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

World Journal of *Gastroenterology*

World J Gastroenterol 2021 November 7; 27(41): 7005-7209



Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

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Weekly Volume 27 Number 41 November 7, 2021

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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: *Ying-Yi Yuan*; **Production Department Director:** *Xiang Li*; **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, Subrata Ghosh

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

PUBLICATION DATE
November 7, 2021

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ONLINE SUBMISSION
https://www.f6publishing.com

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World Journal of Gastroenterology

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World J Gastroenterol 2021 November 7; 27(41): 7005-7013

DOI: 10.3748/wjg.v27.i41.7005

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

EDITORIAL

Nucleic acid vaccines: A taboo broken and prospect for a hepatitis B virus cure

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Author contributions: Tsounis PE was responsible for the literature search and analysis, drafting of the manuscript, and interpretation of the data; Mouzaki A and Triantos C were responsible for revising the manuscript for important intellectual content; All authors gave their final approval of the submitted version.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest.

Country/Territory of origin: Greece

Specialty type: Infectious diseases

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

Open-Access: This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution

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Abstract

Although a prophylactic vaccine is available, hepatitis B virus (HBV) remains a major cause of liver-related morbidity and mortality. Current treatment options are improving clinical outcomes in chronic hepatitis B; however, true functional cure is currently the exception rather than the rule. Nucleic acid vaccines are among the emerging immunotherapies that aim to restore weakened immune function in chronically infected hosts. DNA vaccines in particular have shown promising results *in vivo* by reducing viral replication, breaking immune tolerance in a sustained manner, or even decimating the intranuclear covalently closed circular DNA reservoir, the hallmark of HBV treatment. Although DNA vaccines encoding surface antigens administered by conventional injection elicit HBV-specific T cell responses in humans, initial clinical trials failed to demonstrate additional therapeutic benefit when administered with nucleos(t)ide analogs. In an attempt to improve vaccine immunogenicity, several techniques have been used, including codon/promoter optimization, coadministration of cytokine adjuvants, plasmids engineered to express multiple HBV epitopes, or combinations with other immunomodulators. DNA vaccine delivery by electroporation is among the most efficient strategies to enhance the production of plasmid-derived antigens to stimulate a potent cellular and humoral anti-HBV response. Preliminary results suggest that DNA vaccination *via* electroporation efficiently invigorates both arms of adaptive immunity and suppresses serum HBV DNA. In contrast, the study of mRNA-based vaccines is limited to a few *in vitro* experiments in this area. Further studies are needed to clarify the prospects of nucleic acid vaccines for HBV cure.

Key Words: Chronic hepatitis B; Therapeutic vaccination; Nucleic acid vaccines; DNA vaccines; Electroporation; Immunotherapy

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Received: March 18, 2021
Peer-review started: March 18, 2021
First decision: July 3, 2021
Revised: July 7, 2021
Accepted: October 14, 2021
Article in press: October 14, 2021
Published online: November 7, 2021

P-Reviewer: Martin Mateos RM
S-Editor: Ma YJ
L-Editor: Filipodia
P-Editor: Ma YJ



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Core Tip: A nucleic acid vaccine could be of particular value in the field of hepatitis B virus therapies. DNA vaccines have been studied more extensively over the past two decades and have been shown to overcome immune exhaustion in preclinical models of chronic infection. Although vaccination elicited robust humoral and cellular immune responses, it had negligible effects on clinical endpoints. Therefore, the scientific community has focused on optimizing vaccine design and delivery to improve immunogenicity. Electroporation-mediated delivery of multivalent plasmids in combination with molecular adjuvants could efficiently restore adaptive immunity in virally suppressed patients and be part of future combination therapy.

Citation: Tsounis EP, Mouzaki A, Triantos C. Nucleic acid vaccines: A taboo broken and prospect for a hepatitis B virus cure. *World J Gastroenterol* 2021; 27(41): 7005-7013
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7005.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7005

INTRODUCTION

Hepatitis B virus (HBV) is a relatively small (3.2 kb) DNA virus that causes acute or chronic liver infection leading to cirrhosis or hepatocellular carcinoma. Although an effective preventive vaccine containing hepatitis B surface antigen (HBsAg) produced from recombinant DNA has been available for more than two decades[1], chronic hepatitis B (CHB) remains a major burden of liver-related morbidity and mortality, with more than 292000000 infections worldwide[2]. Current treatment strategies, *i.e.* nucleoside or nucleotide (NA) drugs and interferon-α (IFN-α), effectively suppress viral replication, prevent progression of liver disease to end-stage, and improve patient quality of life. However, a truly functional cure, defined as undetectable HBV DNA with concomitant elimination of HBsAg over a limited therapeutic period, is rarely achieved with existing therapies[3]. HBV has evolved several mechanisms to become chronic, and the failure to eradicate HBV is largely due to the inability to address the following issues: (1) Organization of the viral genome into a highly stable, drug-resistant, chromosomal covalently closed circular DNA (cccDNA) conformation in the nuclei of infected cells; (2) Integration of HBV DNA into the host genome; (3) Disruption of innate immunity signaling; and (4) Depletion of HBV-specific T and B cells[4].

There is a growing consensus that elimination of HBV requires a combination of treatments that include traditional therapeutics, novel direct-acting antivirals that target different steps of the HBV life cycle, and immunotherapies to restore the host immune response. A number of immunomodulators are in clinical trials, including pattern recognition receptor agonists, immune checkpoint inhibitors, adaptive transfer of engineered T cells, and therapeutic vaccines[5]. Various vaccine delivery platforms are being explored to optimize immunogenicity and elicit robust responses to rejuvenate the exhausted immune system. In particular, viral vector technology, immune complex platforms, virus-like particle-based vaccines, and nucleic acid vaccines are some of the categories of therapeutic vaccines under development[6].

Although the world is still struggling with a global health crisis, coronavirus disease 2019 has been at the forefront of scientific breakthroughs in the field of vaccinology, bringing nucleic acid vaccine technology to the forefront. Recent technological innovations have improved the delivery, tolerability, and efficacy of DNA- and mRNA-based therapeutics. Two prophylactic mRNA vaccines against severe acute respiratory syndrome coronavirus 2 were the first drugs in this category to be approved for human use and represent the pinnacle of progress[7,8]. Clearly, the role of nucleic acid-based vaccines in restoring immune dysfunction in CHB and their prospects as part of future combination therapy need to be re-evaluated.

HBV-INDUCED IMMUNE DYSREGULATION

Resolution of acute HBV infection requires an alert innate immune system and polyclonal and multispecific cellular and humoral responses. The human innate immune system is equipped with several pattern recognition receptors that recognize pathogen- or damage-associated patterns and initiate intracellular signal transduction leading to the production of antiviral IFNs and proinflammatory cytokines. Previous studies in humans and chimpanzees reported limited IFN type I production during the initial phase of the logarithmic rise in viremia, supporting the view that HBV is a "stealth virus"[9,10]. However, recent data show that hepatocytes express pattern recognition receptors that recognize HBV components, highlighting the role of nucleic acid signaling and the associated activation of NF-κB-dependent pathways[11]. Therefore, it seems more likely that HBV impairs intrinsic immunity to establish chronic infection. Indeed, HBV alters the functional phenotype of monocytes/macrophages by inducing the secretion of anti-inflammatory cytokines interleukin (IL)-10 and transforming growth factor-β and suppressing the production of tumor necrosis factor-α and IL-12 by inhibiting the toll-like receptor-2 downstream signaling pathway[12,13]. In parallel, myeloid-derived suppressive cells are recruited to CHB and contribute to the immunosuppressive cascade by secreting IL-10 and arginase and downregulating IFN-γ expression by T cells[14]. In addition, dendritic cells, which are critical for generating effective adaptive immune responses, exhibit decreased antigen presentation capacity, cell migration capacity, phagocytic activity, and cytokine production, possibly due to inhibition of costimulatory molecule expression[15]. Therefore, HBV creates a tolerogenic microenvironment in the liver infiltrated with IFN-γ-deficient natural killer cells and T regulatory cell populations[16]. This dysregulated milieu has a significant impact on T and B cell maturation and differentiation, resulting in impaired adaptive immune responses. Moreover, prolonged exposure to high concentrations of viral antigens contributes to T cell exhaustion, characterized by reduced cytotoxic capacity, impaired proliferative capacity, and upregulation of inhibitory molecules (programmed death-1 (PD-1), CTLA-4, T cell immunoglobulin and mucin-domain containin-3). Clearly, approaches aimed at restoring or stimulating sustained HBV-specific cellular and humoral responses may be central to the treatment of chronically infected patients.

DNA VACCINES

DNA vaccine technology is based on genetically engineered plasmids containing a potent promoter that triggers increased transcriptional activity *in vivo*, followed by a sequence encoding the preselected immunogenic antigen(s). The corresponding plasmid is administered either systemically or primarily topically *via* intramuscular injection. Taking advantage of the host cellular apparatus, the exogenous DNA is delivered to the nuclei of transfected cells, including resident antigen-presenting cells (APCs).

The expression of plasmid-encoded antigens and their presentation by major histocompatibility complex (MHC) class I and MHC class II molecules are key elements of adaptive immunity. In particular, transfected myocytes or keratinocytes express the plasmid-derived proteins, which are later released into the bloodstream *via* exosomes or apoptotic bodies[17]. APCs play an important role in this process by mediating the presentation of vaccine peptides both on MHC-I molecules, either by direct transfection or cross-presentation, and on MHC-II molecules after uptake of circulating antigens. Subsequently, afferent lymphatic vessels transport APCs to lymph nodes, where they present the antigenic epitopes to naïve T and B cells and provide essential costimulatory signals. This interaction leads to clonal expansion of CD8+ T cells, which elicit a strong cytotoxic response, and CD4+ T cells, which regulate the differentiation of antigen-presenting B cells[18].

DNA vaccine platforms have many advantages: they are fast to develop, easy to replicate, and very stable at room temperature, resulting in low manufacturing and storage costs[19]. Theoretically, they are safer than conventional live attenuated vaccines because they do not elicit potential anti-vector immune responses and by continuously expressing antigens elicit a long-lasting response without being infectious[20]. Moreover, the intracellular synthesis of the encoded antigens enables the endogenous post-translational modifications that generate proteins in their native conformation[18]. In addition, the expression of a broader repertoire of antigenic epitopes is possible by combining two or more plasmids or by constructing polycis-

tronic carriers[19]. Indeed, DNA vaccination has shown promising results in preclinical models of chronic hepatitis virus infection. In HBV transgenic mice, vaccination broke immune tolerance and caused a significant decrease in viral replication [21], while in a duck HBV model it was able to reduce the intranuclear cccDNA pool [22]. Moreover, immunization with a DNA prime adenovirus boost vaccine in transgenic mice showed strong synergistic effects with NAs, eliciting sustained suppression of viral replication and strong and specific CD8+ T cell responses[23].

The initial clinical trials investigating therapeutic vaccination in CHB patients showed moderate efficacy in restoring host T cell responses but dampened enthusiasm by demonstrating moderate immunogenicity. In a phase I clinical trial, administration of a DNA vaccine expressing envelope proteins (S and preS2/S) resulted in transient activation of IFN-γ-producing T cells and a reduction in HBV DNA in 10 viremic patients[24]. Repeated immunization doses were well tolerated, resulted in proliferative responses against HBsAg, and achieved hepatitis B e antigen (HBeAg) seroconversion in 2 participants[24,25]. In another study, DNA-based vaccination resulted in changes in peripheral NK cell populations, with a relative increase in $CD56^{bright}$ NK cells correlating with HBV-specific T cell activity, indicating the importance of $CD56^{bright}$ NK cells in shaping the adaptive immune response[26].

To determine whether therapeutic vaccines are unable to reactivate pre-existing HBV-specific T cells due to persistent antigenemia, DNA vaccines were administered in combination with conventional therapeutics. The efficacy of a preS2/S-expressing DNA vaccine was evaluated in a phase I/II clinical trial in virus-suppressed patients on stable NA therapy. Addition of the vaccine to NA treatment elicited multispecific, polyfunctional, and far more sustained CD4+ T cell responses compared with monotherapy. Nevertheless, the immunostimulatory effect was not strong enough to influence relapse rates after discontinuation of NA[27]. Accordingly, five intramuscular injections of an envelope-expressing DNA vaccine failed to sufficiently restore immune function or reduce relapse risk after treatment discontinuation in a prospective multicenter study of 70 virus-suppressed CHB patients[28]. These results have underscored the need to optimize vaccine immunogenicity, use new delivery systems, and re-evaluate vaccination regimens.

Recently, a new generation of DNA-based vaccines has demonstrated significant immunostimulatory activity with a favorable safety profile. Their preventive or therapeutic value has already been investigated in clinical trials on infectious diseases (Ebola virus, ZIKA virus, HIV, influenza virus) or malignancies (prostate cancer, cervical cancer, and human papillomavirus-related head and neck tumors)[29]. This progress is mainly due to the breakthroughs in biomedical engineering and nanotechnology, which have introduced novel delivery platforms to improve DNA uptake compared to previous needle approaches, *e.g.*, gene gun, jet injection, advanced electroporation (EP), and chemical or biological adjuvant systems[19]. In addition, various molecular tools have been used to improve the immunogenicity of DNA vaccines, *e.g.*, codon optimization, plasmid vector backbone optimization, attachment of virus-derived nuclear localization signals to facilitate nuclear entry, or DNA-complexing nanocarriers to prevent extracellular DNA degradation[17,30]. The development of target-specific plasmid-encoded chimeric molecules and polycistronic vectors that contain genetic loci encoding cytokines or other signaling molecules in addition to immunogenic epitopes are alternative strategies to further stimulate immune responses[19,29].

In this regard, Wang *et al*[31] demonstrated the superior protective effect of a bicistronic DNA vaccine encoding the core protein plus IFN-γ gene sequences compared to a monovalent vaccine expressing the core antigen in a model of HBV infection in marmosets. Experimental *in vivo* models have also shown that coadministration of plasmids encoding cytokine sequences (IL-2, IFN-γ) with core-expressing DNA vaccines increases the production of HBV-specific neutralizing antibodies[32] and that the cccDNA reservoir was depleted in the majority of subjects receiving IFN-γ as an adjuvant[33]. In an attempt to optimize antigen delivery to APCs and thereby increase vaccine efficacy, plasmids were combined with a protein that targets dendritic cells *via* electrostatic coupling (pSVK-HBVA vaccine). In HBV transgenic mice, the pSVK-HBVA vaccine significantly reduced HBV DNA copy number as well as circulating HBsAg[34].

These encouraging preclinical data formed the basis for transferring these techniques from the laboratory bench to clinical trials. Yang *et al*[35] showed that the DNA-based vaccine HB-100, which consists of five different plasmids expressing most HBV antigens as well as a human IL-12 mutant (hIL-12N222L) induced durable CD4+ memory T cell responses when administered to 12 chronically infected Caucasian patients receiving regular lamivudine therapy. Moreover, the DNA vaccine in

combination with an oral antiviral resulted in HBeAg seroconversion in 4 of 6 participants, whereas HBsAg clearance was achieved in only 1 patient, who had the highest concentration of HBV-specific IFN-γ-secreting memory T cells[35]. Long-term control of viremia also correlated with an increase in plasma IL-12 and IL-12/p40 ratio [36].

These positive observations promoted the development of HB-110, a second generation multivalent HBV DNA vaccine comprising three plasmids encoding envelope proteins, core protein/polymerase, and the human IL-12 mutant. HB-110 was administered in a randomized, dose-escalated phase I clinical trial to Korean patients with CHB treated with adefovir dipivoxil. Although HB-110 elicited robust humoral and T cell responses in the HBV mouse model, vaccination in the Korean cohort resulted in weaker HBV-specific T cell responses and lower HBeAg seroconversion rates than HB-100 immunization in Caucasian patients[37]. These unexpected results may be due in part to the fact that HB-110 was tested in Asian patients, most of whom acquire HBV by vertical transmission and have high immune tolerance. It is clear that in addition to improving the immunogenicity of the vaccine, a combination of therapies is required to address compromised immune function and effectively eradicate chronic HBV infection.

In this context, the advent of immune checkpoint inhibitors has paved the way for alternative strategies to overcome T cell exhaustion or anergy. Remarkably, the addition of a PD-1 receptor inhibitor to treatment with NA plus DNA vaccine resulted in significant expansion and activation of virus-specific CD8+ T cells and prolonged suppression of viral replication in marmosets[38]. A phase I clinical trial of the PD-1 blocker nivolumab alone or in combination with the yeast-derived vaccine GS-4774 in 24 HBeAg-negative virally suppressed patients demonstrated not only high-affinity binding of nivolumab to its ligand but also sustained occupancy of the receptor. In addition, significant HBsAg responses were observed without serious adverse events, and HBsAg levels decreased below the limit of detection in 1 participant[39].

ELECTROPORATION-MEDIATED DELIVERY

One of the most efficient methods of DNA vaccine delivery is EP, which uses an electrical pulse to create a potential difference across the cell membrane that creates transient pores, thereby increasing membrane permeability[40]. Compared to conventional injection of plasmid DNA with a syringe, a pulsed electric field dramatically increases cellular uptake, approximately by a factor of 500, resulting in profound immune responses[29]. At the same time, low inflammation and altered vascular permeability cause a local influx of APCs and other immune cells, suggesting robust antigen processing and presentation at the injection site[41]. Cova[42] gave a concise overview of the progress of EP-mediated therapeutic vaccination from experimental models to clinical reality. Initial studies of EP-based DNA vaccination showed that it is capable of eliciting *in vivo* multispecific cytotoxic T cell responses and more effective immune stimulation, resulting in a dose-sparing effect of the vaccine[43]. EP-mediated administration of HB-110 accelerated antigen expression, increased anti-HBs antibody production, and elicited a broader repertoire of multispecific cellular responses in mice against all antigens, including subdominant epitopes[44]. Comparable conclusions emerged from high-pressure injection in combination with EP, to deliver codon-optimized HBc and IL-12 expressing plasmids that elicit polyfunctional T cell responses[45].

In the duck hepatitis B virus model, EP-mediated vaccination increased the production of neutralizing HBV-specific antibodies, expanded the spectrum of targeted epitopes[46] and resulted in T helper type 1 polarization[47]. Short non-coding DNA fragments appear to increase the immunopotency of EP-mediated HBV DNA vaccination[48]. DNA vaccine platforms based on EP show an enhanced ability to activate both arms of adaptive immunity and thus represent an important tool to overcome immunological exhaustion in CHB.

Regarding human studies, a dual plasmid-HBV vaccine consisting of a therapeutic plasmid encoding the preS2/S antigen and an adjuvant plasmid containing the fused sequence of IL-12 and IFN-γ was the first to be administered *via* electroporation against CHB. In total, 6 of 39 HBeAg-positive participants received vaccine monotherapy, while the remaining patients were randomized 1:2 to receive either lamivudine plus placebo or the experimental treatment, lamivudine plus vaccination. The EP-mediated immunization stimulated HBV-addressing IFN-γ-producing T cells, and the combination therapy produced a more profound suppression of viral replication

and a lower risk of virologic breakthrough compared with NA monotherapy[49].

The satisfactory immunostimulatory efficacy followed by a very consistent safety record as demonstrated in the pilot study has led to a phase IIb trial of the EP-mediated dual plasmid DNA vaccine. In this study, 225 previously untreated HBeAg-positive patients were divided 1:1 into groups treated with either lamivudine plus vaccine or lamivudine plus placebo. Although the primary endpoint of undetectable HBV DNA rate or HBeAg seroconversion was not met, four intramuscular doses of the vaccine showed a modest therapeutic effect, with more vaccinated patients achieving a > 2 log10 IU/mL decrease in HBV DNA compared with the control group[50].

Other HBV DNA vaccines administered *via* EP are currently being investigated in clinical trials with different eligibility criteria, combination regimens, or dosing schedules. An open-label phase I study (NCT02431312) evaluating the safety and reactogenicity of dose combinations of INO-1800 (DNA plasmid expressing core and surface antigens) and INO-9112 (DNA plasmid containing IL-12 sequences) in patients who have received stable NA therapy for at least 1 year has been completed, and final results are pending. In addition, a phase I study (NCT03463369) is underway to evaluate the efficacy of the DNA vaccine JNJ-64300535 administered by EP-mediated intramuscular injection in virally suppressed CHB patients on NA treatment. Overall, administration of the DNA vaccine by EP resulted in multispecific humoral and long-lasting memory T cell responses, although efficacy on clinical endpoints was subpar. These results are important for future studies in which careful patient selection is performed and EP-mediated vaccination realizes its full potential to efficiently restore adaptive immune responses in CHB.

MRNA VACCINES

mRNA vaccines are an attractive alternative to traditional vaccine platforms because they allow a rapid and scalable manufacturing process without being infectious or carrying the risk of integration. Compared to plasmid vaccines, mRNA vaccines have a stronger immunostimulatory effect on innate immunity and result in the desired vaccine responses[51]. Optimization of mRNA stability and translation as well as advances in vaccine delivery have led to increased use of mRNA therapeutics in basic research and clinical trials for the treatment of infectious diseases and cancer[52]. Nevertheless, research on mRNA-based vaccines in the field of anti-HBV prevention or control strategies has been sparse.

In an attempt to develop an anti-HBV mRNA vaccine for prophylactic or immunotherapeutic purposes, Lamb[53] developed and implemented an mRNA production process containing all critical HBsAg epitopes. The mRNA lipoplex nanoparticles were designed to protect against exonuclease degradation, promote endocytosis-mediated cellular uptake, and facilitate endosomal escape of the entrapped mRNA. Upon release into the cytoplasm, the mRNA is used by the host translational machinery as a template for the production of S-HBsAg or L-HBsAg, which are amenable to intrinsic post-translational modifications and are either secreted or degraded in a proteasome-dependent manner. *In situ* processing and presentation of the mRNA-derived antigen products would then resemble that of DNA vaccines and elicit robust pathogen-specific humoral and cell-mediated immune responses[53,54]. Interestingly, an mRNA-based vaccine formulation that was efficiently translated into detectable L-HBs and S-HBs in cultured hepatoma cells was produced using a highly reproducible method. However, L-HBs was expressed at lower levels than expected, and its secretion was modest, possibly reducing its immunostimulatory effect[53]. Clearly, in later stages of development, optimization of downstream processes is required to fully exploit the immunogenic benefits of the vaccine.

CONCLUSION

Recently, numerous nucleic acid vaccines have been used in clinical trials to prevent or treat infectious diseases as innovations in biotechnology have improved their immunogenicity and tolerability. Compared to mRNA-based formulations, plasmid vaccines have been studied more intensively in the field of HBV therapeutics. Pioneering preclinical studies have shown that DNA vaccination could suppress HBV transcriptional activity and even affect the cccDNA pool and was able to break CHB immune tolerance. In parallel, the introduction of *in vivo* EP as a delivery platform has dramatically improved the immunostimulatory effect of DNA vaccines without

serious adverse events.

Given these data and despite the moderate results of the initial clinical trials, the design of new studies to clarify the role of DNA vaccination in this field is essential. An ideal vaccine candidate would contain multiple dominant and subdominant HBV epitopes in combination with appropriate adjuvants to elicit potent and multispecific responses and should be administered *via* EP to enhance immunogenicity. Add-on or sequential treatment regimens could be applied to progressively repair the dysregulated adaptive immune responses and eventually achieve HBV eradication. Identifying patients who benefit most from immunization strategies should be a priority of future studies. Preferably, vaccination should be studied in virus-suppressed CHB patients on stable therapy with second-generation NAs that are less susceptible to drug resistance, such as entecavir or tenofovir. In summary, nucleic acid-based immunotherapies still have a long way to go before market approval but may retain a place in the context of combination therapy aimed at a functional cure for HBV.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 November 7; 27(41): 7014-7024

DOI: 10.3748/wjg.v27.i41.7014

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

FRONTIER

Recent insights into the characteristics and role of peritoneal macrophages from ascites of cirrhotic patients

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Author contributions: García-Peñ arrubia P designed the overall concept outline of this manuscript and was responsible for writing, editing, reviewing the bibliography and approving the final version of the article; Ruiz-Alcaraz AJ wrote the manuscript, compiled, reviewed and edited the bibliography, adapted the figures and approved the final version of the article; Martínez-Esparza M wrote the manuscript, compiled and reviewed the bibliography and approved the final version of the article; Ruiz-Ballester M and Rami rez-Pávez TN meet the criteria for authorship established by the International Committee of Medical Journal Editors and approved the final version of the article.

Conflict-of-interest statement: The authors declare no conflict of interest.

Country/Territory of origin: Spain

Specialty type: Gastroenterology

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Abstract

Macrophages are a diverse myeloid cell population involved in innate and adaptive immune responses, embryonic development, wound repair, and regulation of tissue homeostasis. These cells link the innate and adaptive immunities and are crucial in the development and sustainment of various inflammatory diseases. Macrophages are tissue-resident cells in steady-state conditions; however, they are also recruited from blood monocytes after local pathogen invasion or tissue injury. Peritoneal macrophages vary based on their cell complexity, phenotype, and functional capabilities. These cells regulate inflammation and control bacterial infections in the ascites of decompensated cirrhotic patients. Our recent work reported several phenotypic and functional characteristics of these cells under both healthy and pathological conditions. A direct association between cell size, CD14/CD16 expression, intracellular level of GATA-6, and expression of CD206 and HLA-DR activation/maturation markers, indicate that the large peritoneal macrophage $CD14^{high}CD16^{high}$ subset constitutes the mature phenotype of human resident peritoneal macrophages during homeostasis. Moreover, elevated expression of CD14/CD16 is related to the phagocytic capacity. The novel large $CD14^{high}CD16^{high}$ peritoneal subpopulation is increased in the ascites of cirrhotic patients and is highly sensitive to lipopolysaccharide (LPS)-induced activation, thereby exhibiting features of inflammatory priming. Thus, phosphorylation of ERK1/2, PKB/Akt, and c-Jun is remarkably increased in response to LPS *in vitro*, whereas that of p38 MAPK is reduced compared with the monocyte-derived macrophages from the blood of healthy controls. Furthermore, *in vitro* activated monocyte-derived macrophages from ascites of cirrhotic patients secreted significantly higher levels of IL-6, IL-10, and TNF-α and lower amounts of IL-1β and IL-12 than the corresponding cells from healthy donor's blood. Based on these results, other authors have recently reported that the surface expression level of CD206 can be used to identify

mature, resident, inflammatory peritoneal macrophages in patients with cirrhosis. Soluble CD206 is released from activated large peritoneal macrophages, and increased concentrations in patients with cirrhosis and spontaneous bacterial peritonitis (SBP) indicate reduced odds of survival for 90 d. Hence, the level of soluble CD206 in ascites might be used to identify patients with SBP at risk of death. In conclusion, peritoneal macrophages present in ascites of cirrhotic patients display multiple phenotypic modifications characterized by reduced ratio of cells expressing several membrane markers, together with an increase in the ratios of complex and intermediate subpopulations and a decrease in the classic-like subset. These modifications may lead to the identification of novel pharmaceutical targets for prevention and treatment of hepatic damage.

Key Words: Cirrhosis; Inflammation; Peritoneal macrophages; Phenotypic markers; Activation routes

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Core Tip: This frontier article is based on a summary of recent relevant publications on the biology of mouse, as the main animal model used, and human peritoneal macrophages under the perspective of its future clinical translation to the role that these cells can play on several human liver diseases. Concretely, we have reviewed recent findings on several characteristics of human peritoneal macrophages obtained from the ascites of cirrhotic patients compared with those obtained from healthy donors. Featured article: Role of MAP kinases and PI3K-Akt on the cytokine inflammatory profile of peritoneal macrophages from the ascites of cirrhotic patients.

Citation: García-Peñarrubia P, Ruiz-Alcaraz AJ, Ruiz-Ballester M, Ramírez-Pávez TN, Martínez-Esparza M. Recent insights into the characteristics and role of peritoneal macrophages from ascites of cirrhotic patients. *World J Gastroenterol* 2021; 27(41): 7014-7024
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7014.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7014

BIOGRAPHY

Pilar García-Peñarrubia (Figure 1), MD, PhD is a Professor in the Departments of Biochemistry and Molecular Biology B and Immunology in the School of Medicine, at the University of Murcia, Spain. She received her MD degree from the School of Medicine at the University of Murcia, Spain in 1975. She certified as MD specialist in Microbiology and Parasitology in 1980. She received her PhD degree from the School of Medicine at the University of Murcia, Spain in 1979 with honor "cum laude".

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Her subjects of interest include the immunopathology of hepatic cirrhosis and endometriosis, the physiology of human NK cells and peritoneal macrophages, and theoretical models of biological systems, especially the immune system.

INTRODUCTION

Liver cirrhosis is the end stage of various different chronic hepatic diseases, characterized by a gradual substitution of the liver structure by fibrotic tissue[1]. The role of monocytes and macrophages in the physiopathology of liver cirrhosis has been extensively reported[2-5]. Rapid mobilization of these cells to peritoneum or hepatic tissue is an important mechanism of defense against incidental bacterial infection translocated from the gut[6]. Pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), peptidoglycan, mannan, glucans, bactDNA, and many

and hepatology

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

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Received: April 14, 2021
Peer-review started: April 14, 2021
First decision: June 23, 2021
Revised: July 2, 2021
Accepted: October 11, 2021
Article in press: October 11, 2021
Published online: November 7, 2021

P-Reviewer: Ferrarese A, Zhang L
S-Editor: Wang LL
L-Editor: A
P-Editor: Wang LL



Figure 1 Pilar García-Peñarrubia, MD, PhD is a Professor in the Departments of Biochemistry and Molecular Biology B and Immunology in the School of Medicine, at the University of Murcia, Spain. She received her MD degree from the School of Medicine in the University of Murcia, Spain in 1975. She certified as MD specialist in Microbiology and Parasitology in 1980. She received her PhD degree from the School of Medicine in the University of Murcia, Spain in 1979 with honor "cum laude". From 1986 to 1989 she was a Research Associate with the Department of Medicine, School of Medicine, University of New Mexico, Albuquerque, United States, and a Fellow of the Fulbright Foundation (1986-1987). She was Vice Dean of the School of Medicine (University of Murcia) since 1999 to 2002. Her subjects of interests include the immunopathology of hepatic cirrhosis and endometriosis, the physiology of human NK cells and peritoneal macrophages, and theoretical models of biological systems, especially the immune system.

others, induce the secretion of cytokines from myeloid-derived monocytes and macrophages[7-10]. Chronic inflammation and fibrosis are crucial features associated with macrophage accumulation in the liver[2,10]. Moreover, marked hepatic and systemic damage in cirrhotic patients is associated with high secretion of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6, as well as anti-inflammatory cytokines such as IL-10 and TGF-β[11]. In this sense, recent large-scale observational studies have pointed to systemic inflammation as a hallmark of acute decompensation of cirrhosis. Hence, a recent hypothesis proposes that systemic inflammation is the key mechanism in the progression from compensated to decompensated cirrhosis, as well as in the development of acute episodes of decompensation, which are associated with generalized dysfunction or even with extrahepatic multiorgan failure[4].

The study of human tissue-resident macrophages presents several challenges. First, it is necessary to carry out surgical interventions to obtain these macrophages; second, the cell count obtained is low; and third, these cells are difficult to grow *in vitro*. Thus, accumulated research on resident macrophages in both homeostasis and pathology has been performed in animal models, particularly in mice. Nevertheless, it is not always possible to extrapolate from mice to man, in particular in this topic[12,13]. Thus, it is essential to verify whether the tissue factors and macrophage transcription molecules identified in murine models play similar roles in the origin and biology of human tissue-resident macrophages. Additionally, research on the role of the immune system in human inflammatory diseases is principally carried out with peripheral blood leukocytes; however, the study of macrophages from an inflammatory setting can provide relevant information for a better understanding of the physiopathology of numerous human diseases[8,14]. Hence, human peritoneal macrophages (pMϕ) are a potential option for studying the biological characteristics of this cell type[15]. Peritoneal leukocytes play a crucial role in the defense against microbial infections within the peritoneal cavity, and they also contribute to endometriosis and cancer pathologies. Thus, while knowledge about the inflammatory status in peripheral blood and liver of cirrhotic patients has increased dramatically in the last few years, less is known about its correlation with the inflammatory status in ascites fluid (AF), and limited information is available on differences related to cirrhotic etiology[16]. Recent studies have demonstrated that pMϕ are crucial in regulating inflammation and controlling peritoneal infections in decompensated cirrhotic patients. The accurate phenotypic characterization of macrophages obtained from AF of cirrhotic patients helps us to understand and presumably prognosticate the risk of experiencing episodes of spontaneous bacterial peritonitis (SBP), which impairs the outcome in this clinical condition[17,18]. Furthermore, as macrophages are implicated in the physiopathology associated with hepatic cirrhosis, these cells are also studied as targets of new therapies expected to avert the progression of hepatic damage[2,3,14].

Very recently, novel findings on the ontogeny, phenotype, function, specific transcription factors, migratory activity[19-21] and sex differences[22], of mouse resident pMϕ have been reported. These findings open new avenues for the study of human pMϕ in both health and disease conditions.

ORIGIN OF HUMAN PERITONEAL MACROPHAGES

Macrophages are a heterogeneous myeloid cell population involved in innate and adaptive immune responses, as well as in embryonic development, regulation of tissue homeostasis, and wound repair. These immune cells link innate and adaptive immunities by acting as antigen-presenting cells and are crucial in the development and persistence of various inflammatory conditions.

Macrophages are found in all tissues as resident cells in steady-state conditions, and also as immigrant foreign cells derived from peripheral blood monocytes in response to microbial invasion, tissue injury, or inflammation[7,8]. The contribution of monocytes to resident macrophages is highly tissue-dependent, and until recently it was admitted that most of the homeostatic murine pMϕ are terminally differentiated and replenished by blood monocytes[8]; however, groundbreaking findings revealed that the majority of tissue-resident macrophages do not arise from hematopoietic progenitors, as they directly originate from embryonic precursors (yolk sac and fetal liver) and are able to proliferate and self-renew[19]. Thus, local proliferation reestablishes the normal macrophage number after inflammation-induced loss of resident pMϕ[20]. Moreover, it has been reported that the expression of transcription factor GATA-binding protein 6 (GATA-6) is mostly limited to the long-lived murine F4/80^{hi}CD11b^{hi} large peritoneal macrophages (LPMs) of embryonic origin, whereas the subpopulation of F4/80^{low}MHC-II^{hi}, namely small peritoneal macrophages (SPMs), arise from inflammatory monocytes[21]. Recently, Louwe *et al*[23] reported that the fate and function of inflammation-activated pMϕ seem to be regulated by environmental changes. Thus, moderate inflammation-elicited murine pMϕ survive for 5 mo, although they do not acquire the GATA-6^{hi} resident signature. In contrast, high inflammation results in depletion of resident macrophages for a sustained period, although ultimately, stimulated cells achieve a mature GATA-6^{hi} expression.

