrated that this combination regimen could be beneficial as a form of chemoprevention against alcohol-related liver fibrosis.

ARTICLE HIGHLIGHTS

Research background

Liver fibrosis related to alcoholic liver disease (ALD) is one of the most critical health issues. Alcohol cessation is the therapeutic mainstay for patients with all stages of ALD, whereas pharmacological strategies for liver fibrosis have not been established. It has been recognized that the gut-derived endotoxin lipopolysaccharide (LPS), which is a key player of gut-liver axis, particularly exacerbates the inflammation and fibrosis *via* activation of toll-like receptor 4 (TLR4)/nuclear factor kB (NF-kB) signaling pathway in ALD. Thus, blockage of the transfer of LPS to the liver by maintaining gut barrier has gained attention for a therapeutic strategy to prevent ALD-related liver fibrosis.

Research motivation

Currently, zinc acetate and rifaximin are often used for the cirrhotic patients in the clinical practice. Several clinical and basic studies have demonstrated that both agents also could suppress the intestinal hyperpermeability. Although these evidences suggest that combination of zinc acetate and rifaximin should exert beneficial effects on the ALD-related liver fibrosis through inhibition of LPS/TLR4/NF-kB signaling, its effects on ALD-related liver fibrosis remain to be fully elucidated.

Research objectives

To determine the efficacy of dual therapy with zinc acetate and rifaximin for liver fibrosis and explore its underlying mechanisms with the linkage of gut barrier function in a mouse ALD model.

Research methods

Female C57BL/6J mice were fed a 2.5% ethanol-containing liquid diet and administered carbon tetrachloride (CCl4) twice weekly (1 mL/kg; ip) for 8 wk to induce ALD-related liver fibrosis, and zinc acetate (100 mg/L) and/or rifaximin (100 mg/L) were orally administered during experimental period. Histological changes in hepatic steatosis, inflammation and fibrosis, oxidative markers, and LPS/TLR4/NF-kB signaling as well as intestinal permeability and tight junction proteins (TJPs) were evaluated. Additionally, *in vitro* assays were performed to investigate the direct effects of both agents on Caco-2 barrier function.

Research results

The ethanol plus CCl_4-treated mice showed significantly increased transaminases, hepatic fat accumulation, lipid peroxidation (malondialdehyde), F4/80-positive Kupffer cell expansion and increased proinflammatory response, liver fibrosis development and HSC activation. The combination with zinc acetate and rifaximin attenuated these phenotypic changes with with blunted hepatic exposure of LPS and the TLR4/NF-kB signaling pathway. This combination therapy improved the atrophic changes and permeability in the ileum and restored the TJPs (ZO-1, Occludin, Claudin1 and Claudin4) with decreased levels of tumor necrosis factor α and myosin light chain kinase. Moreover, *in vitro* assay revealed that zinc acetate and rifaximin directly reinforced ethanol or LPS-stimulated paracellular permeability and upregulated TJPs in Caco-2 cells by modulating different pathways, *i.e.*, induction of AKT phosphorylation by zinc acetate and pregnane X receptor activation by rifaximin.

Research conclusions

The combination of zinc acetate and rifaximin exerted a preventive effect on the ALD-related liver fibrosis in a mouse ALD model by maintaining intestinal barrier integrity and reduce hepatic LPS exposure, thereby, leading to Kupffer cell expansion and HSC activation by inhibition of the TLR4 signaling pathway.

Research perspectives

By indicating that zinc acetate and rifaximin inhibits ALD-related liver fibrosis development through the gut-liver axis, the results of this study demonstrated that this combination regimen could be beneficial as a form of chemoprevention against ALD-related liver fibrosis.

REFERENCES

1 **Younossi Z**, Henry L. Contribution of Alcoholic and Nonalcoholic Fatty Liver Disease to the Burden of Liver-Related Morbidity and Mortality. *Gastroenterology* 2016; **150**: 1778-1785 [PMID: 26980624 DOI: 10.1053/j.gastro.2016.03.005]

2 **Williams R**, Alexander G, Armstrong I, Baker A, Bhala N, Camps-Walsh G, Cramp ME, de Lusignan S, Day N, Dhawan A, Dillon J, Drummond C, Dyson J, Foster G, Gilmore I, Hudson M, Kelly D, Langford A, McDougall N, Meier P, Moriarty K, Newsome P, O'Grady J, Pryke R, Rolfe L, Rice P, Rutter H, Sheron N, Taylor A, Thompson J, Thorburn D, Verne J, Wass J, Yeoman A. Disease burden and costs from excess alcohol consumption, obesity, and viral hepatitis: fourth report of the Lancet Standing Commission on Liver Disease in the UK. *Lancet* 2018; **391**: 1097-1107 [PMID: 29198562 DOI: 10.1016/S0140-6736(17)32866-0]

3 **Liangpunsakul S**, Haber P, McCaughan GW. Alcoholic Liver Disease in Asia, Europe, and North America. *Gastroenterology* 2016; **150**: 1786-1797 [PMID: 26924091 DOI: 10.1053/j.gastro.2016.02.043]

4 **Seitz HK**, Bataller R, Cortez-Pinto H, Gao B, Gual A, Lackner C, Mathurin P, Mueller S, Szabo G, Tsukamoto H. Alcoholic liver disease. *Nat Rev Dis Primers* 2018; **4**: 16 [PMID: 30115921 DOI: 10.1038/s41572-018-0014-7]

5 **Louvet A**, Mathurin P. Alcoholic liver disease: mechanisms of injury and targeted treatment. *Nat Rev Gastroenterol Hepatol* 2015; **12**: 231-242 [PMID: 25782093 DOI: 10.1038/nrgastro.2015.35]

6 **Bajaj JS**. Alcohol, liver disease and the gut microbiota. *Nat Rev Gastroenterol Hepatol* 2019; **16**: 235-246 [PMID: 30643227 DOI: 10.1038/s41575-018-0099-1]

7 **Szabo G**. Gut-liver axis in alcoholic liver disease. *Gastroenterology* 2015; **148**: 30-36 [PMID: 25447847 DOI: 10.1053/j.gastro.2014.10.042]

8 **Ciesielska A**, Matyjek M, Kwiatkowska K. TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. *Cell Mol Life Sci* 2021; **78**: 1233-1261 [PMID: 33057840 DOI: 10.1007/s00018-020-03656-y]

9 **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]

10 **Maret W**. Zinc biochemistry: from a single zinc enzyme to a key element of life. *Adv Nutr* 2013; **4**: 82-91 [PMID: 23319127 DOI: 10.3945/an.112.003038]

11 **Zhou Z**. Zinc and alcoholic liver disease. *Dig Dis* 2010; **28**: 745-750 [PMID: 21525759 DOI: 10.1159/000324282]

12 **Wu J**, Meng QH. Current understanding of the metabolism of micronutrients in chronic alcoholic liver disease. *World J Gastroenterol* 2020; **26**: 4567-4578 [PMID: 32884217 DOI: 10.3748/wjg.v26.i31.4567]

13 **Shen YC**, Chang YH, Fang CJ, Lin YS. Zinc supplementation in patients with cirrhosis and hepatic encephalopathy: a systematic review and meta-analysis. *Nutr J* 2019; **18**: 34 [PMID: 31279342 DOI: 10.1186/s12937-019-0461-3]

14 **Himoto T**, Masaki T. Associations between Zinc Deficiency and Metabolic Abnormalities in Patients with Chronic Liver Disease. *Nutrients* 2018; **10** [PMID: 29342898 DOI: 10.3390/nu10010088]

15 **Moriya K**, Nishimura N, Namisaki T, Takaya H, Sawada Y, Kawaratani H, Kaji K, Shimozato N, Sato S, Furukawa M, Douhara A, Akahane T, Mitoro A, Yamao J, Yoshiji H. Zinc Administration and Improved Serum Markers of Hepatic Fibrosis in Patients with Autoimmune Hepatitis. *J Clin Med* 2021; **10** [PMID: 34199421 DOI: 10.3390/jcm10112465]

16 **Zhong W**, Wei X, Hao L, Lin TD, Yue R, Sun X, Guo W, Dong H, Li T, Ahmadi AR, Sun Z, Zhang Q, Zhao J, Zhou Z. Paneth Cell Dysfunction Mediates Alcohol-related Steatohepatitis Through Promoting Bacterial Translocation in Mice: Role of Zinc Deficiency. *Hepatology* 2020; **71**: 1575-1591 [PMID: 31520476 DOI: 10.1002/hep.30945]

17 **Sun J**, Zhang C, Zhang B. Research Note: Effects of organic zinc on broiler intestinal permeability and integrity in Clostridium perfringens-challenged condition. *Poult Sci* 2020; **99**: 6653-6656 [PMID: 33248581 DOI: 10.1016/j.psj.2020.09.032]

18 **Lambert JC**, Zhou Z, Wang L, Song Z, McClain CJ, Kang YJ. Prevention of alterations in intestinal permeability is involved in zinc inhibition of acute ethanol-induced liver damage in mice. *J Pharmacol Exp Ther* 2003; **305**: 880-886 [PMID: 12626662 DOI: 10.1124/jpet.102.047852]

19 **Tan HK**, Streeter A, Cramp ME, Dhanda AD. Effect of zinc treatment on clinical outcomes in patients with liver cirrhosis: A systematic review and meta-analysis. *World J Hepatol* 2020; **12**: 389-398 [PMID: 32821337 DOI: 10.4254/wjh.v12.i7.389]

20 **DuPont HL**. Travelers' diarrhea: antimicrobial therapy and chemoprevention. *Nat Clin Pract Gastroenterol Hepatol* 2005; **2**: 191-8; quiz 1 p following 198 [PMID: 16265184 DOI:

10.1038/ncpgasthep0142]
21 **Moran S**, López-Sánchez M, Milke-García MDP, Rodríguez-Leal G. Current approach to treatment of minimal hepatic encephalopathy in patients with liver cirrhosis. *World J Gastroenterol* 2021; **27**: 3050-3063 [PMID: 34168407 DOI: 10.3748/wjg.v27.i22.3050]
22 **Kaji K**, Takaya H, Saikawa S, Furukawa M, Sato S, Kawaratani H, Kitade M, Moriya K, Namisaki T, Akahane T, Mitoro A, Yoshiji H. Rifaximin ameliorates hepatic encephalopathy and endotoxemia without affecting the gut microbiome diversity. *World J Gastroenterol* 2017; **23**: 8355-8366 [PMID: 29307995 DOI: 10.3748/wjg.v23.i47.8355]
23 **Esposito G**, Nobile N, Gigli S, Seguella L, Pesce M, d'Alessandro A, Bruzzese E, Capoccia E, Steardo L, Cuomo R, Sarnelli G. Rifaximin Improves Clostridium difficile Toxin A-Induced Toxicity in Caco-2 Cells by the PXR-Dependent TLR4/MyD88/NF-kB Pathway. *Front Pharmacol* 2016; **7**: 120 [PMID: 27242527 DOI: 10.3389/fphar.2016.00120]
24 **Ishida K**, Kaji K, Sato S, Ogawa H, Takagi H, Takaya H, Kawaratani H, Moriya K, Namisaki T, Akahane T, Yoshiji H. Sulforaphane ameliorates ethanol plus carbon tetrachloride-induced liver fibrosis in mice through the Nrf2-mediated antioxidant response and acetaldehyde metabolization with inhibition of the LPS/TLR4 signaling pathway. *J Nutr Biochem* 2021; **89**: 108573 [PMID: 33388347 DOI: 10.1016/j.jnutbio.2020.108573]
25 **Roychowdhury S**, Chiang DJ, McMullen MR, Nagy LE. Moderate, chronic ethanol feeding exacerbates carbon-tetrachloride-induced hepatic fibrosis *via* hepatocyte-specific hypoxia inducible factor 1α. *Pharmacol Res Perspect* 2014; **2**: e00061 [PMID: 25089199 DOI: 10.1002/prp2.61]
26 **Johnson JK**, Harris FL, Ping XD, Gauthier TW, Brown LAS. Role of zinc insufficiency in fetal alveolar macrophage dysfunction and RSV exacerbation associated with fetal ethanol exposure. *Alcohol* 2019; **80**: 5-16 [PMID: 30580016 DOI: 10.1016/j.alcohol.2018.11.007]
27 **Kitagawa R**, Kon K, Uchiyama A, Arai K, Yamashina S, Kuwahara-Arai K, Kirikae T, Ueno T, Ikejima K. Rifaximin prevents ethanol-induced liver injury in obese KK-A^{y} mice through modulation of small intestinal microbiota signature. *Am J Physiol Gastrointest Liver Physiol* 2019; **317**: G707-G715 [PMID: 31509430 DOI: 10.1152/ajpgi.00372.2018]
28 **Her Z**, Tan JHL, Lim YS, Tan SY, Chan XY, Tan WWS, Liu M, Yong KSM, Lai F, Ceccarello E, Zheng Z, Fan Y, Chang KTE, Sun L, Chang SC, Chin CL, Lee GH, Dan YY, Chan YS, Lim SG, Chan JKY, Chandy KG, Chen Q. CD4^{+} T Cells Mediate the Development of Liver Fibrosis in High Fat Diet-Induced NAFLD in Humanized Mice. *Front Immunol* 2020; **11**: 580968 [PMID: 33013934 DOI: 10.3389/fimmu.2020.580968]
29 **Luciano-Mateo F**, Cabré N, Fernández-Arroyo S, Baiges-Gaya G, Hernández-Aguilera A, Rodríguez-Tomàs E, Muñoz-Pinedo C, Menéndez JA, Camps J, Joven J. Chemokine C-C motif ligand 2 overexpression drives tissue-specific metabolic responses in the liver and muscle of mice. *Sci Rep* 2020; **10**: 11954 [PMID: 32686726 DOI: 10.1038/s41598-020-68769-7]
30 **Suzuki M**, Kon K, Ikejima K, Arai K, Uchiyama A, Aoyama T, Yamashina S, Ueno T, Watanabe S. The Chemical Chaperone 4-Phenylbutyric Acid Prevents Alcohol-Induced Liver Injury in Obese KK-A^{y} Mice. *Alcohol Clin Exp Res* 2019; **43**: 617-627 [PMID: 30748014 DOI: 10.1111/acer.13982]
31 **Matheus VA**, Monteiro L, Oliveira RB, Maschio DA, Collares-Buzato CB. Butyrate reduces high-fat diet-induced metabolic alterations, hepatic steatosis and pancreatic beta cell and intestinal barrier dysfunctions in prediabetic mice. *Exp Biol Med (Maywood)* 2017; **242**: 1214-1226 [PMID: 28504618 DOI: 10.1177/1535370217708188]
32 **Hossain KFB**, Akter M, Rahman MM, Sikder MT, Rahaman MS, Yamasaki S, Kimura G, Tomihara T, Kurasaki M, Saito T. Amelioration of Metal-Induced Cellular Stress by α-Lipoic Acid and Dihydrolipoic Acid through Antioxidative Effects in PC12 Cells and Caco-2 Cells. *Int J Environ Res Public Health* 2021; **18** [PMID: 33671655 DOI: 10.3390/ijerph18042126]
33 **Wang Y**, Tong J, Chang B, Wang B, Zhang D. Effects of alcohol on intestinal epithelial barrier permeability and expression of tight junction-associated proteins. *Mol Med Rep* 2014; **9**: 2352-2356 [PMID: 24718485 DOI: 10.3892/mmr.2014.2126]
34 **Shao Y**, Wolf PG, Guo S, Guo Y, Gaskins HR, Zhang B. Zinc enhances intestinal epithelial barrier function through the PI3K/AKT/mTOR signaling pathway in Caco-2 cells. *J Nutr Biochem* 2017; **43**: 18-26 [PMID: 28193579 DOI: 10.1016/j.jnutbio.2017.01.013]
35 **Lin W**, Wang YM, Chai SC, Lv L, Zheng J, Wu J, Zhang Q, Wang YD, Griffin PR, Chen T. SPA70 is a potent antagonist of human pregnane X receptor. *Nat Commun* 2017; **8**: 741 [PMID: 28963450 DOI: 10.1038/s41467-017-00780-5]
36 **Nishii N**, Oshima T, Li M, Eda H, Nakamura K, Tamura A, Ogawa T, Yamasaki T, Kondo T, Kono T, Tozawa K, Tomita T, Fukui H, Miwa H. Lubiprostone Induces Claudin-1 and Protects Intestinal Barrier Function. *Pharmacology* 2020; **105**: 102-108 [PMID: 31536982 DOI: 10.1159/000503054]
37 **Tsuji Y**, Kaji K, Kitade M, Kaya D, Kitagawa K, Ozutsumi T, Fujinaga Y, Takaya H, Kawaratani H, Namisaki T, Moriya K, Akahane T, Yoshiji H. Bile Acid Sequestrant, Sevelamer Ameliorates Hepatic Fibrosis with Reduced Overload of Endogenous Lipopolysaccharide in Experimental Nonalcoholic Steatohepatitis. *Microorganisms* 2020; **8** [PMID: 32575352 DOI: 10.3390/microorganisms8060925]
38 **Kumar H**, Mandal SK, Gogoi P, Kanaujia SP. Structural and functional role of invariant water molecules in matrix metalloproteinases: a data-mining approach. *J Biomol Struct Dyn* 2021; 1-12 [PMID: 34121627 DOI: 10.1080/07391102.2021.1938683]
39 **Fleming S**, Toratani S, Shea-Donohue T, Kashiwabara Y, Vogel SN, Metcalf ES. Pro- and anti-inflammatory gene expression in the murine small intestine and liver after chronic exposure to alcohol. *Alcohol Clin Exp Res* 2001; **25**: 579-589 [PMID: 11329499]

40 **Chen P**, Stärkel P, Turner JR, Ho SB, Schnabl B. Dysbiosis-induced intestinal inflammation activates tumor necrosis factor receptor I and mediates alcoholic liver disease in mice. *Hepatology* 2015; **61**: 883-894 [PMID: 25251280 DOI: 10.1002/hep.27489]

41 **Wang R**, Tang R, Li B, Ma X, Schnabl B, Tilg H. Gut microbiome, liver immunology, and liver diseases. *Cell Mol Immunol* 2021; **18**: 4-17 [PMID: 33318628 DOI: 10.1038/s41423-020-00592-6]

42 **Trebicka J**, Macnaughtan J, Schnabl B, Shawcross DL, Bajaj JS. The microbiota in cirrhosis and its role in hepatic decompensation. *J Hepatol* 2021; **75** Suppl 1: S67-S81 [PMID: 34039493 DOI: 10.1016/j.jhep.2020.11.013]

43 **Assimakopoulos SF**, Tsamandas AC, Tsiaoussis GI, Karatza E, Triantos C, Vagianos CE, Spiliopoulou I, Kaltezioti V, Charonis A, Nikolopoulou VN, Scopa CD, Thomopoulos KC. Altered intestinal tight junctions' expression in patients with liver cirrhosis: a pathogenetic mechanism of intestinal hyperpermeability. *Eur J Clin Invest* 2012; **42**: 439-446 [PMID: 22023490 DOI: 10.1111/j.1365-2362.2011.02609.x]

44 **Bishehsari F**, Magno E, Swanson G, Desai V, Voigt RM, Forsyth CB, Keshavarzian A. Alcohol and Gut-Derived Inflammation. *Alcohol Res* 2017; **38**: 163-171 [PMID: 28988571]

45 **Bala S**, Marcos M, Gattu A, Catalano D, Szabo G. Acute binge drinking increases serum endotoxin and bacterial DNA levels in healthy individuals. *PLoS One* 2014; **9**: e96864 [PMID: 24828436 DOI: 10.1371/journal.pone.0096864]

46 **Bode C**, Bode JC. Effect of alcohol consumption on the gut. *Best Pract Res Clin Gastroenterol* 2003; **17**: 575-592 [PMID: 12828956 DOI: 10.1016/s1521-6918(03)00034-9]