Bain *et al*[22] reported that murine pMϕ exhibit sexual dimorphism, determined by different microenvironmental cues and a differential replenishment rate from the bone marrow. After sexual maturity, the time of residency and local tissue factors seem to result in increased expression of immune function-related genes in F4/80^{hi}CD102+ macrophages, particularly CD209b, in female mice, which more efficiently control the peritoneal infection with *Streptococcus pneumoniae*. In contrast, the rate of replenishment from the bone marrow is higher in male animals. In this regard, Oh *et al*[24] identified the mTORC2-FOXO1 axis as crucial for integrating microenvironmental signs to regulate metabolic reprogramming, differentiation, and activity of peritoneal tissue-resident macrophages.

PERITONEAL MACROPHAGES CAN MIGRATE VIA A NONVASCULAR ROUTE TO THE INJURED LIVER

Studies conducted in mice have revealed the crucial role played by pMϕ in homeostasis as well as in the physiopathology of multiple systemic or abdominal diseases[25,26]. In this regard, Wang and Kubes[27] reported that murine mature pMϕ F4/80^{hi}GATA-6^{+} rapidly (within 12 h post-injury) infiltrate the injured liver through a non-vascular route across the mesothelium layer, thereby adopting an alternatively activated phenotype with an increased expression of arginase 1, and protect against acute liver damage. The recruitment of pMϕ toward the sites of liver injury was dependent neither on chemokine receptor signaling nor on β1 or β2 integrins, which indicates that this mechanism differs from that of the recruitment of immune cells *via* an intravascular route. Recruitment guidance was dependent on ATP and hyaluronan in the injured tissues, as well as on macrophage expression of CD44, which is a known receptor for the last molecule.

These findings challenge the present assumption that tissue-resident macrophages are stationary cells, and suggest that rapid mobilization of pMϕ, with ability to induce tissue repair, into the damaged liver, can be an important defense mechanism against infections, trauma, metabolic diseases, fibrosis, and tumor diseases.

CHARACTERISTICS OF HUMAN PERITONEAL MACROPHAGES IN HOMEOSTASIS

Human pMϕ are the best choice for carrying out studies on the biological properties of tissue-resident macrophages under homeostatic conditions; moreover, these macrophages can be used as a healthy control group to compare data from individuals suffering from various pathologies affecting the peritoneal cavity, such as cirrhotic or cancer ascites. For this purpose, peritoneal fluid (PF) samples must be obtained either from healthy people or from individuals whose disease does not affect the peritoneal compartment. The most frequent control samples referred to in the corresponding literature were collected from patients on continuous ambulatory peritoneal dialysis (CAPD), not affected by SBP, as well as from exploratory gynecological laparoscopies/laparotomies performed in healthy women[28-31].

Nevertheless, CAPD patients are not healthy people; moreover, it has been reported that the fluid flow through the rat omentum increases with peritoneal dialysis, thereby leading to a dramatic enlargement of the leukocyte aggregates called "milky spots", which are rich in macrophages, lymphoid B and T cells, mast cells, and stromal cells [32,33], and promoting omental fibrosis[34]. Thus, due to these objections, peritoneal cells from CAPD do not really qualify as representative of homeostatic peritoneal cells to be used as healthy control.

We have recently described an optimized method for obtaining human pMϕ from the PF of healthy women[35], and studied several characteristics of healthy human pM ϕ compared with the well-known CD14/CD16 blood monocyte subsets, in order to analyze common properties or tissue-specific differences[36]. Hence, PF from 79 healthy women was acquired from the Gynecological Unit of the HCUVA, Murcia, Spain. Cell samples from blood and PF were obtained during exploratory or therapeutic laparoscopies for benign gynecological pathology (simple ovarian cysts or uterine fibroids) or tubal ligation. Under physiological conditions, a small amount of 5-20 mL PF is present in the peritoneal cavity. It is produced by mesothelial cells and contains a mix of plasma transudate, ovarian exudates, tubal fluid, and macrophages' secretions[37-39]. The physiological functions of PF include lubricating the friction of the intestinal loops and other organs contained in the peritoneal cavity, allowing the exchange of nutrients, repairing injured tissues, and eliminating detritus and microorganisms. In our experience, the first PF obtained by the endoscopic aspirator is quite scarce; with a mean of 6.85 ± 2.6 mL (range 5-8.7 mL). Moreover, this fluid has practically no polymorphonuclear (PMN) leukocytes, which is indicative of the absence of local inflammatory signals. Among human peritoneal leukocytes, macrophages are the predominant cell type (45-90%) followed by T lymphocytes (predominantly T effector/memory cells, CD45RO) (45%), NK cells (8%), dendritic cells (2-6%), B lymphocytes (2%) and less than 5% of PMN cells[31,35,40-43].

Our results revealed that primary human pMϕ have phagocytic and oxidative activities, and they respond to activation of the main proinflammatory routes such as Toll-like receptors and inflammasomes, which further results in the secretion of different proinflammatory cytokines[35]. Furthermore, we demonstrated that pMϕ are heterogeneous with respect to their morphology and CD14/CD16 cell expression. This peritoneal population is made up of akin proportions (approximately 42%) of classic ($CD14^{++}CD16^{-}$) and intermediate ($CD14^{++}CD16^{+}$) small cells, and a novel subset of complex $CD14^{high}CD16^{high}$ cells (approximately 16%), which are not found in the peripheral blood. In contrast, nonclassical blood monocyte-like cells are not detected in the peritoneal cavity[36]. Moreover, pMϕ reveal higher expression of CD14 and CD16 than blood monocytes, which makes them more competent or available for phagocytosis in the presence of LPS or microorganisms. Notably, the percentages of these cell subpopulations are modulated under inflammatory processes. Thus, besides describing the presence of a novel human $CD14^{high}CD16^{high}$ LPM subpopulation (33% ± 2.4%) in the ascites of decompensated cirrhotic patients for the first time, we also found that the percentage of intermediate $CD14^{++}CD16^{+}$ subset was predominant (49% ± 2.0%), whereas the classic $CD14^{++}CD16^{-}$ subset revealed lowest values (18% ± 1.3%) [44]. These modifications in pathological versus steady-state conditions strengthen the importance of these results.

We also analyzed the expression of several monocyte/macrophage-associated membrane receptors implicated in phagocytosis of IgG-opsonized (CD64, high affinity FcγRI) and complement-opsonized microorganisms (CD11b and CD11c, the α chains of Complement receptors, CR3 and CR4); adhesion to activated endothelial cells and tissue recruitment (CR3, CR4, CD62L, and 6-sulfo LacNAc (Slan)), antigen presentation (MHC class II molecule HLA-DR), costimulatory markers (CD80, CD86, CD40),

cytokines receptors (CD116, GM-CSFR and CD119, IFNγR1 or IFNγ chain α receptor), and the mannose receptor (CD206), reported as a M2 polarized marker and denotative of activation/maturation[45]. In comparison with the complete population of blood monocytes, CD86, CD64, and CD11b revealed similar expression on pMϕ; whereas, small significant differences were observed for a higher expression of HLA-DR, CD116 and CD119 on pMϕ. The most compelling differences were found for CD40, CD80, CD11c, CD206, Slan, and CD62L, of which CD62L was the only receptor expressing higher levels of blood monocytes. These findings suggest that human pMϕ could exert remarkable antimicrobial (also high phagocytic and oxidative capacity), antigen-presenting, and T-cell costimulatory capacities; however, this remains to be further explored. Conversely, the steady increase in the percentages and density of CD206 expression from 28.2% in $CD14^{++}CD16^{-}$ to 60.3% in $CD14^{++}CD16^{+}$ and 92.8% in $CD14^{high}$ $CD16^{high}$ suggested that human pMϕ may also exhibit features and functional characteristics of M2 macrophages, as previously described in CAPD[46,47] and endometriosis patients[48]. Nevertheless, the most remarkable differences between blood and pMϕ subsets were detected on selectin CD62L expression, that is, percentages of pMϕ expressing CD62L in each subpopulation increase in parallel with the expression of CD16, whereas the corresponding expression of CD62L in blood monocytes diminishes as CD16 increases. Moreover, it was observed that the percentages of cells expressing Slan were statistically higher in the peritoneal subset[49]. These differences in adhesion molecules could be associated with a differential pattern of cell-tissue recruitment (endothelium/mesothelium)[27]. Expression of GATA-6 in the three subsets of pMϕ was similar, whereas it was absent in blood monocytes. Nevertheless, we found a high correlation between the increment of GATA-6 and the cell membrane expression of CD14 and CD16; suggesting that monocyte migration to the peritoneal compartment in steady-state is scarce, or that the GATA-6 expression in recently arrived peritoneal monocytes is rapid. The homeostatic state of this cell population was confirmed by the low percentages of cells exhibiting intracellular IL-6, TNF-α, and IL-10 cytokines. Notably, the intermediate subset revealed the highest level of intracellular cytokines, whereas the $CD14^{high}CD16^{high}$ LPM subset presented a higher number of IL-10 positive cells related to the named proinflammatory cytokines, supporting the hypothesis related to its M2 polarization tendency. Eventually, we found a linear relationship between CD14/CD16 cell expression and activation/maturation markers, such as CD206 and HLA-DR, intracellular level of GATA-6, phagocytic/oxidative capacity, and intracellular level of IL-6, TNF-α, and IL-10. These data suggest that the population of LPM $CD14^{high}CD16^{high}$ could act as the phenotypic marker of mature differentiated human-resident pMϕ in homeostasis, whereas the intermediate $CD14^{++}CD16^{+}$ subset could be a transitional cell type also integrated by newly recruited blood monocytes.

CHARACTERISTICS OF HUMAN PERITONEAL MACROPHAGES FROM THE ASCITES OF CIRRHOTIC PATIENTS

In the last decade, our group has also focused on the study of pMϕ characteristics in patients with decompensated cirrhosis and culture-negative ascites. We found that these pMϕ display a preactivated status at baseline, with elevated expression of HLA-DR, CD86 and CD54 membrane markers, increased phosphorylated levels of PKB (Akt), ERK1/2 and c-Jun intracellular signaling molecules, and high secretion of IL-6 [50]. These findings presumably indicate that repeated events of bacterial translocation (BT) promote a sustained immune response, even in the temporary absence of PAMPs. This primed state could enhance an IL-6-regulated fast response to intermittent BT events[50]. Further studies performed *in vitro* with pMϕ from ascites of cirrhotic patients revealed that the secretion of proinflammatory cytokines TNF-α, IL-1β, and IL-6 are regulated by the MAPK signaling intracellular cascades, whereas the PI3K-Akt pathway plays an important role in regulating the anti-inflammatory activity of IL-10[51,52].

The inhibitors of MEK1 and c-Jun N-terminal kinases (JNK) decreased the synthesis of TNF-α, IL-1β and IL-6, and could thus be assayed as therapeutic compounds to reduce hepatic damage associated with liver failure[16,35,52]. Conversely, inhibitors of PI3K-Akt blocked the secretion of IL-10 and augmented the production of IL-1β, mainly by inducing the secretion of intracellular IL-1β and caspase-1 to the extracellular compartment (Figure 2). Based on these results, PI3K-Akt inhibitors are excluded as potential drugs for the treatment of hepatic fibrosis, since these agents may enhance the inflammatory status[51].

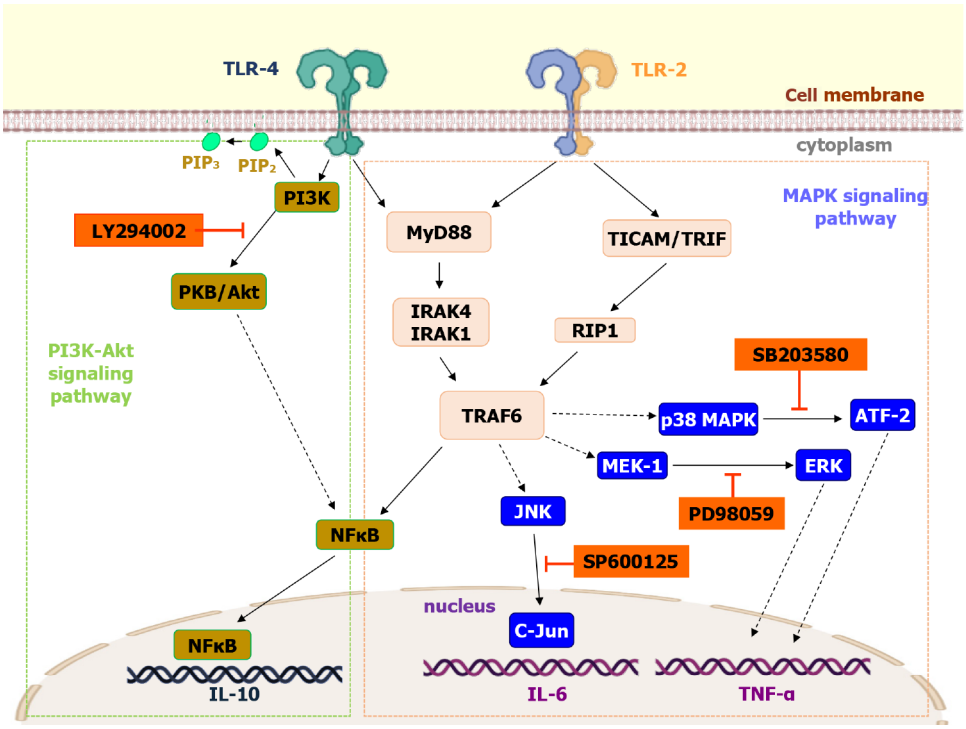


Figure 2 TLR4 and TLR2 cell signaling pathways in normal subjects. TLR2 and TLR4 engagement induce activation of PKB-Akt and MAPK intracellular signaling pathways leading to the phosphorylation of several molecules, which control the expression levels of pro- and anti-inflammatory cytokines. The targets of PD98059, SB203580, SP600125, and LY294002 inhibitors (orange boxes) are indicated by dashed arrows. Adapted from Tapia-Abellán *et al*[51] with permission from John Wiley and Sons, Inc. Citation: Tapia-Abellán A, Ruiz-Alcaraz AJ, Hernández-Caselles T, Such J, Francés R, García-Peñarrubia P, Martínez-Esparza M. Role of MAP kinases and PI3K-Akt on the cytokine inflammatory profile of peritoneal macrophages from the ascites of cirrhotic patients. *Liver Int* 2013; 33: 552-560. Copyright© John Wiley and Sons, Inc.

Peritoneal macrophages from non-infected AF present basal activation of caspase-1 and an increased expression of IL-1β, IL-18, and AIM2 compared to peripheral blood macrophages. The inflammasome activation *in vitro* did not need a priming signal, which supports the preactivated status of these pMϕ[52,53]. As mentioned above, our group reported that a novel $CD14^{high}CD16^{high}$ LPM subpopulation in the AF of cirrhotic subjects is highly sensitive to stimulation with LPS. The $CD14^{++}CD16^{+}$ intermediate subpopulation is augmented in the blood of decompensated cirrhotic patients (from 4% to 11%) and is prevalent in ascites (49%). Baseline hyperactivation of ERK and JNK/c-Jun routes found in ascites pMϕ was associated with cell subsets expressing high levels of CD14/CD16, whereas PI3K/PKB was correlated with CD16 low expressing cells. *In vitro* stimulated pMϕ from ascites of cirrhotic individuals generated statistically higher levels of TNF-α, IL-6, and IL-10, and lower amounts of IL-1β and IL-12 than monocyte-derived macrophages (M-DM) from the blood of controls[44] (Figure 3).

Moreover, Irvine *et al*[54] reported two subsets of pMϕ in AF from decompensated cirrhotic patients: that is, a more phagocytic subset expressing high levels of *VSIG4* (encoding CRIg) and Tim4, and a second less phagocytic subset exhibiting low levels of *VSIG4*, high levels of CCR2a, and responsiveness to retinoic acid. Our unpublished data revealed that these subsets are equivalent to our $CD14^{high}CD16^{high}$ LPMs and $CD14^{++}CD16^{+/-}$, respectively.

More recently, Stengel *et al*[18] have reported that LPMs from the AF of cirrhotic patients present a proinflammatory signature based on the expression of $CD14^{+}$, $CD16^{+}$, $CD206^{+}$, $CD163^{+}$, $MERTK^{+}$, $CD40^{+}$, $CCR2^{-}$, and on *in vitro* transcriptomic analysis and cytokine secretion in the presence and absence of LPS or viable *Escherichia coli* stimulation, respectively. Meanwhile, the corresponding subset of SPMs from AF expresses $CD14^{+}$, $CD16^{+}$, $CD206^{-}$, $CD163^{+}$, $MERTK^{+}$, $CCR2^{+}$. As normal control group, they used macrophages from effluents of CAPD patients with end-stage renal disease, not affected by SBP. These control LPMs displayed a similar phenotype to that of the corresponding subset from cirrhotic patients AF. Interestingly, during SBP episodes, LPMs change to a more inflammatory phenotype characterized by low CD206, low MERTK, and normal CD163 cell surface expression. In particular, LPMs shed surface-bound CD206 as soluble CD206 (sCD206) in response to bacterial peritonitis as well as

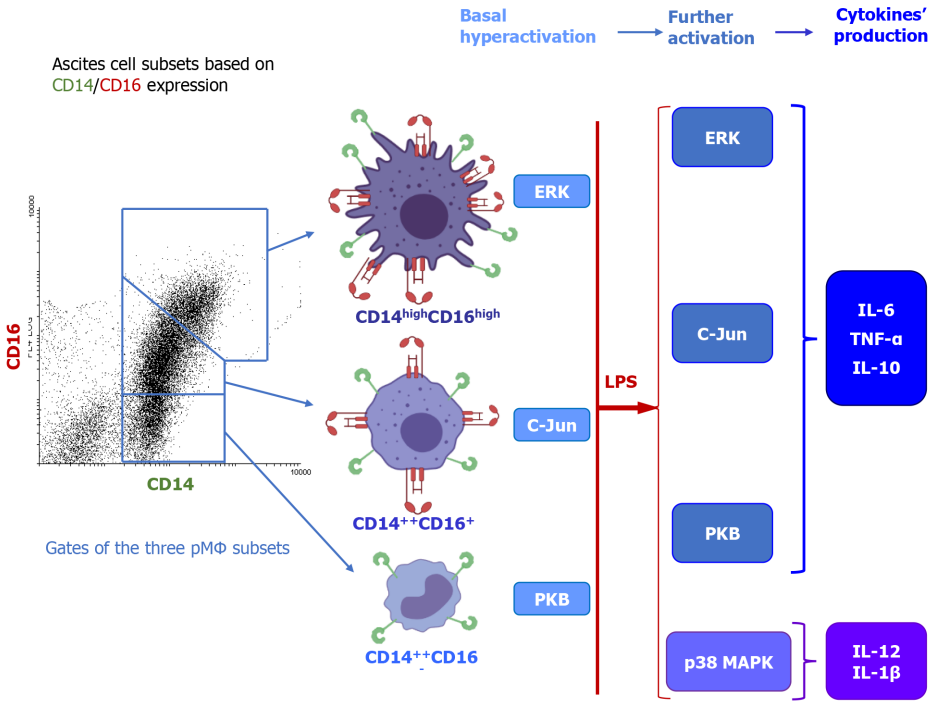


Figure 3 Peritoneal macrophage subsets from cirrhotic patients. The ascitic fluid of cirrhotic patients presents three different subpopulations of peritoneal macrophages based on their cell morphology and CD14/CD16 expression. Baseline hyperactivation of ERK and JNK/c-Jun signaling routes detected in ascites peritoneal macrophage (pMϕ) correlates with CD14/CD16 high expressing subsets, whereas PI3K/PKB correlated with the CD16 low expressing cells. *In vitro* treatment with LPS drastically increases PKB/Akt, ERK1/2, and c-Jun activation, whereas the corresponding p38 MAPK is lowered in pMϕ from ascites cells compared to monocyte-derived macrophages (M-DM) from the control blood. *In vitro* LPS-activated macrophages from cirrhotic ascites also produce statistically higher levels of TNF-α, IL-6, and IL-10, as well as lower levels of IL-1β and IL-12 than the control blood M-DM. Adapted from Ruiz-Alcaraz *et al*[44] with permission from Elsevier. Citation: Ruiz-Alcaraz AJ, Tapia-Abellán A, Fernández-Fernández MD, Tristán-Manzano M, Hernández-Caselles T, Sánchez-Velasco E, Miras-López M, Martínez-Esparza M, García-Peñarrubia P. A novel CD14(high) CD16(high) subset of peritoneal macrophages from cirrhotic patients is associated to an increased response to LPS. *Mol Immunol* 2016; 72: 28-36. Copyright© Elsevier.

in vitro response to LPS and *E. coli*. AF sCD206 is an independent predictor of death in patients with SBP. Concentrations of AF sCD206 of > 0.53 mg/L prognosticate a lower 90-day survival rate. In contrast, the rapid loss of CD206+ LPMs in cirrhotic patients in response to SBP is consistent with a process of macrophage depletion, which could allow for blood monocyte settlement.

Previous studies from episodes of SBP have shown that the ascites from negative-culture SBP and positive-culture SBP patients had significantly more macrophages than those from patients with sterile ascites. Furthermore, pMϕ from positive-culture SBP showed poor bactericidal capacity[55,56] and a tolerant state[57], which is consistent with the hypothesis of systemic inflammation. Moreover, high ascites bacterial burden was associated with reduced pMϕ HLA-DR expression. The presence of pMϕ (CD14+/HLA-DR+) in ascites was associated with a lower number of neutrophils and a tendency towards a lower bacterial burden[58]. Given the scarcity of studies on the role of pMϕ in SBP, new lines of research may be opened in this regard to provide new knowledge about the pathophysiology and potential treatments of liver cirrhosis.

CONCLUSION

These new findings can pave way for several important questions: (1) Are human resident peritoneal macrophages able to migrate through the new described nonvascular route as those cells in mice; (2) Are resident peritoneal macrophages able to migrate to virus or bacterial infected liver; (3) Are human resident peritoneal macrophages able to migrate toward other abdominal organs, such as pancreas, spleen, ovary, or gut; (4) Could omentum comprise a reservoir of mature peritoneal macrophages, ready to move toward other peritoneal organs by detecting danger signs in order to repair tissue damage and maintain health; (5) Are there any differences in GATA-6 expression depending on the type or stage of distinct liver pathologies; (6)

Are there differences in the pattern of cytokine secretion between the three CD14/CD16 cell populations identified in ascites of decompensated cirrhotic patients; (7) Could data of proinflammatory potential of CD206+ LPM in the AF of cirrhotic patients be reproduced in cohorts of cirrhosis from other etiologies; (7) Is AF sCD206 a useful marker to prognosticate mortality risk from decompensated cirrhotic patients; and (8) Could AF sCD206 be used as a useful marker to prognosticate evolution of other peritoneal diseases, such as endometriosis, ovarian cancer, or others?

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7025-7040

DOI: 10.3748/wjg.v27.i41.7025

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

FRONTIER

Involvement of parathyroid hormone-related peptide in the aggressive phenotype of colorectal cancer cells

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Author contributions: Calvo N and Gentili C supervised this project; Gentili C was responsible for the project administration and funding acquisition and provided the resources; All authors were involved in conceptualization, methodology, investigation, formal analysis, visualization, writing original draft, writing-review and editing.

Conflict-of-interest statement: Authors declare no conflict of interest for this article.

Supported by Agencia Nacional de Promoción Científica y Tecnoló gica, No. PICT-2013-1441; Consejo Nacional de Investigaciones Científicas y Técnicas, No. PIP11220150100350; Instituto Nacional del Cáncer, Asistencia financiera II, No. 12002-4395-14-1; Instituto Nacional del Cáncer, Asistencia Financiera III, No. RESOL-2016-1006-E-APN-MS; and Universidad Nacional del Sur, Argentina, No. PGI: 24/B230; PGI: 24/B303.

Country/Territory of origin:

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Abstract

Colorectal cancer (CRC) remains one of the leading causes of mortality from malignant diseases worldwide. In general terms, CRC presents high heterogeneity due to the influence of different genetic and environmental factors; also, the neoplastic cells are strongly influenced by the extracellular matrix and several surrounding cells, known together as the tumor microenvironment (TME). Bidirectional communication takes place between the tumor and the TME through the release of autocrine and paracrine factors. Parathyroid hormone-related peptide (PTHrP) is a cytokine secreted by a wide variety of tissues and is able to regulate several cellular functions both in physiological as well as in pathological processes. It exerts its effects as a paracrine/autocrine factor, although its mode of action is mainly paracrine. It has been shown that this peptide is expressed by several tumors and that the tumor secretion of PTHrP is responsible for the malignant humoral hypercalcemia. Eight years ago, when our research group started studying PTHrP effects in the experimental models derived from intestinal tumors, the literature available at the time addressing the effects of PTHrP on colorectal tumors was limited, and no articles had been published regarding to the paracrine action of PTHrP in CRC cells. Based on this and on our previous findings regarding the role of PTH in CRC cells, our purpose in recent years has been to explore the role of PTHrP in CRC. We analyzed the behavior of CRC cells treated with exogenous PTHrP, focalizing in the study of the following events: Survival, cell cycle progression and proliferation, migration, chemoresistance, tumor-associated angiogenesis, epithelial to mesenchymal transition program and other events also associated with invasion, such us the induction of cancer stem cells features. This work summarizes the major findings obtained by our investigation group using *in vitro* and *in vivo* CRC models that evidence the parti-

Argentina

Specialty type: Gastroenterology and hepatology

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

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Received: April 27, 2021
Peer-review started: April 27, 2021
First decision: June 13, 2021
Revised: June 14, 2021
Accepted: August 23, 2021
Article in press: August 23, 2021
Published online: November 7, 2021

P-Reviewer: Rojas A, Zhu Y
S-Editor: Wu YXJ
L-Editor: Filipodia
P-Editor: Liu JH

cipation of PTHrP in the acquisition of an aggressive phenotype of CRC cells and the molecular mechanisms involved in these processes. Recently, we found that this cytokine induces this malignant behavior not only by its direct action on these intestinal cells but also through its influence on cells derived from TME, promoting a communication between CRC cells and surrounding cells that contributes to the molecular and morphological changes observed in CRC cells. These investigations establish the basis for our next studies in order to address the clinical applicability of our findings. Recognizing the factors and mechanisms that promote invasion in CRC cells, evasion to the cytotoxic effects of current CRC therapies and thus metastasis is decisive for the identification of new markers with the potential to improve early diagnosis and/or to predict prognosis, to predetermine drug resistance and to provide treatment guidelines that include targeted therapies for this disease.

Key Words: Parathyroid hormone-related protein; Colorectal cancer; Tumor biomarkers; Neoplastic processes; Drug resistance; Tumor microenvironment

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Core Tip: In colorectal cancer (CRC) cells, we found that parathyroid hormone-related peptide (PTHrP) promotes survival, cell cycle progression, proliferation, migration and chemoresistance and also modulates markers expression associated with invasion, angiogenesis, epithelial to mesenchymal transition and cancer stem cells features. *In vivo* tests indicate that PTHrP administration increases the expression of several markers related to tumorigenic events. Recently, we observed that PTHrP influences on tumor microenvironment cells, inducing events associated with the progression of CRC. Our project focuses in understanding the biology of CRC and the underlying mechanisms related to the aggressive behavior of CRC cells with the aim to identify new markers that improve the diagnosis, prognosis and therapy of CRC.

Citation: Novoa Díaz MB, Carriere PM, Martín MJ, Calvo N, Gentili C. Involvement of parathyroid hormone-related peptide in the aggressive phenotype of colorectal cancer cells. *World J Gastroenterol* 2021; 27(41): 7025-7040
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7025.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7025

INTRODUCTION

Colorectal cancer (CRC) remains one of the leading causes of mortality from malignant diseases worldwide[1]. In general terms, CRC presents high heterogeneity due to the influence of different genetic and environmental factors[2,3]. Among the factors associated with colorectal tumorigenesis are the damage to intestinal tissue, the presence of harmful microorganisms and the persistence of inflammatory reactions, which can lead to pre-malignant lesions that progress towards a neoplasm[4]. These conditions result in the deregulation of several signaling pathways, a fact that directly alters the cell survival and induces the transformation of the normal epithelium into a hyperproliferative mucosa, causing the development of adenomatous polyps[3,5]. These processes can result in tumor progression, metastasis and resistance to drug therapy and are accompanied by alterations in the DNA repair mechanism, epigenetic changes, genomic instability and several mutations[6,7]. In this context, it is important to highlight that the neoplastic cells are strongly influenced by the extracellular matrix and several surrounding cells, known together as the tumor microenvironment (TME)[8,9]. Bidirectional communication takes place between the tumor and the TME through the release of autocrine and paracrine factors. As a consequence, in the neoplastic cells, multiple molecular mechanisms are triggered to promote their aggressive capacities[10,11]. Simultaneously, the extracellular matrix undergoes modifications that facilitate invasiveness and migration of tumor cells to other tissues where they metastasize[12]. In this instance, the tumor cells show changes in their cell polarity and acquire a mesenchymal-like phenotype, a process known as epithelial to

mesenchymal transition (EMT)[13]. Cumulative evidence associates the EMT process with the acquisition of cancer stem cell (CSC) features[13,14].

CSCs are a fraction of cells in the tumor with the ability of self-renewal, differentiation and drug resistance[14,15]. Another important parameter in tumor development is angiogenesis. This process is stimulated by the production and secretion of pro-angiogenic molecules from tumor cells and the TME[10,11]. In consequence, fibroblasts, mesenchymal cells and even tumor cells can differentiate into cells with an endothelial phenotype and form new blood vessels[16]. The above mentioned events are implicated in the development of a more aggressive phenotype of CRC cells.

Parathyroid hormone (PTH)-related peptide (PTHrP) is a cytokine described for the first time by Fuller Albright in 1941. He suggested that some tumors might cause hypercalcemia by ectopic PTH production or by secreting a very similar molecule[17]. Then, it was discovered that although both molecules present biochemical similarities, PTH and PTHrP have distinct roles and modes of action. PTH is a hormone secreted by the parathyroid gland and is an important mediator of bone remodeling; it acts together with calcitriol and calcitonin as an essential regulator of calcium and phosphate homeostasis[18]. In contrast, PTHrP can be secreted by a wide variety of tissues and can regulate several cellular functions both in physiological as well as in pathological processes[19]. This cytokine exerts its effects as a paracrine/autocrine factor, although its mode of action is mainly paracrine[17]. It has been shown that this peptide is expressed by several tumors such as breast, prostate, lung, kidney, skin and stomach[20] and that the tumor secretion of PTHrP is responsible for the malignant humoral hypercalcemia[21]. Moreover, bone resorption and tumor establishment and expansion are effects closely related to the over-expression of PTHrP by the tumor[19,22,23].

Regarding its production by normal or tumor cells, PTHrP messenger RNA is translated into a long peptide that undergoes an extensive post-translational process from which several functional fragments are derived (Figure 1). Each of these peptide fragments acts through one or more receptors on the cell surface. However, only those fragments that contain the N-terminal region (1-34) show homology with PTH and share the type 1 PTH receptor (PTHR1)[20,24,25].

Eight years ago, when our research group started studying PTHrP effects in experimental models derived from intestinal tumors, the literature at the time described how the behavior of various types of malignant cells is affected by this factor[20,22,23,26]. However, only limited research had addressed the effects of PTHrP on colorectal tumors. The fact that PTHrP and PTHR1 were detected in the normal rectal and colonic epithelium[27] clearly indicated that PTHrP is a cytokine that acts as a local regulator through a paracrine/autocrine pathway[26]. According to this finding, clinical data revealed that PTHrP over-expressed in the tumors of CRC patients correlated with a poor prognosis[20]. Moreover, *in vitro* investigations showed that the proliferation and migration of LoVo cells derived from CRC were increased when these cells over-expressed PTHrP[28,29]. These *in vitro* assays together with others performed in that decade[30,31] provided knowledge about how this cytokine acts through the autocrine/intracrine modes of action, but until that date, no articles had been published regarding to the paracrine action of PTHrP in CRC cells.

Before we began to evaluate the effects of PTHrP in CRC models, we had demonstrated an anti-tumor role of PTH in the Caco-2 cell line derived from human CRC that expresses PTHR1; through much evidence we had found that the hormone exerts its effects by the modulation of the well-known pathways involved in CRC[32]. These previous investigations led us to consider the need to expand the knowledge about the biology of cells derived from this disease. Since several factors regulate the events involved in the aggressiveness of CRC cells, we inquired if PTHrP could be one of those involved in the malignant behavior. Therefore, the purpose of our next investigations was to explore the role of PTHrP in CRC and to accomplish this broad objective, it was necessary to incorporate new experimental models by including more CRC-derived cell lines and an *in vivo* study model.

This work summarizes the major findings obtained in recent years by our investigation group using *in vitro* and *in vivo* CRC models that evidence the role of the cytokine PTHrP in the acquisition of an aggressive phenotype of CRC cells and the molecular mechanisms involved in these processes. We analyzed the behavior of CRC cells under PTHrP action, focalizing in the study of the following events: Cell cycle progression and proliferation, migration, chemoresistance, tumor-associated angiogenesis and morphological changes related to EMT, a key program associated with invasion. Due to this, the readers will find our results described and discussed in the next four sections. These investigations establish the basis for our next studies to address the clinical applicability of our findings. Recognizing the factors and

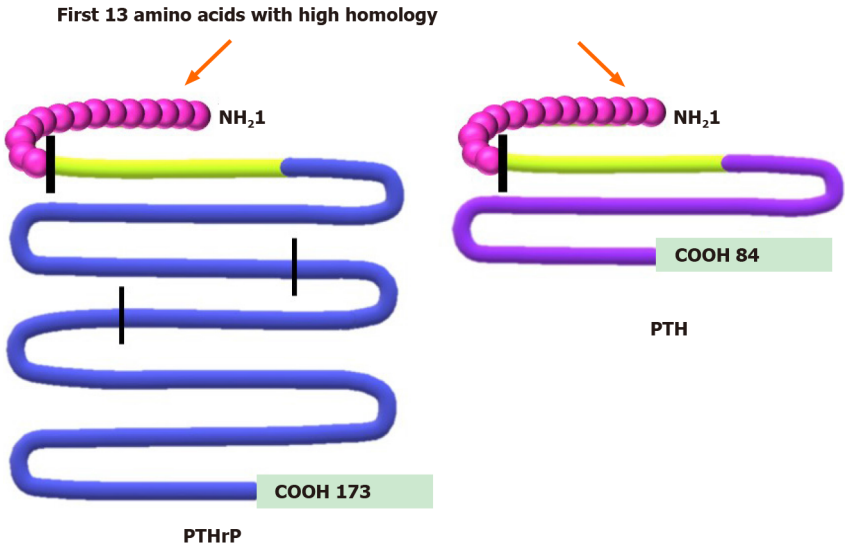


Figure 1 Comparison between protein structure of parathyroid hormone-related peptide and parathyroid hormone. Parathyroid hormone-related peptide (PTHrP) peptide (left side) undergoes a complex post-translational process, obtaining several secreted forms. The N-terminal region 1-34 (green) shows high homology with parathyroid hormone (PTH) and shares more than 60% of the first 13 amino acids (first vertical line). This region allows PTHrP to interact with the type 1 PTH receptor. The PTHrP 36-86, region between N-terminal domain and the second vertical line, is related to placental calcium transport. The 87-107 domain contains a nuclear localization signal (domain between the second and third vertical lines), and the remaining COOH region corresponds to the osteostatin domain[20,24,25]. This figure is original for this work and is based on data published in Soki *et al*[20], Wysolmerski JJ[24] and Goltzman D[25].

mechanisms that promote in CRC cells the invasion, evasion to the cytotoxic effects of current CRC therapies and thus metastasis is decisive for the identification of new markers with the potential to improve early diagnosis and/or to predict prognosis, to predetermine drug resistance and to provide treatment guidelines that include targeted therapies for this disease.