47 **Furuya S**, Cichocki JA, Konganti K, Dreval K, Uehara T, Katou Y, Fukushima H, Kono H, Pogribny IP, Argemi J, Bataller R, Rusyn I. Histopathological and Molecular Signatures of a Mouse Model of Acute-on-Chronic Alcoholic Liver Injury Demonstrate Concordance With Human Alcoholic Hepatitis. *Toxicol Sci* 2019; **170**: 427-437 [PMID: 30517762 DOI: 10.1093/toxsci/kfy292]

48 **Garg A**, Zhao A, Erickson SL, Mukherjee S, Lau AJ, Alston L, Chang TK, Mani S, Hirota SA. Pregnane X Receptor Activation Attenuates Inflammation-Associated Intestinal Epithelial Barrier Dysfunction by Inhibiting Cytokine-Induced Myosin Light-Chain Kinase Expression and c-Jun N-Terminal Kinase 1/2 Activation. *J Pharmacol Exp Ther* 2016; **359**: 91-101 [PMID: 27440420 DOI: 10.1124/jpet.116.234096]

49 **He S**, Guo Y, Zhao J, Xu X, Wang N, Liu Q. Ferulic Acid Ameliorates Lipopolysaccharide-Induced Barrier Dysfunction *via* MicroRNA-200c-3p-Mediated Activation of PI3K/AKT Pathway in Caco-2 Cells. *Front Pharmacol* 2020; **11**: 376 [PMID: 32308620 DOI: 10.3389/fphar.2020.00376]

50 **Mencarelli A**, Renga B, Palladino G, Claudio D, Ricci P, Distrutti E, Barbanti M, Baldelli F, Fiorucci S. Inhibition of NF-kB by a PXR-dependent pathway mediates counter-regulatory activities of rifaximin on innate immunity in intestinal epithelial cells. *Eur J Pharmacol* 2011; **668**: 317-324 [PMID: 21806984 DOI: 10.1016/j.ejphar.2011.06.058]

51 **Shao YX**, Lei Z, Wolf PG, Gao Y, Guo YM, Zhang BK. Zinc Supplementation, *via* GPR39, Upregulates PKCζ to Protect Intestinal Barrier Integrity in Caco-2 Cells Challenged by *Salmonella enterica* Serovar Typhimurium. *J Nutr* 2017; **147**: 1282-1289 [PMID: 28515165 DOI: 10.3945/jn.116.243238]

52 **Sarkar P**, Saha T, Sheikh IA, Chakraborty S, Aoun J, Chakrabarti MK, Rajendran VM, Ameen NA, Dutta S, Hoque KM. Zinc ameliorates intestinal barrier dysfunctions in shigellosis by reinstating claudin-2 and -4 on the membranes. *Am J Physiol Gastrointest Liver Physiol* 2019; **316**: G229-G246 [PMID: 30406698 DOI: 10.1152/ajpgi.00092.2018]

53 **Zhong W**, Li Q, Sun Q, Zhang W, Zhang J, Sun X, Yin X, Zhang X, Zhou Z. Preventing Gut Leakiness and Endotoxemia Contributes to the Protective Effect of Zinc on Alcohol-Induced Steatohepatitis in Rats. *J Nutr* 2015; **145**: 2690-2698 [PMID: 26468492 DOI: 10.3945/jn.115.216093]

54 **Szuster-Ciesielska A**, Plewka K, Daniluk J, Kandefer-Szerszeń M. Zinc supplementation attenuates ethanol- and acetaldehyde-induced liver stellate cell activation by inhibiting reactive oxygen species (ROS) production and by influencing intracellular signaling. *Biochem Pharmacol* 2009; **78**: 301-314 [PMID: 19376089 DOI: 10.1016/j.bcp.2009.04.009]

55 **Zhang B**, Shao Y, Liu D, Yin P, Guo Y, Yuan J. Zinc prevents Salmonella enterica serovar Typhimurium-induced loss of intestinal mucosal barrier function in broiler chickens. *Avian Pathol* 2012; **41**: 361-367 [PMID: 22834550 DOI: 10.1080/03079457.2012.692155]

56 **Foligné B**, George F, Standaert A, Garat A, Poiret S, Peucelle V, Ferreira S, Sobry H, Muharram G, Lucau-Danila A, Daniel C. High-dose dietary supplementation with zinc prevents gut inflammation: Investigation of the role of metallothioneins and beyond by transcriptomic and metagenomic studies. *FASEB J* 2020; **34**: 12615-12633 [PMID: 32729971 DOI: 10.1096/fj.202000562RR]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 December 28; 27(48): 8343-8356

DOI: 10.3748/wjg.v27.i48.8343

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

ORIGINAL ARTICLE

Case Control Study

Combination of squamous cell carcinoma antigen immunocomplex and alpha-fetoprotein in mid- and long-term prediction of hepatocellular carcinoma among cirrhotic patients

Antonio Gil-Gómez, Ángela Rojas, Chang-Hai Liu, Rocio Gallego-Duran, Rocio Muñoz-Hernandez, Giorgio Fassina, Patrizia Pontisso, Javier Ampuero, Manuel Romero-Gómez

ORCID number: Antonio Gil-Gómez 0000-0001-9622-1761; Ángela Rojas 0000-0002-6853-627X; Chang-Hai Liu 0000-0003-3901-3039; Rocio Gallego-Duran 0000-0002-9452-1661; Rocio Muñoz-Hernandez 0000-0003-3765-6276; Giorgio Fassina 0000-0003-1845-2657; Patrizia Pontisso 0000-0003-2077-9202; Javier Ampuero 0000-0002-8332-2122; Manuel Romero-Gómez 0000-0001-8494-8947.

Author contributions: Gil-Gómez A led the formal analysis and writing-original draft; Rojas A equally contributed to the data curation and supported the formal analysis; Liu CH supported formal analysis and writing-original draft; Gallego-Duran R equally contributed to data curation, and led the resources; Muñoz-Hernandez R supported the data curation and validation; Fassina G and Pontisso P equally contributed to the validation; Ampuero J supported the conceptualization, led the supervision, and equally contributed to the writing-review and editing; Romero-Gómez M led the conceptualization, and equally contributed to the writing-review and editing.

Institutional review board

Antonio Gil-Gómez, Ángela Rojas, Rocio Gallego-Duran, Rocio Muñoz-Hernandez, Javier Ampuero, Manuel Romero-Gómez, SeLiver Group, Institute of Biomedicine of Seville, Seville 41013, Spain

Antonio Gil-Gómez, Ángela Rojas, Rocio Gallego-Duran, Rocio Muñoz-Hernandez, Javier Ampuero, Manuel Romero-Gómez, CIBERehd, Instituto de Salud Carlos III, Madrid 28029, Spain

Antonio Gil-Gómez, Mucosal Immunity Lab, IRCCS Humanitas Research Hospital, Milan 20089, Italy

Chang-Hai Liu, Center of Infectious Diseases, West China Hospital of Sichuan University, Chengdu 610017, Sichuan Province, China

Chang-Hai Liu, State Key Laboratory of Biotherapy and Center of Infectious Diseases, West China Hospital, Chengdu 610017, Sichuan Province, China

Giorgio Fassina, Life Biotechnology, Padua University, Venice 30175, Italy

Patrizia Pontisso, Department of Clinical and Experimental Medicine, University of Padova, Padova 35123, Italy

Javier Ampuero, Manuel Romero-Gómez, UCM Digestive Diseases, Virgen del Rocío University Hospital, Seville 41014, Spain

Corresponding author: Manuel Romero-Gómez, MD, Full Professor, SeLiver Group, Institute of Biomedicine of Seville, Avda. Manuel Siurot sn, Seville 41013, Spain.
mromerogomez@us.es

Abstract

BACKGROUND

The combination of alpha-fetoprotein (AFP) and squamous cell carcinoma antigen immunocomplex (SCCA-IgM) have been proposed for its use in the screening of hepatocellular carcinoma (HCC). Current screening programs for all cirrhotic patients are controversial and a personalized screening is an unmet need in the

statement: Human samples were collected after obtaining a signed informed consent as approved by the Ethical Committee both hospitals (C330020).

Informed consent statement: Human samples were collected after obtaining a signed informed consent.

Conflict-of-interest statement: The authors declare no conflict of interest.

Data sharing statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy reasons.

Supported by Sara Borrell postdoctoral fellowships from Instituto de Salud Carlos III to support Ángela Rojas postdoctoral contract; Consejería de Salud y Familias, Junta de Andalucía supporting Antonio Gil-Gómez contract; PI19/01404 Grant from Spanish Ministry of Economy, Innovation and Competition, the Instituto de Salud Carlos III; PI19/00589/Spanish Ministry of Economy, Innovation and Competition, the Instituto de Salud Carlos III; and the Xeptagen, Italy, provided the ELISA kits for the measurements of SCCA-IgM. None of the founders had a role in the design, writing or interpretation of the study.

Country/Territory of origin: Spain

Specialty type: Gastroenterology and Hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Open-Access: This article is an

precision medicine era.

AIM

To determine the role of the combination of SCCA-IgM and AFP in predicting mid- and long-term appearance of HCC.

METHODS

Two-hundred and three cirrhotic patients (Child A 74.9%, B 21.2%, C 3.9%) were followed-up prospectively every six months to screen HCC by ultrasound and AFP according to European Association for the Study of the Liver guidelines. The estimation cohort was recruited in Italy (30.5%; 62/203) and validation cohort from Spain (69.5%; 141/203). Patients underwent to evaluate SCCA-IgM by enzyme-linked immunosorbent assay (Hepa-IC, Xeptagen, Italy) and AFP levels at baseline. Patients were followed-up for 60 mo, being censored at the time of the appearance of HCC.

RESULTS

There were 10.8% and 23.1% of HCC development at two- and five-years follow-up. Patients with HCC showed higher levels of SCCA-IgM than those without it (425.72 ± 568.33 AU/mL *vs* 195.93 ± 188.40 AU/mL, $P = 0.009$) during the five-year follow-up. In multivariate analysis, after adjusting by age, sex, aspartate transaminase and Child-Pugh, the following factors were independently associated with HCC: SCCA-IgM [Hazard ratio (HR) = 1.001, 95%CI: 1.000-1.002; $P = 0.003$], AFP (HR = 1.028, 95%CI: 1.009-1.046; $P = 0.003$) and creatinine (HR = 1.564 95%CI: 1.151-2.124; $P = 0.004$). The log-rank test of the combination resulted in 7.488 ($P = 0.024$) in estimation cohort and 11.061 ($P = 0.004$) in the validation cohort, and a 100% of correctly classified rate identifying a low-risk group in both cohorts in the two-year follow-up.

CONCLUSION

We have constructed a predictive model based on the combination of SCCA-IgM and AFP that provides a new HCC screening method, which could be followed by tailored HCC surveillance for individual patients, especially for those cirrhotic patients belonging to the subgroup identified as low-risk of HCC development.

Key Words: Squamous cell carcinoma antigen; Hepatocellular carcinoma prediction; Precision medicine; Stratification of cirrhotic patient

Core Tip: Current screening programs of hepatocellular carcinoma (HCC) for all cirrhotic patients are controversial and a personalized strategy is an unmet need in the precision medicine era. By studying circulating biomarkers in two-hundred and three cirrhotic patients followed-up for 60 mo, we found that the combination of circulating alpha-fetoprotein and squamous cell carcinoma antigen immunocomplex resulted in a 100% of correctly classified rate identifying a low-risk group of HCC at two years of follow-up in two different cohorts. This predictive model provides a new screening method, which could be followed by tailored HCC surveillance for individual patients.

Citation: Gil-Gómez A, Rojas Á, Liu CH, Gallego-Duran R, Muñoz-Hernandez R, Fassina G, Pontisso P, Ampuero J, Romero-Gómez M. Combination of squamous cell carcinoma antigen immunocomplex and alpha-fetoprotein in mid- and long-term prediction of hepatocellular carcinoma among cirrhotic patients. *World J Gastroenterol* 2021; 27(48): 8343-8356
URL: https://www.wjgnet.com/1007-9327/full/v27/i48/8343.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i48.8343

Received: April 22, 2021
Peer-review started: April 22, 2021
First decision: June 13, 2021
Revised: June 27, 2021
Accepted: December 8, 2021
Article in press: December 8, 2021
Published online: December 28, 2021

P-Reviewer: Shousha HI
S-Editor: Wang JL
L-Editor: A
P-Editor: Wang JL

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common malignant primary liver tumor and the second leading cause of cancer-related death in the world, according to the World Health Organization[1].

Up to 90% of HCCs in the Western world seem to occur in patients with cirrhosis, with an annual incidence ranging from 2% to 4% with differences in age, gender, etiology and duration of the cirrhosis[2,3]. According to the Barcelona Clinic Liver Cancer stratification, patients diagnosed on stage 0 and A of HCC have a tremendously better five-year HCC-free rate (93%) than those patients diagnosed on the advanced stage (5%) due to the availability of curative therapies such as surgical resection or liver transplantation[4]. However, the vast majority of HCC patients are diagnosed at advanced stages[5] and only a small proportion of new HCC patients are diagnosed through the surveillance[6]. Tumor stage at diagnosis can be impacted by several factors in clinical practice, including low surveillance rates and compliance and delays in follow-up of abnormal screening tests[4]. Therefore, in order to diagnose HCC at the early stage, besides having an accurate diagnostic tool, an appropriate strategy of HCC surveillance specifically focusing on well-defined high-risk population is essential and indispensable.

Current guidelines[7,8] recommend HCC screening by abdominal ultrasound at 6-month intervals in cirrhotic patients. However, the practice guideline-recommended "one-size-fits-all" HCC screening program for early tumor detection is performed in less than 20% of the target population and its implementation in clinical practice is far from satisfactory due to multiple patient- and provider-related factors[9]. More importantly, the risk of developing HCC is likely not uniform across all cirrhotic patients[10,11]. Therefore, an individual HCC risk prediction followed by tailoring the personalized surveillance strategy is expected to overcome the challenge in the era of precision medicine[9,12].

SERPINB3 and SERPINB4, formerly known as squamous cell carcinoma antigen 1-2 (SCCA1/2), are two isoforms of Clade B Serine Protease Inhibitors that are found physiologically in the spinous and granular layers of normal squamous epithelium such as tongue, esophagus, lung and uterus among others, while become highly expressed in squamous cell carcinomas of these organs[13,14]. Recent evidences found the plasma levels of both SCCA[15] and immunoglobulin M complex (SCCA-IgM)[16] associated with liver tumor development, suggesting that monitoring of SCCA and SCCA-IgM levels might be useful for identifying cirrhotic patients at higher risk of developing HCC[15]. A large number of studies further supported the usefulness of SCCA-IgM for the diagnosis[17] and monitoring of chronic liver disease[18-20] including the histological response after antiviral treatments. A recent meta-analysis concluded that both SCCA and SCCA-IgM had a similar moderate diagnostic accuracy (0.7-0.9) for HCC screening; however, a combination of SCCA and SCCA-IgM was the best diagnostic option[17]. Pozzan *et al*[21] proved that SCCA-IgM alone was able to predict HCC-free and progression-free survival for intermediate-stage patients treated by transcatheter arterial chemoembolization. Lately, Biasiolo *et al*[22] showed that SCCA-IgM alone but not AFP was significant to predict the HCC-free survival in a prospective cohort. However, the previous study did not assess the combination of SCCA-IgM and AFP, and there was no external validation study that further confirmed those results. More importantly, the majority of previous studies were performed only in Italian cohorts with a dominant hepatitis C etiology by a uni-center design. The present study aims to evaluate the potential role of the combination of SCCA-IgM and AFP as a biomarker in the mid-term and long-term prediction of HCC among patients with cirrhosis by using a multi-center and internal-external-validation study design.

MATERIALS AND METHODS

Patients

From January 2007 to March 2016, 62 cirrhotic patients (30.5%; 62/203) were enrolled from the outpatient clinics of the *Azienda Ospedaliera di Padova* (Padova, Italy) as estimation cohort and 155 cirrhotic patients (69.5%; 141/203) were included at Valme University Hospital (Seville, Spain) as validation cohort. The study was retrospectively performed on prospectively collected sera. Patients were followed-up every six months for HCC screening according to European Association for the Study of the Liver guidelines[7]. The study was performed by following the ethical guidelines

expressed in the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice. Human samples were collected after obtaining a signed informed consent as approved by the Ethical Committee of both hospitals.

Cirrhosis was diagnosed by documenting at least one of the following: clinical (esophageal varices, liver dysfunction, or previous ascites or variceal bleeding), pathological (liver biopsy) or radiological (coarse/nodular/lobar redistribution on ultrasound) markers of cirrhosis. Demographic, clinical and laboratory parameters were recorded at the first visit including age, sex, etiology of cirrhosis, aspartate transaminase (AST), alanine aminotransferase, bilirubin, albumin, creatinine and platelet levels. Patients with both chronic viral hepatitis and a history of alcohol intake were categorized as having viral hepatitis. Similarly, patients with steatohepatitis were included as alcoholic cirrhosis if alcohol was determined as the cause of liver disease in the clinical record. Non-alcoholic steatohepatitis, as well as autoimmune liver diseases such as autoimmune hepatitis, primary biliary cirrhosis or primary sclerosing cholangitis, were categorized as "Others". Follow-up time was censored at the last clinic visit, death, liver transplantation or diagnosis of HCC within the term of 60 mo. HCC was diagnosed without biopsy in the majority of the cases because of current clinical diagnostic approaches, including ultrasonography, computed tomography, magnetic resonance imaging were sufficient to diagnose HCC[7,8].

Sample storage and assays

Peripheral blood sample was collected from each patient at the time of the first clinic visit. Plasma and serum aliquots were stored in cryovials at -80°C after centrifugation for 10 min at 1500 ×*g* at 4°C. Serum AFP and SCCA-IgM were measured for each patient by an experienced technician who was blind to the clinical information. AFP levels were determined by an electrochemiluminescence immunoassay using an automatized analyzer Elecsys (Roche, Switzerland) and SCCA-IgM was measured in duplicate using commercially available enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (Hepa-IC, Xeptagen, Venice, Italy). The amount of SCCA-IgM immune complexes was expressed in arbitrary units (AU)/mL by interpolation of samples absorbance on the calibration curves plotted with SCCA-IgM calibrators.

Statistical analysis

Cox proportional hazards regression was used to estimate the hazard ratio (HR) and CI. Comparisons between categorical variables were made by the Chi-square or Fisher test. Results are presented as frequencies and percentages for categorical variables, means ± SDs for normal continuous variables and median, quartile 1 and 3 for not normal continuous variables. Missing data was listwise deleted (complete-case analysis). Those factors showing statistical ($P < 0.05$) association to HCC in univariate analyses were combined in a backwards stepwise multivariable model. Factors not significant but of potential clinical relevance such as age and sex were also included in order to avoid confounding. In the estimation cohort, we used two-year follow-up data to perform the univariate and multivariate analysis to assess the factors independently associated with HCC-free survival because cirrhotic patients need to be screened at least every two years. Akaike's information criterion (AIC) was additionally computed to select the most robust predictors. The predictive cut-off of SCCA-IgM was established by means of receiver operating characteristic (ROC) curve method at a value that maximized specificity and sensitivity according to Youden index. The same AFP cut-off value derived from estimation cohort (5 ng/mL) was used in validation cohort. Categorical variables were compared by means of the Kaplan-Meier method, with curves compared using the log-rank test. The Harrell's concordance index (C-index) was used to assess the score's discrimination ability. C-index values and the corresponding 95%CIs were estimated for each main study time point. The sensitivity, specificity, positive predictive value and negative predictive value were calculated to demonstrate the predictive ability. SPSS (version 25.0; SPSS Inc., IL, the United States) and Stata 11 (StataCorp, College Station, TX) statistical packages were used.

RESULTS

Identification of the study cohort and baseline characteristics

The baseline characteristics and biochemical parameters of the overall cohort, as well as estimation and validation cohorts, are shown in Table 1. Briefly, a total of 203 patients with liver cirrhosis were included in the study, with 74.9% Child-Pugh A, 21.2%B, and 3.9% Child-Pugh C. The most common etiology of cirrhosis was alcohol (54.2%), followed by HCV (27.1%) and HBV (8.4%). HCC development was observed in 22 patients (10.8%) during the two-year follow-up (22.1 ± 5.11) and 47 patients (23.2%) during the five-year follow-up (41.9 ± 16.0 mo). The baseline values of serum SCCA-IgM were significantly higher in patients who developed HCC than in those who did not (514.17 ± 714.43 AU/mL *vs* 216.92 ± 233.51 AU/mL, $P < 0.001$) during the two-year follow-up, as well as AFP (23.91 ± 41.37 ng/mL *vs* 6.16 ±10.49 ng/mL, $P < 0.001$).

Identification of risk factors for HCC development

Univariate analysis showed that the levels of SCCA-IgM ($P = 0.004$), AFP ($P < 0.001$), AST ($P = 0.021$) and creatinine ($P = 0.018$) were associated with two-year HCC-free survival in the estimation cohort (Table 2). Nevertheless, Child-Pugh classification, platelets count and other biochemical parameters were similar between both groups of patients. By using a multivariate Cox regression, after adjusting for age, gender, AST and Child-Pugh, SCCA-IgM (HR = 1.001, 95%CI: 1.000-1.002; $P = 0.003$), AFP (HR = 1.028, 95%CI: 1.009-1.046; $P = 0.003$) and creatinine (HR = 1.564, 95%CI: 1.151-2.124; $P = 0.004$) were independently associated with increased two-year risk of HCC.

Internal estimation of the combination of SCCA-IgM and AFP

After multivariate analysis, the model including SCCA-IgM, AFP and creatinine was the most robust for the prediction of HCC development (AIC: 44.83); however, no statistical significance was observed in ROC curve analysis ($P = 0.234$) so the second model consisting of the combination of SCCA-IgM and AFP was chosen (AIC: 55.54). Therefore, we performed ROC curve to explore the ability of SCCA-IgM and AFP in predicting the patients with cirrhosis to develop HCC during the two-year follow-up. By establishing a cut-off of 124 AU/mL for SCCA-IgM (sensitivity of 75% and specificity of 76%) and using a cut-off of 5 ng/mL for AFP (sensitivity of 75% and specificity of 48%), we obtained AUROCs of 0.74 (95%CI: 0.55-0.93; $P = 0.029$) and 0.73 (95%CI: 0.52-0.95; $P = 0.034$), respectively. However, although the predictive ability of the combination of SCCA-IgM and AFP was also significant [AUROC 0.77 (95%CI: 0.63-0.92; $P = 0.013$)], we observed no statistical significance when comparing the combinatory model to SCCA-IgM ($P = 0.669$) or AFP ($P = 0.715$) alone (Figure 1).

This combination allowed us to stratify the cohort into low-risk group (AFP < 5 ng/mL and SCCA-IgM < 124 AU/mL), intermediate-risk group (AFP > 5 ng/mL or SCCA-IgM > 124 AU/mL) and high-risk group (AF P> 5 ng/mL and SCCA-IgM > 124 AU/mL). The predicted mean survival curves were compared by Kaplan-Meier at two- and five-years follow-up in the estimation cohort (Figure 2). Notably, we found that the low-risk group that was stratified by the combination of SCCA-IgM and AFP correctly identified a 100% of HCC-free survival rate in two-year followed-up which was further confirmed in the five-year follow-up (100%) (Figure 2C).

External validation

The same cut-off values were used for the validation cohort to confirm the results of the predictive ability of HCC-free survival. Again, the low-risk group showed a 100% of two-year and 96.2% of five-year follow-up of HCC-free survival rate (Figure 3C). However, there were no differences between the combination and SCCA-IgM or AFP alone in the comparative C-index estimates for the validation data cohort (Table 3), as are the results of the confirmatory analysis of the predictive ability of both the two- and five-year HCC-free survival.

For practical applications, we calculated sensitivity, specificity, positive predictive value (PPV), negative predictive value and likelihood ratio (LR) of the combination of SCCA-IgM and AFP to demonstrate the predictive ability (Table 4). An LR- of 0 were obtained in both estimation and validation cohort in two-year follow-up, so the low-risk group of patients who did not develop HCC could be accurately ruled-out. The correctly classified rate increased from 75.3% (estimation cohort) to 78.8% (validation cohort) in two-year follow-up and from 61.1% (estimation cohort) to 68.5% (validation cohort) in five-year follow-up.

Table 1 Characteristics of included patients

	Global (*n* = 203)	Italian (*n* = 62) (Estimation cohort)	Spanish (*n* = 141) (Validation cohort)	Univariable analysis
Gender (Male)	73.4% (149/203)	74.2% (46/62)	73.0% (103/141)	0.865
mean age (yr)	57.93 ± 9.76	55.77 ± 10.51	58.87 ± 9.22	
Etiology				0.001
Alcohol	54.2% (110/203)	41.9% (26/62)	59.6% (84/141)	
HCV	27.1% (55/203)	38.7% (24/62)	22.0% (31/141)	
HBV	8.4% (17/203)	16.1% (10/62)	5% (7/144)	
Others	10.3% (21/203)	3.2% (2/62)	13.5% (19/141)	
Child-Pugh				0.340
A	74.9% (152/203)	64.5% (40/62)	79.4% (112/141)	
B	21.2% (43/203)	27.4% (17/62)	18.4% (26/141)	
C	3.9% (8/203)	8.1% (5/62)	2.1% (3/141)	
AST (IU/mL)	51.69 ± 38.49	69.17 ± 47.74	44.50 ± 31.44	0.001
ALT (IU/mL)	42.54 ± 38.68	61.09 ± 56.11	34.91 ± 25.16	0.000
Tot. Bilirubin (mg/dL)	1.60 ±1.87	1.94 ± 2.87	1.45 ± 1.22	0.215
Creatinine (mg/dL)	0.86 ± 0.68	0.98 ± 1.21	0.81 ± 0.22	0.292
Platelets ($\times 10^9$/mL)	116.00 ± 58.10	100.53 ± 43.11	122.45 ± 62.32	0.005
Albumin (mg/dL)	3885.19 ± 586.66	3810.34 ± 613.79	3916.34 ± 574.96	0.248
AFP (ng/mL)	8.09 ± 17.50	12.00 ± 25.43	6.69 ± 12.82	0.101
SCCA-IgM (AU/mL)	249.13 ± 332.01	197.73 ± 431.13	271.73 ± 276.35	0.144
Two-year HCC (Yes)	10.8% (22/203)	12.9% (8/62)	9.9% (14/141)	0.530
Five-year HCC (Yes)	23.2% (47/203)	21.0% (13/62)	24.1% (34/141)	0.625

Comparisons between groups were made using the Mann-Whitney *U* test or the Student *t*-test for continuous variables, and the χ^2 test or the Fisher's exact test for categorical data. *P* values represent the statistical significance of the differences between both subsets. Data are expressed as numbers of patients (%) or mean ± SD. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HCV: Hepatitis C virus; HBV: Hepatitis B virus; AFP: Alpha-fetoprotein; SCCA-IgM: Squamous cell carcinoma antigen and its immune complexes; Child-Pugh: The Child–Turcotte–Pugh score or Child Criteria; HCC: Hepatocellular carcinoma.

DISCUSSION

In the present study, we revealed an enhanced HCC risk assessment by using the combination of SCCA-IgM and AFP serum levels. A low-risk subgroup of cirrhotic patients with 100% of internal-external validated two-year follow-up (mid-term) of HCC-free survival rate was correctly identified. This strategy may enable to personalize intensity of HCC screening. Moreover, a high HCC-free survival rate (96.2%) at five-year follow-up (long-term) further confirmed our proposed surveillance strategy with patients at low-risk of HCC development. Although prior studies have proposed SCCA-IgM for HCC prediction[21,22], our study is the first to internal-externally validate the proposed biomarkers. Validation is an important aspect of predictive model development, because of the performance of regression models is generally substantially higher in the estimation cohort than in validation cohort[23]. An inconsistency of correctly classified rate from estimation to validation cohorts further explains and highlights the urgent need of a well-defined cut-off developed by multi-center larger-population based studies in the future[17].

Combination of clinical symptoms, laboratory variables and molecular biomarkers have been investigated to develop HCC risk predictive models; however, their performance is still debated and not yet adopted in clinical practice. A recent disease-specific Toronto HCC Risk Index revealed that the 10-year cumulative incidence of HCC differed from etiologic category ranging from 22% to 5%, and further allowed to stratify patients into three groups according to the HCC risk estimation with a 10-year

Table 2 Univariable and multivariable analysis regarding two-year hepatocellular carcinoma disease-free survival in the estimation cohort

Covariate	Non-HCC (*n* = 54)	HCC (*n* = 8)	Univariable analysis HR (95%CI; *P* value)	Multivariable analysis HR (95%CI; *P* value)
Gender (Male)	75.9% (41/54)	62.5% (5/8)	0.571 (0.137-2.392; 0.444)	
mean age (yr)	55.96 ± 10.82	54.5 ± 8.5	0.987 (0.924-1.055; 0.706)	
Etiology (alcohol/HCV/HBV/other)	25/17/10/2	1/7/0/0	1.075 (0.481-2.405; 0.859)	
Child-Pugh (A/B/C)	35/14/5	5/3/0	0.922 (0.290-2.935; 0.891)	
AST (IU/mL)	63.94 ± 42.21	107.29 ± 69.83	1.013 (1.002-1.024; 0.021)	
ALT (IU/mL)	58.86 ± 55.48	77.29 ± 62.52	1.004 (0.993-1.015; 0.452)	
Tot. Bilirubin (mg/dL)	1.97 ± 3.05	1.75 ± 1.02	0.983 (0.734-1.316; 0.906)	
Creatinine (mg/dL)	0.83 ± 0.20	2.04 ± 3.44	1.363 (1.055-1762; 0.018)	1.564 (1.151-2.124; 0.004)
Platelets ($\times 10^9$/mL)	102.25 ± 43.28	88.00 ± 42.89	0.992 (0.974-1.010; 0.387)	
Albumin (mg/dL)	3833 ± 625	3642 ± 525	1.000 (0.998-1.001; 0.394)	
AFP (ng/mL)	7.80 ± 9.25	40.38 ± 62.71	1.024 (1.010-1.038; 0.001)	1.028 (1.009-1.046; 0.003)
SCCA-IgM (AU/mL)	136.83 ± 163.44	608.75 ± 1093.53	1.001 (1.000-1.002; 0.004)	1.001 (1.000-1.002; 0.003)

Cox proportional hazards model was used to estimate the hazard ratios and CIs in the multivariable analysis. Data are numbers of patients (%) or mean ± SD. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HCV: Hepatitis C virus; HBV: Hepatitis B virus; AFP: Alfa-fetoprotein; SCCA-IgM: Squamous cell carcinoma antigen and its immune complexes; Child-Pugh score: The Child–Turcotte–Pugh score or Child Criteria; HCC: Hepatocellular carcinoma; HR: Hazard ratio.

Table 3 Predictive discrimination ability of the combination of squamous cell carcinoma antigen immunocomplex and alfa-fetoprotein as compared with squamous cell carcinoma antigen immunocomplex or alfa-fetoprotein alone in both estimation and validation cohorts

Total patients (*n* = 203)	Combination of SCCA-IgM and AFP (95%CI)	SCCA-IgM (95%CI; *P* value)	AFP (95%CI; *P* value)
Estimation cohort (*n* = 62)			
Two-year HCC-free survival	0.787 (0.620-0.955)	0.727 (0.526-0.927; 0.451)	0.705 (0.464-0.946; 0.398)
Five-year HCC-free survival	0.744 (0.613-0.876)	0.686 (0.535-0.837; 0.299)	0.705 (0.539-0.871; 0.581)
Validation cohort (*n* = 141)			
Two-year HCC-free survival	0.773 (0.659-0.887)	0.706 (0.588-0.827; 0.122)	0.748 (0.617-0.880; 0.701)
Five-year HCC-free survival	0.730 (0.648-0.813)	0.706 (0.623-0.788; 0.297)	0.646 (0.548-0.734; 0.067)

C-index values and the corresponding 95% CIs were estimated for each main study time point to assess the model's discrimination ability. *P* values represent the statistical significance of the differences between the combination and the squamous cell carcinoma antigen immunocomplex or alfa-fetoprotein alone. AFP: Alfa-fetoprotein; SCCA-IgM: Squamous cell carcinoma antigen and its immune complexes; HCC: Hepatocellular carcinoma.

incidence of HCC of 3%, 10% and 32%, respectively[10]. The AFP has been currently removed from the clinical practice guidelines because of its low PPV, which potentially results in "overdoing" the follow-up testing (*e.g.*, computed tomography, magnetic resonance imaging), in the frequently encountered patients with mildly elevated AFP[24]. However, El-Serag *et al*[24] constructed an AFP-based algorithm to identify patients at risk for HCC, and further suggested that the wide availability of AFP tests, high level of laboratory standardization and low cost made AFP still a feasible strategy to predict HCC. Moreover, three recent meta-analyses have proved the usefulness of the combination of AFP with SCCA-IgM[17], Des-gamma-carboxyprothrombin and Golgi protein 73[25,26] for hepatocellular carcinoma diagnosis, suggesting the combinations of biomarkers a feasible strategy of HCC screening. Therefore, the consideration remaining to us is not whether to use AFP for HCC screening and predicting or not, but how to use it appropriately.

Table 4 Operating characteristics for the combination of squamous cell carcinoma antigen immunocomplex and alfa-fetoprotein regarding two- and five-year hepatocellular carcinoma disease-free survival

Variables	Two-year incidence in validation cohort				Five-year incidence in validation cohort			
	Estimation cohort		Validation cohort		Estimation cohort		Validation cohort	
	Low-risk	High risk	Low-risk	High risk	Low-risk	High risk	Low-risk	High risk
Cut-off	AFP < 5 ng/mL and SCCA-IgM < 124 AU/mL	AFP > 5 ng/mL and SCCA-IgM > 124 AU/mL	AFP < 5 ng/mL and SCCA-IgM < 124 AU/mL	AFP > 5 ng/mL and SCCA-IgM > 124 AU/mL	AFP < 5 ng/mL and SCCA-IgM < 124 AU/mL	AFP > 5 ng/mL and SCCA-IgM > 124 AU/mL	AFP < 5 ng/mL and SCCA-IgM < 124 AU/mL	AFP > 5 ng/mL and SCCA-IgM > 124 AU/mL
True positive	8	4	14	7	13	5	33	12
False positive	33	8	101	21	28	7	82	16
True negative	21	46	26	106	21	42	25	91
False negative	0	4	0	7	0	8	1	22
Sensitivity	100%	50%	100%	50%	100%	38%	96%	35%
Specificity	39%	85%	20%	83%	43%	86%	23%	85%
PPV	20%	33%	12%	25%	32%	42%	29%	43%
NPV	100%	92%	100%	94%	100%	84%	96%	81%
LR+	1.64	3.38	1.26	3.02	1.75	2.69	1.27	2.36
LR-	0.00	0.59	0.00	0.60	0.00	0.72	0.13	0.76
Correctly classified	75.8%		78.8%		61.1%		68.5%	

PPV: positive predictive values; NPV: negative predictive values; LR: likelihood ratio.

By using the present combination of SCCA-IgM and AFP, we will enable rational allocation of the limited medical resources to the high-risk patients who most need to be screened, and avoid wasteful and unnecessary distribution to low-risk individuals who had 100% of HCC-free survival rate in the two-year follow-up. Moreover, the disordered PPV that was influenced by the low prevalence of HCC development through using current "one-size-fits-all" surveillance program, further strengthen the necessity of altering surveillance to a subgroup of high-risk population inside the cirrhotic patients that will ensure a high pre-test probability[27]. Currently there have not been any randomized controlled trial of HCC surveillance in patients with cirrhosis[6]. Cirrhotic patients are older, have more comorbidities and abdominal ultrasound has low sensitivity for HCC detection in a nodular cirrhotic liver. Several cohort studies demonstrated that surveillance was associated with increased early tumor detection, curative treatment option and it improved the overall survival[28]. In contrast, other studies reported that HCC surveillance was not associated with decreased HCC-related mortality, adding to the existing controversy surrounding the benefits of HCC surveillance[29,30]. Nevertheless, modifying HCC screening frequency according to estimated individual HCC risk by using the present combination of biomarkers may enable more efficient early tumor detection because of high-risk subjects are more likely develop HCC.

In this sense, the combination of SCCA-IgM and AFP, classifying a low-risk group with 100% of HCC-free survival, will enable us to exclude those patients from surveillance programs or to extend the intensity of screening to two years. This strategy will enable rational allocation of medical resources, cost-effective and accurate preventive intervention, which will substantially improve the dismal prognosis of HCC and will uphold the spirit of advancing with time in the era of precision medicine. Furthermore, a recent cost-effectiveness study has further verified that tailored HCC surveillance strategies according to estimated patient's risk stratification indeed revealed superior cost-effectiveness[31]. The present strategy of SCCA-IgM and AFP should be further implemented and verified in the clinical setting through future well-designed prospective studies. Moreover, an easy-to-use and outpatient-based

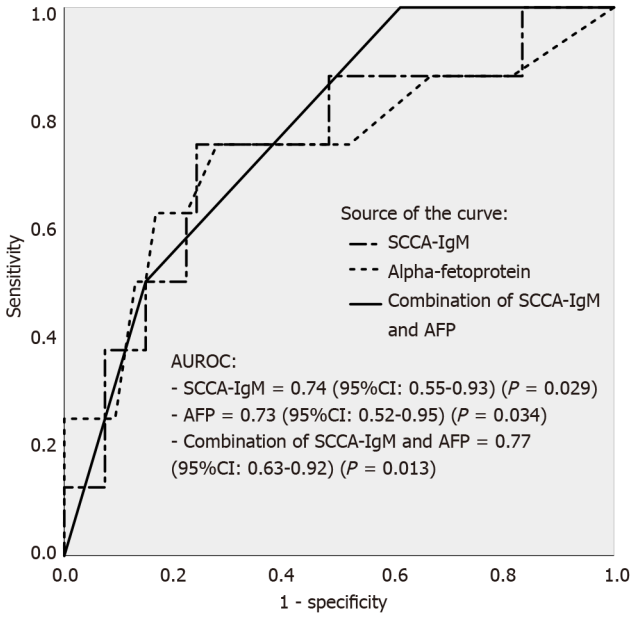

Figure 1 Receiver operating characteristic curves of the combination of squamous cell carcinoma antigen immunocomplex and alpha-fetoprotein as compared to squamous cell carcinoma antigen immunocomplex and alpha-fetoprotein in predicting two-year mortality in the estimation cohort. The clinical relevance of squamous cell carcinoma antigen immunocomplex and alpha-fetoprotein in patients with cirrhosis was determined by the calculation of the area under the receiver operating characteristic. Baseline serum levels distribution above the cut-off of the two biomarkers in patients who developed hepatocellular carcinoma vs patients who did not was compared. AUROC, area under the receiver operating characteristic. Comparison of the AUROCs estimated for each set. AFP: Alpha-fetoprotein; SCCA-IgM: Squamous cell carcinoma antigen immunocomplex.

instead of laboratory-based kit will optimize the performance of the combination of the present biomarkers.

There were several limitations in the present study. First, the present study did not used biopsy to ultimately confirm HCC. Second, the definition of cirrhosis was not reached from liver biopsies. This can lead to an underestimation of subclinical cirrhosis of the population studied. However, according to the current clinical practice guidelines there is no need to perform biopsy for the diagnosis of HCC and cirrhosis, and the ethic concern prohibited certain studies design to perform the biopsy[32]. In fact, the recent technological approach with typical radiological characteristics on contrast-enhanced cross-sectional imaging have a positive predictive value of almost 100%[33]. Third, lead time bias and length time bias were always a crucial consideration of diagnostic accuracy experimental design.

CONCLUSION

In summary, we have proved that the combination of SCCA-IgM and AFP enhanced the predictive value for detecting HCC, which could be followed by tailored HCC surveillance for individual patients, especially for those cirrhotic patients belonging to the subgroup identified as low-risk of HCC development.