PTHrP PROMOTES CELL CYCLE PROGRESSION, PROLIFERATION AND MIGRATION OF CRC CELLS

As mentioned in the Introduction section, we previously observed that PTH exerts anti-tumor effects in Caco-2 cells through PTHR1[32]. Given that PTHrP (1-34) also binds to the same receptor on the plasma membrane[19,21,33], our first objective was to analyze the actions of this fragment in cell lines derived from colorectal tumors and the associated molecular mechanisms. As previously stated, PTHrP is a factor whose mode of action is mainly paracrine, and, for this reason, all our *in vitro* experiments were performed with the addition of exogenous PTHrP to cells in culture. Regarding the selection of the concentration of this cytokine, we decided to start our investigations employing doses similar to those used with PTH[32] and considering studies carried out in other experimental models[34]. Since we observed that PTH (1-34) (10^{-8} M) induced apoptosis in Caco-2 cells[32], we investigated whether PTHrP employed at the same dose (10^{-8} M) is able or not to induce this response in this cell line. Surprisingly, PTHrP exerts the opposite effect to PTH, since we obtained evidence that PTHrP through a paracrine pathway increases the survival of Caco-2 cells under apoptotic conditions[35]. According to our findings, it was observed in other tumor cells such as breast, renal and prostate cancer cells that PTHrP also increases the resistance of death through the inhibition of apoptosis[20] It is known that the malignant cell transformation involves enhanced cell proliferation, enhanced cell survival by evasion of apoptosis or a combination of both processes[36]. Considering this important concept and based on our initial and interesting result concerning the PTHrP effect on Caco-2 cells, the following goal was to study further the role of this cytokine employing the same concentration in these tumor cells.

By the implementation of multiple assays, we found that PTHrP (1-34) stimulates the cell cycle progression and proliferation of Caco-2 cells[37,38]. In line with these results, we reported similar effects induced by the cytokine in HCT116 cells, a CRC-derived cell line more undifferentiated and aggressive concerning Caco-2 cells, which also expresses PTHR1[39]. Despite the notable differences between Caco-2 cell and

HCT116 cell phenotypes, the molecular mechanisms leading to these responses to the peptide in both CRC cells were similar and implied the activation of well-known deregulated pathways in CRC. Specifically, we found the activation by PTHrP of the non-receptor tyrosine kinase Src, protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), protein kinase B (PKB or Akt), extracellular signal-regulated kinase (ERK) 1/2 and p38, both members of the mitogen-activated protein kinases family (MAPK), p90 ribosomal S6 kinase (RSK) and β-catenin signaling pathways in CRC cells[37-40]. Figure 2 shows the molecular mechanisms modulated by PTHrP and the complex cross-talk among the pathways when this cytokine acts on CRC cells. The upstream/downstream relations between the proteins of these cascades were analyzed employing specific inhibitors that block the protein activity. So, using Ro-318220, PP1 and LY294002, which are the inhibitors of PKC, Src and PI3K, respectively, we found that the activity of these three kinases converges in the phosphorylation/activation of Akt in CRC cells exposed to PTHrP. Furthermore, the specific inhibitor of Akt, GSK690693, suppressed the phosphorylation/activation of ERK1/2 MAPK induced by PTHrP, suggesting the role of Akt in the activation of this MAPK[38,39].

Although the upstream cascades that regulate the activation of p38 MAPK by PTHrP are still unknown, we think that the mechanisms involved are triggered immediately after the activation of PTHrP receptor. We suppose this hypothesis because, in CRC cells exposed to PTHrP, we observed that p38 MAPK is phosphorylated and subsequently activated faster than Src, PKC and Akt. More studies are needed to confirm our idea. RSK is a serine/threonine kinase associated with several types of cancer, including CRC. Its activation is complex and involves various signaling pathways such as MAPKs[41]. We found that RSK is activated by PTHrP and to investigate the involvement of ERK1/2 MAPK and p38 MAPK in this activation, we used PD98059 (a specific inhibitor of MEK1/2, which are the upstream kinases of ERK1/2 MAPK) and SB203580 (a p38 MAPK inhibitor). Experimental data revealed that ERK1/2 inhibitor totally blocked the phosphorylation of RSK induced by PTHrP, whereas the inhibition of p38 MAPK did not reverse the effect of PTHrP over RSK phosphorylation. These results indicate that PTHrP activates RSK *via* ERK1/2 MAPK signaling pathway but not through p38 MAPK[40].

Additionally, as shown in Figure 2, the signaling pathways regulated by PTHrP are responsible for modulating several cell cycle regulators. Our data demonstrated that PTHrP increases the protein expression of cyclin D1, cyclin D-dependent kinase 6 and c-Myc. As PTHrP treatment in CRC cells increases β-catenin protein expression and its subsequent nuclear translocation[38] and as it is known that c-Myc is a target gene of β-catenin[42,43], we suggest that the positive modulation of c-Myc by PTHrP in CRC cells may be *via* the β-catenin pathway. More experiments are needed to confirm this hypothesis. On the other hand, PTHrP paracrine action diminishes the expression of the following negative cell cycle regulators: p27Kip1, p15INK4B and p53. The inhibition of ERK1/2 MAPK, p38 MAPK, PI3K, Akt and RSK pathways suppressed the changes in the protein expression of all the mentioned molecular markers[37-40]. Other transcription factors related to cell proliferation were also activated by exogenous PTHrP, such as cAMP response element-binding protein (CREB) and activating transcription factor 1 (ATF-1). The pre-incubation of CRC cells with the specific MAPK inhibitors suppressed the activation of these transcription factors induced by PTHrP[38]. Taken together, our results demonstrated that in CRC cells, PTHrP positively modulates cell cycle progression and proliferation through the modulation of several mitogenic pathways such as PI3K, Akt, ERK1/2 MAPK, p38 MAPK and RSK.

In order to assess PTHrP effects in a more complex CRC model, we also performed *in vivo* investigations. The studies employing subcutaneous murine xenografts of HCT116 cells revealed that the intratumor administration of PTHrP also stimulates ERK 1/2 MAPK pathway among other mitogenic markers. These data validated part of the results that we observed *in vitro*[39,40]. One difficulty encountered when implementing the study of *in vivo* models was that xenografts of HCT116 cells grew rapidly in the subcutaneous area of the mice, and this situation led to insufficient blood irrigation in the center of the tumors. This caused some of the tumors to show internal areas of necrosis that were detrimental from the experimental point of view, since we were unable to observe differences in tumor volume growth due to treatment with PTHrP. Due to this, and in order to preserve the welfare of the animals, all of the *in vivo* assays were forced to finish at 20 d of the initial administration of PTHrP or vehicle solution. Although at the end of the trials the differences between the volumes and weights of tumors from untreated and treated animals were not significant, we are sure that if we had continued the assays the size of the tumors in the mice treated with PTHrP would be higher than that observed in control mice. We support this idea

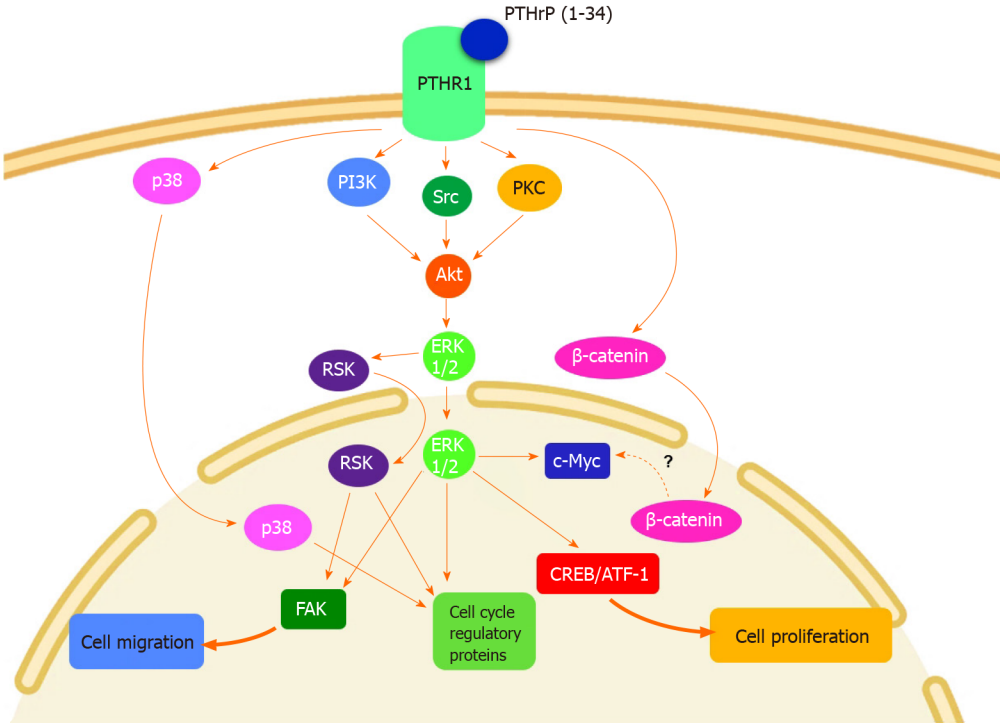


Figure 2 Molecular mechanisms involved in parathyroid hormone-related peptide effects on colorectal cancer cells. Parathyroid hormone-related peptide (PTHrP) induces cell cycle progression and proliferation of colorectal cancer (CRC) cells through non-receptor tyrosine kinase Src (Src), extracellular signal-regulated kinase (ERK) 1/2 and p38, both members of the mitogen activated protein kinases family (MAPK), PI3K/protein kinase B (Akt), p90 ribosomal S6 kinase (RSK) and β-catenin pathways. This cytokine also promotes CRC cell migration and focal adhesion kinase (FAK) protein expression through ERK/RSK signaling pathway[37-40]. This figure is original for this work and shows the results published in Calvo *et al*[37], Martín *et al*[38], Martín *et al*[39], and Calvo *et al*[40]. ATF-1: Activating transcription factor 1; CREB: cAMP response element binding protein; PI3K: Phosphoinositide 3-kinase;PKC: Protein kinase C; PTHR1: Parathyroid hormone receptor 1.

because we observed that the continued administration of PTHrP in nude mice xenografts increased the protein levels not only of the mentioned ERK 1/2 MAPK but also of Ki67, which is a marker of CRC cell proliferation, and the following markers: Cyclin D1, CREB/ATF-1, RSK[39,40] and others[44,45], which we will mention in the next sections due to their relation with the tumor-associated angiogenesis and invasion.

As several of these studied signaling pathways are also involved in cell migration [46], we then decided to study this process. As shown in Figure 2, we observed that PTHrP enhances the motility of CRC-derived cell lines[40]. However, contrary to the results obtained studying tumor proliferation, the effect of this cytokine on migration was higher in the more aggressive cell line, HCT116, than in Caco-2 cells. Furthermore, our investigations revealed that under PTHrP action, ERK 1/2 MAPK and RSK pathways have a relevant role in the increased expression of the focal adhesion kinase and in the migration of CRC-derived cells (Figure 2)[40].

An aspect that we want to comment is about the experiments we performed at that moment to confirm that our findings were exclusively mediated by PTHrP (1-34) and involved only the activation of PTHR1. We used an antibody against PTHR1 to block the PTHrP/PTHR1 interaction and then we evaluated the status of the active ERK1/2 under these conditions since this is a kinase that is involved in most of the processes induced by PTHrP and evaluated by us. We found that the antibody against PTHR1 totally suppressed the response of both Caco-2 cells and HCT116 cells to PTHrP, indicating that ERK activation in cells derived from CRC is due PTHrP/PTHR1 interaction.

Together, these results confirmed that PTHrP is not only involved in the humoral hypercalcemia syndrome but also participates in other responses that may contribute to the progression of CRC. Besides, although PTH and PTHrP interact with the same receptor, these ligands exert opposite effects on intestinal tumor cells. This fact is today explained by the ability of PTHR1 to adopt two different active conformations in response to PTH or PTHrP binding, which will differ markedly in the signaling pathway triggered[47].

PTHrP IS INVOLVED IN THE CHEMORESISTANCE OF CRC CELLS

The incidence and mortality rates related to CRC have decreased in the last decades thanks to the implementation of prevention programs, the early diagnoses by regular testing starting at age 55 and the development of new therapies[48]. Despite all these efforts, late diagnoses are very frequent, and 20% of cases present severe symptoms and a poor or non-existent response to therapy regimens, implying poor survival[15,49-52]. In addition to this situation, in the last decade, there has been an increase in the detection of CRC in patients younger than 55 of age and the appearance of tumor subtypes with poor response to the currently employed treatments[53]. The processes that we have mentioned and analyzed in the previous section suggested that PTHrP can participate in other events associated with the malignant behavior of CRC cells. Since the tumor progress to the most advanced stages mostly implicates the acquisition of resistance to chemotherapy, our next investigations focused to elucidate whether PTHrP also participates in the chemoresistance to drugs commonly used in CRC therapy. One of the drugs most used as first and second-line chemotherapy for advanced or recurrent CRC is Irinotecan (also denominated CPT-11). The combination of CPT-11 with other drugs has shown to increase significantly the survival of patients who have not responded to the initial treatment[54,55]. However, resistance to these improved chemotherapeutic schemes has also been registered[55,56]. As we mentioned earlier, several mechanisms are involved in the development of drug resistance by tumor cells. Regarding recent evidence, the EMT program, the induction of CSC properties and angiogenesis stand out as key events in this process[57]. In a process known as EMT, tumor cells change their morphology acquiring a mesenchymal phenotype to evade the cytotoxic effects of the therapy[58]. In addition to these morphological changes, neoplastic cells acquire CSC properties. These cells are capable of maintaining their population and differentiate into several types of tumor cells, favoring tumorigenesis, the metastatic process, drug resistance and disease recurrence[59,60]. Furthermore, the formation of new vasculature from pre-existing ones, a process known as angiogenesis, is essential not only for tumor growth but also to drug delivery. Factors associated with this neovascularization, such as hypoxia-inducible factor 1-alpha (HIF-1α), may participate in drug resistance[61]. The main processes that participate in CRC chemoresistance are summarized in Figure 3.

The concept of PTHrP decreasing the sensitivity to therapeutic drugs in CRC-derived cells had not been described at the time that we started our studies related to chemoresistance. Also, there was only limited information about the effect of this cytokine on the induction of chemoresistance in other tumor cells[26,62]. Considering this background, we decided to study whether PTHrP may be an underlying factor in the observed chemoresistance to CPT-11. To achieve this objective, Caco-2 and HCT116 cell lines were treated with PTHrP followed by exposure to CPT-11 (10 μM). We found that the exogenous addition of the peptide attenuated the cytotoxic effect of the cytostatic agent in both cell lines. These results suggest that PTHrP favors the chemoresistance of CRC cells to CPT-11. Furthermore, ERK 1/2 MAPK, an enzyme that we mentioned in the previous section due to its involvement in the proliferation and migration of CRC cells, also participates in this pro-tumor response[39]. In line with our results, other researchers reported that the activation of ERK 1/2 MAPK in the HCT116 cell line can generate resistance to other antitumor agents, such as oxaliplatin[63].

So far, we could demonstrate the PTHrP-induced resistance to topoisomerase inhibitors such as CPT-11. The question that immediately emerged after this analysis was whether this cytokine also exerts resistance to other kinds of anti-tumor drugs or a combination of them. Investigations involving the participation of other signaling pathways and the resistance to other drugs in the treatment of CRC, such as oxaliplatin and 5-fluorouracil, are actually in study in our laboratory. This information will be relevant in determining to what extent PTHrP is able to promote drug resistance.

EFFECTS OF PTHrP ON TUMOR-ASSOCIATED ANGIOGENESIS

As previously mentioned, tumor angiogenesis is one of the main mechanisms by which tumors can generate blood vessels and is an essential process for cancer growth and metastasis that can influence therapeutic effectiveness. It is highly regulated by a fine balance between pro-angiogenic and anti-angiogenic factors and is modulated by different signaling pathways. In cancer this balance is lost due to an increased release

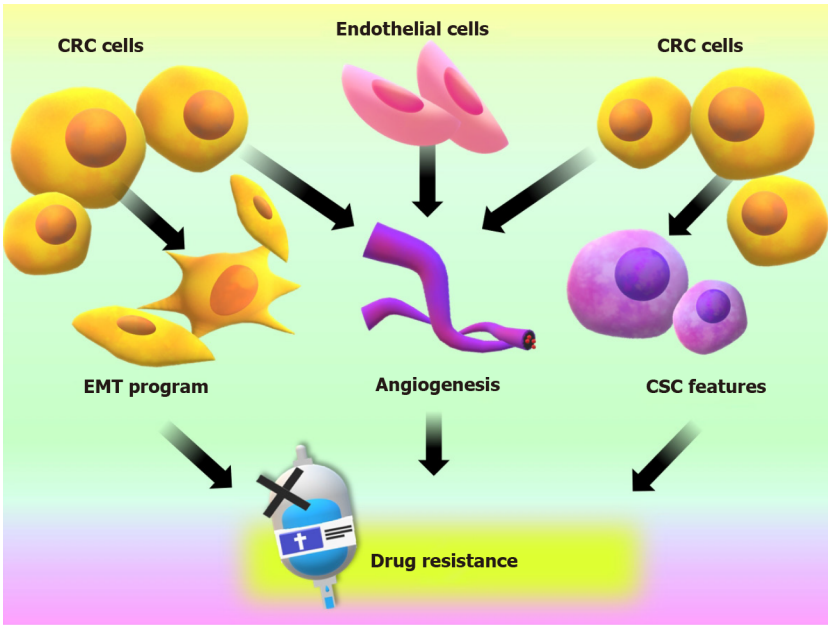


Figure 3 Events that promote an aggressive phenotype on colorectal cancer cells and are related to chemotherapeutic drug resistance and treatment failure. CRC: Colorectal cancer; CSC: Cancer stem cell; EMT: Epithelial to mesenchymal transition.

of pro-angiogenic factors, such as the vascular endothelial growth factor (VEGF), that are produced by tumor cells and the tumor microenvironment, stimulating endothelial cells and promoting tumor angiogenesis[64]. Because of this imbalance, the tumor vessels do not form completely, are abnormal, tortuous, irregular, dilated and permeable, have weak unions, few pericytes and incomplete basal membrane and do not differ in venules, capillaries or arterioles. In addition, blood lakes are formed, and thus the flow becomes irregular, slow and oscillating, a fact that leads to a decrease in oxygen levels and can contribute to the difficulty of successful therapy[65].

Based on this background and our previous results obtained during the years 2013 to 2018, we initially set out to explore whether PTHrP regulates the expression of pro-angiogenic factors in Caco-2 and HCT 116 cell lines to evaluate the effect of this cytokine in the angiogenesis associated to tumor progression. We observed that PTHrP increases messenger RNA levels of VEGF, HIF-1α and matrix metalloproteinase 9 *via* ERK1/2 and PI3K/Akt pathways in both cell lines. Moreover, and as we mentioned in the previous section, we evidenced increased levels of VEGF in HCT116 xenograft tumors treated with PTHrP concerning to control tumors. These results were complemented with the presence of cells forming structures with characteristics of neoformed vessels and stained positively for the vascular endothelial marker cluster of differentiation 31[44]. The ability to distinguish quantitatively between tumor neovascularization and pre-existing vessels is important because these data provide more accurate information in the assessment of tumor angiogenesis. Altogether, these results suggested a pro-angiogenic role of PTHrP in CRC. In line with our results, other authors had found that PTHrP can modulate the expression of factors involved in angiogenesis in tumor cells, resulting in the stimulation of this process. This cytokine stimulates VEGF expression in MDA-MB-231 cells from breast cancer *via* protein kinase A, PKC, ERK 1/2 MAPK and p38 MAPK signaling pathways, and this tumor cell response increased the proliferation and migration of endothelial HUVECs cells [66]. PTHrP modulates the expression of other factors involved in the angiogenesis associated with breast tumors such as the connective tissue growth factor (CTGF/CCN2)[67] and the factor VIII[22]. Moreover, PTHrP can induce the expression of the angiogenic factor interleukin-8 in PCa prostate cancer cells[68].

Our findings suggested an interaction between tumor cells and their microenvironment through pro-angiogenic factors. So, we decided to continue these investigations to evaluate further the molecular crosstalk between tumor cells and endothelial cells. From this goal, we employed conditioned media from CRC tumor cells (TCMs) and indirect co-culture with transwell inserts, and we incorporated the HMEC-1 cell line as an endothelial cell model. We found that the TCMs from colorectal cancer cells exposed to PTHrP exhibit increased proliferation, migratory capacity and formation of tube-like structures of these endothelial cells[44]. Besides, TCMs that were pre-

incubated with the anti-VEGF antibody decreased the stimulating effects of TCMs on endothelial cells[44]. This finding indicates that PTHrP increases the expression of VEGF in Caco-2 cells and HCT116 cells with its subsequent release into the culture medium. This factor in turn exerts its pro-angiogenic effects on endothelial cells. These data broadened the view concerning to the mechanism of action of PTHrP since this cytokine not only acts directly on CRC cells but also exerts its effects acting as a mediator between the tumor and its microenvironment (Figure 4).

Until that moment the progress of our research had demonstrated that PTHrP exerts its effects on the endothelial cells in a tumor cell-dependent manner. Despite the available literature mentioned earlier in this work[22,66,67] that demonstrated, like our research, a pro-angiogenic role of PTHrP, other authors had suggested an inhibitory role for this cytokine in this process[69]. In view of this controversial information, our next challenge was to evaluate if PTHrP acting directly on the endothelial cells also promotes angiogenesis. First, we evidenced the presence of PTHR1 in HMEC-1 cells. Then, we observed that PTHrP increases the phosphorylation of ERK 1/2 MAPK and the proliferation of these endothelial cells. Nevertheless, this cytokine did not stimulate the migration or tube formation of HMEC-1 cells[44]. Assays performed to evaluate cell motility and tube formation on a matrix are widely used to study angiogenesis *in vitro*[70]. However, it would be necessary to carry out *ex vivo* or *in vivo* assays to study further if PTHrP has or not a direct action on endothelial cells with the aim to promote angiogenesis. Using xenografts of HCT116, we cannot discern whether the effect observed in vessel formation is due to the direct or indirect role of PTHrP. We plan to use in the future other experimental models to continue the study of this part of our project.

EFFECTS OF PTHrP ON THE MODULATION OF EMT PROGRAM AND OTHER EVENTS ASSOCIATED WITH INVASION

The fact that PTHrP promotes the chemoresistance of CRC cells and the angiogenesis associated with these tumor cells led us to explore if this cytokine is also involved in other events associated with tumor progression. The process of invasion requires the acquisition of characteristics by tumor cells and the presence of various environmental factors that participate in the remodeling of extracellular matrix, such as matrix metalloproteinases (MMPs)[71]. Therefore, we focalized in the study of MMP-7 because it is overexpressed in 80% of patients with CRC[71], and we found that the treatment with PTHrP induces an increase in MMP-7 transcription in CRC cells. In view of this, our next goal was to investigate if the CRC cells undergo morphological changes under PTHrP action that are related to events associated with tumor progression. EMT is a cellular program that is observed in embryonic development, tissue remodeling and wound healing, and it has also been established as a crucial step in the progression of different tumors. As mentioned, during EMT, epithelial cells reduce intercellular adhesion and acquire mesenchymal properties that increase their migration and invasion capacity, recognized characteristics of tumor cells[58]. The results from the study of the EMT program were incorporated in our work recently accepted for publication[45]. In this manuscript, we reported that PTHrP modulates the expression of factors and favored morphological changes associated with EMT in the CRC-derived HCT116 cell line (Figure 5); we also show that the key molecular mechanisms associated with EMT observed in this cell line in response to PTHrP were not found in the more differentiated and less aggressive Caco-2 cells. The difference in the response of both CRC-derived cell lines raises an interesting new scenario for the action of PTHrP where its effect would depend on the different aggressiveness of the cell line.

Our findings described herein showed that PTHrP through a paracrine manner is involved in events related to the aggressive behavior of CRC cells. The fact that this cytokine establishes a communication between CRC cells and endothelial HMEC cells through molecular factors promoting tumor-associated angiogenesis (see Figure 4), motivated us in this last time to continue evaluating how PTHrP promotes the interaction between the tumor cell and cells from its microenvironment. In our recent work[45] we demonstrated that this cytokine acts on these endothelial cells promoting the release of factors that contribute to EMT program in CRC-derived cells (Figure 5).

After investigating the effects of PTHrP on the EMT program, we began to inquire about other programs strictly associated with malignant progression and chemoresistance, such as CSC. The evidence suggests that the EMT program is tightly associated with the CSC phenotype. This process is recognized for altering not only the

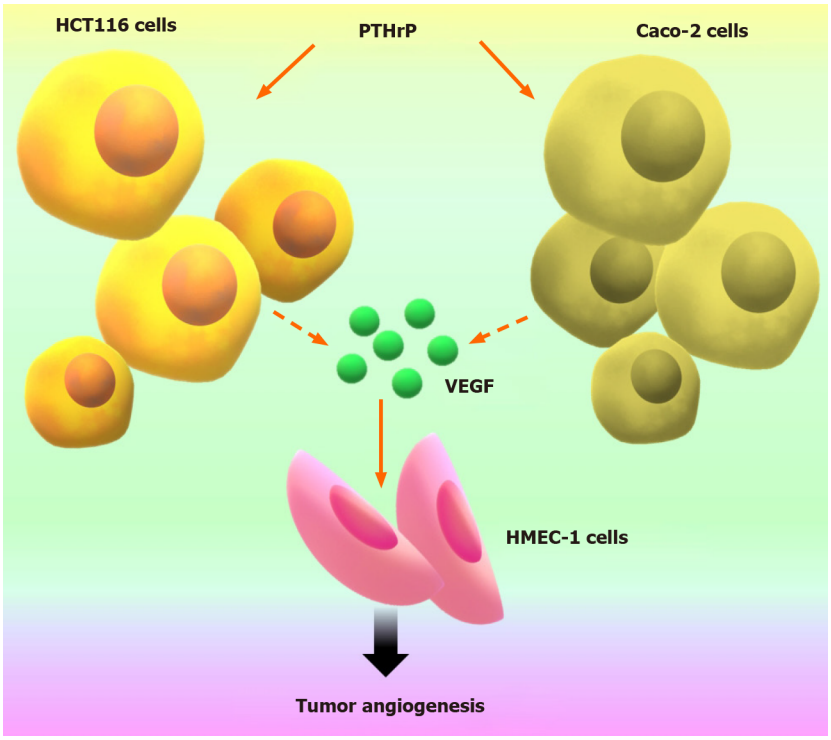


Figure 4 Parathyroid hormone-related peptide induces tumor-associated angiogenesis through the pro-angiogenic factor vascular endothelial growth factor released from colorectal cancer cells. This figure is original for this work and shows results published in Calvo *et al*[44]. PTHrP: Parathyroid hormone-related peptide; VEGF: Vascular endothelial growth factor.

phenotype of the tumor cells but its microenvironment, inducing the initiation of CSC and regulating their features[13,14]. Recent findings from our research group suggest that, in CRC-derived cells, PTHrP modulates the protein expression of cell surface markers widely linked to colon CSC, possibly participating in the initiation and reprogramming of this cell subpopulation. Accumulating evidence associates these cells with resistance to cytotoxic drugs through several mechanisms[15].

Given all these results, we postulate that PTHrP participates in the modulation of several events related to an aggressive phenotype of colorectal tumor cells. The action of autocrine and paracrine factors derived from the tumor and their stroma can promote several events contributing to the phenotypic and genetic heterogeneity of tumor cells and affecting the response to currently used treatments.

Together, these investigations made it possible to project PTHrP as a mediator in the tumor microenvironment and delineate new lines of research.

FUTURE APPROACHES OF PTHrP

Despite the contributions from our research group regarding the role of PTHrP in the modulation of events associated with the aggressive phenotype of CRC cells, we consider that other key aspects of the action of this cytokine are necessary to evaluate. In fact, we are now designing new experiments to analyze if PTHrP also confers chemoresistance to other drugs for CRC treatment and to elucidate how this cytokine contributes to the aggressive behavior of CRC cells through its action on the TME. Another challenge in our project is to analyze the clinical relevance of our observations.

The TME is a factor that is acquiring more and more evidence regarding its relevant role in the progression of CRC and drug resistance[72]. Solid tumors consist of tumor cells that are surrounded by a stroma composed of a variety of cells such as fibroblasts, myofibroblasts, endothelial cells, lymphocytes, mast cells and macrophages. The stroma interacts with tumor cells through cytokines, integrins and proteases to influence functions such as proliferation, apoptosis, migration and angiogenesis[73]. Although it is well defined that PTHrP is expressed by tumor cells in CRC[74-76], it is still unknown the sources from the TME where PTHrP is produced and secreted. The fact that PTHrP is expressed in the stromal cells of other types of tumors contributing

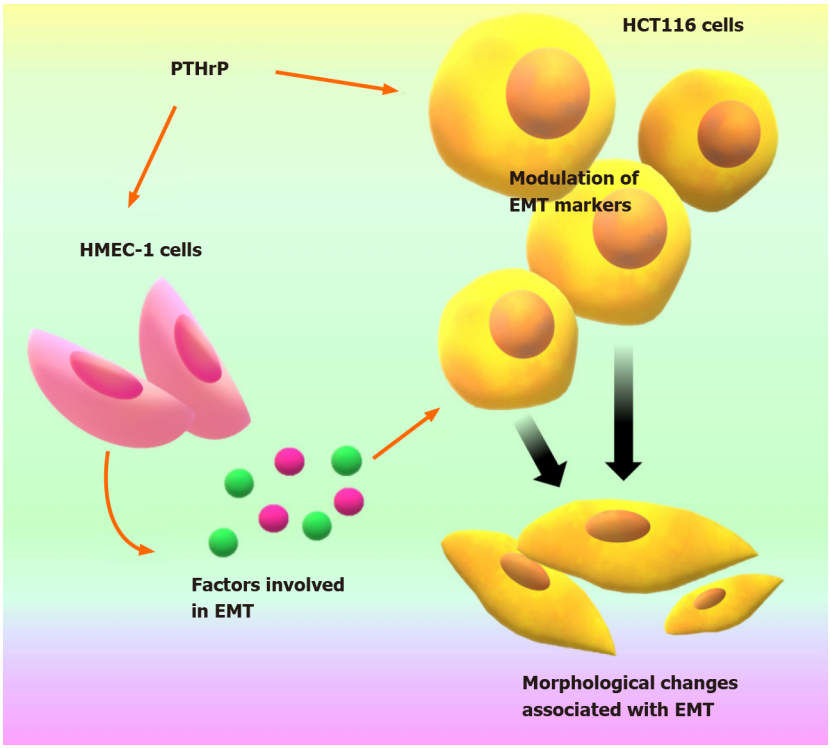


Figure 5 Parathyroid hormone-related peptide establishes a communication between colorectal cancer cells and endothelial HMEC cells through molecular factors modulating markers expression and morphological changes associated with cellular programs that promote the invasive phenotype in HCT116 cells. This figure is original for this work and shows results published in Carriere *et al*[45]. EMT: Epithelial to mesenchymal transition; PTHrP: Parathyroid hormone-related peptide.

to their aggressive behavior[77,78] supports the hypothesis that the cytokine derived from the TME may play a role in the pathogenesis and progression of CRC. Perhaps PTHrP from TME through a paracrine manner also induces its own expression in tumor cells. The influence of the TME in the expression of PTHrP in tumor cells was shown in the work of Yan and collaborators[79]; they reported that the cytokine TGF-β derived from the TME stimulated the secretion of PTHrP from prostate cancer cells promoting the progression of the disease.

As we mentioned in previous sections of this work, our approaches recently revealed that PTHrP, through molecular factors, establishes a communication between CRC cells and endothelial cells derived from TME that leads to the promotion of events related to the aggressive behavior of tumor cells[44,45]. Despite the advances by our research group highlighting the impact of PTHrP on TME cells, it still remains to study key aspects of the action of this peptide, especially regarding the origin of PTHrP in the tumor niche and its effect on CRC cells through its influence on other types of TME cells and also on the ECM.

According to this, we are now planning to study the chemoresistance and other events associated with the aggressive behavior of CRC cells with the focus on the role of TME to understand further the implication of PTHrP in this complex process.

All the experimental models that we used heretofore allowed us to evaluate the signaling pathways triggered as well as the molecular and phenotypic changes in response to PTHrP. Despite this, to evaluate functional aspects regarding TME and drug therapy, we consider it relevant to implement new techniques and models to extrapolate reliably these results. We recognize that two-dimensional *in vitro* cultures present several limitations when studying the interaction networks of TME and drug resistance since they do not represent the three-dimensional character of the tissues. Considering this aspect and following the National Institutes of Health standards for the use and care of laboratory animals that seek to reduce the use of experimental animals, we will perform our next experiments incorporating cell co-cultures in our experimental models. We planned to do new experiments using several tumor cell lines derived from CRC co-cultured with stromal cells (such as fibroblasts, endothelial cells and macrophages) in a three-dimensional model of spheroids to evaluate the complex interaction between neoplastic cells and TME cells under PTHrP action. To validate the results obtained in these experiments, it will be necessary to implement

new *in vivo* methods that allow us to evaluate the drug resistance and the progression of angiogenesis, invasion and malignancy programs considering the impact of tumor stroma. Athymic nude mice have been very useful so far since they have allowed us to extrapolate the results observed in the cell lines to a complete organism. Nevertheless, this model represents a challenge since the deficient immune response of these animals makes it difficult to assess the interactions between the tumor and the stroma; the subcutaneous xenograft models are limited because tumors cells interact poorly with the surrounding stroma and also, they do not metastasize. An alternative model is the co-injection of tumor and stromal cells with matrigel or the orthotopic model.