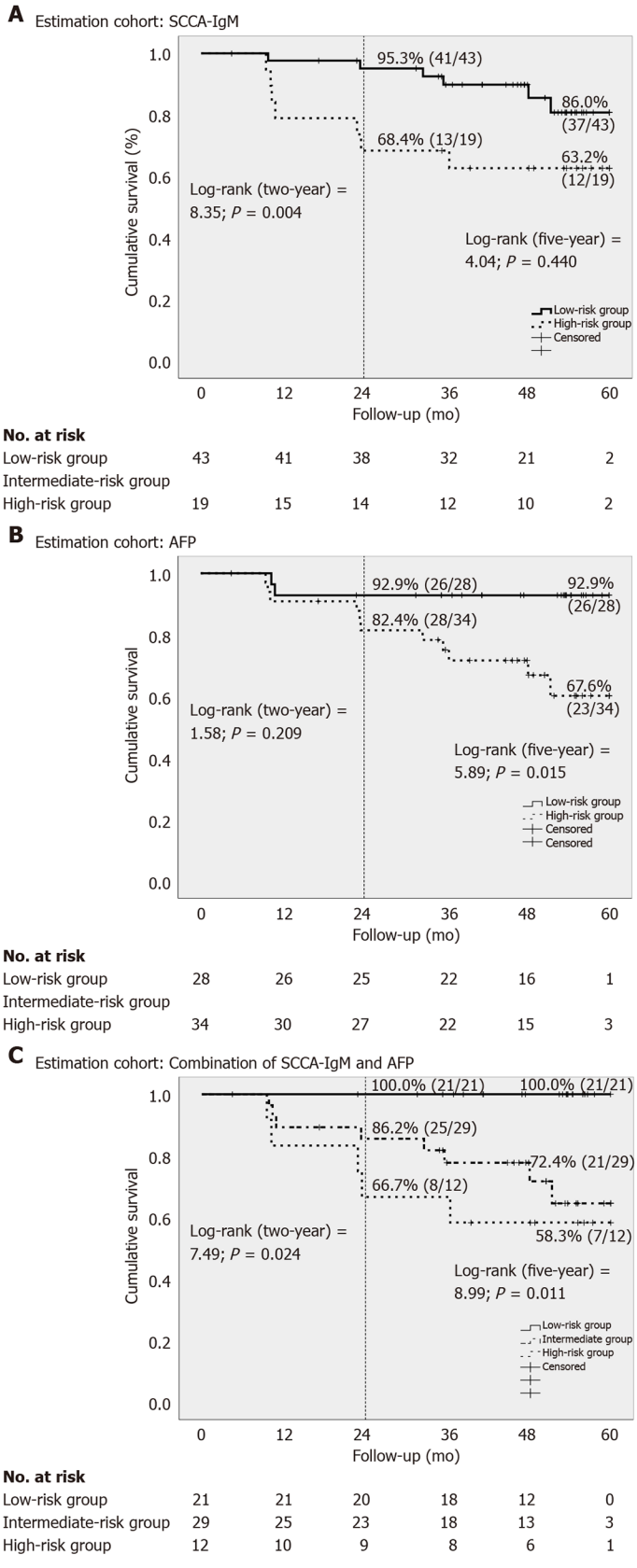

Figure 2 Estimating two- and five-year hepatocellular carcinoma disease-free survival by using Kaplan-Meier method according to the squamous cell carcinoma antigen immunocomplex, alpha-fetoprotein and combination of those in estimation cohort. A: Squamous cell carcinoma antigen immunocomplex (SCCA-IgM); low-risk: < 124 AU/mL, high-risk: > 124 AU/mL; B: Alpha-fetoprotein (AFP); low-risk: < 5 ng/mL, high-risk: > 5 ng/mL; C: Combination of SCCA-IgM and AFP. AFP: Alpha-fetoprotein; SCCA-IgM: Squamous cell carcinoma antigen immunocomplex.

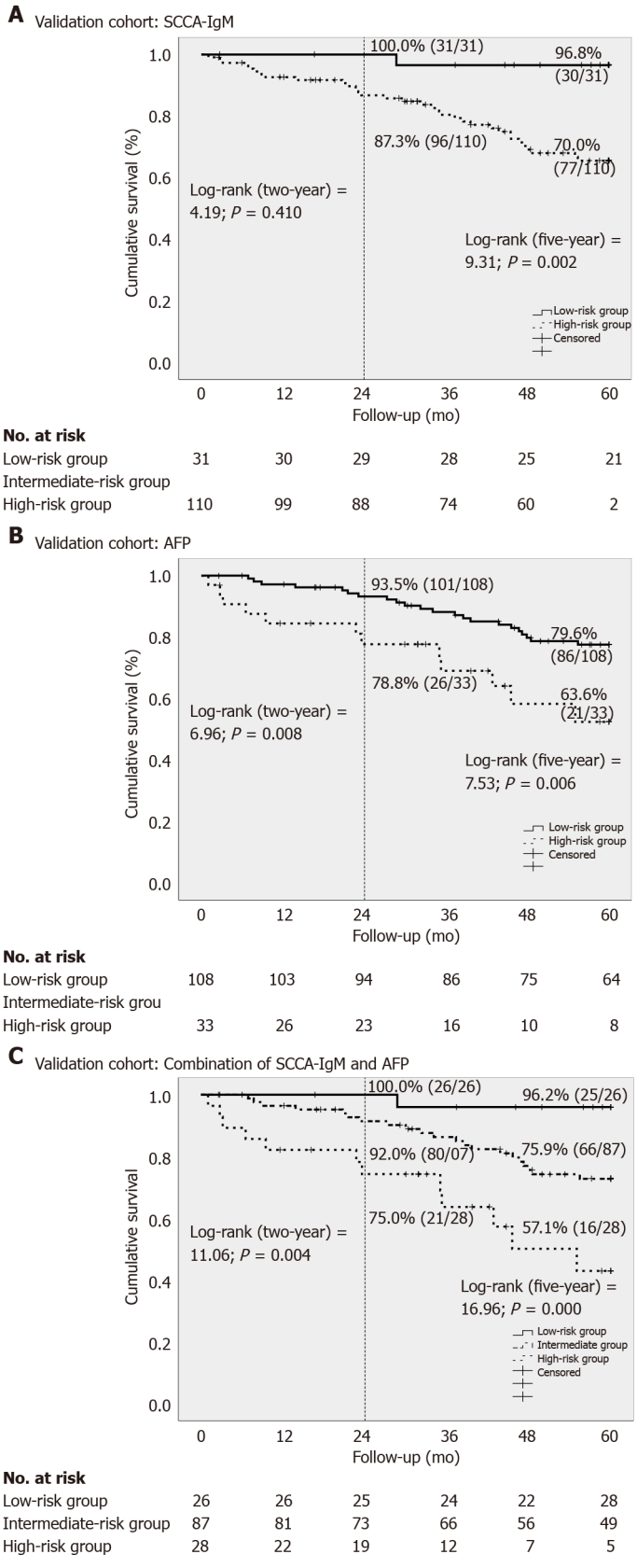

Figure 3 Estimating two- and five-year hepatocellular carcinoma disease-free survival by using Kaplan-Meier method according to the squamous cell carcinoma antigen immunocomplex, alpha-fetoprotein and combination of those both in validation cohort. A: Squamous cell carcinoma antigen immunocomplex (SCCA-IgM); low-risk: < 124 AU/mL, high-risk: > 124 AU/mL; B: Alpha-fetoprotein (AFP); low-risk: < 5 ng/mL, high-risk: > 5 ng/mL; C: Combination of SCCA-IgM and AFP. AFP: Alpha-fetoprotein; SCCA-IgM: Squamous cell carcinoma antigen immunocomplex.

ARTICLE HIGHLIGHTS

Research background

Early diagnosis or prediction of hepatocellular carcinoma (HCC) development would have a major impact on the prognosis of patients under surveillance.

Research motivation

Current screening programs for HCC are far from being satisfactory due to patient- and provider-related factors. Individualizing the program according to the risk of HCC development could be a strategy to overcome these challenges in the era of precision medicine.

Research objectives

This study aimed to evaluate non-invasive biomarkers in the prediction of HCC among patients with cirrhosis.

Research methods

Retrospective cohort study analyzing the association of baseline serum biomarkers with the development of HCC in the mid- and long-term in cirrhotic patients of different etiologies.

Research results

Squamous cell carcinoma antigen immunocomplex (SCCA-IgM) serum levels are associated to the development of HCC at mid- long-term, independently of previously known predictors.

Research conclusions

A predictive model based on the combination of alpha-fetoprotein and SCCA-IgM levels could provide a new HCC screening method, optimizing surveillance for individual patients, especially for cirrhotic patients allocated in the low-risk group.

Research perspectives

Tailored HCC surveillance assessed by non-invasive biomarkers in individual patients would help to better allocate the resources to those patients at higher risk of developing HCC.

REFERENCES

1 **Kulik L**, El-Serag HB. Epidemiology and Management of Hepatocellular Carcinoma. *Gastroenterology* 2019; **156**: 477-491.e1 [PMID: 30367835 DOI: 10.1053/j.gastro.2018.08.065]

2 **Bruix J**, Reig M, Sherman M. Evidence-Based Diagnosis, Staging, and Treatment of Patients With Hepatocellular Carcinoma. *Gastroenterology* 2016; **150**: 835-853 [PMID: 26795574 DOI: 10.1053/j.gastro.2015.12.041]

3 **Serper M**, Taddei TH, Mehta R, D'Addeo K, Dai F, Aytaman A, Baytarian M, Fox R, Hunt K, Goldberg DS, Valderrama A, Kaplan DE; VOCAL Study Group. Association of Provider Specialty and Multidisciplinary Care With Hepatocellular Carcinoma Treatment and Mortality. *Gastroenterology* 2017; **152**: 1954-1964 [PMID: 28283421 DOI: 10.1053/j.gastro.2017.02.040]

4 **Forner A**, Reig M, Bruix J. Hepatocellular carcinoma. *Lancet* 2018; **391**: 1301-1314 [PMID: 29307467 DOI: 10.1016/S0140-6736(18)30010-2]

5 **Vitale A**, Trevisani F, Farinati F, Cillo U. Treatment of Hepatocellular Carcinoma in the Precision Medicine Era: From Treatment Stage Migration to Therapeutic Hierarchy. *Hepatology* 2020; **72**: 2206-2218 [PMID: 32064645 DOI: 10.1002/hep.31187]

6 **Kanwal F**, Singal AG. Surveillance for Hepatocellular Carcinoma: Current Best Practice and Future Direction. *Gastroenterology* 2019; **157**: 54-64 [PMID: 30986389 DOI: 10.1053/j.gastro.2019.02.049]

7 **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* 2018; **69**: 182-236 [PMID: 29628281 DOI: 10.1016/j.jhep.2018.03.019]

8 **Marrero JA**, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, Roberts LR, Heimbach JK. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. *Hepatology* 2018; **68**: 723-750 [PMID: 29624699 DOI: 10.1002/hep.29913]

9 **Fujiwara N**, Liu PH, Athuluri-Divakar SK, Zhu S, Hoshida Y. Risk Factors of Hepatocellular Carcinoma for Precision Personalized Care. 2019 Aug 6. In: Hoshida Y, editor. Hepatocellular Carcinoma: Translational Precision Medicine Approaches [Internet]. Cham (CH): Humana Press,

2019: Chapter 1 [PMID: 32078275 DOI: 10.1007/978-3-030-21540-8_1]
10 **Sharma SA**, Kowgier M, Hansen BE, Brouwer WP, Maan R, Wong D, Shah H, Khalili K, Yim C, Heathcote EJ, Janssen HLA, Sherman M, Hirschfield GM, Feld JJ. Toronto HCC risk index: A validated scoring system to predict 10-year risk of HCC in patients with cirrhosis. *J Hepatol* 2017 [PMID: 28844936 DOI: 10.1016/j.jhep.2017.07.033]
11 **Singal AG**, Mukherjee A, Elmunzer BJ, Higgins PD, Lok AS, Zhu J, Marrero JA, Waljee AK. Machine learning algorithms outperform conventional regression models in predicting development of hepatocellular carcinoma. *Am J Gastroenterol* 2013; **108**: 1723-1730 [PMID: 24169273 DOI: 10.1038/ajg.2013.332]
12 **Fujiwara N**, Friedman SL, Goossens N, Hoshida Y. Risk factors and prevention of hepatocellular carcinoma in the era of precision medicine. *J Hepatol* 2018; **68**: 526-549 [PMID: 28989095 DOI: 10.1016/j.jhep.2017.09.016]
13 **Sun Y**, Sheshadri N, Zong WX. SERPINB3 and B4: From biochemistry to biology. *Semin Cell Dev Biol* 2017; **62**: 170-177 [PMID: 27637160 DOI: 10.1016/j.semcdb.2016.09.005]
14 **Turato C**, Pontisso P. SERPINB3 (serpin peptidase inhibitor, clade B (ovalbumin), member 3). *Atlas Genet Cytogenet Oncol Haematol* 2015; **19**: 202-209 [PMID: 25984243 DOI: 10.4267/2042/56413]
15 **Pontisso P**, Calabrese F, Benvegnù L, Lise M, Belluco C, Ruvoletto MG, Marino M, Valente M, Nitti D, Gatta A, Fassina G. Overexpression of squamous cell carcinoma antigen variants in hepatocellular carcinoma. *Br J Cancer* 2004; **90**: 833-837 [PMID: 14970861 DOI: 10.1038/sj.bjc.6601543]
16 **Pontisso P**, Quarta S, Caberlotto C, Beneduce L, Marino M, Bernardinello E, Tono N, Fassina G, Cavalletto L, Gatta A, Chemello L. Progressive increase of SCCA-IgM immune complexes in cirrhotic patients is associated with development of hepatocellular carcinoma. *Int J Cancer* 2006; **119**: 735-740 [PMID: 16550605 DOI: 10.1002/ijc.21908]
17 **Liu CH**, Gil-Gómez A, Ampuero J, Romero-Gómez M. Diagnostic accuracy of SCCA and SCCA-IgM for hepatocellular carcinoma: A meta-analysis. *Liver Int* 2018; **38**: 1820-1831 [PMID: 29704434 DOI: 10.1111/liv.13867]
18 **Biasiolo A**, Tono N, Ruvoletto M, Quarta S, Turato C, Villano G, Beneduce L, Fassina G, Merkel C, Gatta A, Pontisso P. IgM-linked SerpinB3 and SerpinB4 in sera of patients with chronic liver disease. *PLoS One* 2012; **7**: e40658 [PMID: 22808225 DOI: 10.1371/journal.pone.0040658]
19 **Cagnin M**, Biasiolo A, Martini A, Ruvoletto M, Quarta S, Fasolato S, Angeli P, Fassina G, Pontisso P. Serum Squamous Cell Carcinoma Antigen-Immunoglobulin M complex levels predict survival in patients with cirrhosis. *Sci Rep* 2019; **9**: 20126 [PMID: 31882893 DOI: 10.1038/s41598-019-56633-2]
20 **Martini A**, Fattovich G, Guido M, Bugianesi E, Biasiolo A, Ieluzzi D, Gallotta A, Fassina G, Merkel C, Gatta A, Negro F, Pontisso P. HCV genotype 3 and squamous cell carcinoma antigen (SCCA)-IgM are independently associated with histological features of NASH in HCV-infected patients. *J Viral Hepat* 2015; **22**: 800-808 [PMID: 25611978 DOI: 10.1111/jvh.12394]
21 **Pozzan C**, Cardin R, Piciocchi M, Cazzagon N, Maddalo G, Vanin V, Giacomin A, Pontisso P, Cillo U, Farinati F. Diagnostic and prognostic role of SCCA-IgM serum levels in hepatocellular carcinoma (HCC). *J Gastroenterol Hepatol* 2014; **29**: 1637-1644 [PMID: 24635038 DOI: 10.1111/jgh.12576]
22 **Biasiolo A**, Trotta E, Fasolato S, Ruvoletto M, Martini A, Gallotta A, Fassina G, Angeli P, Gatta A, Pontisso P. Squamous cell carcinoma antigen-IgM is associated with hepatocellular carcinoma in patients with cirrhosis: A prospective study. *Dig Liver Dis* 2016; **48**: 197-202 [PMID: 26614642 DOI: 10.1016/j.dld.2015.10.022]
23 **Toll DB**, Janssen KJ, Vergouwe Y, Moons KG. Validation, updating and impact of clinical prediction rules: a review. *J Clin Epidemiol* 2008; **61**: 1085-1094 [PMID: 19208371 DOI: 10.1016/j.jclinepi.2008.04.008]
24 **El-Serag HB**, Kanwal F, Davila JA, Kramer J, Richardson P. A new laboratory-based algorithm to predict development of hepatocellular carcinoma in patients with hepatitis C and cirrhosis. *Gastroenterology* 2014; **146**: 1249-55.e1 [PMID: 24462733 DOI: 10.1053/j.gastro.2014.01.045]
25 **Gatselis NK**, Tornai T, Shums Z, Zachou K, Saitis A, Gabeta S, Albesa R, Norman GL, Papp M, Dalekos GN. Golgi protein-73: A biomarker for assessing cirrhosis and prognosis of liver disease patients. *World J Gastroenterol* 2020; **26**: 5130-5145 [PMID: 32982114 DOI: 10.3748/wjg.v26.i34.5130]
26 **Song T**, Wang L, Xin R, Zhang L, Tian Y. Evaluation of serum AFP and DCP levels in the diagnosis of early-stage HBV-related HCC under different backgrounds. *J Int Med Res* 2020; **48**: 300060520969087 [PMID: 33135527 DOI: 10.1177/0300060520969087]
27 **Ampuero J**, Romero-Gómez M. Editorial: looking for patients at risk of cirrhosis in the general population-many needles in a haystack. *Aliment Pharmacol Ther* 2018; **47**: 692-694 [PMID: 29417625 DOI: 10.1111/apt.14517]
28 **Singal AG**, Pillai A, Tiro J. Early detection, curative treatment, and survival rates for hepatocellular carcinoma surveillance in patients with cirrhosis: a meta-analysis. *PLoS Med* 2014; **11**: e1001624 [PMID: 24691105 DOI: 10.1371/journal.pmed.1001624]
29 **Moon AM**, Weiss NS, Beste LA, Su F, Ho SB, Jin GY, Lowy E, Berry K, Ioannou GN. No Association Between Screening for Hepatocellular Carcinoma and Reduced Cancer-Related Mortality in Patients With Cirrhosis. *Gastroenterology* 2018; **155**: 1128-1139.e6 [PMID: 29981779 DOI: 10.1053/j.gastro.2018.06.079]
30 **Singal AG**, Murphy CC. Hepatocellular Carcinoma Surveillance: An Effective But Complex Process. *Gastroenterology* 2019; **156**: 1215 [PMID: 30543799 DOI: 10.1053/j.gastro.2018.08.066]

31 **Goossens N**, Singal AG, King LY, Andersson KL, Fuchs BC, Besa C, Taouli B, Chung RT, Hoshida Y. Cost-Effectiveness of Risk Score-Stratified Hepatocellular Carcinoma Screening in Patients with Cirrhosis. *Clin Transl Gastroenterol* 2017; **8**: e101 [PMID: 28640287 DOI: 10.1038/ctg.2017.26]

32 **Di Tommaso L**, Spadaccini M, Donadon M, Personeni N, Elamin A, Aghemo A, Lleo A. Role of liver biopsy in hepatocellular carcinoma. *World J Gastroenterol* 2019; **25**: 6041-6052 [PMID: 31686761 DOI: 10.3748/wjg.v25.i40.6041]

33 **Zhou J**, Sun H, Wang Z, Cong W, Wang J, Zeng M, Zhou W, Bie P, Liu L, Wen T, Han G, Wang M, Liu R, Lu L, Ren Z, Chen M, Zeng Z, Liang P, Liang C, Yan F, Wang W, Ji Y, Yun J, Cai D, Chen Y, Cheng W, Cheng S, Dai C, Guo W, Hua B, Huang X, Jia W, Li Y, Liang J, Liu T, Lv G, Mao Y, Peng T, Ren W, Shi H, Shi G, Tao K, Wang X, Xiang B, Xing B, Xu J, Yang J, Yang Y, Ye S, Yin Z, Zhang B, Zhang L, Zhang S, Zhang T, Zhao Y, Zheng H, Zhu J, Zhu K, Shi Y, Xiao Y, Dai Z, Teng G, Cai J, Cai X, Li Q, Shen F, Qin S, Dong J, Fan J. Guidelines for the Diagnosis and Treatment of Hepatocellular Carcinoma (2019 Edition). *Liver Cancer* 2020; **9**: 682-720 [PMID: 33442540 DOI: 10.1159/000509424]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 December 28; 27(48): 8357-8369

DOI: 10.3748/wjg.v27.i48.8357

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

ORIGINAL ARTICLE

Retrospective Study

New prognostic model for patients with advanced gastric cancer: Fluoropyrimidine/platinum doublet for first-line chemotherapy

Dong-Hoe Koo, Min-Hee Ryu, Mi-Yeon Lee, Mee-Sun Moon, Yoon-Koo Kang

ORCID number: Dong-Hoe Koo 0000-0001-9913-8883; Min-Hee Ryu 0000-0002-1033-1263; Mi-Yeon Lee 0000-0003-2119-9226; Mee-Sun Moon 0000-0003-4613-9713; Yoon-Koo Kang 0000-0003-0783-6583.