Another aspect that we decided to evaluate is the clinical relevance of our *in vitro* and *in vivo* results by a retrospective study that is currently in process. To this end, we are using diagnostic biopsies preserved in paraffin plugs from patients who received the diagnosis of CRC and subsequently adjuvant chemotherapy. The objective of this part of our project is to correlate the expression of PTHrP and other markers (that were relevant in the cell and animal models studied by us) with the characteristics of the tumor, the tumor response to the established treatment, the progression-free time and the overall survival of the CRC patient in order to identify PTHrP and its effectors as possible prognostic markers and/or predictive of CRC. In these retrospective studies, we will analyze 300 to 400 samples to consider the data as statistically significant. These samples are from the Hospital Provincial de Neuquén (Province of Neuquén, Argentina) and from the Hospital Dr. José Penna, Bahia Blanca (Province of Buenos Aires, Argentina), which are two hospitals with a high attendance of patients, and therefore we consider that our work will have a regional scope because we will evaluate biopsies of CRC patients from two different provinces of our country.

Following our proposed studies in the clinical context, we plan to resort to strategies such as *in silico* modeling methods because they are tools that allow a comprehensive approach of published genomic and proteomic data related to CRC progression and can predict the clinical efficacy of treatments. It is clear that innovative models are required to translate data involving biological and genomic/proteomic networks into suitable therapeutic schemes. With this approach, we plan to evaluate our experimental system against available databases to contrast our findings and predict the effect of PTHrP in the response of patients to the chemotherapy employed.

CONCLUSION

Recent publications denote the importance of the tumor microenvironment in the response in different stages of CRC[80]. It is known that cytokines in the tumor stroma critically influence the development and progression of CRC by direct stimulation of neoplastic cells or by altering the function and activity of cells in the microenvironment[81].

These antecedents, together with the promising results obtained by our research group throughout these years, allow us to hypothesize that PTHrP is a cytokine that acts through a paracrine manner to play an important role in the acquisition of an aggressive phenotype of intestinal tumor cells. By its action on CRC cells and on its microenvironment, this peptide promotes an interaction between cells from the tumor niche favoring the tumor aggressive behavior. Our findings suggest that PTHrP and its effectors could be involved in the tumorigenesis and/or progress of CRC disease and also could influence the success of chemotherapeutic treatment.

ACKNOWLEDGEMENTS

The authors would like to thank Silvana Gigola for her helpful advice. Being an English Professor graduated from Universidad Nacional de Quilmes (Argentina) and currently working at Instituto Superior Juan XXIII (from Bahia Blanca city, Argentina), she revised the paper and made useful comments regarding spelling, grammar and punctuation of this manuscript. Novoa Díaz MB, Martín MJ and Carriere PM appreciate CONICET for the fellowships.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 November 7; 27(41): 7041-7064

DOI: 10.3748/wjg.v27.i41.7041

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

REVIEW

Over-feeding the gut microbiome: A scoping review on health implications and therapeutic perspectives

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Author contributions: Barone M, D'Amico F, and Fabbrini M performed the literature search; Barone M, D'Amico F, Fabbrini M, Rampelli S and Turroni S drafted the manuscript; Barone M, Brigidi P, and Turroni S edited and corrected the manuscript; all authors have read and approved the final manuscript.

Conflict-of-interest statement: The authors declare no conflict of interest.

Country/Territory of origin: Italy

Specialty type: Gastroenterology and hepatology

Peer-review report's scientific quality classification

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

Open-Access: This article is an open-access article that was selected by an in-house editor and

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Abstract

The human gut microbiome has gained increasing attention over the past two decades. Several findings have shown that this complex and dynamic microbial ecosystem can contribute to the maintenance of host health or, when subject to imbalances, to the pathogenesis of various enteric and non-enteric diseases. This scoping review summarizes the current knowledge on how the gut microbiota and microbially-derived compounds affect host metabolism, especially in the context of obesity and related disorders. Examples of microbiome-based targeted intervention strategies that aim to restore and maintain an eubiotic layout are then discussed. Adjuvant therapeutic interventions to alleviate obesity and associated comorbidities are traditionally based on diet modulation and the supplementation of prebiotics, probiotics and synbiotics. However, these approaches have shown only moderate ability to induce sustained changes in the gut microbial ecosystem, making the development of innovative and tailored microbiome-based intervention strategies of utmost importance in clinical practice. In this regard, the administration of next-generation probiotics and engineered microbiomes has shown promising results, together with more radical intervention strategies based on the replacement of the dysbiotic ecosystem by means of fecal microbiota transplantation from healthy donors or with the introduction of synthetic communities specifically designed to achieve the desired therapeutic outcome. Finally, we provide a perspective for future translational investigations through the implementation of bioinformatics approaches, including machine and deep learning, to predict health risks and therapeutic outcomes.

Key Words: Gut microbiome; Microbial metabolites; Obesity; Next-generation probiotics; Fecal microbiota transplantation; Deep learning

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Received: April 29, 2021
Peer-review started: April 29, 2021
First decision: June 17, 2021
Revised: July 2, 2021
Accepted: October 14, 2021
Article in press: October 14, 2021
Published online: November 7, 2021

P-Reviewer: Cheungpasitporn W
S-Editor: Fan JR
L-Editor: Webster JR
P-Editor: Fan JR

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Core Tip: The gut microbiome (GM) has gained increasing attention in recent years due to its key role in contributing to host health, potentially serving as a target for personalized precision medicine. This review summarizes the current evidence for the involvement of the GM in the regulation of various pathophysiological aspects, particularly in obesity and related comorbidities. The influence of diet and the molecules produced by commensal microorganisms is discussed, together with traditional and innovative microbiome-based strategies in the prevention and treatment of obesity, up to the development of machine and deep learning bioinformatics tools for the prediction of health risks and therapeutic outcomes.

Citation: Barone M, D'Amico F, Fabbrini M, Rampelli S, Brigidi P, Turroni S. Over-feeding the gut microbiome: A scoping review on health implications and therapeutic perspectives. *World J Gastroenterol* 2021; 27(41): 7041-7064
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7041.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7041

INTRODUCTION

Over the past two decades, the trillion-member community that resides in the human gastrointestinal tract (*i.e.*, the gut microbiome-GM) has emerged as a key regulator of host physiology, supporting overall host health or, vice versa, contributing to triggering and sustaining pathological conditions when altered. The wide range of metabolic activities and the multiple levels of bidirectional interaction with the host strongly support GM as a strategic therapeutic target, laying the foundations for the development of innovative microbiome-tailored intervention strategies aimed at restoring an eubiotic layout. In parallel, advances in sequencing techniques and bioinformatics tools are proving crucial to deepen our understanding of the complex interactions established by the GM with the host, as well as to rationally fine-tune and successfully translate personalized microbiome-based interventions into clinical practice.

Our scoping review aims to discuss the state of the art on research and application aspects related to the role of GM in obesity, a complex and multifactorial disease that represents a major health risk factor. In particular, we first discuss the influence of GM on human physiology and its contribution to the pathogenesis of numerous enteric and non-enteric diseases when imbalances occur. Next, we focus on evidence for a link between GM and obesity and discuss the growing literature on the impact of diet on GM structure, and the key role played by GM-produced or derived bioactive compounds [*e.g.*, fatty acids, amines, bile acids (BAs) and neuroactive metabolites] in affecting host physiology and metabolism, at both local and systemic level. We then review the adjuvant interventions currently available to manipulate GM and alleviate the obesity phenotype, which are based on diet modulation and the supplementation of prebiotics, probiotics and synbiotics. Since these traditional approaches have shown only moderate ability to induce sustained change in the complex and dynamic GM ecosystem, we stress the need to develop innovative and tailored microbiome-based intervention strategies, such as the administration of next-generation probiotics (NGPs) and engineered microbiomes, to be organically integrated into clinical practice. More radical approaches involving replacement of the dysbiotic microbial ecosystem through fecal microbiota transplantation (FMT) or the infusion of synthetic microbial communities, rationally designed to meet the desired therapeutic outcome and patient needs, are also discussed as promising alternatives for personalized clinical applications. Finally, we provide a perspective for future translational investigations through the implementation of bioinformatics approaches, including machine and deep learning, to predict health risks and therapeutic outcomes. Current clinical practice is increasingly leveraging new artificial intelligence technologies and refined bioinformatics approaches, but still little has been done in relation to GM. Therefore, we conclude by discussing the possibilities offered by machine and deep learning for the development of microbiome-based strategies, especially in the context of obesity and related morbidities. All articles included in this review were identified through

the PubMed platform, the full-text archive of biomedical and life sciences journal literature at the United States National Institutes of Health's National Library of Medicine. The most pertinent and relevant articles for each aforementioned topic were selected and commented on.

HUMAN GM: WHERE DO WE STAND?

From the development of next-generation sequencing approaches and their application to the microbiome field, the analysis of the intestinal microbial community has had a strong burst, as evidenced by > 3 million hits returned by typing "gut microbiota" on the NCBI database (as accessed on April 26, 2021). Of course, this is not surprising when one considers the plethora of actors and functions involved in the whole GM field[1,2]. Indeed, GM is composed of a multitude (over trillions) of microbial entities from all domains of life (*i.e.*, bacteria, archaeabacteria and micro-eukaryotes), which encode a number of genes probably more than 500 times greater than the human genome. This genetic heritage, still largely underestimated, allows them to perform various functions recognized as instrumental to maintaining host homeostasis[3,4]. With specific regard to the bacterial counterpart, indisputably the most explored to date, despite the thousands of different species identified so far, most of them belong to 2 phyla, Firmicutes and Bacteroidetes, which together account for approximately 90% of an adult-like community, with Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia representing the most commonly found subdominant taxa[3]. This ecosystem is characterized by essential ecological features, such as stability, resistance and resilience, associated with high diversity and functional redundancy, the loss of which can lead to unhealthy microbe-microbe and microbe-host interactions[5]. Established at birth, GM develops structurally and functionally over time, in relation to the personal exposome (*i.e.*, the totality of exposures that individuals experience in their lives), while always providing the host with a series of fundamental immunological and metabolic ecosystem services for a mutualistic GM-host relationship[6,7]. In particular, GM is known to act as a protective barrier against infectious threats (the so-called "colonization resistance" by occupying niches, taking up resources and producing antimicrobials) and play an active role in the development and modulation of immune responses[8,9]. On the other hand, GM is also called "metabolic organ" as it provides numerous bioactive molecules from the degradation of dietary compounds, which are the main actors of the well-known local and systemic functions attributed to GM, just to name a few: (1) Nutritional support for the intestinal epithelium; (2) Synthesis of vitamins and balance of energy intake; (3) Lipid and carbohydrate metabolism-related effects; (4) Immune system modulation; and (5) Enteric and central nervous system regulation through the gut-brain axis[10]. Moreover, recent findings on GM-drug interactions have indicated its role in influencing the response to treatments, including the occurrence of side effects, with potentially groundbreaking implications in precision medicine[11].

That said, it is not hard to imagine how strategic it is for our health to maintain this intricate and complex balance with our microbial inhabitants. Indeed, when this balance fails, the so-called dysbiosis is established, *i.e.*, a disease-promoting or associated GM alteration[12]. Several endogenous (*e.g.*, immune dysregulation and inflammation) and exogenous (unbalanced diet, antibiotic intake, pathogen infection, *etc*.) factors are capable of promoting GM dysbiosis, and several studies have tried to explain the mechanisms underlying these events[13-15]. Generally speaking, dysbiosis may present with one or more of the following characteristics: (1) Loss of biodiversity (a recognized hallmark of healthy gut); (2) Depletion of beneficial, health-associated taxa, typically short-chain fatty acid (SCFA) producers from the *Lachnospiraceae* and *Ruminococcaceae* families; and (3) Enrichment in pathogens or pathobionts[16]. To date, dysbiotic profiles have been associated with a plethora of enteric and non-enteric disorders, including metabolic, hepatic, respiratory, cardiovascular, immunological and oncological disorders, and are supposed to cause some of these[12]. However, it is of the utmost importance to corroborate claims on GM-related causality in human diseases, as only a few of them have been validated[17]. Regardless, a very hot topic in the field now is the development of GM modulatory strategies, to increase the resilience of healthy states (prevention) or overcome that of unhealthy states (treatment) to alleviate the disease phenotype and restore eubiosis.

EVIDENCE FOR A LINK BETWEEN GUT MICROBIOTA AND OBESITY

The prevalence of overweight and obesity has dramatically risen over the past four decades[18]. Combined with polygenic host susceptibility, increased food consumption and sedentary lifestyles lay the foundation for a widespread obesity epidemic[19]. Recognized as a multifactorial disease, obesity represents a major risk factor for health, with dramatic consequences on quality of life and healthcare costs [20-22]. Comorbidities linked to obesity (*e.g.*, diabetes, cardiovascular disease, cancer) are indeed among the leading causes of premature death, and researchers are striving to find more effective treatments for these conditions[23]. Over the past 15 years, pioneering studies have proposed GM as a key factor involved in energy storage and fat mass gain. Among the first, Bäckhed *et al*[24,25] demonstrated that germ-free mice gained less body weight and fat mass than conventional mice harboring a GM[24], as well as providing proof of concept by showing that the lack of a microbial ecosystem confers resistance to obesity induced by a high-fat diet[25]. In addition, a pivotal study by Turnbaugh *et al*[26] showed that the obese phenotype can be induced in lean mice by transferring the GM of obese animals[26], thus suggesting a causality between unbalanced GM and the development of obesity. Subsequently, the GM of obese individuals was shown to have reduced microbial richness and biodiversity, combined with an altered representation of the two main phyla, namely Bacteroidetes and Firmicutes, compared to lean subjects[27]. These findings have paved the way for several epidemiological studies focused on showing differences in the GM of obese and lean individuals, which have led to accumulating evidence of the GM role in mediating the impact of environmental factors on obesity pathogenesis[28-31]. As expected, given the high taxonomic level, not all studies have been successful in validating the association between the Firmicutes/Bacteroidetes ratio and obesity, and the biological relevance of this ratio is now highly controversial. On the other hand, greater resolution was gradually provided in the description of the bacterial composition, as well as in the understanding of the underlying mechanisms through which a dysbiotic layout might contribute to triggering host metabolic imbalances. For instance, species-level characterization of GM in twins highlighted a positive association of SCFA producers *Eubacterium ventriosum* and *Roseburia intestinalis* with obesity[32]. Similar correlations were observed for *Collinsella* spp.[33], which were also found to be overrepresented in type 2 diabetes (T2D) and atherosclerosis[34,35]. The hypothesized mechanisms include reduced expression of tight junction proteins, possibly leading to gut leakage and metabolic endotoxemia, as well as impaired cholesterol absorption, decreased hepatic glycogenesis and increased triglyceride synthesis[36,37]. It is therefore not surprising that *Collinsella* has been proposed as a target in future GM intervention studies for the improvement of metabolic parameters [38]. On the other hand, the most common methanogenic archaeon found in GM, *Methanobrevibacter smithii*, as well as butyrate producers, such as potentially heritable *Oscillospira* spp., were found to be more represented in lean individuals[39]. *Bacteroides thetaiotaomicron* was depleted as well in obesity-related GM configurations and inversely correlated with serum concentration of glutamate, a common food additive able to induce obesity and insulin resistance[40]. *In vivo* studies have highlighted the ability of this glutamate-fermenting commensal to protect against adiposity[40], suggesting its potential application in probiotic-based intervention strategies in obese individuals. Similarly, *Parabacteroides goldsteinii*, whose levels are reduced in high-fat diet-fed mice, has been proposed as an anti-obesogenic probiotic, capable of promoting adipose tissue thermogenesis and intestinal integrity, and reducing inflammation and insulin resistance[41]. Finally yet importantly, the mucin-degrading bacterium *Akkermansia muciniphila* has been repeatedly and consistently found to be inversely correlated with body fat mass, fasting blood glucose levels and subcutaneous adiposity in mice and humans[42,43], potentially representing a NGP or live biotherapeutic candidate for obesity treatment. In human studies, *A. muciniphila* has shown protective effects against gut permeability and endotoxemia, as well as improving glucose homeostasis and promoting better overall health[43]. Very recently, in a proof-of-concept exploratory study, Depommier *et al*[44] demonstrated that its daily oral administration, as live or pasteurized bacteria, for three months to overweight/obese insulin-resistant volunteers, was safe and well tolerated, and led to the improvement of multiple metabolic parameters[44]. However, it should also be remembered that microorganisms interact with each other in complex syntrophic networks, the understanding of which could help guide more rational preventive and therapeutic strategies. In an attempt to take a step forward in this direction, Tavella *et al*[45] recently identified a distinct GM compositional structure (with elevated proportions of *Christensenellaceae*, *Porphyromonadaceae* and *Rikenellaceae*) associated with reduced

visceral adipose tissue and healthier metabolic profile in elderly Italians[45]. It has also been hypothesized that peculiar "steady states" of GM, combined with long-term dietary habits, may predict the development of childhood obesity[31].

INFLUENCE OF DIET ON THE GUT MICROBIOTA

Diet is a major driver of GM variation and undoubtedly plays a central role in promoting and maintaining GM diversity, a strategic element to ensure eubiosis and resilience. Indeed, GM can rapidly shift its composition and functionality in response to dietary changes, contributing to the generation of health-relevant metabolic outputs [46]. In recent years, interest in understanding the relationship between dietary habits, GM and host physiology has grown remarkably. Different dietary patterns, such as Western-type diets, vegetarian or vegan diets and Mediterranean-style diets have been explored and each has been found to be associated with quite distinct GM profiles, which obviously affect host metabolism in a distinct way[47]. In particular, the advent of a Westernized lifestyle, with the fast-paced globalization of food, excessive sanitation and modern medicines, has led to the introduction of a dietary pattern mostly based on saturated fat, sugar and salt, and dramatically low in fiber, otherwise known as "microbiota-accessible carbohydrates"[13]. Several studies have shown that this type of diet is associated with alterations in the GM structure and functionality, in particular enrichment in mucus degraders, bile-tolerant and antibiotic-resistant species ("BloSSUM", *i.e.*, bloom or selected in societies of urbanization/modernization) and the loss of diversity, ancestral fiber-degrading taxa and related functions ("VANISH", *i.e.*, volatile and/or associated negatively with industrialized societies of humans)[48]. This has collectively been referred to as the "microbiota insufficiency syndrome" and is supposed to result in broad dysfunctions, including obesity and chronic inflammation, thus contributing to the emergence of non-communicable chronic diseases. In contrast, high-fiber dietary patterns are typically associated with highly diverse GMs, enriched in fibrolytic SCFA-producing bacteria, *e.g.*, *Ruminococcus*, *Faecalibacterium*, *Eubacterium* and *Roseburia*, along with high fecal levels of SCFAs[49,50], to which multiple beneficial effects are attributed, as detailed below. Moreover, a microbial footprint of this dietary habit is the greater abundance of *Prevotella*, as also found in hunting-gathering and rural populations who consume a plant-based diet with unprocessed foods[51-53]. It is also worth noting that these dietary habits involve increased intake of polyphenols, which are known to have important GM-mediated health benefits[54], In a recent study in Italian individuals habitually following omnivore, vegetarian or vegan diets[55], we found that high-level adherence to a Mediterranean diet (rich in fruit, legumes and vegetables) was associated with beneficial GM and metabolome profiles, *i.e.*, increased proportions of fiber-degrading bacteria, higher levels of fecal SCFAs and lower urinary levels of trimethylamine N-oxide (TMAO), a risk factor for cardiovascular disease, potentially helping to explain the effectiveness of this diet against obesity, T2D and other inflammatory disorders. Consistently, a 1-year Mediterranean diet intervention was found to positively modulate GM and metabolome (including lower production of secondary BAs and p-cresols) and reduce frailty in elderly subjects, thus paving the way for novel intervention strategies possibly based on Mediterranean diet-responsive taxa and/or metabolites[56]. All this considered, it is not surprising that in recent years, the Paleolithic diet, with a high intake of plant foods while totally excluding industrially processed products and refined sugars, has received a lot attention. Despite some concerns about its long-term adherence, especially due to the consumption of fat and meat[57], it appears to lead to improved metabolic parameters in obese and T2D patients[58] and high levels of GM diversity, similar to those found in traditional rural populations[59].

MICROBIOME-DERIVED COMPOUNDS

SCFAs, protein metabolites, TMAO and BAs

SCFAs, mainly acetate, propionate and butyrate, are the best-known examples of diet-derived microbial metabolites with several local and systemic functions. Indeed, the human genome encodes only a limited number of carbohydrate-active enzymes (CAZymes), thus requiring complementation by the GM for the degradation of otherwise indigestible dietary fibers (*e.g.*, glycans, xylans, *etc.*)[60]. SCFAs are the end-

products of fermentation of these complex polysaccharides. These metabolites can be variously beneficial to health, as local (butyrate) and peripheral (acetate and propionate) energy sources, inflammation modulators, regulators of gut motility, vasodilators and even wound healing promoters[4]. SCFAs also affect the proliferation and differentiation of colonic epithelial cells, modulate their gene expression, reinforce the epithelial barrier (through increased mucus production and strengthening tight junctions), and influence the expansion and function of other cell lineages, including hematopoietic lineages[61]. With specific regard to metabolic health, they control the expression and secretion of appetite and glucose regulatory peptides, such as peptide YY (PYY) and glucagon-like peptide-1 (GLP-1), by enteroendocrine L-cells, and activate intestinal gluconeogenesis mainly by the regulation of gene expression[10,61]. They have also been attributed functions involved in lipid metabolism, with acetate exerting an anti-lipolytic effect, which could be beneficial in the long term by reducing systemic lipid spillover[62]. Their immunomodulatory and anti-inflammatory activity is also extremely important, for maintaining the delicate balance between tolerogenic and immunogenic signals[61].

On the other hand, branched-chain fatty acids, such as isobutyrate, 2-methylbutyrate and isovalerate, resulting from protein fermentation, have been associated with insulin resistance[63], probably through activation of mammalian target of rapamycin complex 1 (mTORC1)[64]. Protein metabolism by GM may also lead to other potentially harmful compounds, including: (1) P-cresyl and indoxyl sulfate, both of which are associated with cardiovascular morbidity and mortality; (2) 4-ethylphenylsulfate, implicated in promoting autism-like behavior in animal models; and (3) Phenylacetate, which has been shown to contribute to the development of fibrosis and non-alcoholic fatty liver disease (NAFLD)[65].

An admirable but unfortunate example of GM-host co-metabolism of dietary compounds is TMAO. Several gut microbes, *e.g.*, *Campylobacter*, *Shigella* and *Ruminococcus gnavus*, can in fact convert choline and L-carnitine present in seafood, cheese, eggs and red meat, into trimethylamine (TMA) that, once absorbed, circulates to the liver where it is oxidized by host enzymes of the flavin monooxygenase family to TMAO[66-68]. TMAO has recently emerged as a candidate risk factor for cardiovascular disease, as it is proatherogenic, increases platelet hyperreactivity and therefore the risk of thrombosis[69]. In particular, TMAO can reduce reverse cholesterol transport and BA synthesis, interfering with the normal pathway of cholesterol metabolism and elimination[70]. However, it is worth noting that some condiments, such as cold-pressed extra virgin olive oil, grape seed oil and balsamic vinegar, along with some red wines contain a structural analogue of choline, 3,3-dimethyl-1-butanol, which inhibits TMA lyase (*i.e.*, the microbial enzyme involved in TMA formation), thus paving the way for the use of selective enzyme inhibitors for the prevention and treatment of cardiometabolic diseases.

The metabolism of BAs is another example of GM-host co-metabolism, through which the GM can modify the composition of the pool of primary and secondary BAs available to the host and therefore modulate their signaling, meaning not only the traditional role in fat absorption but various effects related to glucose, lipid and energy homeostasis, thermogenesis, insulin signaling, immune responses and inflammation [71].

Finally, it is worth mentioning that GM is increasingly recognized as a major, still largely underestimated, player in determining the toxicity of environmental pollutants [72]. With specific regard to diet, for example it can mediate the adverse metabolic effects of non-calorie artificial sweeteners, whose chronic consumption increases the risk of glucose intolerance[73]. In contrast, some GM components have been shown to be involved in the bioremediation of common food processing products, including Maillard reaction products and advanced glycation end-products, which have been implicated in a wide variety of civilization disorders, *e.g.*, atherosclerosis and diabetes [74].

Neuroactive metabolites

GM is well known to interact with the enteric and central nervous systems *via* the bidirectional gut-brain axis[75]. On the one hand, GM can be directly influenced by mental health, through the luminal secretion of endocrine mediators capable of interacting with microbial receptors, thus having direct effects on microbial gene expression and signaling mechanisms. At the same time, the microbial community can be indirectly modulated as a result of induced changes in the gut environment. On the other hand, as anticipated above, the GM is capable of producing neuroactive metabolites in a diet-dependent manner, *e.g.*, SCFAs and conjugated fatty acids, which besides exerting peripheral effects can modulate the central nervous system through

direct or indirect mechanisms, involving a complex network of neuroendocrine factors and their receptors. Interestingly, these interactions have been shown to affect central appetite, food reward signaling and energy balance[76-79]. GM has also been found to influence eating behaviors through vagal nerve stimulation and immune activation [80]. Perturbations of the gut-microbiome-brain axis could therefore compromise the inhibitory mechanisms normally involved in the regulation of food intake, resulting in unbalanced eating patterns towards cravings, overeating and hedonic-driven eating behavior[79,81]. Regarding neuroactive GM metabolites, it is worth noting that propionate modulates reward pathways by reducing anticipatory reward responses to high-energy foods *via* striatal pathways[82]. Moreover, tryptophan metabolites have been closely implicated in the modulation of gut-microbiome-brain interactions, and indole propionate has recently been associated with increased food addiction behaviors in obese individuals[83,84]. GM metabolites may also interact with the endocannabinoid system, affecting the homeostatic and hedonic control of appetite and food intake[85], while the dopaminergic mesolimbic system, involved in reward mechanisms and hypothesized to play an important role in the development of obesity, is influenced by GM through the modulation of gut hormone secretion[86]. Not least, it should be remembered that microbes can even produce neurotransmitters, *e.g.*, serotonin and GABA, potentially affecting our mood and feeding[87,88].

In conclusion, particular GM layouts with distinct metabolic activities might have pleiotropic effects on host physiology, including eating behaviors, thus strongly contributing to the development of obesity and eating disorders. In this context, GM modulation or its replacement could be valuable tools to implement and increase the success of current preventive or therapeutic interventions against obesity and related comorbidities, as detailed below.

MICROBIOME-BASED STRATEGIES FOR PREVENTION AND TREATMENT OF OBESITY

The main microbiome-based strategies that are or could be effective for the prevention and treatment of obesity are discussed below and summarized in Figure 1. In short, traditional (prebiotics, probiotics and synbiotics) and innovative (NGPs and engineered microbes) interventions were considered, along with microbiome replacement strategies, based on FMT and synthetic ecology approaches. Finally, in the next paragraph, the potential of bioinformatics tools, such as machine learning and deep learning, for health risk or outcome prediction is discussed.

Prebiotics, probiotics and synbiotics

Prebiotics are typically referred to as "a substrate that is selectively utilized by host microorganisms conferring a health benefit"[89]. Prebiotic supplementation has been proposed as a means of driving changes in GM while benefitting the host in the context of various disorders, including obesity, for improved glucose homeostasis and enteroendocrine L-cell activity. For instance, dietary supplementation with whole-grain barley and brown rice improved GM diversity, increasing the Firmicutes/Bacteroidetes ratio and the relative abundance of *Blautia* (an acetate producer from the *Lachnospiraceae* family), as well as attenuating postprandial blood glucose levels and decreasing plasma interleukin (IL)-6 levels in healthy individuals[90]. Oligosaccharide supplementation has shown promising anti-obesity effects *via* the SCFA and BA pathways. In particular, several animal studies have confirmed that SCFAs produced following oligosaccharide fermentation stimulate the secretion of PYY and GLP-1 *via* the G-protein-coupled receptors (GPRs) GPR-41 and GPR-43 expressed on enteroendocrine L cells[91-93]. These appetite-decreasing intestinal hormones help reduce food intake[94], increase satiety and energy expenditure[95] and improve glucose metabolism and insulin secretion[96]. Microbial fiber metabolism has additional, SCFA-independent effects, mediated by ferulic acid, a plant cell wall component with antioxidant, anti-inflammatory and anti-diabetic effects, and by alteration of the intestinal BA pool, with downstream implications in terms of energy and glucose homeostasis[97]. Not least, adding fermentable fiber to a high-fat diet in mice has been shown to result in IL-22 induction, increased enterocyte proliferation, reduced microbiota encroachment into the mucosa and pro-inflammatory gene expression, and increased antimicrobial gene expression, thereby protecting against high-fat diet-induced metabolic syndrome[97]. Although current prebiotics are mainly carbohydrate-based, other substances, such as polyphenols and polyunsaturated fatty acids (PUFAs), could exert prebiotic effects as well, as tested in both mice and humans

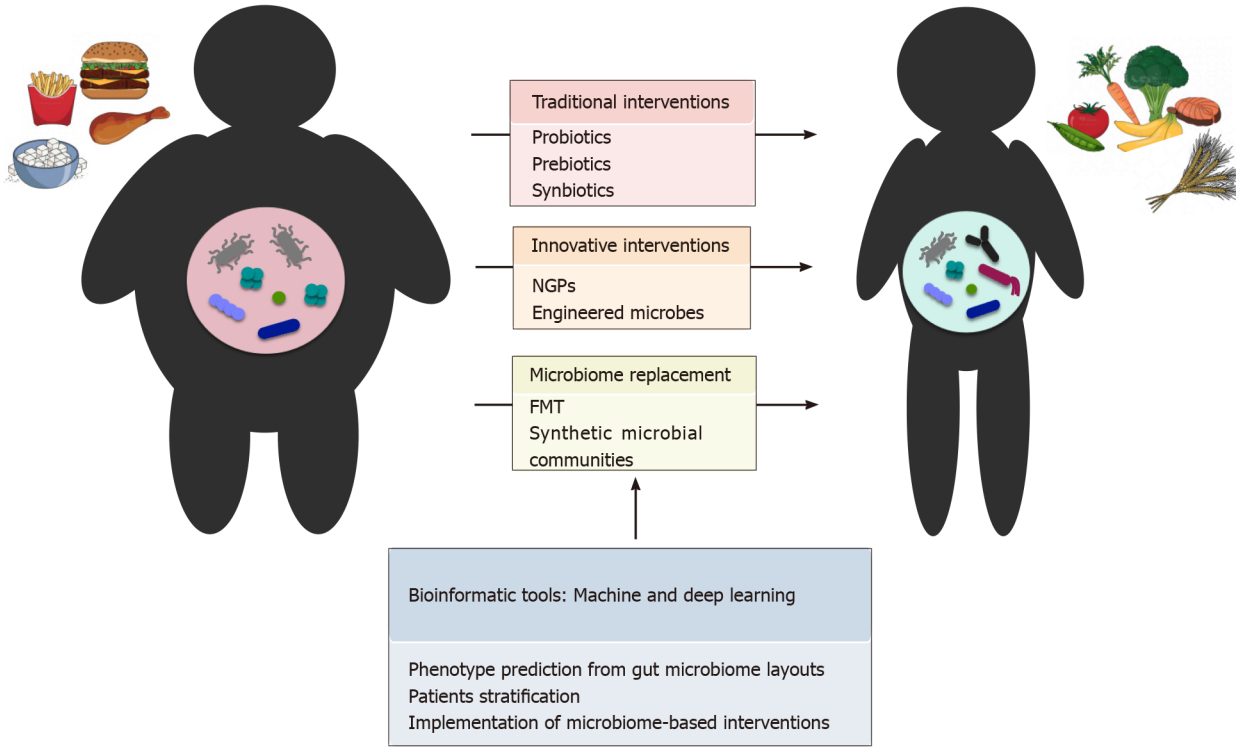


Figure 1 Overview of the main microbiome-based strategies currently in use or potentially effective in the prevention and treatment of obesity. Traditional intervention strategies include dietary supplementation with prebiotics, probiotics and synbiotics, which have generally been shown to be moderately effective for the prevention and amelioration of obesity. Innovative strategies have therefore been implemented for improved treatment efficacy, using next-generation probiotics and non-pathogenic engineered microorganisms designed for the *in-situ* delivery of specific modulators. More recently, more direct modulation strategies based on gut microbiome replacement by means of fecal microbiota transplantation or synthetic communities, are being considered. In this scenario, bioinformatic tools, including machine and deep learning, could be crucial not only for the rational design of synthetic communities, but also for stratifying patients based on disease-associated phenotypes and thus predicting their health risks and outcomes. In the near future, all the accumulating knowledge about the gut microbiome and technological advances should lead to a rational implementation of innovative microbiome-based interventions geared towards personalized precision medicine. Food items were obtained from the Mind the Graph platform (https://mindthegraph.com/). NGPs: Next-generation probiotics; FMT: Fecal microbiota transplantation.

[89]. Polyphenols, *i.e.*, secondary metabolites derived from plant sources, are extensively metabolized in the intestine and converted into phytoestrogens with multiple beneficial effects[50,98]. Following regular consumption of polyphenols, a reduced risk of cardiometabolic diseases has been observed, together with antioxidant, anti-inflammatory and anti-obesogenic effects[99,100]. However, the prebiotic effects of polyphenols can be affected by the food source and although these compounds are generally recognized as GM compositional modulators, further research is needed to validate their prebiotic potential[101]. Multiple health benefits have also been reported for nutritional supplementation with PUFAs, such as eicosapentaenoic acid and docosahexaenoic acid, including anticancer activity[102,103], secondary prevention of ischemic heart disease[104] and prevention of cardiovascular diseases[105], as well as a reduction of mucosal inflammation and modulation of the GM composition in patients with ulcerative colitis[106]. Dietary supplementation with PUFAs may therefore be useful not only for obesity mitigation but also for the treatment of obesity-associated comorbidities[107,108]. In addition, PUFA-derived mediators, such as resolvins and protectins, have shown the potential to counteract inflammation in the context of obesity[109]. In this regard, a recent study in obese diabetic *db/db* mice demonstrated a marked improvement in insulin sensitivity following the administration of protectin D1[110]. In light of these findings, prebiotic supplementation should be considered a potential integrative therapy for the prevention and treatment of obesity.

Probiotics, *i.e.*, "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host"[111], represent one of the most widely used GM manipulation tools, for which an increasing number of clinical studies have been carried out in subjects with various pathological conditions, including obesity[112, 113]. However, it should be noted that conflicting results have emerged in relation to the ability of probiotics to counterbalance weight loss and obesity-related features. In particular, two meta-analyses of randomized controlled clinical trials[114,115] found

almost no efficiency in terms of weight and body mass index (BMI) reduction in obese individuals, especially in short-term interventions. On the other hand, the meta-analysis carried out by Zhang *et al*[116] on a large number of clinical trials reported substantially different results and a significant reduction in body weight and BMI, with consistent maximum outcomes with multi-strain product supplementation for at least 8 wk. Similar conclusions were drawn by John *et al*[117] in a similar meta-analysis, showing that probiotic administration was associated with a reduction in all considered parameters (*i.e.*, body weight, BMI and fat mass). As expected, several studies have found strain-specific probiotic effects on body weight and metabolism, with only a few species belonging to *Lactobacillus* (*e.g.*, *L. acidophilus*, *L. casei*, *L. rhamnosus*, *L. reuteri*) and *Bifidobacterium* (*e.g.*, *B. bifidum*, *B. lactis*, *B. longum*) proven effective in overweight/obese individuals[104,118,119]. The greatest decreasing effect on BMI and body weight was reported with high doses of single-strain probiotics[117, 120], although John *et al*[117] observed a considerable reduction in both parameters even at lower doses when interventions continued for more than 12 wk[117]. More recently, a study by our group showed that administering a multi-strain probiotic mixture along with a hypocaloric Mediterranean diet led to weight loss, improvement in oxidative stress markers and an increase in *Akkermansia* in elderly obese women, even in 15 d[33]. Regardless of the strain, the beneficial effects of probiotics in the treatment of obesity are also attributable to the following general mechanisms of action: (1) Antimicrobial activity, by inhibiting the growth of pathogenic microorganisms and exerting antagonistic effects against colonization of the intestinal mucosa and epithelium adherence; (2) Improvement of the barrier function, reducing intestinal permeability and increasing mucus production; and (3) Immunomodulation, through interaction with innate and adaptive components of the immune system[120]. Taken together, these mechanisms contribute to positively modulate the GM composition towards restoring a health-associated layout, which in turn can help alleviate host metabolic imbalances.

Synbiotics refer to "a mixture comprising live microorganisms and substrates selectively used by host microorganisms that confers a health benefit on the host" [121]. Accumulating evidence has reported the stronger effect of synbiotics in terms of GM modulation than either probiotics or prebiotics alone[122,123], with an overall improvement in lipid metabolism, glycemic status and inflammatory mediators[124, 125]. Although the appropriate dose, duration of administration and the composition of a synbiotic product necessary to confer a health benefit are influenced by several factors (*e.g.*, baseline GM layout, medications, habitual diet and lifestyle), the randomized clinical trial conducted by Dao *et al*[42] on 225 overweight and obese adults resulted in a 4.5% reduction in body fat mass when administering a combination of *B. animalis* subsp. *Lactis* 420 and polydextrose[42]. The control of body fat mass in overweight or obese individuals by the aforementioned synbiotic was also confirmed in a second clinical trial, along with a peculiar rearrangement in the GM composition that included an increased abundance of *Akkermansia*, *Christensenellaceae* and *Methanobrevibacter*, all taxa related to improved metabolic health and leanness [126]. GM modulation with increased proportions of potentially beneficial microbial groups (*e.g.*, *Lactobacillus*) was also observed in a clinical trial in obese individuals following administration of a synbiotic consisting of a multi-strain probiotic formulation (*i.e.*, *B. lactis*, *B. longum*, *B. bifidum* and *L. acidophilus*) and galactooligosaccharides as a prebiotic component[127].

In conclusion, prebiotics, probiotics and synbiotics are promising dietary agents for the modulation of human GM also in the context of obesity and metabolic disorders. However, given the still conflicting data in the scientific literature on probiotics, further studies are needed to rationally include their prescription as a preventive or therapeutic supplement for obesity.

NGPs and engineered microbes

Moving from the "one-size-fits-all" concept related to traditional probiotics, researchers are striving to thoroughly elucidate the role of each commensal member within the complex ecosystem of the human gastrointestinal tract in influencing host health. Compared to the modest ameliorative effects generally shown by traditional probiotics in obese individuals, emerging NGPs are beginning to reveal their great potential as novel preventive and therapeutic tools[128]. In this perspective, several studies have shown differences in the GM composition between obese and lean individuals, pointing out that an increased abundance of *A. muciniphila* could lead to an improvement in obesity and metabolic disorders[129,130]. Subsequently, the mechanism underlying the beneficial effect of *A. muciniphila* was investigated and the involvement of an immunomodulatory membrane protein "Amuc_1100" was

proposed, which showed the same beneficial effects as live bacteria[131]. Cani *et al* [132] demonstrated the ability of this promising NGP to modulate the endocannabinoid system[132], a crucial regulatory system involved in controlling glucose metabolism in obesity, T2D and inflammatory conditions[132]. More recently, as anticipated above, the daily oral administration of live or pasteurized *A. muciniphila*, for three months to overweight/obese insulin-resistant volunteers, has been shown to be safe and well tolerated, while leading to improved metabolic parameters[44]. However, since some animal studies have reported an increase in *A. muciniphila* in multiple sclerosis[133] and Parkinson's disease[134], further studies are needed to fully unravel its effects on host health.

Christensenella minuta also showed potential probiotic effects by ameliorating obesity and associated metabolic disorders through modulation of dysbiotic GM layouts[135], and its abundance was greater in lean individuals with low BMI[136]. However, Yang *et al*[137] recently highlighted potential pathogenic features (*e.g.*, LPS-mediated triggering of a mild inflammatory response *via* NF-kB pathway) of *C. minuta*, suggesting that its application should be limited to therapeutic interventions focused on obesity control[137]. As mentioned above, *P. goldsteinii* is also a promising anti-obesity and anti-inflammatory NGP candidate. Being selectively enriched in the GM of mice fed a high-fat diet supplemented with oriental medicinal fungi, this commensal bacterium has been associated with increased adipose tissue thermogenesis, reduced levels of inflammation, enhanced intestinal integrity and amelioration of insulin resistance[41]. While promising, these *in vivo* results have not yet been translated into clinical trials. On the other hand, *Faecalibacterium prausnitzii*, one of the most promising NGPs due to its well characterized anti-inflammatory activity[138,139], showed a lower clade diversity in intestinal diseases and obese individuals[140]. In light of this finding, caution must be taken in selecting the most suitable strain for the development of therapeutic interventions. Despite the growing amount of NGP candidates so far isolated and characterized, further strain-level functional analyses are required to fully assess the underlying mechanisms by which they could confer health benefits to the host, before pushing them for clinical application.

Synthetic biology approaches have recently been exploited to address disease-specific mechanisms and meet medical needs by designing non-pathogenic and commensal bacteria to deliver therapeutic effectors[141,142]. Regarding the feasibility of using engineered probiotics to alleviate obesity-related characteristics, Long *et al* [143] demonstrated a decrease in body weight gain in overweight mice given an engineered *Bifidobacterium* strain secreting oxyntomodulin, an anorexigenic hormone that reduces appetite and food intake[143]. In a similar study, Chen *et al*[144] developed an engineered probiotic strain of *Escherichia coli* with increased secretion of N-acyl-phosphatidylethanolamine, the immediate precursor of the anorexigenic metabolite N-acylethanolamide, resulting in reduced weight gain and less accumulation of fat mass in mice fed a high-fat diet[144]. Another potential strategy to alleviate obesity and metabolic disorders has recently been proposed by Bai *et al*[145]. Administration of *Bacillus subtilis* SCK6 strain BsS-RS066550 engineered to increase butyric acid production resulted in decreased body weight and food intake in high-fat diet-fed mice, along with beneficial effects on insulin resistance, blood glucose and hepatic biochemistry[145]. In addition, Wang *et al*[146] showed that administration of genetically engineered *Lactococcus lactis* expressing GLP-1 significantly reduced body weight and blood glucose of obese mice fed a high-fat diet[146]. Anti-obesity mechanisms included the promotion of fatty acid oxidation and the restoration of GM biodiversity[146]. To date, numerous therapeutic interventions based on engineered live bacteria have entered the early or mid-stage of clinical development[141]. If successfully completed, such studies will be crucial in providing the missing proof of concept required to pave the way for a new class of precision therapeutics. Once their efficacy and risk ratio have been verified and approved for use in humans, engineered bacterial therapeutics will enable specific disease mechanisms and unmet medical needs to be addressed, even in obese patients.

FMT

Consisting of the transfer of microbes from healthy individuals to recipients hosting a dysbiotic GM layout with the aim of restoring eubiosis[147,148], FMT has attracted considerable attention in clinical practice, particularly for the treatment of recurrent infections by antibiotic-refractory *Clostridioides difficile*[149,150]. Recently, the potential of FMT in treating a large number of conditions, including obesity, has been explored. In a pivotal study, Ridaura *et al*[63] showed that mice receiving GM from obese individuals developed obesity, while those receiving GM from healthy individuals remained lean[63]. Sequencing analysis of post-treatment stools confirmed the

successful engraftment of the donor microbiota, along with the transfer of functions associated with either "lean" or "obese" microbial communities. Shortly before, a clinical study on diabetic and obese adult males by Vrieze *et al*[151] demonstrated improved microbial diversity and insulin sensitivity, along with the expansion of Bacteroidetes and butyrate-producing taxa, following GM transplantation from lean donors[151].

As of April 2021, there were 15 registered clinical trials (ClinicalTrials.gov search terms: "gut microbiota", "obesity" and "fecal microbiota transplantation") on obese individuals undergoing FMT for the replacement of the obesogenic microbial community (Table 1). Of these, four have been completed and only two have made the results publicly available. Yu *et al*[152] performed FMT on 24 obese individuals with the aim of improving metabolic outcomes[152]. Administration of FMT capsules ensured engraftment, persisting for at least 12 wk after treatment, although no clinically significantly metabolic effects were observed. Allegretti *et al*[153] performed FMT on 22 obese, metabolically uncompromised patients, and although no significant changes in BMI occurred, a sustained shift of obesity-associated microbiomes towards the donor microbiome layout was observed, along with improved BA profiles and decreased fecal levels of taurocholic acid[153]. In a secondary analysis focused on the prevention of metabolic syndrome within the same patient group, the authors found a significant change in glucose and insulin levels after FMT[154]. Similar to other microbiome-based therapeutics, while promising, FMT is still at an early stage for the treatment of obesity and associated comorbidities. Therefore, it should be recognized as a separate pharmacological category, consisting of an entirely novel class of agents and requiring systematic research to fill knowledge gaps, thereby facilitating the development of standardized next-generation microbiota therapeutic interventions with improved safety and efficacy.

Microbial replacement therapy through synthetic ecology approaches

It is generally believed that the administration of multi-species microbial consortia is more useful than a single probiotic organism[155], as it probably retains some properties of the community structure, with community members continuously interacting and communicating with each other. Furthermore, a consortium of bacteria possesses a larger gene pool than monocultures, resulting in greater diversity in metabolic pathways. This richness is reflected in a greater ability of the consortium to perform more complex tasks than single organisms, thus exploiting the resources available in the surroundings more efficiently and better adapting to the environment, also through self-organization to form spatial patterns in response to substrates and metabolite gradients[156-160].

Synthetic ecology indicates the rational design of ecosystems, where two or more defined microbial populations are assembled in a well-characterized and controlled environment[161]. This approach requires *in vitro* controlled environments, biologically relevant bacterial strains and mathematical models of ecological interactions [162] to simulate community behavior in response to several factors. The idea is to shape a complex microbial community in order to obtain a desired compositional and functional profile that meets the needs of specific industrial production processes or pharmacological interventions.

Synthetic microbial communities are systems of known and trimmed-down complexity that can undergo experimental treatments and mathematical modelling, enabling a system-level understanding of the consortium[163,164]. These communities are not only a way to study how the microbial consortium structure emerges and the conditions necessary to generate specific interaction networks among its components, but they can also elucidate the overall function, resistance and resilience of microbial systems[10]. By the so-called microbial resource management[165,166], *i.e.*, the management of consortium parameters such as richness, evenness, predation, abiotic factors, quorum sensing and spatial disposition, the community can be steered to the desired functionality, in order to obtain novel products and processes or potentially improve human health by transplanting the desired synthetic community into a recipient patient.

To date, many studies[167-169] have reported the *in vitro* assembly of synthetic GM communities with at least two bacterial strains, but clinical applications are still limited. Petrof *et al*[170] obtained a synthetic consortium consisting of 33 individual microbial species derived from a stool sample from a healthy donor and demonstrated the potential of such synthetic microbiota in the treatment of *C. difficile* infection. Furthermore, they reported that some of the administered bacteria that were forming the synthetic community stably colonized the recipient's colon, as opposed to most commercially available probiotics, which only transiently colonize the intestine.

Table 1 Registered clinical trials on ClinicalTrials.gov (as accessed on April 2021) focused on fecal microbiota transplantation for the replacement of obesogenic microbial communities. Search terms included "gut microbiota", "obesity" and "fecal microbiota transplantation"

Rank	Title	Status	Results	Condition	Intervention	Location	URL
1	Fecal Microbiota Transplantation for the Treatment of Obesity	Completed	Available	Obesity	FMT *vs* placebo	United States	https://ClinicalTrials.gov/show/NCT02741518
2	Faecal Microbiota Transplantation in Obesity	Recruiting	Not available	Obesity	FMT *vs* placebo	Finland	https://ClinicalTrials.gov/show/NCT03391817
3	Randomized Controlled Trial of Fecal Microbiota Transplantation in Severe Obesity	Enrolling by invitation	Not available	Obesity	FMT *vs* placebo	Norway	https://ClinicalTrials.gov/show/NCT03273855
4	Fecal Microbiota Transplant (FMT) to Induce Weight Loss in Obese Subjects	Active, not recruiting	Not available	Obesity	FMT and mucosal microbiota assessment	China	https://ClinicalTrials.gov/show/NCT03789461
5	FMT and Fiber in Patients With Metabolic Syndrome	Completed	Not available	Obesity, Metabolic Syndrome	FMT and dietary supplement with fiber (cellulose) *vs* placebo	Canada	https://ClinicalTrials.gov/show/NCT03727321
6	Assessment of the Health Improvement of Obese Patients After Fecal Microbiota Transplantation (FMT)	Completed	Not available	Obesity, Type 1 and 2 Diabetes	FMT	Russian Federation	https://ClinicalTrials.gov/show/NCT04579263
7	Fecal Microbiota Transplantation for Diabetes Mellitus Type II in Obese Patients	Unknown status	Not available	T2DM, Obesity	FMT and dietary intervention (high-fat low-fiber diet, sham diet or low-fat high-fiber diet)	Israel	https://ClinicalTrials.gov/show/NCT02346669
8	Fecal Microbial Transplantation and Fiber Supplementation in Participants With Obesity and Metabolic Syndrome	Active, not recruiting	Not available	Obesity, Metabolic Syndrome	FMT and dietary supplement with fiber (cellulose) or FMT only *vs* placebo	Canada	https://ClinicalTrials.gov/show/NCT03477916
9	Randomised Placebo-controlled Study of FMT to Impact Body Weight and Glycemic Control in Obese Subjects With T2DM	Active, not recruiting	Not available	T2DM, Obesity	FMT and lifestyle modification program or FMT only *vs* placebo	China	https://ClinicalTrials.gov/show/NCT03127696
10	Fecal Microbiota Transplant for Improvement of Metabolism	Completed	Available	Obesity	FMT *vs* placebo	United States	https://ClinicalTrials.gov/show/NCT02530385
11	The Role of Microbiome in Recurrent Obesity	Not yet recruiting	Not available	Obesity	FMT *vs* placebo	Israel	https://ClinicalTrials.gov/show/NCT04697550
12	Effects of Fecal Microbiota Transplantation on Weight in Obese Patients With Non-alcoholic Fatty Liver Disease	Recruiting	Not available	NAFLD	FMT, dietary intervention and physical activity *vs* placebo	India	https://ClinicalTrials.gov/show/NCT04594954
13	Proposal to Examine the Effect of Fecal Transplantation on Obesity	Unknown status	Not available	Obesity	FMT *vs* placebo	Israel	https://ClinicalTrials.gov/show/NCT02336789
14	Safety and Efficacy of Fecal Microbiota	Recruiting	Not available	IBD, IBS, Obesity,	FMT	China	https://ClinicalTrials.gov/show/NCT04014413

	Transplantation			Metabolic Syndrome, Infections, Others			
15	Transplantation of Microbes for Treatment of Metabolic Syndrome & NAFLD	Completed	Not available	Type 1 and 2 Diabetes, NAFLD, Obesity	FMT	Canada	https://ClinicalTrials.gov/show/NCT02496390

FMT: Fecal Microbiota transplantation; IBD: Inflammatory bowel disease; IBS: Inflammatory bowel syndrome; NAFLD: Non-alcoholic fatty liver disease; T2DM: Type 2 diabetes mellitus.

Despite its proven efficacy for the treatment of *C. difficile* infection, FMT hides some uncertainties that may be resolved by a synthetic stool replacement strategy. Indeed, the synthetic ecology approach has multiple advances over the canonical use of fecal matter from a donor: (1) The exact composition of the administered bacteria is known and can be reproduced; (2) The bacterial composition can be virtually tailored to the specific patient's needs; (3) The absence of pathogens and viruses can be more reliably guaranteed, improving safety; and (4) The administered microorganisms forming the consortia can be selected based on their sensitivity to antimicrobials, resulting in further improvement of the safety profile.

Applications of synthetic communities have been reported in other fields, such as bioremediation[157] and chemical production[171,172]. Notwithstanding the potential of the synthetic ecology approach in GM replacement therapy, there is still some way to go before synthetic ecology can be translated into the clinic. One major limitation is the lack of a truly representative *in vitro* system to mimic the *in vivo* microbiota, but steps in this direction have been achieved by the gut-on-a-chip model[173] and the HuMiX system[173]. In addition, a large and well-documented collection of cultures of human microbiota members will be extremely useful for improving ecology experiments and building mathematical models. In this regard, since 2015, culturomics has led to the discovery of 232 novel human gut species[174] and it is expected that this number will increase in the coming years. The technique is based on the multiplexing of bacterial isolation conditions through serial addition of specific growth promoters and/or inhibitors, coupled with high-throughput identification with MALDI-TOF mass spectrometry, and is capable of overcoming the limitations of conventional single-medium strategies[175-177]. The resulting knowledge could then be used to improve our understanding of the complex gut ecosystem and rationally design efficient and sophisticated synthetic communities, tailored for the treatment of the disease of interest.

THE FUTURE OF MICROBIOME-BASED PRECISION MEDICINE: HEALTH RISK OR OUTCOME PREDICTION THROUGH DEEP LEARNING

Machine learning and deep learning application to microbiome data

Machine learning and, in particular, deep learning have been the subject of intense media hype in recent years. Thanks to the explosion of available data and the rapid growth in the number and size of databases, they have accomplished nothing short of a revolution in the field of modern artificial intelligence, with notable progress in perceptual problems, such as facial and speech recognition[178,179], and have the potential to do the same in medical disciplines[180,181]. However, we are still exploring the full extent of what machine learning and deep learning can do and have just begun to apply them to a wide variety of problems outside of classical algorithms.

In light of the rapid increase in data from microbiome studies induced by the concomitant decrease in sequencing costs (< 10000 times in the past 10 years)[182], there are requirements to apply machine learning and deep learning algorithms to host-microbiome data, exploiting their associations in various diseases. Improved data analytical tools are needed to explore all the information contained in those datasets and identify key features that represent different aspects of the microbiome and that can be linked to host phenotypes. In particular, the possibility of predicting the patient's phenotype from one's GM is an integral part of personalized medicine, as it represents not only a way to overcome individual variability, but also a potential therapeutic target for pharmacological interventions. In this field, machine learning

might provide new insights, by developing models capable of stratifying individual patients into therapeutic classes, thus paving the way for the fine-tuning of interventions based on the GM structure. An example of machine learning algorithms are Support Vector Machines (SVMs), which were implemented by Cui and Zhang [183] to classify metagenomic samples into inflammatory bowel disease (IBD) and non-IBD classes. More recently, Pasolli *et al*[184] used SVMs to predict diseases such as liver cirrhosis, colorectal cancer and IBD from fecal metagenomes. To date, deep learning is the most advanced machine learning technique for a variety of applications [185] and has already achieved several results in the microbiome field[186-189], including predicting the microbiome structure in terms of bacterial relative abundance and metabolic layout. Other machine learning methods include Ensemble Methods, which combine multiple classifiers for better performance. The best known ensemble method is Random Forest[190], which has been widely used in microbiome studies for patient stratification[191,192] and biomarker search[193,194].

Machine learning, GM and obesity

In a prospective study on obesity in European children, Rampelli *et al*[31] underscored the importance of the microbiome–host–diet configuration as a possible predictor of obesity. In particular, GM was found to mediate dietary impact on individual metabolic and immunological homeostasis, which, in the context of other individual lifestyle and genetic variables, may be involved in the development of the multifactorial obese phenotype. Such results were based on experimental observations and no machine learning/deep learning algorithms were applied or developed. However, the study suggested that applying a machine learning algorithm to microbiome data could be a feasible method of predicting obesity, when other variables concerning host physiology, diet and lifestyle (*e.g.*, physical activity) are included.

Some attempts have been made in recent years, with poor results, mainly due to a lack of data (often lifestyle and dietary information is not collected or collected in a non-systematic and non-standardized way). Specifically, Pasolli *et al*[184] developed a Random Forest-based approach that exclusively utilized metagenome data without success in predicting obesity and T2D. A few years later, Fernández-Navarro *et al*[195] have implemented several machine learning methods, such as decision tree-based methods, ensemble methods and SVMs, to identify predictors of obesity. Starting from serum free fatty acid levels, microbial quantitative polymerase chain reaction information and dietary intake interviews, their model revealed a non-obese profile related to serum eicosapentaenoic acid levels and *Bacteroides* amount in feces.

Nevertheless, this represents just the tip of the iceberg, as the full deployment of machine learning methods in the human GM sphere for full integration into the field of personalized precision medicine requires additional efforts. Indeed, machine learning often runs like black boxes, which makes it difficult to conduct feature selection. In addition, a large amount of data and computational power are required to train powerful and reliable machine learning-based algorithms. In general, novel technologies have dramatically increased our ability to characterize the human GM, but the way to effectively harness that information is uncertain and presents several key challenges. For example, there is a high need for dimensionality reduction to handle the information of hundreds of thousands of gene markers for just a few hundred samples. In this regard, neural encoder-decoder networks[196] based on a deep learning architecture have proved effective[187], but further efforts are still needed to fully exploit microbiome data. What is certain is that machine learning has already shown its great potential when applied to the microbiome field. In the next years, cutting-edge machine learning-based models might enable a further step towards the microbiome implementation in personalized precision medicine.

LIMITATIONS OF CURRENT EVIDENCE AND NEXT STEPS

As discussed in the respective paragraphs, there are several limitations to the design and application of microbiome-based strategies in clinical routine, with particular reference to the prevention/treatment of obesity and related comorbidities. Although promising data come from the field of NGPs/engineered microbes, synthetic ecology and even FMT, it should be remembered that the evidence is still too little to support the clinical benefit of these novel microbiome modulation tools. Added to this are the sometimes inconsistent results on GM compositional and functional changes in the pathological context, and the lack of a full understanding of the underlying mecha-

nisms. Of course, several steps forward have been made, especially methodological ones, but there is still no standardization of study designs and the way of reporting the results, which makes it difficult to compare different studies. In parallel with the implementation of internationally recognized standard operating procedures for GM analysis, it is expected that in the future: (1) -Omics approaches, including metagenomics, metatranscriptomics, metaproteomics and metabolomics are combined to provide mechanistic insights; (2) The mechanisms are possibly validated in animal models; (3) Culturomics approaches are increasingly exploited to unravel the dynamics and ecological rules that govern the establishment of microbial networks; (4) The accumulating microbiome knowledge allows to fine-tune the design of precision strategies to achieve specific objectives, whether based on traditional or next-generation tools; and (5) Progressively generated datasets, including host and microbiome data, enable machine and deep learning technologies to maximize translational impact, through accurate prediction of health outcomes and thus provision of high-quality personalized care. As expected, the same limitations and implications also apply in other pathological contexts, where the modulation of GM can be impactful as well.

CONCLUSION

The emerging role of GM as a contributor to various pathological conditions is fascinating even if not yet easy to untangle. Diseases resulting from multiple factors, such as obesity and metabolic diseases in general, may be difficult to prevent or treat effectively solely relying on currently available therapies. In this scenario, gut microbes and their influence on the host constitute a piece of an intricate puzzle, to be exploited for novel integrated intervention strategies. In a systems biology approach, the advent of –omics technologies and the development of bioinformatics tools are pushing microbiome research to the next level, allowing to extrapolate general principles on community structure and translate the results into rationally designed, personalized microbiome-based interventions aimed at restructuring dysbiotic layouts, thereby contributing to the restoration and maintenance of host health.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 November 7; 27(41): 7065-7079

DOI: 10.3748/wjg.v27.i41.7065

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

REVIEW

Gut microbiota in a population highly affected by obesity and type 2 diabetes and susceptibility to COVID-19

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Conflict-of-interest statement: The authors report no conflicts of interest.

Supported by Secretaría de

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Abstract

Coronavirus disease 2019 (COVID-19) is a disease produced by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and it is currently causing a catastrophic pandemic affecting humans worldwide. This disease has been lethal for approximately 3.12 million people around the world since January 2020. Globally, among the most affected countries, Mexico ranks third in deaths after the United States of America and Brazil. Although the high number of deceased people might also be explained by social aspects and lifestyle customs in Mexico, there is a relationship between this high proportion of deaths and comorbidities

Relaciones Exteriores México (SRE), No. SRE/027/2021; Agencia Mexicana de Cooperación Internacional para el Desarrollo (AMEXCID), No. AMEXCID 2020-5; CONACyT for Doctoral Fellowships, No. 777953 (KC-C), No. 635676 (TB-G), No. 291236 (FH-Q), and No. 336296 (LEV-F); CONACyT for Master Fellowships, No. 997494 (NGZ-T), and No. 997152 (JMV-I); Fellows from the Sistema Nacional de Investigadores, Mexico, No. 43142 (PZ-S), No. 225525 (FB-G), No. 47399 (CP-C), and No. 19815 (JG-M).

Country/Territory of origin: Mexico

Specialty type: Gastroenterology and hepatology

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

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Received: May 1, 2021
Peer-review started: May 1, 2021
First decision: June 12, 2021
Revised: June 25, 2021
Accepted: September 30, 2021
Article in press: September 30, 2021
Published online: November 7, 2021

P-Reviewer: Verma AK, Wang CY
S-Editor: Fan JR
L-Editor: A

such as high blood pressure (HBP), type 2 diabetes, obesity, and metabolic syndrome. The official epidemiological figures reported by the Mexican government have indicated that 18.4% of the population suffers from HBP, close to 10.3% of adults suffer from type 2 diabetes, and approximately 36.1% of the population suffers from obesity. Disbalances in the gut microbiota (GM) have been associated with these diseases and with COVID-19 severity, presumably due to inflammatory dysfunction. Recent data about the association between GM dysbiosis and metabolic diseases could suggest that the high levels of susceptibility to SARS-CoV-2 infection and COVID-19 morbidity in the Mexican population are primarily due to the prevalence of type 2 diabetes, obesity, and metabolic syndrome.

Key Words: SARS-CoV-2; COVID-19; High blood pressure; Hypertension; Type 2 diabetes; Obesity; Metabolic syndrome; Gut microbiota; Immunity

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Core Tip: This work reviews recent data about gut microbiota (GM) diversity in Mexico, a country in which more than 18.4% of adults present high blood pressure, 39.1% are overweight, 36.1% are obese, and more than 10.3% suffer from type 2 diabetes. This review highlights the link between GM dysbiosis and severe acute respiratory syndrome coronavirus 2 prevalence, which ranks Mexico third in cumulative coronavirus disease 2019 deaths in the world.

Citation: García-Mena J, Corona-Cervantes K, Cuervo-Zanatta D, Benitez-Guerrero T, Vélez-Ixta JM, Zavala-Torres NG, Villalobos-Flores LE, Hernández-Quiroz F, Perez-Cruz C, Murugesan S, Bastida-González FG, Zárate-Segura PB. Gut microbiota in a population highly affected by obesity and type 2 diabetes and susceptibility to COVID-19. *World J Gastroenterol* 2021; 27(41): 7065-7079
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7065.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7065

INTRODUCTION

Bacteria maintain the immune response in the gut

The human body harbors approximately 100 trillion cells belonging to commensal microorganisms[1], and they are primarily concentrated in the intestine[2]. The term gut microbiota (GM) refers to the symbiotic intestinal collection of bacteria, archaea, and some eukaryotes with an important influence on health and disease[3]. Among the several functions in the host, the GM participates in the synthesis of water-soluble vitamins, the supply of quinones[4], the metabolism of xenobiotics[5], neurotransmitter modulation[6], the production of energy substrates from dietary fiber[7] and the regulation of immune homeostasis[8].

A functional microbiota promotes the host's immunity[9]. For example, the polysaccharide A in *Bacteroides fragilis*' directs lymphoid organogenesis and corrects systemic T lymphocyte (TL) deficiencies and TL-helper Th1/Th2 imbalances through mechanisms such as interleukin (IL)-12/Stat4-mediated Th1 differentiation. Moreover, *B. fragilis*' polysaccharide A presentation by intestinal dendritic cells (DCs) activates clusters of differentiation in CD4+ TLs, eliciting appropriate cytokine production[10]. Commensal GM is also required for Th17 cell differentiation in the small intestine by activating the transforming growth factor (TGF)-β[11] and influences gut immunoglobulin (Ig) repertories and B lymphocyte (BL) development in the intestinal mucosa [12]. Elevated serum levels of IgE through BL isotype switching at mucosal sites have been reported for germ-free (GF) mice in a CD4+ TL- and IL-4-dependent manner, suggesting that a healthy GM is required to inhibit high IgE induction[13].

The GM plays a vital role in the innate immune system[14]. A total lack of TL and DC under GF conditions in the jejunum of piglets was reverted by *Escherichia coli* colonization, favoring the recruitment of both cell types to the lamina propria[15]. GM

P-Editor: Fan JR

metabolites such as trimethylamine N-oxide and butyrate drive macrophage polarization using the NLRP3 inflammasome as a proteolytic activator[16], they promote monocyte to macrophage differentiation by inhibiting histone deacetylase (HDAC3), and they amplify antimicrobial host defense[17]. Furthermore, GF mice lack IL-22-producing natural killer (NKp46+) cells[18] and have lower levels of mast cell densities in the small intestine than conventional mice due to the absence of CD182 ligands from gut epithelial cells[19]. All this evidence illustrates the vital function of the GM in relation to innate immunomodulation.

The interactions of the host with the microbiota are complex, numerous, and bidirectional. The GM significantly regulates the development and function of the innate and adaptive immune systems[20]. Intestinal bacterial commensals secrete antimicrobial peptides and compete for nutrients and habitat sites, thereby aiding in the state of homeostasis[21]. The GM and immune homeostasis have a reciprocal relationship and are a topic of great interest and intense research investigation in the field of infectious diseases. Additionally, GM-derived signals modulate immune cells for pro- and anti-inflammatory responses, thereby affecting susceptibility to various diseases[22]. Immune gut homeostasis is orchestrated by fine adjustments in the regulatory balance of pro-inflammatory responses such as Th17 cells *vs* inflammatory regulatory T cells (Tregs), whose function is influenced by commensal microorganisms [23]. During the process of launching a response against pathogenic infections and etiological agents such as coronavirus, a healthy gut microbiome is pivotal to maintaining an optimal immune system to prevent an array of excessive immune reactions that eventually become detrimental to the lungs and vital organ systems. Under those circumstances, it becomes crucial to have a balanced immune response as opposed to an overreactive or an under reactive response that could aggravate the disease, causing clinical complications such as pneumonia and/or even acute res-piratory distress syndrome to occur in response to viral diseases such as coronavirus disease 2019 (COVID-19).

Several studies have linked the GM with adaptative immune system homeostasis [24]. For instance, *B. fragilis* induces CD4+ TL differentiation to Th1 and interferon (IFN)-γ production[10], whereas segmented filamentous bacteria favor this process of Th17 differentiation and IL-17 and IL-22 production[8]. However, bacteria such as indigenous *Clostridium* spp. promote this differentiation to CD4+ T regulatory cells and the production of IL-10 and IL-35 through the induction of the TGF-β cytokine and the FOXP3 transcription factor expression[8,25].

An essential role of the GM in the host's susceptibility to viral infection has been suggested by some reports[26]. For example, while *Bifidobacterium breve* and prebiotic oligosaccharides prevented rotavirus infection through IFN-γ, IL-4, tumor necrosis factor (TNF-α), and Toll-like receptor (TLR2) expression[27], human milk oligosaccharides (HMOs) increased *Enterobacter*/*Klebsiella* abundance and rotavirus infectivity, possibly through the viral structural stability conferred by HMOs[28] and lipopolysaccharides[29]. Moreover, there is an interesting report showing that short-chain fatty acids produced by GM protect against allergic inflammation in the lungs[30].

ASSOCIATION OF COVID-19 SEVERITY WITH HIGH BLOOD PRESSURE, TYPE 2 DIABETES, OBESITY, AND METABOLIC SYNDROME

Non-communicable diseases (NCDs) are the leading cause of mortality and premature disability worldwide, with over 36 million deaths *per* year[31]. Obesity (OB) is considered a major risk factor for NCDs, and it is associated with an estimated loss of 5–20 years of life expectancy[32]. OB also increases the risk of metabolic diseases such as fatty liver disease and type 2 diabetes mellitus (T2DM)[33]. From 2000 to 2019, there was an increase in global T2DM prevalence from 151 to 463 million, and this number is expected to grow to 700 million by 2045[34]. It is estimated that T2DM accounts for 87 to 91% of diabetes cases, while type 1 diabetes is only considered to be responsible for 7 to 12% of global diabetes cases[35]. Although there are some reports indicating that in general, the prevalence of diabetes is stabilizing in some populations, overall, it keeps increasing in non-Hispanic black and Hispanic populations[36].

The OB prevalence in Mexico is one of the highest in the world, corresponding to 36.1% of the Mexican population[37]. The number of cases of T2DM in Mexico is 12.8 million people, with 101257 deaths due to related complications, and T2DM is second in Latin America and sixth in the world[34,38].