Author contributions: Koo DH, Ryu MH, and Kang YK contributed to the conception and design of the study; Kang YK, Ryu MH, and Moon MS collected the data; Koo DH, Lee MY, Kang YK, and Ryu MH performed the analysis; Koo DH, Ryu MH, and Kang YK wrote the paper; all authors contributed to manuscript revision and read and approved the submitted version.

Institutional review board statement: The Institutional Review Board of Asan Medical Center approved the study protocol (2020-0574).

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: No conflicts of interest relevant to this article are reported.

Data sharing statement: The data

Dong-Hoe Koo, Division of Hematology/Oncology, Department of Internal Medicine, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul 03181, South Korea

Min-Hee Ryu, Mee-Sun Moon, Yoon-Koo Kang, Department of Oncology, Asan Medical Center, University of Ulsan College of Medicine, Seoul 05505, South Korea

Mi-Yeon Lee, Division of Biostatistics, Department of R&D Management, Kangbuk Samsung Hospital, Seoul 03181, South Korea

Corresponding author: Yoon-Koo Kang, MD, PhD, Professor, Department of Oncology, Asan Medical Center, University of Ulsan College of Medicine, 88, Olympic-ro 43-gil, Songpa-gu, Seoul 05505, South Korea. ykkang@amc.seoul.kr

Abstract

BACKGROUND

New prognostic factors have been reported in patients with metastatic or recurrent gastric cancer (MRGC), necessitating modifications to the previous prognostic model.

AIM

To develop a new model, MRGC patients who received fluoropyrimidines/platinum doublet chemotherapy between 2008 and 2015 were analyzed.

METHODS

A total of 1883 patients was divided into a training set (n = 937) and an independent validation set (n = 946).

RESULTS

Multivariate analysis showed that the following six factors were associated with poor overall survival (OS) in the training set: Eastern Cooperative Oncology Group performance score ≥ 2 and bone metastasis (2 points each), peritoneal metastasis, high alkaline phosphatase level, low albumin level, and high neutrophil-lymphocyte ratio (1 point each). A prognostic model was developed by stratifying patients into good (0-1 point), moderate (2-3 points), and poor (≥ 4 points) risk groups. In the validation set, the median OS of the three risk groups was 15.8, 10.1, and 5.7 mo, respectively, and those differences were significant (P < 0.001).

CONCLUSION

presented in this study are available on request from the corresponding author. The data are not publicly available due to a privacy issue from the patients.

Country/Territory of origin: South Korea

Specialty type: Oncology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: July 12, 2021
Peer-review started: July 12, 2021
First decision: October 3, 2021
Revised: October 9, 2021
Accepted: November 30, 2021
Article in press: November 30, 2021
Published online: December 28, 2021

P-Reviewer: Endo S, Moradi L
S-Editor: Yan JP
L-Editor: A
P-Editor: Yan JP

We identified six factors readily measured in clinical practice that are predictive of poor prognosis in patients with MRGC. The new model is simpler than the old and more easily predicts OS.

Key Words: Stomach neoplasms; Chemotherapy; Prognosis; Validation; Gastric cancer

Core Tip: A new prognostic model for patients with metastatic or recurrent gastric cancer was developed using six clinicopathological elements (poor Eastern Cooperative Oncology Group performance score, bone metastasis, peritoneal metastasis, high alkaline phosphatase level, low albumin level, and high neutrophil-lymphocyte ratio).

Citation: Koo DH, Ryu MH, Lee MY, Moon MS, Kang YK. New prognostic model for patients with advanced gastric cancer: Fluoropyrimidine/platinum doublet for first-line chemotherapy. *World J Gastroenterol* 2021; 27(48): 8357-8369
URL: https://www.wjgnet.com/1007-9327/full/v27/i48/8357.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i48.8357

INTRODUCTION

Gastric cancer is one of the most common causes of cancer-related mortality worldwide and the fifth-ranked cancer in terms of associated mortality in Korea[1,2]. When gastric cancer is diagnosed at an advanced stage or in recurrent status, systemic therapy is considered the primary treatment; however, its outcome often is unsatisfactory[1,3].

Many novel agents that inhibit several pathways, combination strategies, and strict patient selection criteria are being evaluated in clinical trials to improve patient response to systemic therapies and to achieve better clinical outcomes[4]. It is necessary to allocate evenly patients with similar clinical characteristics and expected survival times to derive reliable results from clinical trials. Therefore, many investigators have attempted to develop prognostic models to predict accurate overall survival (OS). Nonetheless, existing prognostic models have certain limitations, such as lack of validation[5] or enrolling patients who do not represent patients in real practice[6]. In addition, some patients were included regardless of type of chemotherapy (*e.g.*, single, doublet, or triplet chemotherapy with/without trastuzumab)[7].

Systemic chemotherapy for metastatic or recurrent gastric cancer (MRGC) has undergone significant changes in terms of standard treatment. Although various kinds of drugs have been trialed for use as first-line chemotherapy[8], the fluoropyrimidines plus platinum combination doublet has become the standard of care[9]. Second-line chemotherapy has emerged as another standard treatment[10]. The use of immuno-oncology agents has been accepted as a standard of care during third-line treatment and is emerging as a standard of care in the first-line setting based on positive results [11,12]. Furthermore, the use of human epidermal growth factor receptor 2 (HER2)-targeted therapies in select patients has shown excellent therapeutic efficacy and prolonged survival[13,14]. Overall, patient prognosis varies according to type of treatment[9]. Therefore, prognostic factors should be investigated in each treatment group, particularly patients who receive fluoropyrimidine/platinum doublet chemotherapy, which is considered the standard first-line treatment for HER2-negative MRGC.

Early in the 2000s, we developed a prognostic model for MRGC[7]. That model used a scoring system with eight prognostic factors [Eastern Cooperative Oncology Group (ECOG) performance score (PS) ≥ 2, bone metastasis (2 points each), no gastrectomy, peritoneal metastasis, lung metastasis, alkaline phosphatase (ALP) > 120 IU/L, albumin < 3.3 g/dL, and total bilirubin > 1.2 mg/dL (1 point each)], and patients were divided into good (0-1 point), moderate (2-3 points), and poor (≥ 4 points) risk groups. However, those factors were identified when few active chemotherapeutic agents were

available and no standard chemotherapy had been established. Furthermore, those eight factors might need to be reduced to enable easier prognostic model application in clinical practice.

The neutrophil-lymphocyte ratio (NLR) is a representative blood marker of the systemic inflammatory response that reflects tumor progression, invasion, and metastasis in cancer patients[15]. The NLR is a relatively new prognostic factor that has been applied to several solid tumors[16]. Recent studies have demonstrated a close relationship between NLR status and poor prognosis in MRGC; even NLR changes during immuno-oncologic therapy can predict poor outcomes[17]. In addition, a recent meta-analysis reported that histologic type was a significant variable for OS in the first-line treatment setting[18].

Therefore, we modified our previous prognostic model by introducing NLR and histology using a cohort of MRGC patients who received first-line fluoropyrimidine/platinum doublet chemotherapy, and we validated our new model in a different cohort.

MATERIALS AND METHODS

Patients and data collection

We previously reported trends in chemotherapy patterns and survival in MRGC patients during the 16 years from 2000-2015, separated into four-year intervals[9]. During the last two of those intervals (2008-2015), more than 60% of MRGC patients received doublet treatment, and more than 55% underwent second-/third-line anticancer therapies. We developed our new model from those recent cohorts. The Stomach Cancer Registry was examined to identify all patients who received first-line palliative chemotherapy for advanced gastric cancer at Asan Medical Center (Seoul, South Korea) between January 2008 and December 2015. Patients aged 18 years or older with histologically confirmed adenocarcinoma of the stomach who received at least one palliative chemotherapy cycle were included. Patients were excluded if they received treatment other than doublet chemotherapy (such as single, triplet, or doublet with trastuzumab) or a novel agent in clinical trials, if they had a history of other malignancies, if they started first-line chemotherapy at another hospital, or if they underwent R1 resection for microscopic residual tumors just before chemotherapy. Of the 2931 patients screened, 1883 met our criteria. Patients' medical records, stored in a prospectively collected registry, were reviewed for demographic data, tumor characteristics, treatment types, treatment responses, and survival. Patients were followed until the date of death or cessation of follow-up in October 2018. The Institutional Review Board of Asan Medical Center approved the study protocol (2020-0574). Our analysis was a retrospective design using fully anonymized data, so the IRB waived the requirement for informed consent.

Statistical analysis

Model development and validation were based on a split-sample method according to time period. During the last four-year period (2012-2015), trastuzumab in HER2-positive MRGC had been accepted as a standard of care in Korea, and ramucirumab and immunotherapy had been introduced as second-/third-line anticancer therapies. Therefore, study participants were separated by treatment period and assigned to a training set (2012-2015; n = 937) or an independent validation set (2008-2011; n = 946). The prognostic model was developed using the training set. OS was measured from the date of first-line chemotherapy until death from any cause. Progression-free survival (PFS) was measured from the date of first-line chemotherapy until tumor progression or death from any cause other than the cancer. The Kaplan–Meier method was used to estimate OS and PFS. Laboratory variables were dichotomized, using the normal value for each as the cutoff point, and survival rates were compared using the log-rank test. NLR was defined as the neutrophil count divided by the lymphocyte count. The sensitivity and specificity values of NLR were evaluated in the training set using receiver operating characteristic (ROC) curve analysis [area under the ROC curve (AUC): 0.651; 95% confidence interval (CI): 0.60-0.71]. The optimal value of NLR was 3.11 (sensitivity: 41.2%; specificity: 83.1%) according to Youden's J statistic. We selected 3.0 as the cutoff value, which had sensitivity and specificity values of 42.6% and 80.9%, respectively, for all further analyses (Supplementary Figure 1). We developed a new prognostic model by adding and deleting variables from our previous model, analyzing those variables through univariate analyses, and performing multivariate analysis using a Cox proportional hazards regression model.

A risk score based on the hazard ratio (HR) was developed from the final multivariate model and validated using the validation set. A nomogram to predict OS probability was established in the training set, and its calibration was accomplished by comparing the predicted and observed probabilities. The prediction accuracy of the old and new prognostic models was compared using Harrell's C-index; an ROC curve analysis; and a decision curve analysis (DCA), which is a method for evaluating prognostic strategies that can visualize the clinical effectiveness of a prediction model[19]. A two-sided P value < 0.05 was considered statistically significant, and 95%CIs were calculated. All statistical analyses were performed using R language (R Core Team, R Foundation for Statistical Computing, Vienna, Austria) and the Statistical Package for the Social Sciences version 25.0 (IBM Corporation, Armonk, NY, United States).

RESULTS

Patient baseline characteristics

A total of 1883 patients received palliative doublet chemotherapy as first-line treatment for MRGC between 2008 and 2015. Overall, 1746 patients (92.7%) died, and the median survival time was 11.9 mo (95%CI: 11.3-12.5). The median follow-up duration of the 137 surviving patients was 54.6 mo (interquartile range: 35.7-84.3 mo). When we compared patient characteristics between training and validation sets, proportion of men, histology findings, and occurrence of liver metastasis differed significantly between the two groups (Table 1).

Development of a new prognostic model and nomogram

In the training set of 937 patients, 848 (90.5%) died. Univariate analyses for OS were performed for NLR (≥ 3 *vs* < 3), histologic type (poorly differentiated/signet-ring cell/undifferentiated *vs* well or moderately differentiated), and the eight factors in the previous model. A high NLR was statistically significant in the training set, but poor histology, no prior gastrectomy, lung metastases, and high total bilirubin were not. Multivariate analysis confirmed that six factors were significantly associated with poor OS (Table 2): Poor ECOG PS, peritoneal metastasis, bone metastasis, high ALP level, low albumin level, and high NLR. Risk scores were assigned based on HRs from the final multivariate model, with two points awarded for HR > 1.5 and one point awarded for HR < 1.5. Based on the resulting scores, patients were assigned to three risk categories: good (0-1 point), moderate (2-3 points), and poor (≥ 4 points). The C-index for the new model was 0.657 (95%CI: 0.637-0.677). In addition, we built a nomogram using those six factors to establish a more convenient and accurate method for survival prediction and used calibration plots to verify it (Figure 1).

Validation and comparison of survival prediction with the previous model

We validated the new model using a separate validation set of 946 patients (2008-2011). Among them, 898 patients (94.9%) died. The proportions of patients classified into each risk category were similar. The observed OS and PFS curves in patients in each risk category showed significant differences in both the training and validation sets (P < 0.001, log-rank test) (Table 3 and Figure 2). The old prognostic model using eight factors also had significantly different OS and PFS outcomes in each risk category. When we compared the OS predictions of the new and old models using the validation set, the C-indexes of the two models were similar [0.638 (95%CI: 0.618-0.658) and 0.635 (95%CI: 0.615-0.655), respectively]. DCA and ROC curve analyses were performed to compare the prediction accuracies of each of the six prognostic factors and the old and new models. The DCA curve showed that the old and new models both had stronger predictive accuracy than the individual prognostic factors, and the performance of the two models was similar (Figure 3). The ROC curve analysis also showed that the two models had similar AUCs at one year [0.598 (95%CI: 0.581-0.617) and 0.600 (95%CI: 0.582-0.620), respectively]. Interestingly, NLR had the largest AUC at one year (0.567; 95%CI: 0.552-0.582) among the six prognostic factors. Although the explanatory power of the two models did not differ, the new model uses two fewer factors and might be more feasible for use in clinical trials or real practice.

Risk group reclassification in the new model

When we compared how the new and old models assigned the patients in the validation set to risk groups, we found that most patients were classified similarly (Supplementary Table 1). However, 35% of the moderate risk group in the old model

Table 1 Patient characteristics during first-line doublet chemotherapy according to treatment period

Clinical characteristics	Training set (2012-2015), *n* = 937	Validation set (2008-2011), *n* = 946	*P* value
Sex, male, *n* (%)	583 (62.2)	637 (67.3)	0.020
Age			
Median, range	56 (19-91)	57 (20-85)	0.785
≥ 65 yr, *n* (%)	257 (27.4)	259 (27.4)	0.981
ECOG PS, *n* (%)			
0/1	799 (85.6)	817 (86.6)	0.531
2/3	134 (14.4)	126 (13.4)	
Prior gastrectomy performed	389 (41.5)	412 (43.6)	0.372
Histology, *n* (%)			
WD/MD	212 (22.6)	256 (27.1)	< 0.001
PD/SRC/undifferentiated	691 (73.7)	590 (62.4)	
Unclassified	34 (3.6)	100 (10.6)	
Status, *n* (%)			
Recurrent	318 (33.9)	334 (35.3)	0.533
Initial metastatic	619 (66.1)	612 (64.7)	
Metastasis No., 2 or more	385 (41.5)	363 (38.9)	0.249
Peritoneal metastasis	518 (55.6)	524 (55.9)	0.902
Liver metastasis	160 (17.2)	226 (24.1)	< 0.001
Lung metastasis	45 (4.9)	43 (4.6)	0.795
PALN metastasis	346 (37.3)	352 (37.5)	0.942
Bone metastasis	93 (10.0)	70 (7.5)	0.051
ALP > 120 IU/L, *n* (%)	201 (21.5)	197 (21.2)	0.868
Albumin < 3.3 g/dL, *n* (%)	279 (29.8)	249 (26.8)	0.150
Total bilirubin > 1.2 mg/dL, *n* (%)	62 (6.6)	77 (8.3)	0.177
NLR ≥ 3, *n* (%)	381 (40.7)	375 (40.3)	0.881

ECOG PS: Eastern Cooperative Oncology Group performance status; WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated; SRC: Signet ring cell; PALN: Para-aortic lymph node; ALP: Alkaline phosphatase; NLR: Neutrophil-lymphocyte ratio.

(15% of the total patients) was reclassified into the good risk group in the new model, and the median predicted OS of those patients increased to 14.1 mo from 10.6 mo (Supplementary Figure 2).

DISCUSSION

This study evaluated several clinicopathological factors associated with the prognosis of patients with MRGC. We developed a new prognostic model using six clinicopathological elements with a nomogram in a training set and validated its appropriateness using C-index, DCA, and ROC curve analyses in a different cohort. The six factors were poor ECOG PS, bone metastasis, peritoneal metastasis, high ALP level, low albumin level, and high NLR. Combining those factors into a simple prognostic model enabled MRGC patients to be classified into three risk groups. Our old and new models showed similar prediction performance in the validation set; however, the new model is simpler and easier to apply than the old because it uses two fewer factors.

Doublet first-line chemotherapy as a standard of care

Previous prognostic models were developed based on heterogeneous treatment

Table 2 Comparison between the old model and new model developed from the training set

Factors	Old model	Univariate analysis		Multivariate analysis		New model
	Score	HR	*P* value	HR	*P* value	Score
Poor PS	2	1.983	< 0.001	2.005	< 0.001	2
No gastrectomy	1	1.046	0.542	-	-	-
Peritoneal metastasis	1	1.355	< 0.001	1.355	< 0.001	1
Bone metastasis	2	1.605	< 0.001	1.651	< 0.001	2
Lung metastasis	1	1.249	0.188	-	-	-
High ALP	1	1.435	< 0.001	1.406	< 0.001	1
Low albumin	1	1.410	< 0.001	1.447	< 0.001	1
High total bilirubin	1	0.965	0.806	-	-	-
High NLR	-	1.445	< 0.001	1.461	< 0.001	1
Poor histology	-	1.104	0.253	-	-	-

HR: Hazard ratio; poor PS: Performance status 2/3; high ALP: Alkaline phosphatase > 120 IU/L; low albumin: Albumin < 3.3 g/dL; high NLR: Neutrophil-lymphocyte ratio ≥ 3.

groups, but first-line fluoropyrimidine/platinum doublet chemotherapy has become a standard of care. Although 5-fluorouracil (5-FU) is one of the cytotoxic agents most commonly used for MRGC, randomized phase III studies have demonstrated that the oral fluoropyrimidines capecitabine[20] and S-1[21] are just as effective. Therefore, oral fluoropyrimidines (capecitabine or S-1) could be used instead of 5-FU in therapeutic combination with platinum compounds. Also, oxaliplatin-based regimens were suggested to be noninferior to cisplatin-based regimens in terms of OS in the REAL-2 study[22]. Further randomized trials have suggested that oxaliplatin is as effective for prolonging survival and generally better tolerated than cisplatin[23]. Cisplatin-free regimens in combination with oral fluoropyrimidine could offer more convenience by preventing hyperhydration, central catheterization, and hospitalization. On the other hand, triplet chemotherapy, which includes taxane to maximize efficacy, carries a limited survival benefit and increases the risk of grade 3/4 toxicities[24]. Patients treated with a single agent, either fluoropyrimidine or taxane, were considered to be intolerant of combination chemotherapy or to have recurrent disease resistant to prior adjuvant chemotherapy with fluoropyrimidine ± platinum; therefore, those patients receive less frequent subsequent chemotherapy, resulting in poor prognosis[9]. Prognostic factor analyses should be performed in patients receiving the same treatment course because the prognosis varies according to first-line chemotherapy regimen.