The global epidemic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has immediate implications for the therapy of common metabolic disorders such as T2DM, gestational diabetes, OB, metabolic syndrome (MetS), and high blood pressure (HBP). T2DM is associated with an increased risk of severe bacterial and viral respiratory tract infections, including H1N1 and influenza[39]. T2DM was also a comorbidity associated with adverse outcomes in hospitalized patients with SARS-CoV-2 in both China and Italy[40,41]. In the Italian cohort, hyperglycemic COVID-19 patients had a higher risk for mechanical ventilation, shock, and multiple organ failure requiring intensive care unit (ICU) assistance and showed higher mortality rates than normoglycemic COVID-19 patients. Hyperglycemic COVID-19 patients treated with insulin infusion had reduced inflammation and coagulation markers and a better prognosis[41]. In a series of 168 lethal cases of SARS-CoV-2 pneumonia collected from 21 hospitals between January 21 and 30, 2020 in Wuhan, China, 75% were men, with a median age of 70 years old, and T2DM was reported in 25% of cases[42].

In Mexico, the first case of COVID-19 was detected on February 27, 2020, and 64 d after this first diagnosis, the number of patients increased exponentially, reaching 19224 confirmed cases and 1859 (9.67%) deceased; currently, these figures amount to 2667769 confirmed cases and 244081 deaths[43]. An epidemiological study conducted in Mexico from February 27, 2020 to April 30, 2020 showed that most cases of COVID-19 were in Mexico City, and the average age of patients was 46 years old. Among the 12656 confirmed cases, the highest number of infected people occurred in the 30- to 59-year-old range (65.85%), with a higher incidence in men (58.18%) than in women (41.82%). Deceased patients had one or multiple comorbidities, primarily HBP or hypertension (45.53%), T2DM (39.39%), and OB (30.4%)[44]. One of the first reports from Wuhan, China indicated that most hospitalized COVID-19 patients presented underlying diseases, such as diabetes, hypertension, and cardiovascular disease (CVD). The occurrence of hypertension worsened the prognosis and was associated with a higher rate of death[40]. In another study in Mexicans conducted from February 27, 2020 to April 10, 2020, a total of 23593 patient samples were evaluated by a laboratory from the Mexican Institute of Epidemiological Diagnosis and Reference. Of these, 18443 were negative for COVID-19, and 3844 were positive for COVID-19. The results showed that patients diagnosed with COVID-19 who developed a severe condition upon admission had higher proportions of OB (17.4%), T2DM (14.5%), and HBP (18.9%) than those without a confirmed diagnosis[45]. Moreover, OB, T2DM, and HBP conditions were accompanied by an inflammatory status, and some molecular mechanisms induced by inflammation altered the microvasculature, resulting in endothelial dysfunction (ED) and lung damage. Thus, COVID-19 patients with these comorbidities have higher rates of ICU treatment[46].

The Mexican Ministry of Health reported that HBP (17.21%), T2DM (13.25%), OB (13.25%), and smoking (7.33%) were the top 4 risk factors associated with SARS-CoV-2 infection mortality[43]. Many OB cases in Mexico live in geographical areas of increased social vulnerability, which poses a fundamental inequality that might also increase mortality from COVID-19 associated with both T2DM and OB. In a study conducted in Mexico on 177133 subjects with COVID-19, the odds of SARS-CoV-2 positivity were higher in subjects affected by T2DM, HBP, OB, being more than 65 years old, and of male sex[47]. When assessing age, reduced odds of SARS-CoV-2 positivity in patients less than 40 years old were observed, but when exploring its interaction with T2DM, an increased probability of SARS-CoV-2 infection was noted [47].

Having a diagnosis of T2DM has been linked to increased susceptibility and adverse outcomes associated with bacterial, mycotic, parasitic, and viral infections, all of which are attributed to a combination of dysregulated innate immunity and defective inflammatory responses[48]. Pulmonary and systemic coronavirus infections, including SARS-CoV-2, may be complicated by secondary bacterial infection, denoting the importance of the epithelial barrier function in the lungs and gastrointestinal tract. T2DM alone or in combination with older age, HBP, and/or CVD characterized by pro-inflammatory states can contribute to SARS-CoV-2 infection and to a larger pro-inflammatory response, which would lead to a more severe and ultimately lethal form of the disease[49].

OB is also a risk factor for increased severity of SARS-CoV-2-related symptoms. An analysis of 124 consecutive ICU admissions in a single center in Lille, France, from February 27, 2020 to April 5, 2020 revealed a large frequency of OB among SARS-CoV-2 patients in comparison to non-SARS-CoV-2 controls. In this observational study, the frequency of OB was 47.5%, compared to 25.8% in a historical control group of ICU subjects with non-SARS-CoV-2 illness. In this study, the requirement for intubation and mechanical ventilation was higher in subjects with OB[50]. In another report

conducted in Shenzhen, China, with 383 patients with COVID-19, overweight (OW) was associated with 86% and OB with a 142% higher risk of developing severe pneumonia compared to patients of normal weight in a statistical model controlling for potential confounders[51]. In another study conducted in Mexico between April 1, 2020 and May 8, 2020, 167 hospitalized patients (67% male) with an average age of 54-years old were suspicious or confirmed for COVID-19; approximately 75.3% suffered from OW or OB, including 7.8% with grade III OB. An 11% mortality rate among patients with Grade I OB was observed, along with a high 33% mortality rate in underweight or Grade III OB patients[52].

Mexicans exhibiting comorbidities such as CVD, HBP, OB, and T2DM, which are also related to MetS, also show more severe disease and higher mortality related to COVID-19. An additional analytical study including 528651 cases for the period from February 25, 2020 to June 6, 2020, of which 202951 were confirmed for COVID-19, allowed the authors to conclude that the presence of one MetS factor doubles the risk of death from COVID-19, and it was higher among patients affected by HBP and T2DM[53].

With regards to SARS-CoV-2 infection and intestinal health, important enzymes such as dipeptidyl peptidase-4 (DPP-4), angiotensin-converting enzyme 2 (ACE2), and transmembrane serine protease 2 (TMPRRSS2) are substantially expressed outside the lungs in epithelial tissues, including small and large bowel enterocytes[54-56]. Acute hyperglycemia has been shown to upregulate ACE2 expression in cells, which might facilitate viral cell entry, but paradoxically, chronic hyperglycemia downregulates ACE2 expression, making the cells vulnerable to the inflammatory and damaging effects of the virus[57]. In addition, the expression of ACE2 on pancreatic β cells directly affects β cell function, suggesting that T2DM is not only a risk factor for a severe form of COVID-19 disease but also that viral infection could trigger diabetes [58]. A great proportion of insulin requirements in patients with a severe course of the infection has also been observed in different countries affected by COVID-19. Nevertheless, it is not clear whether SARS-CoV-2 has a direct role in insulin resistance. Another aspect to consider is the link between COVID-19 and T2DM involving the DPP-4 enzyme, which is commonly targeted pharmacologically in people with T2DM [59].

The gut plays an important role in metabolic homeostasis, producing metabolically active gut hormones, interacting with the microbiota, and by its potential capacity to contribute to gluconeogenesis[60]. It is crucial to have adequate gut health and microbiota to achieve the best absorption of medicinal drugs designed to lower blood glucose levels in patients with diabetes[61].

There is important evidence supporting the notion that intestinal dysbiosis due to HBP, T2DM, OB, and MetS predisposes a patient to greater clinical severity from COVID-19. However, it cannot go unnoticed that other social aspects and lifestyle customs in Mexico, including vulnerability and undernutrition, might substantially contribute to the probability of hospitalization among individuals with COVID-19 and associated comorbidities, as discussed previously[62].

THE DEATH TOLL FOR COVID-19 IN MEXICO IS NOW MORE THAN TWO HUNDRED THOUSAND CASES

During the last month of 2019, a respiratory-type infectious outbreak emerged in China, and despite the sanitary measures established in that country, the disease continued to expand around the world, becoming a critical health issue[63]. This SARS-CoV-2 outbreak has become more serious, becoming a pandemic with more than 150 million confirmed cases and more than 3 million deaths worldwide[64]. According to the Mexican government, there were more than 2.5 million reported estimated cases with 234178 confirmed deaths by April 2021[43], and an association of comorbidities such as HBP, OB, smoking, and T2DM with COVID-19 disease severity has been reported[45]. Among the most affected countries, Mexico ranks third in deaths worldwide after the United States of America, with more than 30 million estimated positive cases and more than half a million deaths, and Brazil, with more than 14 million positive cases and almost 400 thousand deaths due to COVID-19[65], Table 1).

Research in other countries of the world showed that the most common comorbidities are also HBP, T2DM, CVD, and respiratory disease[66], similar to the panorama in Mexico. In Mexico, the principal comorbidities are HBP, OB, T2DM, and smoking[43]. It notable that according to the Non-Communicable Disease Risk Factor

Table 1 Coronavirus disease 2019 reported cases and deaths

Location	Cases	*per* 100K people	Deaths	*per* 100K people
United States	32152531	9795	573044	175
Brazil	14369423	6809	391936	186
Mexico	2329534	1826	215113	169
India	17636307	1291	197894	14
United Kingdom	4409635	6598	127451	191

The figures are based on data from the Johns Hopkins University Center for Systems Science and Engineering, accessed 2021-04-27 (https://coronavirus.jhu.edu/map.html).

Collaboration (NCD RisC), the United States of America, Brazil, and Mexico rank among the top countries afflicted by some of these maladies. For HBP, there is a 19.9% prevalence in Brazil, 17.3% in Mexico, and 10.5% in the United States of America; as a reference, Nigeria has a prevalence of 35.5%[67]. Regarding T2DM, there is an 11.5% prevalence in Mexico, 8.7% in Brazil, and 6.4% in the United States of America; as a reference, there is a 19.8% prevalence in Egypt[68]. Lastly, for OB, there is 38.2% prevalence in the United States of America, 34.0% prevalence in Mexico, and 26.4% prevalence in Brazil, and as a reference, Qatar has a 44.6% prevalence[69].

UNDER PANDEMIC CONDITIONS, BREASTFEEDING PROVIDES THE BEST SEEDING OF THE GM FOR NEWBORNS

As has been discussed, the importance of the functional GM is critical to contribute to appropriate primary (innate) and secondary immune responses. In the context of the global COVID-19 pandemic, a particular concern about mother and infant health is related to the possibility of vertical transmission from infected mothers to neonates or infants. In the mother-neonate pair, transmission may occur primarily through breastfeeding or the consumption of human milk, which may carry the virus. However, although it is essential to consider the potential role of human milk in SARS-CoV-2 transmission, it is more important to consider the protective effects of targeted antibodies and other immunoprotective components present in human milk against the viral agent of COVID-19. Among the multiple benefits breastfeeding provides to neonates, human milk contains a complex community of bacteria that helps to seed the infant GM[70,71]. This event is extremely important since appropriate initial bacterial colonization is essential for adequate intestinal immune development[72,73]. Whether infective SARS-CoV-2 viruses are present in human milk, the data are still limited, and breastfeeding by women with COVID-19 remains a controversial issue. In a recent work reporting data from 30 COVID-19-positive mothers, only one human milk sample was positive for the SARS-CoV-2 *via* quantitative real-time polymerase chain reaction (RT-qPCR) test, even after repeating the analysis the next day. The authors did not find proof for the transmission of the SARS-CoV-2 virus from mother to child through breastfeeding in the Indian population[74].

Furthermore, there are 37 published studies in which the presence of SARS-CoV-2 RNA was assessed in 68 human milk samples from mothers with a positive COVID-19 diagnosis. Only 9 of the 68 samples (13.23%) had detectable levels of SARS-CoV-2 RNA[75]. However, a previous report analyzing milk from two nursing mothers infected with SARS-CoV-2 reported positive results for the presence of viral RNA in only one of the two sampled mothers. Viral RNA was detected in milk for 4 consecutive days, and its presence coincided with mild COVID-19 symptoms and a SARS-CoV-2-positive diagnostic test for the newborn. However, whether the newborn was infected by breastfeeding or by other modes of transmission remains unclear[76]. In another study performed on two participants, only 50% of human milk samples were positive for SARS-CoV-2 RNA, suggesting that the virus is shed intermittently in the milk[77]. Both works conclude that further studies on milk samples from lactating women are needed to propose recommendations on whether mothers with COVID-19 should breastfeed. In a recent review, the authors concluded that there was no evidence of SARS-CoV-2 transmission through breast milk[75]. Human milk contains antibodies, and a recent publication reports the presence of SARS-CoV-2-specific

Table 2 High abundance bacterial taxa characterizing the gut microbiota dysbiosis of selected diseases

Population	Disease	Relevant taxa	Analysis	Ref.
Mexico	ED	f_Veillonellaceae, f_ S24-7, g_*Ruminococcus*, g_*Bacteroides*, g_*Parvimonas*, g_*Oscillospira*.	MaAsLin	Nirmalkar *et al* [87], 2018
	T2DM	o_Bacteroidales, f_Koribacteraceae, g_*Suterella*, g_*Roseburia*, g_*Pelomonas*, g_*Oscillospira*.	LEfSe	Chávez-Carbajal *et al*[85], 2020
	OB	f_ S24-7, g_*Roseburia*, g_*Succinivibrio*.	LEfSe	Chávez-Carbajal *et al*[86], 2019
		g_*Lachnospira*, g_*Roseburia*, g_*Faecalibacterium*.	UPGMA	Murugesan *et al* [88], 2015
	MetS	g_*Lachnospira*, g_*Coprococus*, g_*Faecalibacterium*, g_*Ruminococcus*, g_*Megamonas*.	LEfSe	Chávez-Carbajal *et al*[86], 2019
United States	HBP	g_Dorea, s_Alistipes finegoldii, s_A. indistinctus.	LEfSe	Kim *et al*[89], 2020
	ED	g_*Bifidobacterium*, g_*Akkermansia*, g_*Oxalobacter*.	Pearson's correlation	Johnson *et al*[90], 2019
		o_Bacteroidales, f_ Prevotellaceae, g_ *Hungatella*, g_*Succiniclasticum*.	Mann Whitney U	Kummen *et al*[91], 2018
	T2DM	g_*Bifidobacterium*, g_*Prevotella*.	Mann-Whitney nonparametric test	Barengolts *et al* [92], 2018
	OB	c_Bacilli, f_Streptococcaceae, f_Lactobacillaceae, g_*Streptococcus*, g_*Blautia*.	Kruskal-Wallis	Peters *et al*[93], 2018
		f_Ruminococcacea, g_*Prevotella*, g_*Gardnerella*, g_*Turicibacter*, g_*Megasphera*.	LEfSe	Sergeev *et al*[94], 2020
	MetS	g_Ruminococcus, g_Haemophilus, g_Varibaculum, g_Veillonella, g_Sarcina, g_Lactobacillus, g_Turicibacter, g_Actinomyces, g_Bifidobacterium, g_Lachnobacterium.	Correlations	Tricò *et al*[95], 2019
		g_Clostridium, g_Ruminococcus, g_Faecalibacterium, g_Oscillospira, g_Coprococcus, g_Prevotella.	Compute core microbiome (95%)	Zupancic *et al*[96], 2012
Brazil	ED	f_Lachnospiraceae g_*Roseburia* g_*Coprococcus*	Mann–Whitney U	Silveira-Nunes *et al* [97], 2020
	T2DM	g_*Gemella* g_*Coprococcus* g_*Desulfovibrio*	Relative Abundance	Al Assal *et al*[98], 2020
	OB	g_*Fusobacterium* g_*Enterococcus* s_*Escherichia coli*	FISH	Sarmiento *et al* [99], 2019
	MetS	p_Firmicutes	RT-qPCR	Miranda *et al*[100], 2019

ED: Endothelial dysfunction; T2DM: Type 2 diabetes; OB: Obesity; MetS: Metabolic syndrome; UPGMA: Unweighted pair group method with arithmetic mean; FISH: Fluorescence *in situ* hybridization.

antibodies in human milk after a COVID-19 vaccination scheme in 84 breastfeeding Israeli mothers[78].

Regarding the human milk that is handled and distributed by human milk banks (HMBs), when this review was written, there was no basis for imposing restrictions on the consumption of human milk by neonates in need. It should be mentioned that a requirement for the use of milk from HMBs is heat treatment aimed at reducing the bacterial load, which might include potential pathogens[79]. The standard heat treatment procedure used is Holder pasteurization, which is reported to inactivate the SARS-CoV-2 virus efficiently[80].

GM DYSBIOSIS IS ASSOCIATED WITH ED, T2DM, AND OB IN MEXICANS AND OTHER POPULATIONS

During the pandemic, the ribonucleic acid of SARS-CoV-2 has been detected in different types of samples around the world, including feces[81]. There is evidence of gastrointestinal infection with the viral agent of COVID-19 under conditions in which

Table 3 Taxa associated with immunological or inflammatory diseases

Taxa	Immunological disease	Ref.
s_*Ruminococcus gnavus*	Crohn's disease	Henke *et al*[101], 2019
	Rheumatoid arthritis	Zhang *et al*[102], 2015
s_*Ruminococcus lactaris*	Inflammatory bowel disease	Forbes *et al*[103], 2016
g_*Faecalibacterium*	Multiple sclerosis	Cantarel *et al*[104], 2015
	Psoriasis	Zhang *et al*[105], 2021
	Inflammatory bowel disease	Gevers *et al*[106], 2014
f_Veillonellaceae	Multiple sclerosis	Cantarel *et al*[104], 2015
g_*Coprococus*	Anti-phospholipid syndrome	Ruff *et al*[107], 2015
g_*Roseburia intestinalis*	Increased risk of HIV infection	Libertucci and Young[108], 2019
g_*Parvimonas*	Acute Kawasaki disease	Chen *et al*[109], 2020
g_*Megamonas*	Psoriasis	Zhang *et al*[105], 2021
	Systemic lupus erythematosus	Hevia *et al*[110], 2014
g_*Bacteroides*	Arthritis susceptibility	Xu *et al*[111], 2019
f_S24-7	Reduction in antibody response	Yang *et al*[112], 2017
g_*Coprococcus*	Reduction in antibody response. Inflammatory bowel disease	Yang *et al*[112], 2017; Said *et al*[113], 2014
g_*Oscillospira*		
g_*Sutterella*		
g_*Gemella*	Asthma	Stiemsma *et al*[114], 2016
g_*Clostridium*	Colitis	Guerri *et al*[115], 2019
	Rheumatoid arthritis	Forbes *et al*[116], 2018
g_*Actinomyces*	Rheumatoid arthritis	Forbes *et al*[116], 2018
g_*Streptococcus*	Rheumatoid arthritis	Alpizar-Rodriguez[117], 2019
g_*Prevotella*	Rheumatoid arthritis. Allergic rhinitis, Asthma	Maeda and Takeda[118], 2019
		Chua *et al*[119], 2018
f_Lachnospiraceae	Rheumatoid arthritis	Forbes *et al*[116], 2018
	Asthma	Cherkasov *et al*[120], 2019
f_Veillonellaceae	Autoimmune hepatitis	Wei *et al*[121], 2020
g_*Veillonella*	Multiple sclerosis	Chen *et al*[122], 2016
g_*Blautia*	Multiple sclerosis	Chen *et al*[122], 2016
g_*Dorea*		
g_*Haemophilus*		
g_*Oscillospira*	Allergies	Hua *et al*[123], 2016
g_*Succinivibrio*		
g_*Suterella*		
o_Bacteroidales		

HIV: Human immunodeficiency virus.

more than 20% of the qPCR tests are positive in feces by the time the respiratory tract results are negative[82]. Based on this information, it is possible that an already established GM dysbiosis, such as that observed in some metabolic diseases, influences SARS-CoV-2 clinical manifestations and outcomes. The diversity of the fecal microbiota is reportedly affected during SARS-CoV-2 infection[83], and supported by additional results, an association between the GM dysbiosis seen in T2DM and OB

with the severity of COVID-19 is proposed[84].

Our group has found evidence of dysbiosis in the distal colon microbiota diversity in Mexican adults affected by T2DM, as characterized by an increased relative abundance of Bacteroidetes in relation to Firmicutes[85] and a decrease in the relative abundance of Bacteroidetes to Firmicutes in OB and MetS[86], three diseases of epidemic proportions among Mexicans. Additionally, the results by our group have also uncovered characteristic dysbiosis in ED among Mexican adolescents[87]. There are also reports of a high abundance of specific bacterial taxa depicting GM dysbiosis in epidemic diseases such as HBP, T2DM, and OB in the USA and Brazil, which, along with Mexico, are the top three countries with the highest COVID-19 mortality (Table 2).

The presence of distal GM dysbiosis, as supported by the bacterial profiles characterized in fecal samples of Mexican subjects affected by ED, T2DM, OB, and MetS, is enriched in different but common bacterial taxa. Moreover, the relative abundances of these taxa were augmented in several disorders associated with defective immune responses, allergies, and susceptibility to viral infections (Table 3).

CONCLUSION

As discussed in this review, there is a clear association between comorbidities such as type 2 diabetes, obesity, and MetS and COVID-19 severity in populations such as Mexicans, in which these diseases are a health problem. There is also a defined association of changes in the bacterial taxa of the GM associated with the same diseases. However, to complete the picture, a further characterization of these bacterial taxa should include their metabolic role in the GM function and the type of mutual interaction they maintain with the immune system of the host. This information should help to develop multidisciplinary strategies to manage the GM to improve the primary and secondary immune responses in the face of viruses such as SARS-CoV-2, the viral agent of COVID-19 disease.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7080-7099

DOI: 10.3748/wjg.v27.i41.7080

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

REVIEW

Role of cell-free network communication in alcohol-associated disorders and liver metastasis

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Author contributions: Kuracha MR and McVicker BL wrote the paper; Thomas P and Tobi M made critical revisions; all authors have read and approve the final manuscript.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

Supported by Biomedical Laboratory Research and Development, VA Office of Research and Development, No. BX004127 (to McVicker BL and Tobi M).

Country/Territory of origin: United States

Specialty type: Biochemistry and Molecular Biology

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 open-access article that was

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Abstract

The aberrant use of alcohol is a major factor in cancer progression and metastasis. Contributing mechanisms include the systemic effects of alcohol and the exchange of bioactive molecules between cancerous and non-cancerous cells along the brain-gut-liver axis. Such interplay leads to changes in molecular, cellular, and biological functions resulting in cancer progression. Recent investigations have examined the role of extracellular vesicles (EVs) in cancer mechanisms in addition to their contribution as diagnostic biomarkers. Also, EVs are emerging as novel cell-free mediators in pathophysiological scenarios including alcohol-mediated gut microbiome dysbiosis and the release of nanosized EVs into the circulatory system. Interestingly, EVs in cancer patients are enriched with oncogenes, miRNA, lipids, and glycoproteins whose delivery into the hepatic microenvironment may be enhanced by the detrimental effects of alcohol. Proof-of-concept studies indicate that alcohol-associated liver disease is impacted by the effects of exosomes, including altered immune responses, reprogramming of stromal cells, and remodeling of the extracellular matrix. Moreover, the culmination of alcohol-related changes in the liver likely contributes to enhanced hepatic metastases and poor outcomes for cancer patients. This review summarizes the numerous aspects of exosome communications between organs with emphasis on the relationship of EVs in alcohol-associated diseases and cancer metastasis. The potential impact of

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Received: May 15, 2021
Peer-review started: May 15, 2021
First decision: June 22, 2021
Revised: July 2, 2021
Accepted: September 30, 2021
Article in press: September 30, 2021
Published online: November 7, 2021

P-Reviewer: Wang Z
S-Editor: Chang KL
L-Editor: A
P-Editor: Chang KL

EV cargo and release along a multi-organ axis is highly relevant to the promotion of tumorigenic mechanisms and metastatic disease. It is hypothesized that EVs target recipient tissues to initiate the formation of prometastatic niches and cancer progression. The study of alcohol-associated mechanisms in metastatic cancers is expected to reveal a better understanding of factors involved in the growth of secondary malignancies as well as novel approaches for therapeutic interventions.

Key Words: Exosomes; Extracellular vesicles; Alcohol-associated liver disease; Colorectal cancer; Liver metastasis; Interorgan communication

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Core Tip: Alcohol consumption is an independent risk factor for cancer development as well as the promotion of metastatic disease, a major cause of morbidity and mortality in cancer patients. The identification of mechanisms and potential therapeutic targets for metastases remains to be determined for many cancers. Interorgan communication involving extracellular vesicles (EVs) is considered a vital process in the promotion of tumorigenic pathways and the spread of disease. Understanding the role of EVs in organ-organ communication networks will likely contribute to the development of future opportunities to combat cancer metastasis.

Citation: Kuracha MR, Thomas P, Tobi M, McVicker BL. Role of cell-free network communication in alcohol-associated disorders and liver metastasis. *World J Gastroenterol* 2021; 27(41): 7080-7099
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7080.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7080

INTRODUCTION

The consumption of alcohol in chronic and/or aberrant drinking patterns correlates with a substantial burden of disease worldwide. A recent study conducted by the National Survey on Drug Use and Health stated that in the United States alone, 73.1% of adults regularly use alcohol and nearly 15 million people have an alcohol use disorder[1]. Based on World Health Organization reports, alcohol use has a negative impact on health and quality of life, creating more than 5% of global disease burden and premature deaths[2,3]. The processing of alcohol in the body significantly affects multiple organs including the liver, gut, lungs, heart and brain[4-7]. A prominent alcohol-related disorder is alcohol-associated liver disease (AALD) that is initially facilitated by ethanol metabolism in the liver[8]. However, AALD is a complex disease with factors from other organs also contributing to its development and progression. Notable contributing factors include cells of the innate immune system and bacteria of the alcohol-altered gut microbiota[9,10]. Overall, the interplay between alcohol-affected organs clearly plays a role in the outcomes of AALD as well as additional adverse consequences such as alcohol-related cancer development and metastatic disease.

Alcohol is an identified carcinogenic factor in several cancers including head and neck, esophageal, liver, breast, pancreatic, and colorectal[11,12]. Recent reports indicate that alcohol consumption is the third and fourth largest contributor of all primary cancers in women and men, respectively[3]. Further, studies have shown that alcohol associates with an increased risk of secondary cancers of the upper aerodigestive tract (*i.e.* oral cavity, pharynx and esophagus) as well as metastases of colorectal cancers[13,14]. Multiple mechanisms are attributed to alcohol-induced cancer risk including toxic products and reactive oxygen species generated by ethanol metabolism. Additionally, cellular factors produced in response to injury such as protein, lipids and microRNAs can be packaged and released in extracellular vesicles (EVs)[15]. The EVs can migrate to modulate neighboring cells and/or distant tissues, acting in many cases as tumorigenic signaling molecules. Multiple cell types including endothelial cells, epithelial cells, neuronal cells, immune cells, and cancer cells can secrete nanosized EVs as part of their normal physiology, as well as during the

pathophysiology of disease[16]. Recent studies have suggested that during pathophysiological conditions exosomes have multiple roles in disease progression. Interestingly, tumor-derived exosomes have been implicated as regulatory factors in cancer progression by promoting cancer cell proliferation, migration, and the establishment of a premetastatic niche for drug-resistant cells[17,18]. Overall, EVs have the capability to contribute to the progression of AALD as well as alcohol-related advanced or secondary cancers. A better understanding of the integrated cell-cell communication between cancer cells and normal cells is critical for the development of new therapeutic options. Research into the complex interactions of diverse organs by EVs is a focus of new and clinically relevant areas of study. Here, we review studies on exosome biology and EV communication networks associated with alcohol-related disorders and metastatic cancers.

EXOSOME CHARACTERISTICS

Exosome biogenesis

Exosomes were first identified in 1981 as cell-derived, membrane-bound enzymatic vesicles[19]. Subsequently, it was demonstrated that exosomes are nano-sized (30 to 150 nm) lumen vesicles that originate from the endosomal system[20]. Further, it was elucidated that EV biogenesis is a sequential process in which multivesicular bodies (MVBs) form following membrane invagination of intraluminal vesicles[18,20]. A small fraction of MVBs fuse with the plasma membrane and are released into the extracellular milieu[21]. The regulation of MVB fusion and release can involve cholesterol content as seen in B-lymphocytes where membrane fusion and exosome release were only observed for the high cholesterol pool of MVBs[22]. Additionally, several reports have shown that exosome release depends on the cell polarity and the contribution of specific components of apical or basolateral membranes[23-25]. Overall, existing evidence indicates that different MVB populations exist inside cells and that select pools are involved in extracellular release[25] as well as the scavenging of plasma membrane proteins[26] to maintain cellular homeostasis during the EV maturation process[27,28].

Endosome pathways identified in the regulation of exosome biogenesis include endosomal sorting complex required for transport (ESCRT)-dependent and independent pathways (Figure 1). Studies have eloquently described ESCRT pathways showing the direct control of ESCRT-mediated membrane machinery[29] and ESCRT-independent regulation of EV budding and release by factors such as sphingolipid ceramide[30,31]. It was also demonstrated that vesicle formation and trafficking involve functional proteins such as Rab GTPases, heat shock proteins (HSP70 or HSP90), tetraspanins (CD9, CD63, and CD81), and integrins[18,32]. Further, the role of sphingomyelin, phosphatidylcholine, diacylglycerol, and ceramide as exosome membrane lipids was described[33]. Altogether, these studies suggest that distinct exosome biogenesis pathways, in addition to specific sorting and cargo mechanisms, dictate diverse biological functions and effects of EVs on recipient cells.

Exosome sorting and cargo delivery

A significant feature of exosomes is the morphological and size profile of the vesicles. Based on size, EVs are classified into large exosome vesicles (90-120 nm), small exosome vesicles (60-80 nm), or non-membranous nanoparticles called exomeres (35 nm)[34]. While both large and small size exosomes can respond to signaling pathways such as IL-2/STAT5, density gradient centrifugation studies revealed differences in lipid compositions between various sized EVs[34]. Moreover, subpopulations of low-density and high-density exosomes can have differential effects on gene expression profiles.

In addition to EV size, the characterization of exosome cargo is important to the understanding of EV effects in healthy and pathophysiological scenarios. Exosomes contain distinct ratios of molecular constituents such as nucleic acids, proteins, lipids, and metabolites that vary depending upon their cellular conditions, cells of origin, epigenetic changes, and metabolomic stages[35]. Moreover, studies have described various RNA species that are components of exosome cargo including microRNAs (miRNAs), rRNAs, tRNAs, or long noncoding RNAs (lncRNAs)[36]. The role of miRNAs as EV cargo is an emerging area of study, especially in oncology. In cancer cells, exosomes are highly enriched in miRNAs compared to parent cells indicating that miRNAs are sorting into the exosome cargo[37-39]. Several studies have identified exosomal miRNAs as serum biomarkers for the prediction of cancer progression and

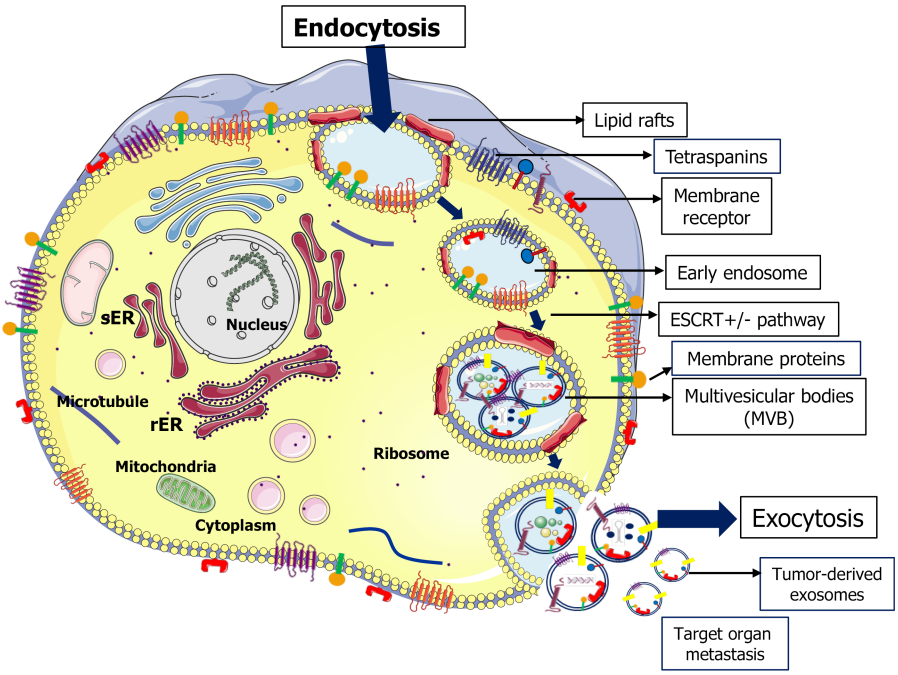


Figure 1 Extracellular vesicle biogenesis. Pathways involved in extracellular vesicle (EV) generation from the endocytosis of cargo components to release of targeting exosomes. EV biogenesis is achieved by endosomal sorting complex required for transport (ESCRT)-dependent or ESCRT-independent pathways. Several cytoplasmic and nuclear molecules can be sorted in the EVs such as ubiquitin-related proteins, heat shock proteins, miRNAs, and cytoskeleton proteins. ESCRT: Endosomal sorting complex required for transport; sER: Smooth endoplasmic reticulum; rER: Rough endoplasmic reticulum.

metastasis[40-42]. Significantly, the differential expression of exosomal miRNA was noted to have a role in the regulation of tumor progression and metastasis in various cancer models[43-45]. However, the mechanisms involved in the loading and sorting of molecules into exosome vesicles remain to be elucidated. Towards those efforts, Villarroya-Beltri *et al*[46], have identified a sequence motif that controls the miRNA loading into exosomes. In addition, Kirsten rat sarcoma (KRAS) oncogene-dependent miRNA sorting into exosomes was found to play a key role in colorectal cancer cell (CRC) since CRC cells expressing mutant KRAS have distinct miRNA profiles compared to wild-type cells[47]. In another study, it was shown that the hyper-activation of mutated KRAS inhibited the localization of the regulatory protein Argonaute 2 into exosomes[48]. The sorting of exosome mRNAs and enrichment of 3′ UTR fragments also demonstrates the importance of exosomal RNA effects in recipient cells[49,50]. Also, tumor-derived exosomes can carry double stranded DNA and genomic DNA fragments that reflect the mutational status of oncogene and tumor suppressor genes[51,52]. And finally, ubiquitination has been noted to have a role in the packaging of target proteins into exosomes[53-55].