NLR as a new prognostic factor

High NLR status, a well-known biomarker of cancer-associated inflammation, has shown a significant correlation with poor prognosis in many solid tumors[16]. NLR can be considered a surrogate of the balance between activation of the protumor inflammatory pathway and antitumor immune function.

Neutrophilia increases the number of inflammatory markers, including proangiogenic factors such as vascular endothelial growth factor, growth factors such as interleukin-8, proteases such as tissue inhibitors of metalloproteinase, and antiapoptotic markers such as nuclear factor kappa B, that support tumor growth and progression[25]. Lymphopenia represents a significant decline in the cell-mediated immune system, which is demonstrated by marked decrease in T4 helper and T8 suppressor lymphocytes. Although no exact NLR cutoff point has been defined, we chose an NLR cutoff value of 3.0 based on our ROC curve analysis. The patients in the validation set who had high NLRs had significantly worse OS and PFS (median: 8.4 and 4.8 mo) than those with low NLRs (14.4 and 6.9 mo; $P < 0.001$) (Supplementary Figure 3). NLR status might be a key factor in predicting the survival outcomes of MRGC patients because it is a surrogate of immune status and is convenient, inexpensive, and reproducible in practice. It also might help clinicians discern when to expect a response to further chemotherapy and immunotherapy in patients with MRGC[26].

Table 3 Survival outcomes of first-line doublet regimens in the training and validation sets according to the new prognostic model

Risk group	Good risk, 0-1 point(s)	Moderate risk, 2-3 points	Poor risk, ≥ 4 points	*P* value
Training set (2012-2015)				
No. of patients	449 (48.8%)	319 (34.7%)	152 (16.5%)	
Hazard ratio (95%CI)	Reference	1.628 (1.40-1.90)	4.013 (3.30-4.88)	< 0.001
Median OS, mo (95%CI)	15.9 (14.5-17.4)	10.6 (9.3-11.9)	4.7 (4.0-5.5)	< 0.001
Median PFS, mo (95%CI)	8.3 (7.4-9.1)	5.9 (5.1-6.6)	2.4 (1.8-2.9)	< 0.001
Survival rate (%)				
At 6 mo	90.0% (87.2-92.8)	74.0% (69.2-78.8)	37.5% (29.8-45.2)	
At 12 mo	63.2% (58.7-67.7)	44.0% (38.6-49.4)	16.1% (10.3-21.9)	
At 18 mo	42.9% (38.3-47.5)	23.4% (18.8-28.0)	6.3% (2.4-10.2)	
At 24 mo	31.2% (26.9-35.5)	16.4% (12.3-20.5)	2.8% (0.2-5.4)	
Validation set (2008-2011)				
No. of patients	474 (52.0%)	291 (31.9%)	147 (16.1%)	
Hazard ratio (95%CI)	Reference	1.634 (1.41-1.90)	2.963 (2.45-3.59)	< 0.001
Median OS, mo (95%CI)	15.8 (14.8-16.9)	10.1 (8.7-11.5)	5.7 (4.7-6.6)	< 0.001
Median PFS, mo (95%CI)	7.0 (6.3-7.7)	5.6 (5.1-6.1)	3.2 (2.5-3.9)	< 0.001
Survival rate (%)				
At 6 mo	88.6% (85.7-91.5)	72.2% (67.1-77.3)	47.6% (39.6-55.7)	
At 12 mo	64.3% (60.0-68.6)	42.3% (36.6-48.0)	17.0% (10.9-23.1)	
At 18 mo	40.1% (35.7-44.5)	22.0% (17.2-26.8)	6.1% (2.2-10.0)	
At 24 mo	25.9% (22.0-29.8)	13.1% (9.2-17.0)	4.8% (1.3-8.3)	
Validation set (2008-2011) according to old model				
No. of patients	393 (41.7%)	390 (41.4%)	160 (16.9%)	
Hazard ratio (95%CI)	Reference	1.493 (1.29-1.73)	3.281 (2.71-3.98)	< 0.001
Median OS, mo (95%CI)	16.2 (15.3-17.1)	10.7 (9.5-12.0)	5.5 (4.5-6.5)	< 0.001
Median PFS, mo (95%CI)	7.1 (6.3-7.9)	5.6 (5.1-6.2)	3.3 (2.5-4.0)	< 0.001
Survival rate (%)				
At 6 mo	90.3% (87.4-93.2)	75.8% (71.5-80.1)	47.5% (39.8-55.2)	
At 12 mo	68.2% (63.6-72.8)	45.5% (40.6-50.4)	16.3% (10.6-22.0)	
At 18 mo	41.3% (36.4-46.2)	26.5% (22.1-30.9)	5.6% (2.0-9.2)	
At 24 mo	27.4% (23.0-31.8)	17.0% (13.3-20.7)	3.1% (0.4-5.8)	

OS: Overall survival; PFS: Progression-free survival; CI: Confidence interval.

Prior gastrectomy as an unneeded prognostic factor

Gastrectomy in this study refers to upfront gastrectomy performed before first-line chemotherapy or prior gastrectomy before recurrence. Several retrospective studies have reported that primary tumor resection in advanced gastric cancer could lessen the tumor burden, or so-called resected metastatic status, and result in a survival benefit[27]. However, most of those studies included patients treated in the early 2000s, when active chemotherapeutic agents were limited, and sequential chemotherapy was not established. Also, most included patients underwent both upfront gastrectomy and conversion surgery after palliative chemotherapy. In a recent prospective randomized study (the REGATTA trial), incurable gastrectomy before chemotherapy failed to show a survival benefit, and so it is no longer recommended [28]. A retrospective comparison study between an initially metastatic group and a recurrent metastatic group reported that prior gastrectomy did not affect prognosis

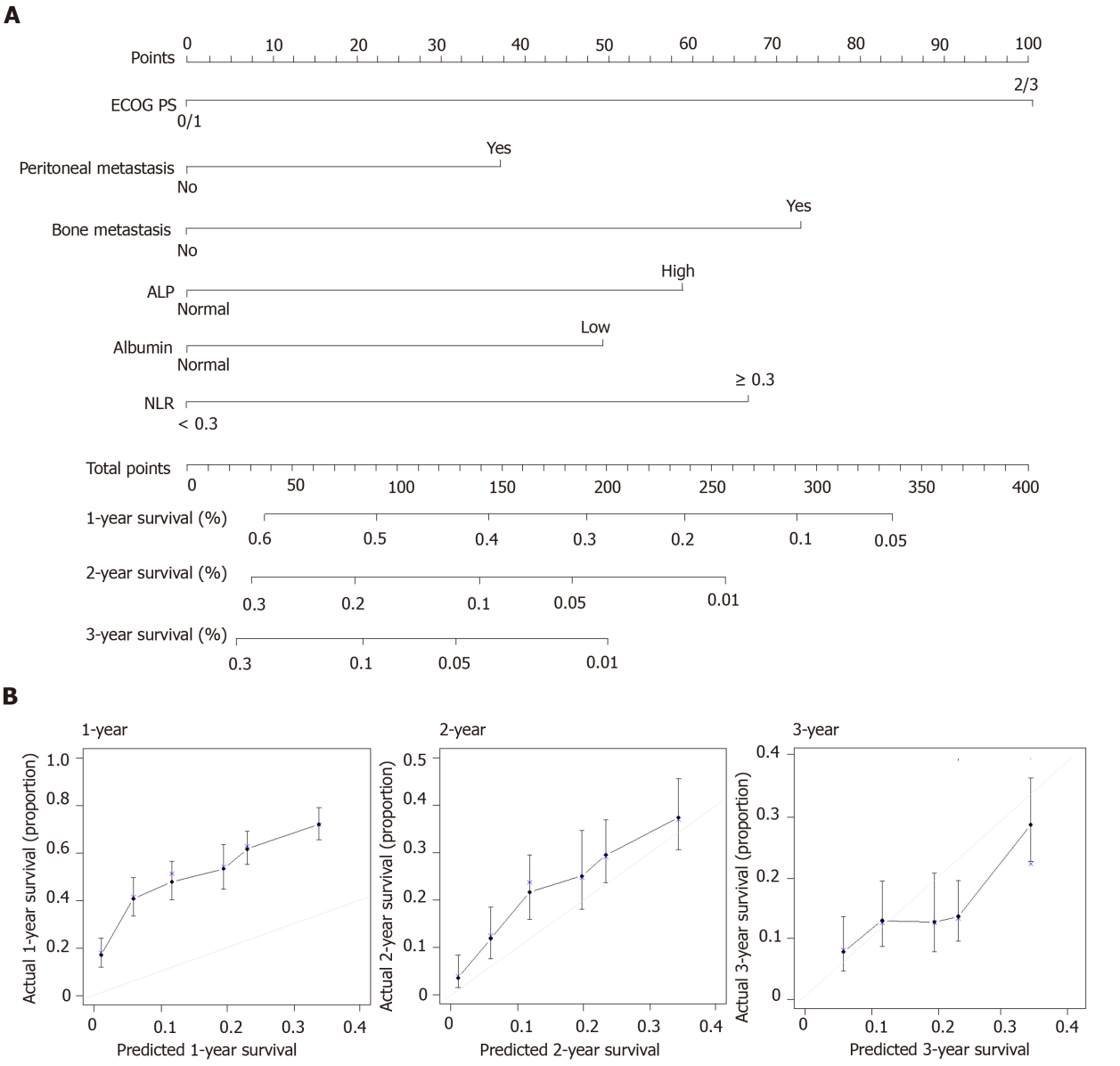

Figure 1 The nomogram using six factors to predict survival rates in the training set. A: The nomogram was applied by summing the scores projected onto the corresponding scale for each factor. The total number of scores projected onto the bottom scale represents the probability of one-, two-, and three-year overall survival; B: The calibration plots of the nomogram, where the X-axis represents the survival rate predicted by the nomogram, and the Y-axis represents the actual survival rate calculated by a Kaplan–Meier analysis. ALP: Alkaline phosphatase; NLR: Neutrophil-lymphocyte ratio; ECOG PS: Eastern Cooperative Oncology Group performance score.

[29]. Because our old model was developed from a cohort treated in the early 2000s, prior gastrectomy might have been a significant favorable prognostic factor. In this study, however, patients in the training set received chemotherapy between 2012 and 2015, when many more active chemotherapeutic agents were available. Therefore, prior gastrectomy would not be expected to significantly affect the prognosis of those patients.

Advantages of the new prognostic model

The new model described herein has several advantages. First, it was derived by analyzing a homogeneous population treated with recent doublet first-line chemotherapy. Second, prognostic factors such as bone metastasis, which are difficult to obtain from electronic medical records, were evaluated based on clinical data sourced from a prospectively collected registry. Third, we validated our new model in a separate cohort of about 1000 patients and found that its performance was as good in the validation set as it was in the training set.

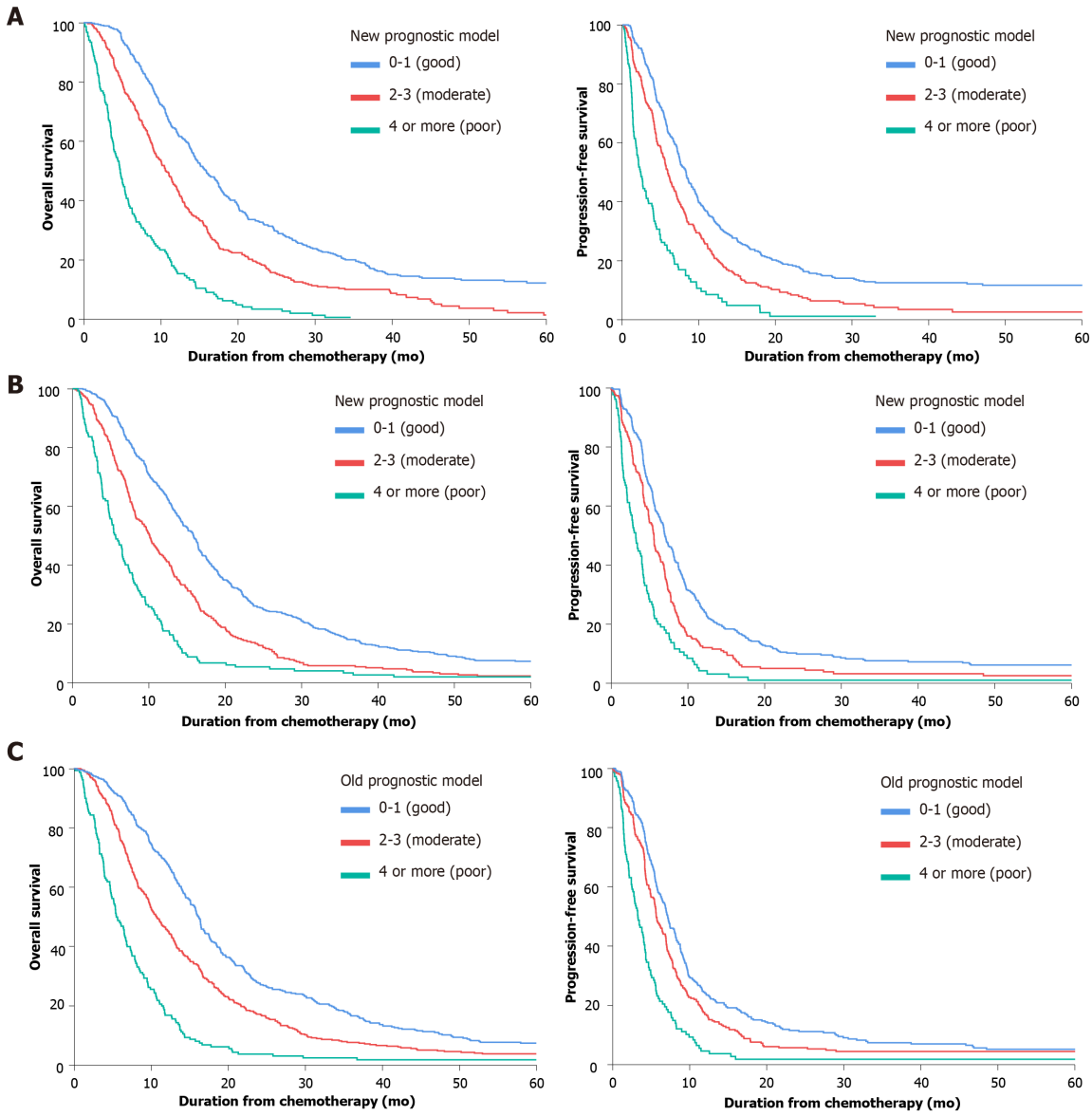

Figure 2 Overall survival and progression-free survival curves according to the new prognostic model. A: In the training set; B: In the validation set; C: According to the old prognostic model in the validation set.

Limitations of this study

Our study also has several limitations. First, despite a large number of patients, the generalizability of this study is limited by its single-center, retrospective design and the single ethnicity of its population. Second, our new prognostic model does not apply to patients who received treatment other than doublet chemotherapy, such as single, triplet, or doublet with trastuzumab. Third, this study does not include other critical factors that affect treatment or prognosis, such as molecular biomarkers.

CONCLUSION

In conclusion, we identified six factors readily measured in clinical practice and predictive of poor prognosis in patients with MRGC. Our new prognostic model uses a scoring system that incorporates those six factors and could be used to classify patients into three groups with significantly different survival outcomes. This model performed well with a validation set and could help to predict life expectancy, guide treatment plans, analyze the findings of clinical studies, and support the design of future clinical trials.

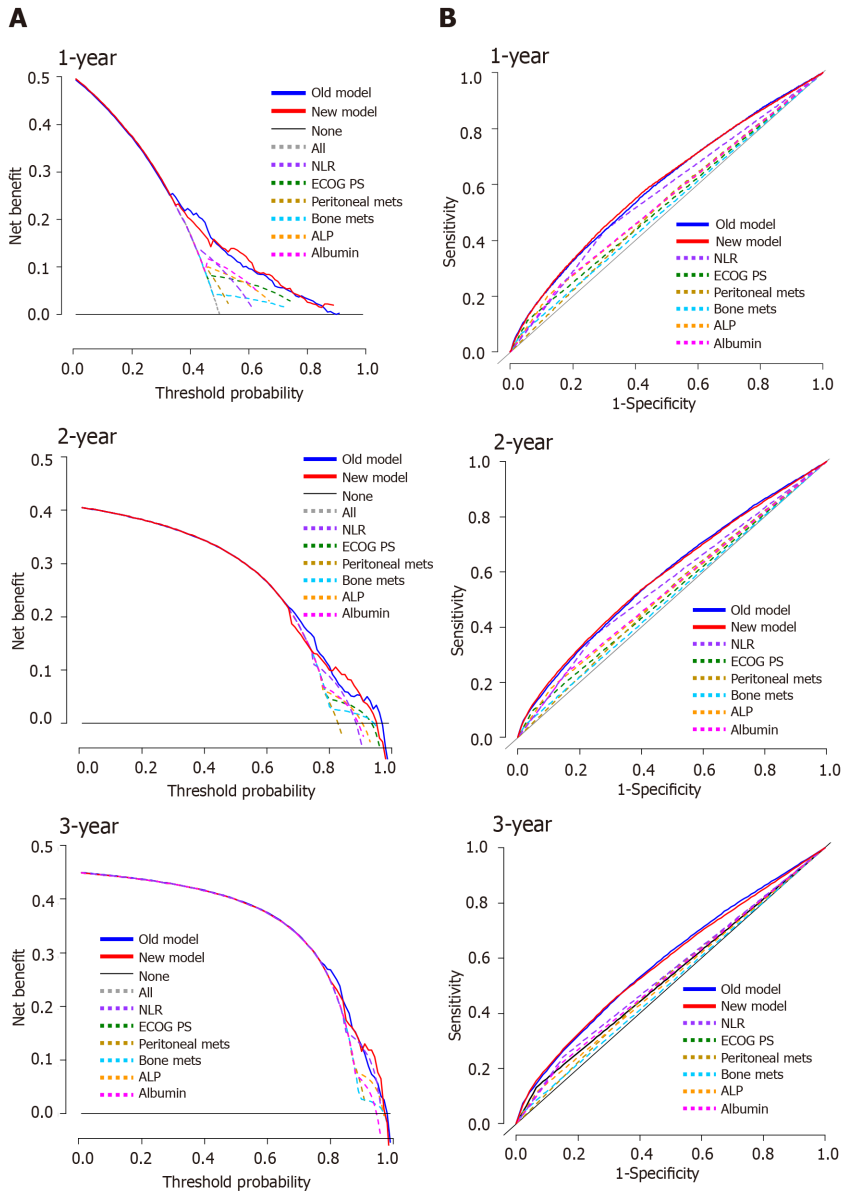

Figure 3 Decision curve analysis curves and time-dependent receiver operating characteristic curves for the nomogram in the validation set. A: The calculated net benefit (Y-axis) corresponds to the threshold probability of survival on the X-axis; B: The time-dependent receiver operating characteristic curve assesses the accuracy of the nomogram. ALP: Alkaline phosphatase; NLR: Neutrophil-lymphocyte ratio; ECOG PS: Eastern Cooperative Oncology Group performance score.

ARTICLE HIGHLIGHTS

Research background

Since systemic chemotherapy for metastatic or recurrent gastric cancer (MRGC) has become standardized, prognostic factors for MRGC patients should be investigated in patients who receive fluoropyrimidine/platinum doublet chemotherapy, which is considered the standard first-line treatment for human epidermal growth factor receptor 2-negative MRGC.

Research motivation

The neutrophil-lymphocyte ratio (NLR) is a representative blood marker of the systemic inflammatory response that reflects tumor progression, invasion, and metastasis in cancer patients. This is a relatively new prognostic factor in MRGC, and its change was reported to predict poor outcomes during immuno-oncologic therapy.

Research objectives

We modified our previous prognostic model by introducing NLR and histology using a cohort of MRGC patients, and we validated our new model in a different cohort.