Another important aspect of exosome cargo and sorting mechanisms is the lipid content of exosome membranes such as cholesterol, sphingomyelin, and glycosphingolipids that have specific roles in protein sorting into exosomes[33,56]. Data indicates that subdomains of the plasma membrane (lipid rafts) enriched with distinct proteins on exosome membranes mediate exosome signaling as well as molecule sorting into exosomes[57,58]. Further, mechanistic studies demonstrated the release of factors such as flotillin-1 and stomatin into the external medium *via* EVs associated with lipid microdomains[59]. Another study showed a positive regulation of sphingosine 1-phosphate (S1P) by sphingosine kinases that enabled S1P receptors to be continuously active on EVs[31]. The continuous activation of S1P has been shown to regulate CD63, CD81, and flotillin-mediated sorting into exosomes through inhibitory G protein-coupled S1P receptors located on MVBs[31]. This suggests that G protein receptor-mediated S1P signaling on MVEs is mainly involved in the ESCRT-independent exosome cargo. Collectively, these studies suggest that distinct molecular constituents such as proteins, lipids, and nucleic acids play an essential role in exosome maturation culminating in effective sorting and extracellular release of EV cargo. The molecular, cellular, and biological functions that result from the released EVs is a critical area of research, especially in the evolving era to understand the mechanisms of alcohol-associated diseases including cancer.

THE EFFECTS OF ALCOHOL ON EXOSOME COMMUNICATION

Alcohol and liver-associated EVs

Clinical manifestations of AALD include steatosis, steatohepatitis, fibrosis, and cirrhosis[8,60]. The liver is sensitized to triggers such as oxidative stress and endotoxins in early phases of AALD resulting in cellular damage and development of advanced disease. Further, consequences of ethanol metabolism lead to alterations in the function of hepatic cells as well as the recruitment of circulating cells and molecules that contribute to organ dysfunction. Previous reviews have comprehensively described the emerging role of EVs during the pathogenesis of alcohol-mediated diseases[61-63]. In brief, alcohol-mediated stresses result in elevated EV generation and release from hepatocytes as well as non-parenchymal cells. The released EVs can modulate gene expression and function of target cells contributing to the perpetuation of liver damage. Examples of the effect of EV cargo (*i.e.* miRNA, proteins, and lipids) include changes in macrophage phenotype and the activation status of hepatic stellate cells. Altogether, EVs generated in the liver are key players in alcohol-mediated liver inflammatory and profibrogenic mechanisms. In addition to EV-mediated intra-organ signaling, communication to extra-hepatic tissues can occur, as well as bidirectional exosome communication between organs such as liver, brain, gut, and lung.

Gut-Liver axis

Alcohol-induced impairments to the intestinal epithelial barrier result in increased gut permeability and release of bacterial products into the circulation[9,64]. The released products can perpetuate gut-barrier dysfunction, as well as contribute to hepatic injury, as the liver is the primary organ to receive and detoxify gut-derived factors. The translocation of intestinal products to the liver is involved in several diseases including obesity, metabolic syndrome, and non-alcoholic and alcoholic liver diseases. In the setting of alcohol, the gut-liver axis sustains bilateral communications between the intestine and the liver leading to gut-dysbiosis and progression of liver injury[65, 66]. Notably, the transfer of gut-derived toxins to the liver due to alcohol consumption is considered a pivotal event in the development and severity of AALD. Clinical data indicates that drinking patterns correlate with processes of the gut-liver axis as changes in intestinal permeability increase with the degree of alcohol consumption [64]. Next-generation sequencing data further confirmed the association between chronic alcohol consumption and altered gut microbiome functions in mice and humans[67,68]. Overall, alcohol consumption is linked to multiple changes in the gut including intestinal epithelial barrier dysfunction, alterations in gut epithelial and mucosal cells, and changes to the intestinal microbiota. As a result, bacterial products (*i.e.* endotoxin and other pathogen-associated molecular patterns) translocate to the liver and contribute to the production of proinflammatory pathways. Despite the current understanding of alcohol's effects on the gut microbiome, the role of EVs in the transfer of gut-derived products is not defined. However, emerging data indicates the EVs significantly contribute to alcohol-related liver inflammation.

The effects of alcohol on the intestinal microbiome and the translocation of injurious factors to the liver is an area of extensive research. It is well characterized that alcohol consumption results in the dysbiosis of bacterial and fungal intestinal species and the release of products including lipopolysaccharide (LPS) from the leaky gut[69,70]. In search of contributing mechanisms, studies have described alcohol-induced reductions in the expression of tight junction proteins as well as direct injury to gut epithelial cells [71,72]. The overexpression miRNA has been implicated in tight junction alterations as the knockdown of miRNA-21 prevented ethanol-induced disruption of tight junctions through the restoration of associated transmembrane proteins such as occludin and zonula occludens-1 (ZO-1)[71,73]. Additionally, the blockade of miRNA-122a was found to be protective against tight junction alterations in Caco-2 cells[74]. It is suggested that EVs generated during alcohol-induced changes to the intestinal barrier contain cargo such as miRNAs, LPS, and bacterial products that target the liver and contribute to AALD. Indeed, a recent study by Lamas-Paz *et al*[75] demonstrated that EVs derived from alcohol-affected intestinal epithelial cells contributed to hepatocellular injury. Further, it is likely that ethanol-mediated changes in intestinal barrier and microbiome composition result in the release of bacterial EVs. For example, in addition to its role as a soluble factor, LPS can also be packaged into EVs for transport from the injured gut. This is supported by a recent report indicating the presence and activity of bacterial EVs in patients with intestinal barrier dysfunction[76]. The role of bacterial EVs in alcohol consuming patients remains to be characterized along with the

therapeutic potential of targeting such EVs.

The mechanistic role of bacterial products in the progression of alcohol-associated diseases has led to the study of the gut microbiota as a therapeutic target in patients with alcohol use disorders[77]. Currently, probiotics (living bacterial cultures), prebiotics (promoters of beneficial or commensal bacteria), and antibiotics, all serve as potential therapies for alcohol-associated diseases[78]. For instance, *Lactobacillus rhamnosus* is protective against alcohol-induced liver injury in mice[79]. Further, the administration of prescribed probiotics is promising as a protective barrier against alcohol-induced gut permeability and AALD[80]. The use of prebiotics may also be beneficial as certain diets (*i.e.* oats, flaxseed) protect against alcohol-induced oxidative stress and hepatic inflammation[81,82]. Similarly, antibiotic treatment can attenuate alcohol-induced endotoxemia by preventing the overgrowth of harmful bacteria in the gut[83]. Overall, insight into the mechanistic utility of targeting exosomes generated by the alcohol-altered gut warrants investigation for the development of effective therapeutics against disease progression related to the gut-liver axis.

Liver–Brain axis

It is well described that manifestations of AALD lead to a spectrum of symptoms in the brain such as cerebral edema and hepatic encephalopathy. Of notable involvement, ammonia and other harmful substances produced by the alcohol-injured liver can reach the brain causing injury and neuroinflammation. However, mechanisms related to exosome communication networks of the liver-brain axis remain to be characterized. Reports to date indicate that the coadministration of alcohol and LPS result in altered profiles of cytokines such as TNF-α, MCP-1, IL-1β in the gut, liver, and brain[84]. Other studies demonstrate that the lack of the tumor necrosis factor receptor 1 results in the accumulation of TNF-α in mouse serum, gut, and liver; and that alcohol intake potentiates long-lasting levels of proinflammatory cytokines in the brain[85]. A recent study demonstrated that TNF-α inhibition reduced systemic inflammation and improved symptoms[86]. Additionally, chronic alcohol consumption not only influences brain inflammation but also interferes with stress-mediated psychiatric behavior through the disruption of the hypothalamic-pituitary-adrenal (HPA) axis [87]. The alcohol-mediated neutralization of the HPA axis could be a potential mechanism by which systemic inflammation continues in individuals who have an addiction to alcohol.

Besides chronic alcohol addiction, the loss of gut barrier integrity is a causative factor of endotoxin transport during sepsis and brain inflammation. Alcohol-induced gut dysbiosis is thought to not only play a role in alcohol dependency but also in the regulation of effects including neuro and endocrine signaling and immune system alterations[64,68]. However, a connective factor such as EVs in the gut-liver-brain axis has yet to be identified. Interestingly, the blood-brain barrier (BBB) serves as a defensive barrier against the extravasation of tumor cells and pathogens[88]. However, cancer cells can destruct the BBB structure to mediate migration during brain metastasis[89]. Overall, it is suggested that EVs facilitate cell network communications through the delivery of their cargo (*i.e.* proteins, mRNA, and miRNAs) to trigger the breakdown of the BBB through EV-induced changes in tight-junction proteins including ZO-1, N-cadherin, and actin.

Liver–Lung axis

Excessive alcohol use is a major factor in the enhanced risk of acute respiratory distress syndrome (ARDS)[90]. Chronic alcohol exposure in the liver-lung axis is linked to hepatopulmonary syndrome, bacterial infection, and increased mortality from ARDS [90,91]. Recently, Siore *et al*[92] reported that pulmonary edema and acute lung damage occur through the activation of inflammatory responses and oxidative stress involving liver-lung axis communications. It was shown that alcohol administration results in elevated levels of the TNF-α responsive chemokines, macrophage inflammatory protein, and keratinocyte chemoattractant. Further, the enhanced chemokine expression is associated with the recruitment of pulmonary neutrophils. Additional studies indicated that the liver-lung axis is bidirectional for the com-munication and effects involved in alcohol-enhanced hepatopulmonary injury. For instance, ventilator-induced lung injury in a mouse model resulted in significant inflammatory responses produced in cultured hepatic sinusoidal endothelial by perfusate from injured lungs [93]. In relationship to the role of the liver-lung axis in alcohol-related cancers, several studies have investigated the role of metastatic determinants[94,95]. In particular, tumor-derived exosomes may have a significant role in cancer cell metastasis that is mediated by cell adhesion molecules such as integrins, tenascin, and periostin[96-98]. In summary, the role of EVs in the interplay between pulmonary disease, AALD and

alcohol-associated cancers is a needed area of research for the identification of potential therapeutic targets.

EXOSOMES AND CANCER: ROLE OF ALCOHOL-MEDIATED EFFECTS

The interorgan communication mediated by EVs is clearly a factor of pathophysiology in various disease states. The role of alcohol-induced EV communication in the development and progression of cancer is not defined and is an area of clinical importance due to the prevalence of alcohol consumption and associated risk of cancers. Thus, investigations into the role of EVs in the initiation and severity of cancers aims to gain insight into the relationship of comorbid conditions related to the effects of alcohol consumption. Moreover, realization of the importance of EVs in cancer progression and metastasis has increased exponentially, as have their potential application in therapy and diagnosis[99]. The contribution of EVs in pathological processes is far reaching since tumor-secreted exosomes can mediate angiogenesis, modulate the immune system, and facilitate the generation of pre-metastatic niches[96, 100]. Indeed, EVs have been identified as key mediators of communication networks within and between organ systems, highlighting the clinical importance of exosome function[18,101,102]. Existing web-based online bioinformatic tools including high-throughput techniques (*i.e.* ExoCarta, EVpedia, Vesiclepedia catalog, and Ingenuity Pathway Analysis, IPA) are beneficial to the scientific community in EV research[103, 104]. These resources assist in the characterization of EV molecular and pathophysiology mechanisms through the identification of key functional elements. Based on IPA data, EV cargo delivery depends on the content of bioactive molecules such as mRNA, enzymes, proteins, DNA and lipids that can dictate the role of EVs in disease progression and diagnostic functions (Figure 2).

The clinical assessment of EVs in body fluids provides another measure towards the understanding of exosomes as diagnostic biomarkers and therapeutic targets. Biomolecule-loaded EVs from blood are stable for more than 90 days under normal storage conditions making EV analyses more useful compared to other less-stable measures of cell-free DNAs and circulating tumor cells that are used as liquid biopsies [105,106]. Examples of exosome-related identification in serum samples include prostate cancer-derived exosomes[107]; and exosome cargo containing an androgen receptor variant that is a biomarker of metastatic prostate cancer[108]. Several studies have also reported the sensitivity of EV miRNA composition as biomarkers in disease identification that can be isolated from various body fluids including blood, saliva, and urine[109,110]. A noted example is the oncogenic signature of miR-21 as a biomarker for various cancers including colorectal[111], breast[112], brain[113], and liver[114]. Concerning diseases of the liver, it has been shown that the concentration of EVs in the circulation is enhanced in the setting of AALD, nonalcoholic fatty liver disease, viral hepatitis, and hepatocellular carcinoma indicating the clinical significance of EV-mediated communication and subsequent effects[66]. Overall, clinical measures as well as bioinformatic programs are valuable in deciphering EV-mediated mechanisms and are useful tools for the characterization of alcohol-associated EVs in development and progression cancers.

Role of exosomes in cancer progression

Mechanisms of tumor development and progression are dynamic, multi-step processes that occur in response to the accumulation of genetic alterations in damaged cells. An integral component of tumor development is thought to be the communication between cancerous and non-cancerous cells that is mediated by nanosized vesicles [115]. Research to date indicates that cancer cell microvesicles actively transfer oncogenic molecules from primary cancer cells to intercellular populations. Indeed, tumor-derived exosomes can regulate cancer progression by stimulating oncogene overexpression, stromal cell remodeling, immune system modulation, and angiogenesis[115]. The transfer of tumorigenic material *via* EVs is implicated in the modulation of morphological changes and the enhancement of anchorage-independent growth capacity of cancer cells. Similarly, tumor-derived exosomes can act as survival factors that bind to and activate anti-apoptotic pathways[116].

The knowledge that exosomes are potential stimulators in cancer progression indicates that EVs can promote angiogenesis and changes in the microenvironment [117]. In this regard, tumor-derived exosomes can influence mesenchymal stem cell differentiation facilitating cancer cell proliferation and disease progression[118]. Moreover, the exosome-mediated transfer of lncRNAs as tumor-promoting material

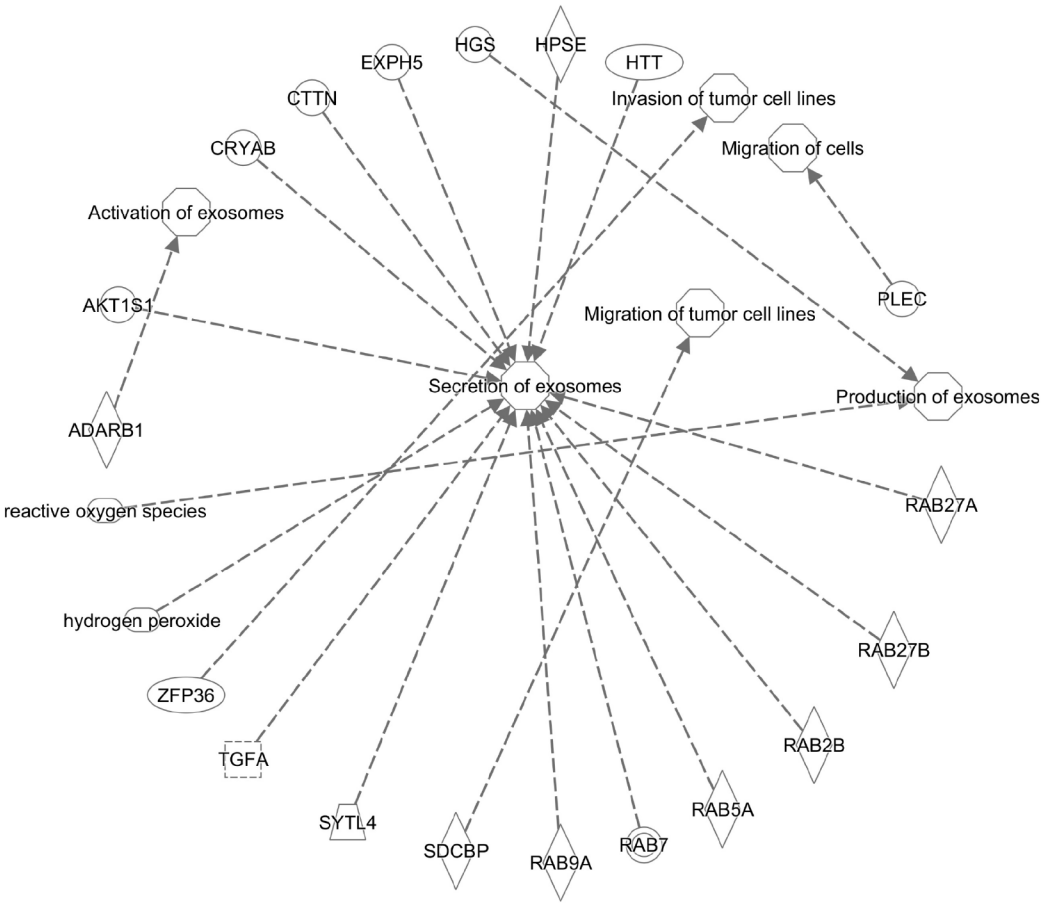


Figure 2 Exosome network analysis. Based on Ingenuity Pathway Analysis, distinct molecules from different tissues and cells can be involved in exosome secretion during cancer progression as well as in diagnostic functions. CRYAB: Chaperone alphaB-crystallin; CTTN: Cortactin; EXPH5: Exophilin 5; HGS: Hepatocyte growth factor; HPSE: Heparinase; HTT: Huntingtin protein; PLEC: Plectin; RAB27A: Ras-related protein Rab-27A; RAB27B: Ras-related protein Rab-27B; RAB2B: Ras-related protein Rab-2B; RAB5A: Ras-related protein Rab-5A; RAB7: Ras-related protein Rab-7; RAB9A: Ras-related protein Rab-9A; SDCBP: Syndecan binding protein; SYTL4: Synaptotagmin like 4; TGFA: Transforming growth factor alpha; ZFP36: Zinc finger protein 36; ADARB1: Adenosine deaminase RNA specific B1; AKT1S1: AKT1 Substrate 1.

has been shown during the transformation of non-malignant cells[119]. The role of EVs enriched with miRNAs has also been shown in cell-cell communications and conversion of cells to populations with enhanced motility[120]. Specific examples include the role of miR-17-92 and miRNA-92a as potent promoters of angiogenesis and oncogenic activity[121]. Likewise, miR-135b-5p[122], miR-30a-5p[40], miR-150-5p [123], miR-183-5p[124], miR-155[125], miR-497[126], miR-181b-5p[127], miR-375[128, 129] and the miR-200 family[110,130,131] have been shown to be effective markers of cancer progression. The clinical evaluation of EV miRNA cargo provides insightful information into processes involved in the various stages of cancer from detection to metastasis as summarized in Table 1.

Another component identified in cancer progression is the release of cancer-associated fibroblasts (CAFs) from exosomes. CAF-derived EVs can play a key role in tumor progression by enabling the transfer of oncogenic molecules such as amino acids, lipids, and TCA-cycle intermediates to confer glycolysis modulation and carboxylation in cancer cells[132]. Tumor-derived exosomes have also been shown to be involved in the stimulation of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 (ICAM-1) enhancing the process of neovascularization in endothelial cells in the microenvironment[133]. Moreover, recent studies suggest that EVs are important in mediating cellular communication between cancer cells and other cells of the microenvironment such as immune cells, neutrophils, natural killer (NK) cells, dendritic cells, T cells, and macrophages. For example, cancer-derived exosomes can alter macrophage polarization[134], induce the recruitment of neutrophils to the tumor site[135], decrease the cytotoxic activity of NK cells[136], or inhibit T-cell proliferation mechanisms[137]. Altogether, it is evident that exosomes can mediate cancer progression through a variety of pathways and cellular communications leading to cancer cell proliferation and spread to distant sites.

Table 1 Summary of exosome miRNA signatures as cancer biomarkers

Exosomal miRNA	Expression profile	Mode of action	Type of cancer	Ref.
miR-320d	Upregulated	Predicts metastasis	CRC	Tang *et al*[41]
miR-106b-3p	Upregulated	Promotes metastasis	CRC	Liu *et al*[163]
miR-6803-5p	Upregulated	Prognosis marker	CRC	Yan *et al*[42]
miR-874	Upregulated	Prognosis marker	GC	Zhang *et al*[164]
miR-30a-5p	Downregulated	Diagnostic tool	CRC	Sun *et al*[40]
miR-21	Upregulated	Diagnostic tool	CRC	Bastaminejad *et al*[111]
miR-135b-5p	Upregulated	Metastatic marker	CRC	Li *et al*[122]
miR-150-5p	Downregulated	Prognosis, marker	CRC	Zou *et al*[123]
miR-183-5p	Upregulated	Angiogenesis	CRC	Shang *et al*[124]
miR-155	Upregulated	Diagnostic tool	CRC	Lv *et al*[125]
miR-16-5p	Upregulated	Regulation of ITGA2	CRC	Xu *et al*[165]
miR-497	Downregulated	Prognosis marker	CRC	Zou *et al*[126]
miR-4461	Downregulated	Regulation of COPB2	CRC	Chen *et al*[43]
miR-146a	Upregulated	Invasion and metastasis	BC	Yang *et al*[45]
miR-125a-3p, miR-320c	Upregulated	Stage I marker	CC	Wang *et al*[166]
miR-4772-3p	Upregulated	Stage II & III marker	CC	Liu *et al*[167]
miR-21, miR-10b	Upregulated	Metastatic marker	HCC	Tian *et al*[44]
miR-1290, miR-375	Upregulated	Prognostic marker	CRPC	Huang *et al*[129]
miR-373, miR-200a, miR-200b, miR-200c	Upregulated	Tumor progression	EOC	Meng *et al*[131]
mir-181b-5p	Downregulated	Diagnostic tool	GC	Yun *et al*[127]

CRC: Colorectal cancer; GC: Gastric cancer; ITGA2: Integrin alpha 2; COPB2: COPI coat complex subunit beta 2; BC: Breast cancer; Stage I, II, III: North American Association of Central Cancer Registries Stages I, II, III and IV; CC: Colon cancer; HCC: Hepatocellular carcinoma; CRPC: Castration-resistant prostate cancer; EOC: Epithelial ovarian cancer.

Exosomes role in cancer metastasis

Metastasis is one of the most common causative factors in cancer-related death. Cancer metastasis is a multi-step process for the development of secondary cancers. In 1889, Stephen Paget described the "seed and soil" theory, in which metastasis depends on the interaction between primary cancer cells as the seed and secondary host microenvironments designated as the soil[138]. Involved mechanisms were found to include changes to the extracellular matrix architecture and associated reprograming of normal cells. Clinically significant interactions between cancer cells and the cells of secondary organ sites have been shown to involve hepatocytes, bone marrow progenitor cells, CAFs, macrophages, and neutrophils. However, regulatory mechanisms of secondary organ-specific metastasis are poorly understood. Towards that understanding, studies have indicated that tumor-derived exosomes assist in the priming of premetastatic niches by delivering prometastatic factors. In particular, the integrin expression profile of tumor derived EVs can act as functional "ZIP codes" during metastatic organotropism to direct metastatic cancer cells to target tissue/organs[96]. Proteomic and clinical data support the role of exosome-sorted integrins as vital players in the development of cancer metastasis. For instance, α6β4 and α6β1 integrins are associated with lung metastasis and αvβ5 is involved with liver metastasis[139]. Also, tumor-derived exosomes are involved in the activation of the Src kinase pathway and the upregulation of pro-inflammatory S100 genes during the establishment of premetastatic niches[140]. Thus, cell-cell communication mediated by EVs appears to be a critical element during premetastatic niche formation in cancer development (Figure 3). Review of the literature indicates the variety of cancer types and stromal cell-derived exosome molecules can initiate signals during the reprogramming of the tumor microenvironment (Table 2). Thus, exosome-mediated

Table 2 Extracellular vesicle components in cancer progression and metastasis

EV cargo	Type of molecule	Action on recipient cells/tissue	Type of cancer	Ref.
CEA	Protein	Inflammation	Colorectal	Yokoyama *et al*[162]
KRAS	Protein	Invasiveness in recipient cells	Colorectal	Beckler *et al*[152]
ITG	Protein	Metastatic organotropism	Breast	Hoshino *et al*[96]
TNC	ECM protein	Stem cell niche formation	Breast	Oskarsson *et al*[97]
MIF	Protein	Liver premetastatic niche formation	Pancreatic	Costa-Silva *et al*[168]
ZFAS1	lncRNA	Cancer growth/metastasis	Gastric	Pan *et al*[119]
Amino acids, lipids, TCA-cycle intermediates	Metabolites	Cancer growth	Prostate	Zhao *et al*[132]

CEA: Carcinoembryonic antigen; KRAS: Kirsten rat sarcoma viral oncogene; ITG: Integrins; TNC: Tenascin C; MIF: Macrophage migration inhibitory factor; ZFAS1: ZNFX1 antisense RNA1; LncRNA: Long non-coding RNA; ECM: Extracellular matrix.

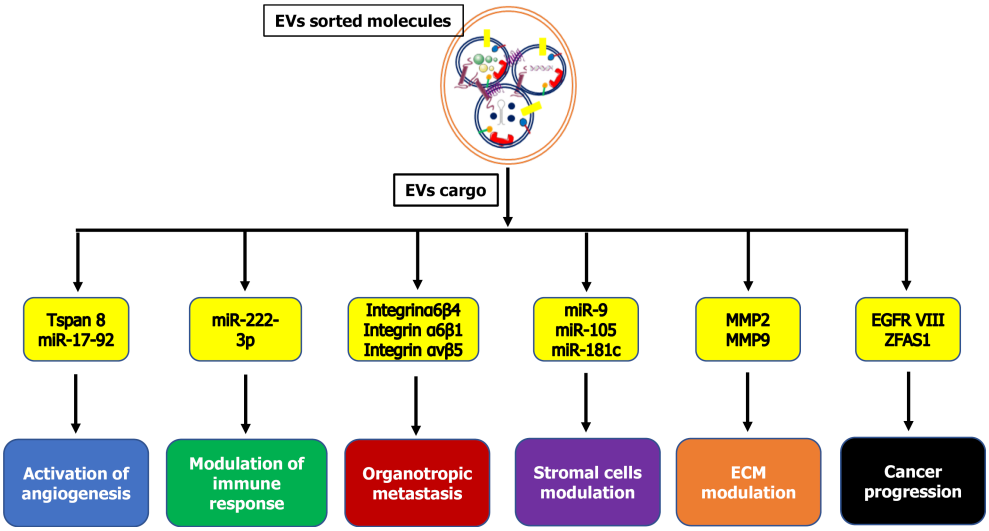


Figure 3 Exosome-mediated functions in the pre-metastatic niche. The role of tumor-derived exosomes along the establishment and progression of metastatic disease. Extracellular vesicle cargo can be involved in the initiation and regulation of cancer by promoting immune responses, angiogenesis, extracellular matrix modulation, stromal cell changes and metastatic organotropism. EVs: Extracellular vesicles; Tspan8: Tetraspanin-8; MMP2: Matrix metallopeptidase 2; MMP9: Matrix metallopeptidase 9; EGFR VIII: Epidermal growth factor receptor variant III; ZFAS1: ZNFX1 antisense RNA1; ECM: Extracellular matrix.

intracellular signaling as well as organ-organ communication can influence cancer progression and changes to host and tumor microenvironments to facilitate metastatic disease.

CLINICAL IMPLICATIONS OF EXOSOME COMMUNICATION NETWORKS

Role of EVs in alcohol and colorectal cancer disease

Recent studies indicate an alarming increased rate of morbidity and mortality from alcohol use disorders in the United States[141]. Of particular clinical significance is the disease burden related to alcohol use in colorectal cancer and associated liver metastasis. CRC is a leading cause of cancer mortality with the majority of deaths due to the development of colorectal liver metastasis (CRLM) as the liver is the foremost site of distant metastatic spread in CRC patients[142,143]. Epidemiological studies suggest that chronic alcohol consumption is one of the major causative factors of colorectal cancer mortality in both men and women[144]. Alcohol use correlates with CRLM at colorectal cancer diagnosis as well as hepatic metastases that occur over time. Further, alcohol-associated CRLM requires intensive follow-up and treatment due to poor liver function and unresectable lesions. Despite advancements in surgical interventions and chemotherapeutics, CRLM morbidity and mortality are leading healthcare concerns

emphasizing the significant need to determine contributing mechanisms. The involvement of EV signaling during CRC progression in the setting of alcohol is not known. Thus, understanding EV communication networks and the role of EVs as biomarkers can significantly contribute to the development of strategies to address the serious public health issues associated with alcohol use and cancers.

The development of CRC is a multi-step process involving the malignant transformation of normal cells of the colon. The contribution of ethanol metabolism and related metabolites in colon carcinogenesis has been investigated for some time. A variety of pathways attributed to the effects of alcohol have been identified in the promotion CRC including genetic abnormities, epigenetic dysregulation, cell signaling, and changes in the tumor microenvironment[13]. However, the role of alcohol during CRC spread to other organs is less understood. In particular, the contribution of alcohol during liver metastasis is emerging as a critical area of study given the substantial mortality associated with CRLM and need to identify targetable mechanisms. Current literature indicates that alcohol creates a hepatic microenvironment susceptible to CRC seeding and growth. Attributable mechanisms include the sensitization of resident macrophages (Kupffer cells, KCs) to endotoxin-induced signaling, the production of inflammatory factors, and the activation of fibroblastic cells that promote disease rather than wound-healing[145]. Moreover, it is likely that targetable mechanisms of CRLM involve communication networks between alcohol-affected macrophages and cancer cell-associated factors. The contribution of EVs in alcohol-associated CRLM is not defined but is clearly considered an important process to characterize.

EVs represent a new form of communication in colorectal cancer progression and liver metastasis. The fact that EVs can deliver cargo (*i.e.* RNAs, lipids, proteins) between cells and organs indicates the potential of playing a key role in metastatic disease[146,147]. CRC proliferation and migration can induce the release of EVs and other tumor-derived factors that can promote prometastatic niche formation, vascular changes, inflammation, and immunosuppression in host microenvironments. Several studies have recently described the contributions of EV cargo as prime mechanisms of CRC metastasis. For example, proteomic data revealed a distinct profile of metastatic factors, signal transduction molecules, and lipid raft-associated components in EVs obtained from metastatic CRC cells[148]. The contribution of mRNA components from CRC-derived EVs in cancer progression has also been shown for miRNAs (i.e. miR-21, miR-192 and miR-221) as well as natural antisense RNAs such as Leucine Rich Repeat Containing 24, MDM2 Proto-Oncogene, and Cyclin Dependent Kinase Inhibitor 1A [149]. Moreover, the role of genetic mutations in CRC patients are of interest. In particular, KRAS mutations are frequently associated with CRC metastasis and the regulation of exosome composition and release in CRC cells[150,151]. In addition, many oncogenic proteins (*e.g.* KRAS, Src family kinases, integrins) are highly enriched in mutant KRAS-derived exosomes indicating a role in CRC progression and metastasis[152]. Together, these observations provide novel insight into the role of EVs and the therapeutic potential of targeting the CRC-generated EVs during metastatic disease.

There is a growing body evidence suggesting that tumor-derived exosomes are crucial factors that influence differentiation in the microenvironment through particular signaling pathways[153]. For example, CRC cell-derived EVs have been shown to promote angiogenesis and tumor growth in the host microenvironment through the hyper-activation of Wnt/β-catenin signaling. As a result, hypoxic metastatic niches provide CRC cells protection from chemotherapy and attack from immune cells[154]. Another signaling pathway implicated in colon tumorigenesis is the activation of proinflammatory cellular kinases. A recent study by Talwar *et al*[155] demonstrated that phosphorylated p38γ is activated in CRC tumorigenesis. Further, it is suggested that the activation of p38γ may be associated with immunoglobulin adhesion molecules such as carcinoembryonic antigen (CEA) and biliary glycoprotein (BGP). In support, the expression of CEA and BGP have been linked to hepatic metastasis in various preclinical models and in CRC patients with ongoing efforts to define the mechanistic role of CEA during CRLM[156-158]. Key studies have shown a direct relationship between CEA and the metastatic potential of CRC cells, and that CEA stimulation results in the production of tumorigenic factors by Kupffer cells[159-161]. Current works are evaluating the role of alcohol on KC function to determine if ethanol-sensitized macrophages are more responsive to CEA leading to advanced metastatic disease. To date, studies have shown that the alcohol-injured liver provides a permissive environment for CRLM and that CEA-mediated inflammatory mechanisms may play a key role[157,162]. However, the role of tumor-derived and alcohol-associated EVs in the process of metastatic mechanisms involving MAPK signaling or

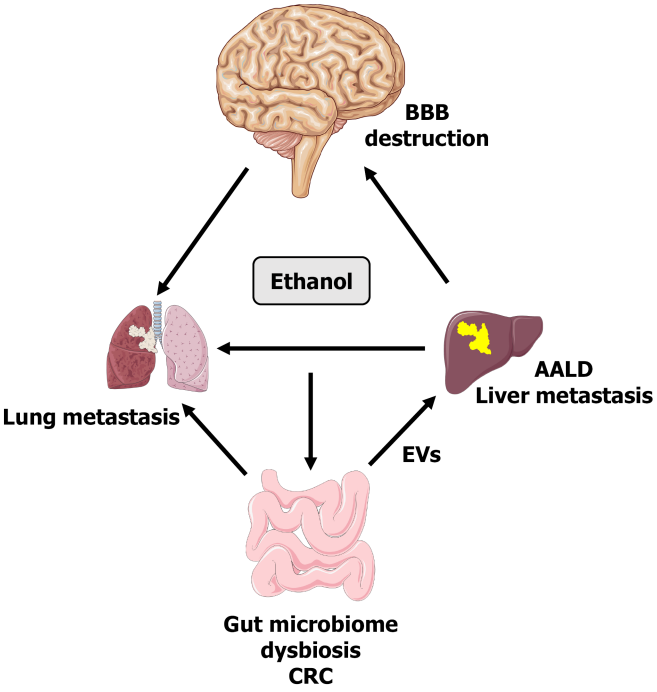


Figure 4 Model of extracellular vesicle interactions along the gut/liver/lung/brain axis during alcohol disorders and cancer progression. Alcohol exposure leads to enhanced gut microbiome dysbiosis, the development of alcohol-associated liver disease, lung inflammation, and neurological manifestations. The potential role of tumor- and alcohol-derived extracellular vesicles (EVs) in advanced malignancies is a potential consequence of EV organ-organ communication during alcohol disorders. BBB: Blood brain barrier; CRC: Colorectal cancer; AALD: Alcohol-associated liver disease.

carcinoembryonic antigen-related cell adhesion molecules is unknown. Further, the effectiveness of blocking EV-mediated communication in the alcohol-injured liver during CRLM also remains to be defined. Overall, the characterization of exosome cargo and communication networks in the transformation of CRC cells and reprogramming of the tumor microenvironment is an important area of translational research, especially in the context of complex comorbidities associated with aberrant alcohol intake.