Research methods

Model development and validation were based on a split-sample method according to time period. Patients were separated by treatment period and assigned to a training set (2012-2015; n = 937) or an independent validation set (2008-2011; n = 946). The prognostic model was developed using the training set.

Research results

Multivariate analysis confirmed that six factors were significantly associated with poor overall survival as follow: poor performance, peritoneal metastasis, bone metastasis, high alkaline phosphatase level, low albumin level, and high NLR. The observed overall survival and progression-free survival curves in patients in each risk category showed significant differences in both the training and validation sets (P < 0.001, log-rank test).

Research conclusions

We identified six factors readily measured in clinical practice and predictive of poor prognosis in patients with MRGC. Our new prognostic model uses a scoring system that incorporates those six factors and could be used to classify patients into three groups with significantly different survival outcomes.

Research perspectives

Our model could help to predict life expectancy, guide treatment plans, analyze the findings of clinical studies, and support the design of future clinical trials in MRGC patients.

REFERENCES

1 **Van Cutsem E**, Sagaert X, Topal B, Haustermans K, Prenen H. Gastric cancer. *Lancet* 2016; **388**: 2654-2664 [PMID: 27156933 DOI: 10.1016/S0140-6736(16)30354-3]

2 **Jung KW**, Won YJ, Hong S, Kong HJ, Lee ES. Prediction of Cancer Incidence and Mortality in Korea, 2020. *Cancer Res Treat* 2020; **52**: 351-358 [PMID: 32178488 DOI: 10.4143/crt.2020.203]

3 **Guideline Committee of the Korean Gastric Cancer Association (KGCA)**; Development Working Group & Review Panel. . Korean Practice Guideline for Gastric Cancer 2018: an Evidence-based, Multi-disciplinary Approach. *J Gastric Cancer* 2019; **19**: 1-48 [PMID: 30944757 DOI: 10.5230/jgc.2019.19.e8]

4 **Arai H**, Nakajima TE. Recent Developments of Systemic Chemotherapy for Gastric Cancer. *Cancers (Basel)* 2020; **12** [PMID: 32354119 DOI: 10.3390/cancers12051100]

5 **Lee J**, Lim T, Uhm JE, Park KW, Park SH, Lee SC, Park JO, Park YS, Lim HY, Sohn TS, Noh JH, Heo JS, Park CK, Kim S, Kang WK. Prognostic model to predict survival following first-line chemotherapy in patients with metastatic gastric adenocarcinoma. *Ann Oncol* 2007; **18**: 886-891 [PMID: 17298958 DOI: 10.1093/annonc/mdl501]

6 **Takahari D**, Boku N, Mizusawa J, Takashima A, Yamada Y, Yoshino T, Yamazaki K, Koizumi W, Fukase K, Yamaguchi K, Goto M, Nishina T, Tamura T, Tsuji A, Ohtsu A. Determination of prognostic factors in Japanese patients with advanced gastric cancer using the data from a randomized controlled trial, Japan clinical oncology group 9912. *Oncologist* 2014; **19**: 358-366 [PMID: 24668328 DOI: 10.1634/theoncologist.2013-0306]

7 **Koo DH**, Ryoo BY, Kim HJ, Ryu MH, Lee SS, Moon JH, Chang HM, Lee JL, Kim TW, Kang YK. A prognostic model in patients who receive chemotherapy for metastatic or recurrent gastric cancer: validation and comparison with previous models. *Cancer Chemother Pharmacol* 2011; **68**: 913-921 [PMID: 21290247 DOI: 10.1007/s00280-011-1561-8]

8 **Koo DH**, Ryu MH, Ryoo BY, Seo J, Lee MY, Chang HM, Lee JL, Lee SS, Kim TW, Kang YK. Improving trends in survival of patients who receive chemotherapy for metastatic or recurrent gastric cancer: 12 years of experience at a single institution. *Gastric Cancer* 2015; **18**: 346-353 [PMID: 24832201 DOI: 10.1007/s10120-014-0385-8]

9 **Koo DH**, Ryu MH, Lee MY, Chae H, Kim EJ, Moon MS, Kang YK. Trends in Chemotherapy Patterns and Survival of Patients with Advanced Gastric Cancer over a 16-Year Period: Impact of Anti-HER2-Targeted Agent in the Real-World Setting. *Cancer Res Treat* 2021; **53**: 436-444 [PMID: 33070558 DOI: 10.4143/crt.2020.725]

10 **Kang JH**, Lee SI, Lim DH, 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]

11 **Janjigian YY**, Shitara K, Moehler M, Garrido M, Salman P, Shen L, Wyrwicz L, Yamaguchi K, Skoczylas T, Campos Bragagnoli A, Liu T, Schenker M, Yanez P, Tehfe M, Kowalyszyn R, Karamouzis MV, Bruges R, Zander T, Pazo-Cid R, Hitre E, Feeney K, Cleary JM, Poulart V, Cullen D, Lei M, Xiao H, Kondo K, Li M, Ajani JA. First-line nivolumab plus chemotherapy versus chemotherapy alone for advanced gastric, gastro-oesophageal junction, and oesophageal adenocarcinoma (CheckMate 649): a randomised, open-label, phase 3 trial. *Lancet* 2021; **398**: 27-40 [PMID: 34102137 DOI: 10.1016/S0140-6736(21)00797-2]

12 **Kang YK**, Boku N, Satoh T, Ryu MH, Chao Y, Kato K, Chung HC, Chen JS, Muro K, Kang WK, Yeh KH, Yoshikawa T, Oh SC, Bai LY, Tamura T, Lee KW, Hamamoto Y, Kim JG, Chin K, Oh DY, Minashi K, Cho JY, Tsuda M, Chen LT. Nivolumab in patients with advanced gastric or gastro-oesophageal junction cancer refractory to, or intolerant of, at least two previous chemotherapy regimens (ONO-4538-12, ATTRACTION-2): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* 2017; **390**: 2461-2471 [PMID: 28993052 DOI: 10.1016/S0140-6736(17)31827-5]

13 **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, randomised controlled trial. *Lancet* 2010; **376**: 687-697 [PMID: 20728210 DOI: 10.1016/S0140-6736(10)61121-X]

14 **Shitara K**, Bang YJ, Iwasa S, Sugimoto N, Ryu MH, Sakai D, Chung HC, Kawakami H, Yabusaki H, Lee J, Saito K, Kawaguchi Y, Kamio T, Kojima A, Sugihara M, Yamaguchi K; DESTINY-Gastric01 Investigators. Trastuzumab Deruxtecan in Previously Treated HER2-Positive Gastric Cancer. *N Engl J Med* 2020; **382**: 2419-2430 [PMID: 32469182 DOI: 10.1056/NEJMoa2004413]

15 **Diakos CI**, Charles KA, McMillan DC, Clarke SJ. Cancer-related inflammation and treatment effectiveness. *Lancet Oncol* 2014; **15**: e493-e503 [PMID: 25281468 DOI: 10.1016/S1470-2045(14)70263-3]

16 **Guthrie GJ**, Charles KA, Roxburgh CS, Horgan PG, McMillan DC, Clarke SJ. The systemic inflammation-based neutrophil-lymphocyte ratio: experience in patients with cancer. *Crit Rev Oncol Hematol* 2013; **88**: 218-230 [PMID: 23602134 DOI: 10.1016/j.critrevonc.2013.03.010]

17 **Grenader T**, Waddell T, Peckitt C, Oates J, Starling N, Cunningham D, Bridgewater J. Prognostic value of neutrophil-to-lymphocyte ratio in advanced oesophago-gastric cancer: exploratory analysis of the REAL-2 trial. *Ann Oncol* 2016; **27**: 687-692 [PMID: 26787231 DOI: 10.1093/annonc/mdw012]

18 **Ter Veer E**, van Kleef JJ, Schokker S, van der Woude SO, Laarman M, Haj Mohammad N, Sprangers MAG, van Oijen MGH, van Laarhoven HWM. Prognostic and predictive factors for overall survival in metastatic oesophagogastric cancer: A systematic review and meta-analysis. *Eur J Cancer* 2018; **103**: 214-226 [PMID: 30268922 DOI: 10.1016/j.ejca.2018.07.132]

19 **Vickers AJ**, Elkin EB. Decision curve analysis: a novel method for evaluating prediction models. *Med Decis Making* 2006; **26**: 565-574 [PMID: 17099194 DOI: 10.1177/0272989X06295361]

20 **Kang YK**, Kang WK, Shin DB, Chen J, Xiong J, Wang J, Lichinitser M, Guan Z, Khasanov R, Zheng L, Philco-Salas M, Suarez T, Santamaria J, Forster G, McCloud PI. Capecitabine/cisplatin versus 5-fluorouracil/cisplatin as first-line therapy in patients with advanced gastric cancer: a randomised phase III noninferiority trial. *Ann Oncol* 2009; **20**: 666-673 [PMID: 19153121 DOI: 10.1093/annonc/mdn717]

21 **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 FLAGS trial. *J Clin Oncol* 2010; **28**: 1547-1553 [PMID: 20159816 DOI: 10.1200/JCO.2009.25.4706]

22 **Cunningham D**, Starling N, Rao S, Iveson T, Nicolson M, Coxon F, Middleton G, Daniel F, Oates J, Norman AR; Upper Gastrointestinal Clinical Studies Group of the National Cancer Research Institute of the United Kingdom. Capecitabine and oxaliplatin for advanced esophagogastric cancer. *N Engl J Med* 2008; **358**: 36-46 [PMID: 18172173 DOI: 10.1056/NEJMoa073149]

23 **Al-Batran SE**, Hartmann JT, Probst S, Schmalenberg H, Hollerbach S, Hofheinz R, Rethwisch V, Seipelt G, Homann N, Wilhelm G, Schuch G, Stoehlmacher J, Derigs HG, Hegewisch-Becker S, Grossmann J, Pauligk C, Atmaca A, Bokemeyer C, Knuth A, Jäger E; Arbeitsgemeinschaft Internistische Onkologie. Phase III trial in metastatic gastroesophageal adenocarcinoma with fluorouracil, leucovorin plus either oxaliplatin or cisplatin: a study of the Arbeitsgemeinschaft Internistische Onkologie. *J Clin Oncol* 2008; **26**: 1435-1442 [PMID: 18349393 DOI: 10.1200/JCO.2007.13.9378]

24 **Mohammad NH**, ter Veer E, Ngai L, Mali R, van Oijen MG, van Laarhoven HW. Optimal first-line chemotherapeutic treatment in patients with locally advanced or metastatic esophagogastric carcinoma: triplet versus doublet chemotherapy: a systematic literature review and meta-analysis. *Cancer Metastasis Rev* 2015; **34**: 429-441 [PMID: 26267802 DOI: 10.1007/s10555-015-9576-y]

25 **Paramanathan A**, Saxena A, Morris DL. A systematic review and meta-analysis on the impact of pre-operative neutrophil lymphocyte ratio on long term outcomes after curative intent resection of solid tumours. *Surg Oncol* 2014; **23**: 31-39 [PMID: 24378193 DOI: 10.1016/j.suronc.2013.12.001]

26 **Ota Y**, Takahari D, Suzuki T, Osumi H, Nakayama I, Oki A, Wakatsuki T, Ichimura T, Ogura M, Shinozaki E, Suenaga M, Chin K, Yamaguchi K. Changes in the neutrophil-to-lymphocyte ratio

during nivolumab monotherapy are associated with gastric cancer survival. *Cancer Chemother Pharmacol* 2020; **85**: 265-272 [PMID: 31907646 DOI: 10.1007/s00280-019-04023-w]

27 **Lee SS**, Lee JL, Ryu MH, Chang HM, Kim TW, Kang HJ, Kim WK, Lee JS, Kang YK. Combination chemotherapy with capecitabine (X) and Cisplatin (P) as first line treatment in advanced gastric cancer: experience of 223 patients with prognostic factor analysis. *Jpn J Clin Oncol* 2007; **37**: 30-37 [PMID: 17272321 DOI: 10.1093/jjco/hyl134]

28 **Fujitani K**, Yang HK, Mizusawa J, Kim YW, Terashima M, Han SU, Iwasaki Y, Hyung WJ, Takagane A, Park DJ, Yoshikawa T, Hahn S, Nakamura K, Park CH, Kurokawa Y, Bang YJ, Park BJ, Sasako M, Tsujinaka T; REGATTA study investigators. Gastrectomy plus chemotherapy versus chemotherapy alone for advanced gastric cancer with a single non-curable factor (REGATTA): a phase 3, randomised controlled trial. *Lancet Oncol* 2016; **17**: 309-318 [PMID: 26822397 DOI: 10.1016/S1470-2045(15)00553-7]

29 **Lee CM**, Choi IK, Kim JH, Park DW, Kim JS, Park SH. Is noncurative gastrectomy always a beneficial strategy for stage IV gastric cancer? *Ann Surg Treat Res* 2017; **92**: 23-27 [PMID: 28090502 DOI: 10.4174/astr.2017.92.1.23]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 December 28; 27(48): 8370-8373

DOI: 10.3748/wjg.v27.i48.8370

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

LETTER TO THE EDITOR

Strategy for the control of drug-induced liver injury due to investigational treatments/drugs for COVID-19

Ken Sato, Yuichi Yamazaki, Toshio Uraoka

ORCID number: Ken Sato 0000-0002-3202-7983; Yuichi Yamazaki 0000-0002-8633-2983; Toshio Uraoka 0000-0002-4425-4331.

Author contributions: Sato K designed the research and drafted the article; Yamazaki Y and Uraoka T analyzed the data and gave critical advice; and Sato K revised the letter and performed the final approval of the version of the article to be published.

Conflict-of-interest statement: We received no financial support for this manuscript.

Country/Territory of origin: Japan

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Ken Sato, Yuichi Yamazaki, Toshio Uraoka, Department of Gastroenterology and Hepatology, Gunma University Graduate School of Medicine, Maebashi 371-8511, Gunma, Japan

Corresponding author: Ken Sato, MD, PhD, Associate Professor, Department of Gastroenterology and Hepatology, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi 371-8511, Gunma, Japan. satoken@gunma-u.ac.jp

Abstract

Investigational treatments/drugs for coronavirus disease 2019 (COVID-19) have been applied, with repurposed or newly developed drugs, and their effectiveness has been evaluated. Some of these drugs may be hepatotoxic, and each monotherapy or combination therapy may increase the risk of drug-induced liver injury (DILI). We should aim to control dysregulation of liver function, as well as the progression of COVID-19, as much as possible. We discussed the potential risks of investigational treatments/drugs and promising drugs for both COVID-19 and DILI due to investigational treatments/drugs.

Key Words: Coronavirus disease 2019; Drug-induced liver injury; Cytochrome P450; Drug-drug interaction; Drug-disease interaction; Cytokine

Core Tip: To cope with dysregulation of liver function in coronavirus disease 2019 (COVID-19), drug-induced liver injury (DILI) due to investigational treatments/drugs or drug-drug or drug-disease interactions should be considered. We described useful information associated with clinical practice. We discussed the potential hepatotoxicity of dexamethasone or remdesivir as representative investigational treatments/drugs for COVID-19. These drugs are predicted to be used for a certain time in monotherapy or combination therapy. We also reported glycyrrhizic acid and ursodeoxycholic acid as therapeutic candidates for the control of DILI due to investigational treatments/drugs, as well as COVID-19.

Citation: Sato K, Yamazaki Y, Uraoka T. Strategy for the control of drug-induced liver injury due to investigational treatments/drugs for COVID-19. *World J Gastroenterol* 2021; 27(48): 8370-8373

Received: July 29, 2021
Peer-review started: July 29, 2021
First decision: November 7, 2021
Revised: November 18, 2021
Accepted: November 10, 2021
Article in press: December 10, 2021
Published online: December 28, 2021

P-Reviewer: Cai J
S-Editor: Wu YXJ
L-Editor: A
P-Editor: Wu YXJ

URL: https://www.wjgnet.com/1007-9327/full/v27/i48/8370.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i48.8370

TO THE EDITOR

We read with great interest the review by Huang *et al*[1], which summarized the current understanding and perspectives on dysregulation of liver function in patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection.We generally agree with the authors' comprehensive review. Additional information regarding the potential hepatotoxicity of investigational treatments/drugs for coronavirus disease 2019 (COVID-19) and the strategy for dealing with drug-induced liver injury (DILI) associated with investigational treatments/drugs is useful in clinical practice.

The investigators[1] cited that the synthetic corticosteroid dexamethasone worsens outcomes in patients with COVID-19 who show milder respiratory symptoms, which was reported in the RECOVERY trial[2]. However, to be technically accurate, dexamethasone therapy had several strengths in reducing the 28-d mortality rate, increasing the rate of patients who were discharged alive from hospital within 28 d, and reducing progression to invasive mechanical ventilation or death in comparison to those with usual care, while these merits were not observed in patients who did not receive oxygen[2]. The World Health Organization (WHO) announced guidelines regarding dexamethasone therapy for COVID-19[3]. Corticosteroids (*i.e.*, dexamethasone, hydrocortisone or prednisone) were recommended for the treatment of patients with severe and critical but not nonsevere COVID-19 on September 2, 2020[3]. The current situation has changed with the emergence of new genetic variants of SARS-CoV-2[4]. SARS-CoV-2 mutation may facilitate transmissibility or virulence, reduce neutralization by antibodies produced in response to natural infection or vaccination, promote the ability to evade detection, or decrease the effectiveness of therapeutics or vaccination[4]. They may affect the disease progression of COVID-19, and thus, we believe that the treatment strategy has a more important role in the control of COVID-19.

The role of dexamethasone is to ameliorate inflammatory organ injury in viral pneumonia[2]. However, dexamethasone is a cytochrome P450 (CYP3A4) inducer and has a high chance of drug-drug interactions with investigational treatments/drugs or agents used to treat comorbidities, especially CYP3A4 substrates. Importantly, CYP enzymes can be inhibited by an increase in infection-related cytokine levels and inflammation[5]. Both investigational treatments/drugs and agents used to treat comorbidities can be affected by compromised CYP-mediated hepatic metabolism, irrespective of the onset/Length of COVID-19 and the extent of liver dysfunction[5]. Subsequently, these drug-drug and drug-disease interactions and dysfunctional CYP-mediated hepatic metabolism might cause dysregulation of liver function, including drug-induced liver injury (DILI)[5]. In addition, dexamethasone therapy caused elevated liver enzymes, increased hepatic lipid peroxidation, and decreased antioxidant activities in rats[6]. On the other hand, dexamethasone is a type of corticosteroid that can be used to treat drug-induced cholestatic hepatitis[7]; in particular, corticosteroids are used for the treatment of DILI associated with hypersensitivity features[8]. The mechanism of dexamethasone against DILI might be involved in alleviation of tissue damage caused by inflammatory responses of the immune system within the liver[7]. Thus, dexamethasone has pros and cons in relation to liver injury. Dexamethasone could be used in combination with antiviral drugs, such as remdesivir (RDV), for COVID-19 patients, although the WHO announced a conditional recommendation against the use of RDV in hospitalized patients on November 20, 2020[9]. As a direct role of RDV in hepatocellular toxicity was suggested [10], combination therapy with dexamethasone and RDV is more likely to cause liver dysfunction, especially for patients with comorbidities, and we should perform careful observation during combination therapy or each monotherapy.

Regarding the treatment of DILI due to investigational treatments/drugs, glycyrrhizic acid was advocated as a treatment candidate for COVID-19 patients, especially those with complex liver injury[11]. In Japan, glycyrrhizic acid has been used for more than 40 years as a treatment for liver diseases[11]. It works as a hepatoprotective drug for a variety of liver diseases, including DILI[11], and has safe and economical features[11]. The possible mechanism of monoammonium glycyrrhizin, the main component of glycyrrhizin, against drug-induced hepatotoxicity involves

Table 1 Anti-coronavirus disease 2019 drugs and drugs for drug-induced liver injury

Name	Type	Mechanisms as anti-COVID-19 drugs and/or drugs for drug-induced liver injury	Mechanisms of hepatotoxicity	Ref.
Anti-COVID-19 drugs				
Dexamethasone	Anti-inflammatory drug	Amelioration of inflammatory organ injury in viral pneumonia. Alleviation of tissue damage caused by inflammatory responses of the immune system within the liver.	Drug-drug interactions due to cytochrome P450 induction. Elevation of liver enzyme levels, increase in hepatic lipid peroxidation, and decrease in antioxidant activities.	[2,6,7]
Remdesivir	Antiviral drug	Inhibition of RNA polymerase, as a nucleotide analog.	Hepatocellular toxicity.	[10]
Drugs for drug-induced liver injury				
Glycyrrhizic acid	Hepatoprotector	Regulation of the expression of hepatobiliary membrane transporters.		[12]
Ursodeoxycholic acid	Hepatoprotector	Anti-inflammatory, antioxidant, immunomodulatory and antiapoptotic profiles. Inhibition of proinflammatory cytokine production.		[14]

COVID-19: Coronavirus disease 2019.

regulating the expression of hepatobiliary membrane transporters[12].

Another therapeutic candidate for DILI due to investigational treatments/drugs is ursodeoxycholic acid (UDCA), which has been used in cholestatic DILI to reduce the time to resolution[13]. UDCA is a hydrophilic bile acid that has anti-inflammatory, antioxidant, immunomodulatory and antiapoptotic profiles[14] and inhibits proinflammatory cytokine production[14]. Thus, UDCA is also beneficial for cytokine storm syndrome, which is caused by a sudden, abnormal release of inflammatory cytokines due to overreaction of innate immunity[14], which is one of the critical pathogeneses of COVID-19. UDCA has been promoted as a candidate therapeutic agent for COVID-19[14,15]. Anti-COVID-19 drugs and drugs for DILI are summarized in Table 1.

We should manage dysregulation of liver function regardless of the association with treatment for COVID-19. We introduced the potential risks of investigational treatments/drugs and promising drugs for both COVID-19 and DILI due to investigational treatments/drugs. Further studies should confirm this hypothesis and may help to establish an effective strategy for the management of COVID-19 and DILI due to investigational treatments/drugs.

REFERENCES

1 **Huang YK**, Li YJ, Li B, Wang P, Wng QH. Dysregulated liver function in SARS-CoV-2 infection: Current understanding and perspectives. *World J Gastoenterol* 2021; **27**: 4358-4370 [PMID: 34366609 DOI: 10.3748/wjg.v27.i27.4358]

2 **RECOVERY Collaborative Group**. Horby P, Lim WS, Emberson JR, Mafham M, Bell JL, Linsell L, Staplin N, Brightling C, Ustianowski A, Elmahi E, Prudon B, Green C, Felton T, Chadwick D, Rege K, Fegan C, Chappell LC, Faust SN, Jaki T, Jeffery K, Montgomery A, Rowan K, Juszczak E, Baillie JK, Haynes R, Landray MJ. Dexamethasone in Hospitalized Patients with Covid-19. *N Engl J Med* 2021; **384**: 693-704 [PMID: 32678530 DOI: 10.1056/NEJMoa2021436]

3 **WHO**. Coronavirus disease (COVID-19): Dexamethasone. Available from: https://www.who.int/news-room/q-a-detail/coronavirus-disease-covid-19-dexamethasone

4 **Cascella M**, Rajnik M, Aleem A, Dulebohn SC, Di Napoli R. Features, Evaluation, and Treatment of Coronavirus (COVID-19). 2021 Sep 2. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan- [PMID: 32150360]

5 **Deb S**, Arrighi S. Potential Effects of COVID-19 on Cytochrome P450-Mediated Drug Metabolism and Disposition in Infected Patients. *Eur J Drug Metab Pharmacokinet* 2021; **46**: 185-203 [PMID: 33538960 DOI: 10.1007/s13318-020-00668-8]

6 **Hasona N**, Morsi A. Grape Seed Extract Alleviates Dexamethasone-Induced Hyperlipidemia, Lipid Peroxidation, and Hematological Alteration in Rats. *Indian J Clin Biochem* 2019; **34**: 213-218 [PMID: 31092996 DOI: 10.1007/s12291-018-0736-z]

7 **Wree A**, Dechêne A, Herzer K, Hilgard P, Syn WK, Gerken G, Canbay A. Steroid and ursodeoxychoclic acid combination therapy in severe drug-induced liver injury. *Digestion* 2011; **84**: 54-59 [PMID: 21304237 DOI: 10.1159/000322298]

8 **Stine JG**, Lewis JH. Current and future directions in the treatment and prevention of drug-induced

liver injury: a systematic review. *Expert Rev Gastroenterol Hepatol* 2016; **10**: 517-536 [PMID: 26633044 DOI: 10.1586/17474124.2016.1127756]

9 **WHO**. WHO recommends against the use of remdesivir in COVID-19 patients. Available from: https://www.who.int/news-room/feature-stories/detail/who-recommends-against-the-use-of-remdesivir-in-covid-19-patients

10 **Zampino R**, Mele F, Florio LL, Bertolino L, Andini R, Galdo M, De Rosa R, Corcione A, Durante-Mangoni E. Liver injury in remdesivir-treated COVID-19 patients. *Hepatol Int* 2020; **14**: 881-883 [PMID: 32725454 DOI: 10.1007/s12072-020-10077-3]

11 **Tian X**, Gan W, Nie Y, Ying R, Tan Y, Chen J, Chen M, Zhang C. Clinical efficacy and security of glycyrrhizic acid preparation in the treatment of anti-SARS-CoV-2 drug-induced liver injury: a protocol of systematic review and meta-analysis. *BMJ Open* 2021; **11**: e051484 [PMID: 34244286 DOI: 10.1136/bmjopen-2021-051484]

12 **Zhou L**, Song Y, Zhao J, Qin H, Zhang G, Zhou Y, Wu X. Monoammonium glycyrrhizinate protects rifampicin- and isoniazid-induced hepatotoxicity *via* regulating the expression of transporter Mrp2, Ntcp, and Oatp1a4 in liver. *Pharm Biol* 2016; **54**: 931-937 [PMID: 26987268 DOI: 10.3109/13880209.2015.1070878]

13 **Garcia-Cortes M**, Robles-Diaz M, Stephens C, Ortega-Alonso A, Lucena MI, Andrade RJ. Drug induced liver injury: an update. *Arch Toxicol* 2020; **94**: 3381-3407 [PMID: 32852569 DOI: 10.1007/s00204-020-02885-1]

14 **Abdulrab S**, Al-Maweri S, Halboub E. Ursodeoxycholic acid as a candidate therapeutic to alleviate and/or prevent COVID-19-associated cytokine storm. *Med Hypotheses* 2020; **143**: 109897 [PMID: 32505909 DOI: 10.1016/j.mehy.2020.109897]

15 **Subramanian S**, Iles T, Ikramuddin S, Steer CJ. Merit of an Ursodeoxycholic Acid Clinical Trial in COVID-19 Patients. *Vaccines (Basel)* 2020; **8** [PMID: 32575350 DOI: 10.3390/vaccines8020320]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2021 December 28; 27(48): 8374-8377

DOI: 10.3748/wjg.v27.i48.8374

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

LETTER TO THE EDITOR

Use of oral contraceptives and risk of pancreatic cancer in women: A recalculated meta-analysis of prospective cohort studies

Jong-Myon Bae

ORCID number: Jong-Myon Bae 0000-0003-3080-7852.

Author contributions: Bae JM designed and performed the study, analyzed the data and wrote the manuscript.

Conflict-of-interest statement: No conflict of interests.

Country/Territory of origin: South Korea

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Jong-Myon Bae, Preventive Medicine, Jeju National University College of Medicine, Jeju-si 63243, Jeju Province, South Korea

Corresponding author: Jong-Myon Bae, MD, PhD, Professor, Preventive Medicine, Jeju National University College of Medicine, No. 102 Jejudaehak-ro, Jeju-si 63243, Jeju Province, South Korea. jmbae@jejunu.ac.kr

Abstract

In a recent systematic review and meta-analysis of observational studies, the author found potential errors in the selection and extraction processes. The recalculated summary relative risks and the results of a dose-response meta-analysis showed that oral contraceptive use may not be associated with the risk of pancreatic cancer in women.

Key Words: Pancreas neoplasms; Oral contraceptives; Risk factor; Meta-analysis; Risk assessment; Systematic review

Core Tip: A systematic review and meta-analysis of observational studies conducted recently concluded that oral contraceptive use was associated with a decreased risk of pancreatic cancer in women. However, the author found potential errors in the selection and extraction processes. The recalculated summary relative risks and the results of a dose-response meta-analysis showed that oral contraceptive use may not be associated with the risk of pancreatic cancer in women. As this conclusion contradicted that reported recently, it is necessary to re-evaluate the direction and statistical significance of this risk through an updated meta-analysis in the future.

Citation: Bae JM. Use of oral contraceptives and risk of pancreatic cancer in women: A recalculated meta-analysis of prospective cohort studies. *World J Gastroenterol* 2021; 27(48): 8374-8377

URL: https://www.wjgnet.com/1007-9327/full/v27/i48/8374.htm

DOI: https://dx.doi.org/10.3748/wjg.v27.i48.8374

Received: July 29, 2021
Peer-review started: July 29, 2021
First decision: October 16, 2021
Revised: October 25, 2021
Accepted: December 16, 2021
Article in press: December 16, 2021
Published online: December 28, 2021

P-Reviewer: Tung TH
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

TO THE EDITOR

I recently read the systematic review and meta-analysis conducted by Ilic *et al*[1] comprising 10 case-control studies and 11 cohort studies, which concluded that the use of oral contraceptives (OCU) was associated with a decreased risk of pancreatic cancer in women (PCW) [summary relative risk (sRR) = 0.85; 95% confidence intervals (CI) = 0.73-0.98; P = 0.03]. Interestingly, the subgroup analysis according to the study design showed no statistical significance in case-control studies but showed borderline statistical significance in cohort studies (sRR = 0.84; 95%CI = 0.70-1.00; P = 0.05).

However, while reviewing the results of the 11 selected cohort studies, I found the following potential errors. First, among the 11 selected studies, the study by Teras *et al* [2] was a cohort study that analyzed the mortality of PCW; therefore, excluding this study would be valid based on the research hypothesis; second, it would be necessary to include the two cohort studies[3,4] that were considered in other studies on the risk of various cancers associated with OCU[5,6]; finally, in the two studies that did not provide an RR for the ever group[7,8], the RR's direction was opposite to that of the forest plot shown in the study by Ilic *et al*[1].

Considering these issues, I recalculated the sRR of the longest duration (LD) group as well as the ever group. The statistical significance disappeared in both groups, and the sRRs were 1 or higher (Figure 1). Egger's test was performed to evaluate publication bias, and no statistical significance was noted in either group (P = 0.439 and 0.817 in the ever group and LD group, respectively).

Eight of the 12 selected cohorts[3,7-13] provided the information necessary for performing a dose-response meta-analysis. A two-stage random-effects dose-response model was used with a dosing unit of 1 year (P of goodness-of-fit = 0.041). The results showed borderline statistical significance with a linear dose-response relationship between OCU duration and PCW risk (sRR = 1.015; 95%CI = 0.999-1.030; P = 0.057) (Figure 2).

Based on the results of the recalculated sRRs and DRMA, the OCU may not be associated with the risk of PCW. Because my conclusion contradicts that reported by Ilic *et al*[1], it is necessary to re-evaluate the direction and statistical significance of risk through an updated meta-analysis in the future.

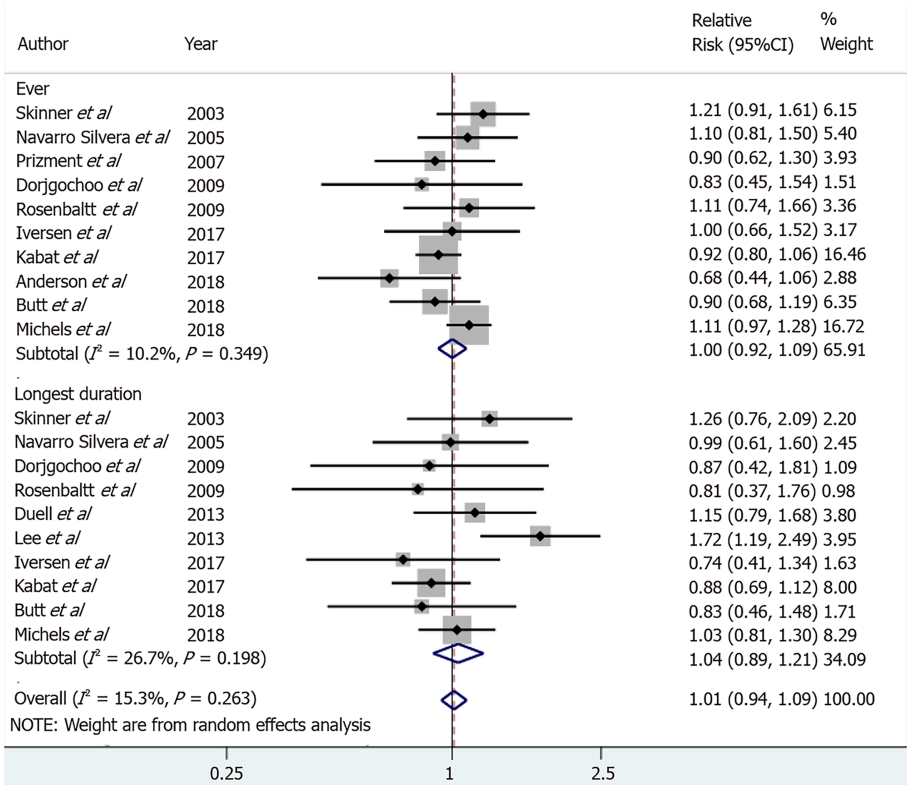

Figure 1 Forest plots in the ever and the longest duration group.

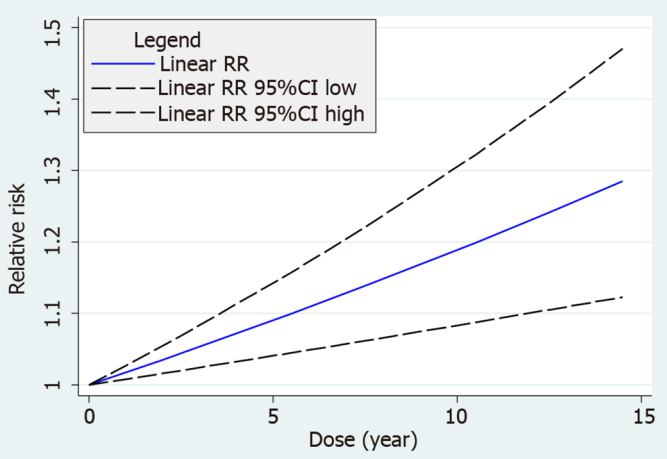

Figure 2 The linear dose-response relationship between duration (year) of oral contraceptive usage and risk of pancreatic cancer in women. RR: Relative risk.

REFERENCES

1 **Ilic M**, Milicic B, Ilic I. Association between oral contraceptive use and pancreatic cancer risk: A systematic review and meta-analysis. *World J Gastroenterol* 2021; **27**: 2643-2656 [PMID: 34092981 DOI: 10.3748/wjg.v27.i20.2643]

2 **Teras LR**, Patel AV, Rodriguez C, Thun MJ, Calle EE. Parity, other reproductive factors, and risk of pancreatic cancer mortality in a large cohort of U.S. women (United States). *Cancer Causes Control* 2005; **16**: 1035-40 [DOI: 10.1007/s10552-005-0332-4]

3 **Rosenblatt KA**, Gao DL, Ray RM, Nelson ZC, Wernli KJ, Li W, Thomas DB. Oral contraceptives and the risk of all cancers combined and site-specific cancers in Shanghai. *Cancer Causes Control* 2009; **20**: 27-34 [PMID: 18704712 DOI: 10.1007/s10552-008-9213-y]

4 **Iversen L**, Sivasubramaniam S, Lee AJ, Fielding S, Hannaford PC. Lifetime cancer risk and combined oral contraceptives: the Royal College of General Practitioners' Oral Contraception Study. *Am J Obstet Gynecol* 2017; **216**: 580.e1-580.e9 [PMID: 28188769 DOI: 10.1016/j.ajog.2017.02.002]

5 **Wu L**, Zhu J. Linear reduction in thyroid cancer risk by oral contraceptive use: a dose-response meta-analysis of prospective cohort studies. *Hum Reprod* 2015; **30**: 2234-2240 [PMID: 26141711 DOI: 10.1093/humrep/dev160]

6 **Rodriguez-Lara V**, Avila-Costa MR. An Overview of Lung Cancer in Women and the Impact of Estrogen in Lung Carcinogenesis and Lung Cancer Treatment. *Front Med (Lausanne)* 2021; **8**: 600121 [PMID: 34079807 DOI: 10.3389/fmed.2021.600121]

7 **Duell EJ**, Travier N, Lujan-Barroso L, Dossus L, Boutron-Ruault MC, Clavel-Chapelon F, Tumino R, Masala G, Krogh V, Panico S, Ricceri F, Redondo ML, Dorronsoro M, Molina-Montes E, Huerta JM, Barricarte A, Khaw KT, Wareham NJ, Allen NE, Travis R, Siersema PD, Peeters PH, Trichopoulou A, Fragogeorgi E, Oikonomou E, Boeing H, Schuetze M, Canzian F, Lukanova A, Tjønneland A, Roswall N, Overvad K, Weiderpass E, Gram IT, Lund E, Lindkvist B, Johansen D, Ye W, Sund M, Fedirko V, Jenab M, Michaud DS, Riboli E, Bueno-de-Mesquita HB. Menstrual and reproductive factors in women, genetic variation in CYP17A1, and pancreatic cancer risk in the European prospective investigation into cancer and nutrition (EPIC) cohort. *Int J Cancer* 2013; **132**: 2164-2175 [PMID: 23015357 DOI: 10.1002/ijc.27875]

8 **Lee E**, Horn-Ross PL, Rull RP, Neuhausen SL, Anton-Culver H, Ursin G, Henderson KD, Bernstein L. Reproductive factors, exogenous hormones, and pancreatic cancer risk in the CTS. *Am J Epidemiol* 2013; **178**: 1403-1413 [PMID: 24008905 DOI: 10.1093/aje/kwt154]

9 **Skinner HG**, Michaud DS, Colditz GA, Giovannucci EL, Stampfer MJ, Willett WC, Fuchs CS. Parity, reproductive factors, and the risk of pancreatic cancer in women. *Cancer Epidemiol Biomarkers Prev* 2003; **12**: 433-438 [PMID: 12750238]

10 **Navarro Silvera SA**, Miller AB, Rohan TE. Hormonal and reproductive factors and pancreatic cancer risk: a prospective cohort study. *Pancreas* 2005; **30**: 369-374 [PMID: 15841050 DOI: 10.1097/01.mpa.0000160301.59319.ba]

11 **Kabat GC**, Kamensky V, Rohan TE. Reproductive factors, exogenous hormone use, and risk of pancreatic cancer in postmenopausal women. *Cancer Epidemiol* 2017; **49**: 1-7 [PMID: 28521283 DOI: 10.1016/j.canep.2017.05.002]

12 **Butt SA**, Lidegaardi Ø, Skovlund C, Hannaford PC, Iversen L, Fielding S, Mørch LS. Hormonal contraceptive use and risk of pancreatic cancer-A cohort study among premenopausal women. *PLoS One* 2018; **13**: e0206358 [PMID: 30376560 DOI: 10.1371/journal.pone.0206358]

13 **Michels KA**, Brinton LA, Pfeiffer RM, Trabert B. Oral Contraceptive Use and Risks of Cancer in the NIH-AARP Diet and Health Study. *Am J Epidemiol* 2018; **187**: 1630-1641 [PMID: 29394309 DOI: 10.1093/aje/kwx388]

Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
Telephone: +1-925-3991568
E-mail: bpgoffice@wjgnet.com
Help Desk: https://www.f6publishing.com/helpdesk
https://www.wjgnet.com