CONCLUSION

In recent years, investigations into the role of EVs in cancer progression and AALD have increased in a remarkable manner. The elucidation of EV communication networks to date have indicated the powerful role of EVs as metastatic cancer markers and inducers of varied biological effects. Extensive work is ongoing to characterize the biogenesis and effects of distinct EV populations generated from different cell types and diseases. The unique features of EV size and cargo contents can produce hallmark effects on recipient cells. Therefore, the heterogeneity of exosome populations will dictate studies on the role and outcomes of exosome networks during disease states. For example, understanding the diversity of EVs released during gut microbiome dysbiosis, migration, and organ-organ communication aims to reveal the association of AALD and hepatic CRC metastasis. The complexity of interorgan communication and the involvement of mediators such as EVs, cytokines, and chemokines is the ongoing focus of translational research. Related to alcohol-associated diseases, it is proposed that EV-mediated communication affects multi-organ damage as well as cancer metastasis along the liver/gut/lung/brain axis (Figure 4). Future studies will likely focus on the characterization of exosomal components involved in alcohol's effects and cancer cell metastasis to secondary organs. Moreover, further investigation is needed to explore the role of exosome-mediated cell-free networks in the detection of alcohol-related tumors and microenvironment interactions for the development of targeted therapeutics.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 November 7; 27(41): 7100-7112

DOI: 10.3748/wjg.v27.i41.7100

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

MINIREVIEWS

DNA diagnostics for reliable and universal identification of *Helicobacter pylori*

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Author contributions: Sulo P designed the outline and wrote most of the manuscript; Šipková B summarized the data, prepared the figures and tables, and wrote some of the manuscript; both authors read and approved the final manuscript.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Supported by Slovak Research and Development Agency, No. PP-COVID-20-0051.

Country/Territory of origin: Slovakia

Specialty type: Gastroenterology and hepatology

Peer-review report's scientific quality classification

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

Open-Access: This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in

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Abstract

Reliable diagnostics are a major challenge for the detection and treatment of *Helicobacter pylori* (*H. pylori*) infection. Currently at the forefront are non-invasive urea breath test (UBT) and stool antigen test (SAT). Polymerase chain reaction (PCR) is not endorsed due to nonspecific primers and the threat of false-positives. The specificity of DNA amplification can be achieved by nested PCR (NPCR), which involves two rounds of PCR. If the primers are properly designed for the variable regions of the 16S rRNA gene, it is not difficult to develop an NPCR assay for the unambiguous identification of *H. pylori*. Elaborate NPCR for a 454 bp amplicon was validated on 81 clinical biopsy, stool, and saliva samples, each from the same individuals, and compared with available *H. pylori* assays, namely histology, rapid urease test, SAT, and ^{13}C-UBT. The assay was much more sensitive than simple PCR, and it was equally sensitive in biopsy samples as the ^{13}C-UBT test, which is considered the gold standard. In addition, it is sufficiently specific because sequencing of the PCR products exclusively confirmed the presence of *H. pylori*-specific DNA. However, due to the threshold and lower abundance, the sensitivity was much lower in amplifications from stool or saliva. Reliable detection in saliva also complicates the ability of *H. pylori* to survive in the oral cavity aside from and independent of the stomach. The reason for the lower sensitivity in stool is DNA degradation; therefore, a new NPCR assay was developed to obtain a shorter 148 bp 16S rRNA amplicon. The assay was validated on stool samples from 208 gastroenterological patients and compared to SAT results. Surprisingly, this NPCR revealed the presence of *H. pylori* in twice the number of samples as SAT, indicating that many patients are misdiagnosed, not treated by antibiotics, and their problems are interpreted as chronic. Thus, it is unclear how to properly diagnose *H. pylori* in practice. In the first approach, SAT or UBT is sufficient. If samples are negative, the 148 bp amplicon NPCR assay should be performed. If problems persist, patients should not be considered negative, but due to threshold *H. pylori* abundance, they should be periodically tested. The advantage of NPCR over UBT is that it can be used universally, including questionable samples taken from patients with achlorhydria, receiving proton pump inhibitors, antibiotics, bismuth compound, intestinal metaplasia, or

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Received: March 20, 2021
Peer-review started: March 20, 2021
First decision: July 3, 2021
Revised: July 11, 2021
Accepted: September 19, 2021
Article in press: September 19, 2021
Published online: November 7, 2021

P-Reviewer: Keikha M, Lee SW
S-Editor: Fan JR
L-Editor: A
P-Editor: Yu HG

gastric ulcer bleeding.

Key Words: Chronic diseases; *Helicobacter pylori*; Diagnostics; Nested polymerase chain reaction; DNA sequencing; Detection limit

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Core Tip: Polymerase chain reaction (PCR) is not endorsed for *Helicobacter pylori* (*H. pylori*) diagnostics due to nonspecific primers and the threat of false-positives. However, a nested PCR that is as specific and equally sensitive in biopsy samples as the ^{13}C-urea breath test was developed. Due to the threshold of *H. pylori* abundance and the ability to survive in the oral cavity, it is not suitable for saliva samples. Despite DNA degradation in stool samples, nested PCR for a shorter 148 bp amplicon identified twice the number of positive samples as stool antigen test, indicating that many patients are misdiagnosed, not treated by antibiotics, explaining why their problems are interpreted as chronic.

Citation: Sulo P, Šipková B. DNA diagnostics for reliable and universal identification of *Helicobacter pylori*. *World J Gastroenterol* 2021; 27(41): 7100-7112
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7100.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7100

INTRODUCTION

Chronic diseases, such as cardiovascular disease (CVD) and cancer, are the leading causes of death worldwide. In the United States alone, they account for 70% of deaths *per* year, and CVD and cancer account for over 50% of all deaths each year. Additionally, diseases of the joints, such as arthritis, Parkinson's disease, and Alzheimer´s disease, reduce the quality of life for the elderly, and the treatment consumes enormous resources (reviewed in[1,2]). In 2005, 133 million Americans had at least one chronic disease. The economic cost was estimated at $1.3 trillion (sic) *per* year[1]. Of the 12.7 million new cases of cancer in 2008, about 2 million were attributed to infectious agents, such as human papilloma virus, hepatitis B virus, hepatitis C virus, and *Helicobacter pylori* (*H. pylori*)[1].

There is clear evidence that *H. pylori* is a major cause of chronic disease in the gastrointestinal tract (GIT). This helical, gram-negative, microaerophilic bacterium is the most successful human pathogen. The route of infection is not fully understood, but it is transmitted between sexual partners and relatives due to gastroesophageal reflux, often in childhood by an oral–oral or oral–fecal route. The infection persists and remains with the host for life[3]. In most cases, the infection causes mild gastritis, which remains mostly asymptomatic. In approximately 10%–20% of infected people, *H. pylori* causes stomach and duodenal ulcers. The chronic state increases the risk of developing duodenal and gastric cancer. Thus, since 1994, the International Agency for Research on Cancer has classified *H. pylori* as a "group 1 (definite carcinogen)" alongside asbestos and benzopyrene[4-6]. Patients with stomach cancer have a poor prognosis. After lung, breast, colorectal, and prostate cancers, stomach cancer is the fifth most common malignancy in the world, and is the third-leading cause of cancer death in both sexes (723000 deaths in 2012, 8.8%)[7]. The high mortality rate is related to early metastatic expansion through the lymphatic system. Since the discovery of *H. pylori* as the causative agent, its eradication has reduced the incidence of ulcers to almost zero. Additionally, a decrease in the incidence of stomach cancer has been recorded in most European countries over the last decade. Despite this trend, the incidence remains high. In some areas of Eastern Europe, it is more than 20 *per* 100000 inhabitants, while it is approximately 7 *per* 100000 in Central and Western Europe, and approximately 2 *per* 100000 in North America[3,7-10]. Recently, *H. pylori* infection has also been associated with a number of extragastric diseases, such as idiopathic thrombocytopenic purpura, iron deficiency anemia, vitamin B12 deficiency, insulin resistance, and metabolic syndrome[4,10,11].

There are several clinical tests for *H. pylori* identification used differentially, depending on the method of medical examination and considering country-specific preferences. The Maastricht V/Florence Consensus Report recommends only the ^{13}C-urea breath test (UBT), the stool antigen test (SAT), or endoscopy for consistent identification[4,10].

The ^{13}C-UBT detects *H. pylori* indirectly by measuring the activity of bacterial urease in the stomach. The principle is based on the hydrolysis of orally administered ^{13}C- or ^{14}C-labelled urea that is hydrolyzed into ammonia and CO_2, which diffuse into the blood and are exhaled through the lungs. The increase in ^{13}C-labelled CO_2 in breath specimens (analyzed before and 30 min after the consumption of the urea) is the proof of urease activity and can be measured by mass spectrometry or by the less expensive infrared spectroscopy and laser-assisted ratio analysis. False-negative test results can occur if the patient has received a proton-pump inhibitor (PPI) two weeks before the examination or antibiotics four weeks before. Bleeding similarly affects the diagnostic reliability of the UBT, and therefore, the assay should be accomplished only when bleeding is suppressed. Corpus-predominant gastritis can also be the reason for false-negatives[11].

SAT relies on the recognition of *H. pylori* antigens in stool. Two types of SAT, the enzyme immunoassay (EIA) and immunochromatography assay (ICA), have been used for *H. pylori* detection. Either polyclonal or monoclonal antibodies are used, but monoclonal antibody-based and EIA-based tests provide more accurate and reliable data. The accuracy can be affected if the patient has taken PPIs, antibiotics, or *N*-acetylcysteine, or has bleeding ulcers. False-negative results may also occur when the *H. pylori* count is low, also due to the use of antibiotics, bismuth, and PPIs. The SAT is a fast, simple, and inexpensive test that is also useful in epidemiological studies and screening programs[11].

Both tests have good sensitivity and specificity, as well as excellent performance if a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) is used[4,10-12]. Nevertheless, extensive study has shown that 3% of cases examined by UBT and 9% of patients tested by *H. pylori*-specific SAT should be taken as false-negatives [13].

In cases where the patient's medical condition requires endoscopy, *H. pylori* infection is examined in gastric biopsies by a rapid urease test (RUT), histology, or cultivation. RUT depends on the ability of *H. pylori* to secrete the enzyme urease. In the mixture containing urea, phenol red or other pH indicators and stomach tissue samples, urea is decomposed into ammonia and carbon dioxide. The presence of ammonia increases the pH, which changes the color of the indicator. The sensitivity and specificity of this method is considered to be > 90%. Patients with achlorhydria and those treated with PPIs, antibiotics, or bismuth compounds may have false-negative results. The test is also not very reliable in patients with intestinal metaplasia or gastric ulcer bleeding[11].

In histology, the biopsy sample is usually stained with Giemsa to identify pathogens and by hematoxylin and eosin to visualize inflammatory cells. If these stains provide inconclusive images, toluidine blue, acridine orange, and Warthin-Starry silver staining can be beneficial. *H. pylori* is unevenly distributed in the mucus layer; therefore, biopsy samples used to be taken from different parts of the stomach. The sensitivity is 80%–95%, and the specificity is 99%–100%. The diagnostic accuracy of histological examination affects many factors, such as the skill and experience of the gastroenterologist performing the sampling and the pathologist observing the biopsy specimens, the staining technique adopted, the use of PPIs or antibiotics, and the bleeding of peptic ulcers[11,12].

The culture of *H. pylori* from gastric biopsy samples is performed only in specialized laboratories, as it is not a routine technique. However, it is rather useful for the detection of antibiotic susceptibility and for scientific research. Sensitivity and specificity are considered to be about 70%–80% and 100%, respectively, but in our hands, it is possible to cultivate *H. pylori* from only about 8% of positive samples[11,14].

Serology, the main approach to detect bacterial infections in blood, is a controversial topic. The ability to recognize active infections of *H. pylori* relies on age, the clinical conditions of the infection, the antigen used for antibody preparation in the ELISA kit, and the prevalence of infection. Serology has a high negative predictive value; despite its low accuracy, it is cost-effective and due to the availability and simplicity, it is commonly used in epidemiological studies[10,11].

Approaches involving DNA amplification have not been widely accepted in medical practice, due to their higher price in comparison to SAT and UBT and the associated technical demands. The other objections are doubts concerning accuracy, as

alterations in the primer binding site may produce false-negatives; on the other hand, nonspecific primers may generate false-positives[12,13,15,16]. This opinion comes from the article by Sugimoto *et al*[17], who examined 26 various PCR reactions with diverse primers, designed to number of different *H. pylori* genes including 16S rRNA. DNA from biopsy and saliva specimens was amplified and compared to the results from cultivation and histological examination[17]. They concluded that none of the amplification systems were consistent in terms of specificity or sensitivity with classical tests and all provided false-positives.

In addition, multiple cases of positive results in PCR assays were reported without confirmation by other methods[16-19]. Furthermore, there are considerable doubts about interpreting PCR from a single gene, although the reliability can be unambiguously verified by sequencing[15,16]. Despite the ability to detect a few *H. pylori* cells or DNA molecules, the PCR approach was not even accepted for proving the eradication by antibiotics, which is again associated with the danger of the identification of cell debris[16]. These misbeliefs are still held, despite the recent meta-analysis reporting that the sensitivity and specificity of stool PCR tests are similar to those of other diagnostic methods[20].

Several PCR modifications, such as real-time PCR, allow the rapid detection and quantification of target DNA[21-24]. However, the disadvantages of real-time PCR include the high equipment cost, the high levels of technical skill required, the increased chance of false-negative results due to operator error resulting from improper assay development, and improper data analysis[21,25,26].

The alternative of choice is nested PCR (NPCR), which includes two rounds of PCR reactions. The first reaction amplifies a larger DNA region that is used as a template in the second reaction, which amplifies a narrower sub-region (Figure 1)[15,27].

Due to the two sets of primers, NPCR is more specific, and DNA can be amplified from samples with a smaller number of target molecules than simple PCR[27-30]. However, this method is prone to spray contamination and false-positives[27,28]. Nevertheless, NPCR has the potential to become the gold standard in diagnostics when sampling difficulties due to the patchy distribution of *H. pylori* and the recurrent incidence of false-positives are properly addressed[15]. The potential of NPCR is reinforced by the detection of antibiotic-resistant mutations in 23S rRNA in stool samples with no reports of false-positives[31,32].

We have been involved in *H. pylori* diagnostics for a while, and during four master's degree theses and one dissertation, we found that simple PCR was much more sensitive than histology and that many samples that were considered negative were actually positive by NPCR performed in our laboratory. However, before designing this assay, 17 different NPCRs available for *H. pylori* detection were evaluated from the point of view of efficiency and selectivity. In most of them, serious limitations and mistakes were found in the design of primers. The first major drawback was the non-specificity of primers, especially at the 3´ ends, which can be proved by the BlastN comparison if the *Helicobacter* TaxId is excluded. This is typical for oligos designed to amplify ribosomal RNAs and protein genes. A lack of specificity was confirmed in two cases from PCR product sequences by the authors themselves[30,33]. The common cause is a mismatch at the 3´ ends of the primer, resulting from polymorphisms found in the *fliI*, *hpaA*, *hsp60*, *ureA*, *ureC/glmM* genes. Frequently, alterations in the melting temperature (T_m) are greater than the accepted 4 °C. Sporadically, primer oligos are very short and their T_m is consequently low. These differences should have an impact on the efficiency of the amplification and could cause a failure in the amplification of positive samples. This issue is profound for the housekeeping gene *glmM*, which was used to confirm qPCR results[34], where the detection rate was only about half of that in the 16S rRNA-positive samples[35-37]. In another two NPCR assays, specificity was assessed through an experiment[31,38] on the *Helicobacter*-free stool samples and the products targeted to the 16S rRNA gene were sequenced. Their GenBank comparison showed 97% or more identity to the unrelated bacteria *Actinomyces naeslundii*, *Bifidobacterium pseudocatenulatum*, and *Varibaculum cambriense*[38] and to *Bacteroides salanitronis* in the case of 23S rRNA amplification[31]. An identity of 97% or higher is the taxonomic criterion that allows isolates to be assigned to the same bacterial species [39]. Apparently, almost all published NPCR systems are not specific or sensitive enough to spot low-density infection in complex specimens. Only *ureA* gene amplification systems passed the BlastN *in silico* test[40,41], but the *ureA* product is not critical for the *H. pylori* persistence in the stomach[42], although it is indispensable for colonization of the GIT in mice[43]. Furthermore, the *ureA* gene PCR systems were not examined on composite samples, and the PCR products were not sequenced.

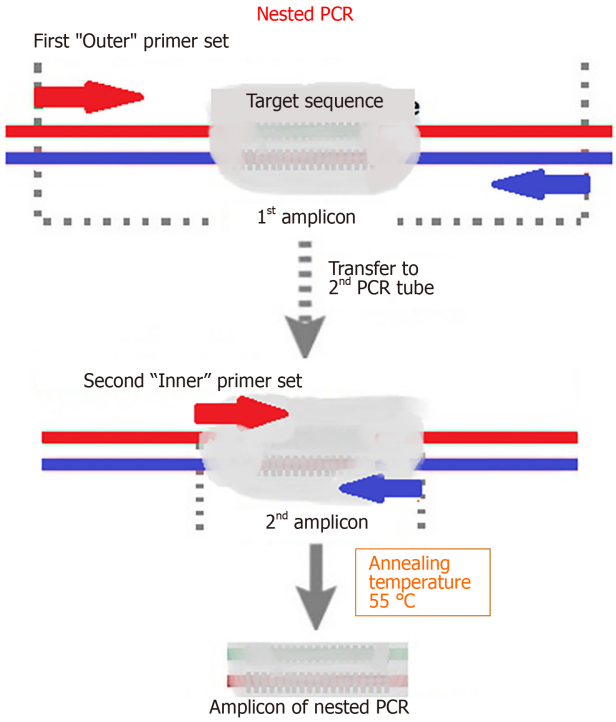


Figure 1 Nested polymerase chain reaction. Nested polymerase chain reaction involves two amplification reactions. The first round targeted a larger DNA region, and the second targeted a narrower sub-region of the products of the first round that were used as a template. PCR: Polymerase chain reaction.

In conclusion, nonspecific primers that amplify the DNA of other biological species are major pitfalls in PCR diagnostics. This current state results from inappropriate primer design, mostly from 30 years ago, and the recurrent use of outdated primers. At that time, only a limited number of sequences were stored in GenBank, so primer design was focused only on unique genes, such as urease genes.

RELIABLE DNA DIAGNOSTICS

Primer design

Many sequences are currently available for various strains, related organisms, and organisms from natural ecological niches, with many for entire genomes. In addition, most of the bioinformatic software offers the option of primer design. However, it is not difficult to design them using common sense.

The first task is gene selection. Various housekeeping genes are considered, but due to habits in taxonomy and clinical microbiology, especially with regard to the identification of new species, our attention should be focus on the 16S rRNA gene for the small ribosomal subunit. This RNA gene present in any bacteria contains conserved regions that are used in metagenomic studies for the universal amplification of bacterial DNA[39,44,45]. In addition, species- or genus-specific hypervariable sections can be found in the 16S rRNA sequence, which favors this gene for primer design[46,47]. Sufficiently selective primers can be designed in the regions discriminating *Helicobacter* from other known bacteria. To select a suitable region, the *H. pylori* 16S rDNA sequence was compared to the corresponding genes from representative stool resident (*Escherichia coli*) and representative of the closely related genera (*Campylobacter jejuni*). If these DNAs are aligned, several unique *H. pylori* regions useful for primer design can be found (Figure 2).

First pick primers were modified for a significant mismatch at the 3′ end, in order to keep the GC content below 50%. Primer length was then trimmed to maintain the T_m at around 55 °C, which can be easily calculated in many programs. These parameters are important to prevent the amplification of false priming sites and to improve efficiency. Primer specificity can be assessed simply *in silico* by BlastN comparison with other GenBank sequences. Good primers match precisely 100 times or more within the *Helicobacter* genus, but exhibit a strong divergence at the 3′ end of other bacteria. This can be done by the exclusion of *Helicobacter* TaxId from the task. According to these

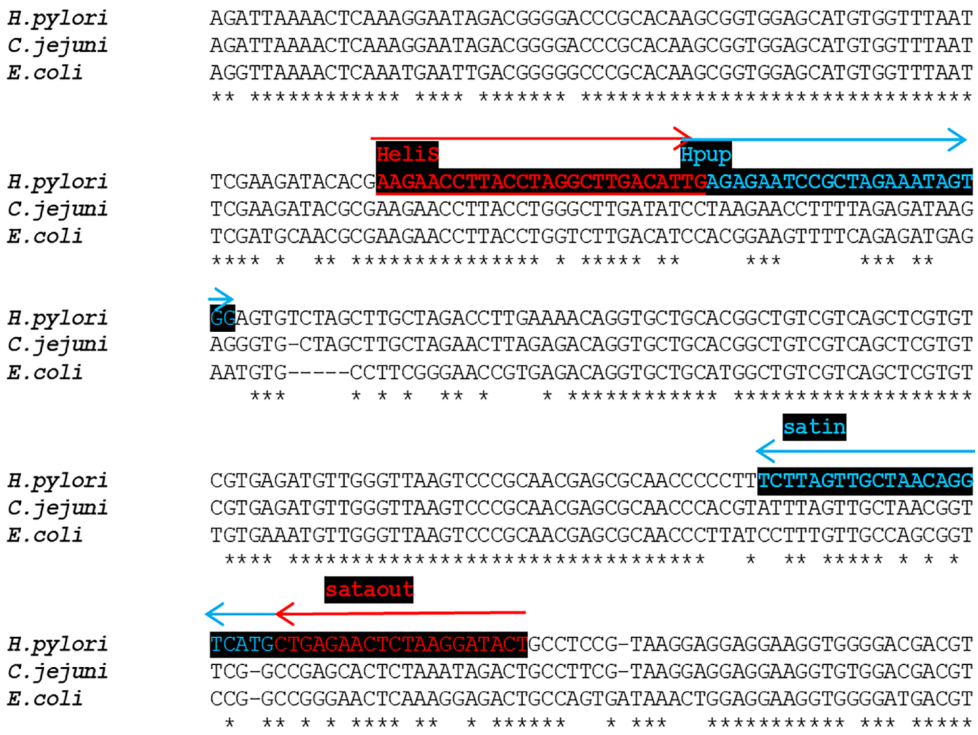


Figure 2 Design of *Helicobacter pylori*-specific primers for shorter 148-bp 16S rRNA amplicon. Alignment of *Helicobacter pylori* (amplified region) to other bacterial species. Selective primers marked in red, and blue were designed in the regions with a high divergence of *Helicobacter* sequence.

principles, two primer sets were selected: External primers for the amplification of a 497 bp region and internal primers for the amplification of a 454 bp fragment. However, the excellent performance of any PCR assay requires the optimization of amplification conditions, such as annealing temperature, the concentration of magnesium and the number of cycles (25–45)[28].

Sensitivity and the limit of detection

The Achilles heel of all identification methods is the absence of a detection limit, which should be understood as the minimal number of cells or DNA copies that can be consistently identified. This can be determined by adding ('spiking') a known number of cells directly into the PCR reaction ('colony PCR') or to the spare samples that previously tested negative. The detection limit for *H. pylori* cells was as low as 0.5 cells in a PCR vial (Figure 3)[28].

This value expresses the smallest DNA amount that can be theoretically amplified, as the *H. pylori* genome contains duplicate 16S rRNA genes[48]. Nevertheless, in reality, when samples are spiked with *H. pylori* culture, the detection threshold is roughly ten times less sensitive; it contained approximately 10 cells in a PCR vial that requires more than 1–5 × 10^3 cells *per* g or mL of biopsy, saliva, or stool specimen[28]. This is apparently the consequence of reduced DNA yield from silica columns. However, due to the unknown elution volumes, these data cannot be compared to those of other studies[17,49]. Nonetheless, the detection limit does not rely on the DNA isolation kit or the enzyme used[28].

Solo PCR is significantly less effective since approximately ten times more cells are needed in the amplification reaction for consistent identification. Another parameter that is extremely important but omitted from almost all diagnostic works is the concentration of target molecules in the analyzed samples. Their actual abundance is possible to determine from dilutions of the sample solutions. The lowest detectable density should be the same for particular NPCRs and specimens as the known threshold limits. The number of target DNA copies in the sample can be estimated by multiplying this value by the dilution factor. The density of *H. pylori* in stomach biopsies was found to be in the range 0.5–2.5 × 10^4 cells/g, while in saliva and feces it corresponded to 5 × 10^3 cells/g or 1 × 10^3 cells/mL, respectively. This was at least 5- to 25-fold lower than that in stomach mucosa[28].

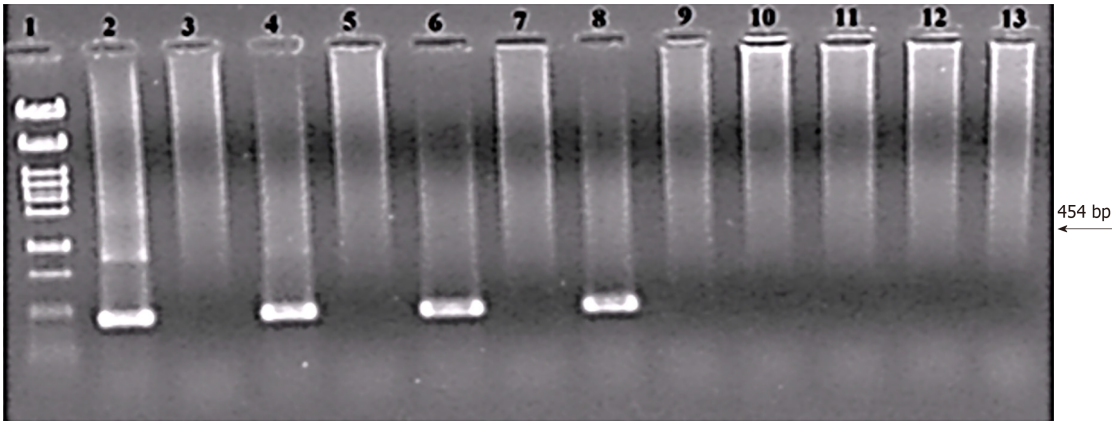


Figure 3 Threshold value of the nested polymerase chain reaction assay for *Helicobacter pylori* detection in cell suspension (colony polymerase chain reaction). Lines: 1: Size marker λ/*Pst1*; 2: 500; 3: Negative control (NC); 4: 50; 5: NC; 6: 5; 7: NC; 8: 0.5; 9: NC; 10: 0.05; 11: NC; 12: 0.005; 13: NC. Numbers express cell counts in the polymerase chain reaction (PCR) reaction. External primers HeliS/HeliN. Internal primers Hpup/Hpdown. Size of PCR product is 454 bp[28,53]. Each sample was tested by PCR separately in two independent experiments, always with the same result. Separated on 2% agarose in TBE.

Reliability of NPCR assays in different specimens

NPCR was validated in biopsy, stool, and saliva samples from the same individuals and compared to other detection methods[28]. Overall, 39.5% of patients were positive for *H. pylori* by UBT considered the gold standard, but only 21% by histopathology, 18.5% by RUT, and 27.2% by immunochromatographic SAT, while 39.5% of the biopsy samples were positive by NPCR (Table 1)[28]. Biopsy specimens were subjected to evaluation by simple PCR (second amplification reaction from NPCR), but only 29.6% were positive (Table 1).

As expected, NPCR was more sensitive than simple PCR. In addition to NPCR, samples that were positive in all other *H. pylori* tests were also positive using UBT. The *H. pylori* origin of PCR products was confirmed by DNA sequencing and their comparison revealed that they belong to at least 32 different strains. The sensitivity of histology, RUT, SAT, and simple PCR tests was lower, so these differences were interpreted as false-negatives (Tables 1 and 2)[28].

Are saliva specimens reliable?

There is an increasing demand for non-invasive diagnostics to circumvent the discomfort of the endoscopic examination required to collect samples[16,17]. The oral cavity is as suitable for a *H. pylori* reservoir as the stomach in adults[18], as are inflamed teeth (pulp) in children[18], but this is still a controversial issue[15,35,50]. It remains unclear whether *H. pylori* colonizes the oral cavity residentially, transiently, or at all[35]. Therefore, saliva and feces from 81 individuals were examined for the presence of *H. pylori*-specific DNA by simple and nested PCR. Simple PCR did not provide specific PCR products from any samples, but NPCR revealed a positive rate of about 12% in stool and 10% in saliva samples. Sequencing confirmed the correct origin, demonstrating high specificity of NPCRs, because several hundred diverse bacteria can be found in saliva[51]. The variability or identity of microbial populations can be distinguished simply by DNA polymorphism. Sequence comparisons of stool and saliva sources confirmed identical strains in the GIT and oral cavity in only three of the eight *H. pylori*-positive samples. Different strains in the stomach and saliva were found in two cases. However, in three individuals, *H. pylori* was identified exclusively in saliva/the oral cavity, but not in stool samples. Apparently, this pathogen can persist in the oral cavity, aside from and independent of the stomach, which was already reported in adolescents[52]. Nevertheless, NPCR of saliva samples appears to be a reproducible, consistent assay because the *H. pylori* 16S rRNA gene can be repeatedly amplified from any positive specimen. To find out how sampling could affect the results, we took advantage of the willingness of one SAT- and saliva-positive volunteer who was keen to provide samples throughout the day. However, besides one sample, which was only positive when the DNA concentration in the reaction was increased to maximum, all other daily saliva assays were negative. This outcome can be explained by variables but especially by the insufficient occurrence of bacteria in the samples. Therefore, saliva cannot be considered as a reliable source to confirm the presence of *H. pylori* in the stomach. This conclusion regarding the consistent detection

Table 1 *Helicobacter pylori* positivity by different diagnostic tests

Patients	^{13}C-UBT	Histology (biopsy)	RUT (biopsy)	SAT (stool)	PCR[1] (biopsy)	NPCR (biopsy)	NPCR (stool)	NPCR (saliva)
5	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	-
7	+	+	+	+	+	+	-	-
2	+	-	-	+	+	+	+	-
5	+	-	-	+	+	+	-	-
2	+	+	-	-	+	+	-	-
6	+	-	-	-	-	+	-	-
2	+	-	-	-	-	-	-	-
2	-	-	-	-	-	+	-	-
3	-	-	-	-	-	-	-	+
44	-	-	-	-	-	-	-	-
Total 81	**32**	**17**	**15**	**22**	**24**	**32**	**10**	**8**

[1]Simple polymerase chain reaction (PCR).

Hpup/HPdown primers; 37 cycles; PCR products sequenced. GenBank database comparisons confirmed the DNA sequence origin as *Helicobacter pylori*. Plus indicates a positive result, minus indicates a negative result[43]. RUT: Rapid urease test; SAT: Stool antigen; PCR: Polymerase chain reaction; NPCR: Nested PCR.

Table 2 Sensitivity and specificity of diagnostic tests

	Histology (biopsy)	RUT (biopsy)	SAT (stool)	PCR (biopsy)	NPCR (biopsy)
Sensitivity (%)	53.1	46.9	68.8	75	100
Specificity (%)	100	100	100	100	95.6
Positive predictive values (%)	100	100	100	100	93.8
Negative predictive values (%)	74.6	72.1	81.5	84.6	91.3

Sensitivity and specificity related urea breath test[43]. RUT: Rapid urease test; SAT: Stool antigen; PCR: Polymerase chain reaction; NPCR: Nested PCR.

also supports a 50-fold lower (threshold) abundance in the oral cavity in comparison to the stomach[28,53].

The detection of H. pylori DNA in stool

Stool contains several thousand different species of bacteria. Nevertheless, we only amplified *H. pylori* DNA from stool samples using NPCR. These data again demonstrate the specificity of NPCR[54]. According to the SAT test, 22 samples were positive but only 10 were positive by NPCR. When stool samples were spiked with dilutions of *H. pylori* culture, the SAT limit was ≥ 2–5 × 10^5/g, which is 100 times less than that of the NPCR assay[28]. This shows strong inconsistencies between the detection limits and detection capabilities of SAT and NPCR. This paradox could be caused by the breakdown of intact *H. pylori* cells and its DNA in the digestive system. During digestion, DNA from food components is degraded to only about 200 bp fragments[55] which are much smaller than the NPCR product (454 bp). Despite our efforts, we were unable to determine which antigen was used to produce antibody components of the immunochromatographic SAT kits. Hypothetically, the SAT test could be more sensitive if antibodies were prepared against secreted antigens such as urease, *CagA*, *VacA* or surface antigens, which are not extensively degraded in the stool. To explain the SAT/NPCR paradox, we designed a new NPCR that allows the amplification of a shorter 148 bp segment of the 16S rRNA gene. SAT and NPCR for the 148 bp amplicon showed that only about 30% of 106 volunteers and 203 gastroenterological patients were positive by SAT, but 60% by short NPCR[53,56]. The origin of

the PCR product was confirmed by DNA sequencing, indicating a sensitivity for SAT of only 50%.

A comparison of SAT and NPCR indicates that many patients are misdiagnosed. They have health problems and host *H. pylori*, but are diagnosed as negative. They have been using proton pump inhibitors for a long time and see a physician regularly, but their problems become chronic. Gastroenterologists are aware of this phenomenon and often consider alternative pathogens[57]. We and others[51] have not identified any another pathogens, even by metagenomic analysis, and the most plausible explanation is the insufficient sensitivity of *H. pylori* tests due to the threshold of abundance[53,56,58].

Pitfalls of NPCR and H. pylori diagnostics

The major drawback of NPCR is false-positives due to the spray effect, as the tubes are opened after the first PCR to add aliquots to the second amplification reaction[27,59,60]. To avoid contamination, instead of a single negative control, we included two negative controls after each sample and analyzed the samples in triplicate. Only cases with a signal in the sample and without the signal in the negative control were considered positive.

This arrangement is good for the amplification of longer fragments (400–500 bp) but not for the amplification of shorter DNA (100–200 bp). Testing for a short 148 bp amplicon is not routine. The rules of the forensic laboratory and a number of rules, especially for pipetting, must be followed, not all of which can be reported in the protocol. Apparently, this assay cannot be used in practice in medical laboratories for the routine analysis of tens or hundreds of samples. The major source of the spray effect and thus of false-positives is the opening of tubes containing DNA amplified in the first reaction. However, it is possible to simplify NPCR so that it can take place in a single tube according to the rules described in previous studies[61-63]. Moreover, the assay can be modified for real-time PCR using both SYBR Green and TaqMan detection. Preliminary data are promising, and even the SYBR Green variant was shown to be more robust and as sensitive as the 148 bp amplicon NPCR assay. This modification has the potential for use in medical practice in the future. However, there are several other emerging methods, such as CRISPR-based detection, new imaging techniques, and novel fluorescent methods in histology[10,64,65].

CONCLUSION

The diagnosis of *H. pylori* can be divided into two basic categories. The first includes culture, RUT, and UBT and relies more on the good physiological state of metabolically active bacteria than on their abundance. However, this feature cannot be neglected; although in the case of UBT, it involves the extent of stomach colonization. However, these methods have a number of limitations, as errors can occur in patients receiving PPIs, antibiotics, and bismuth compounds or those with intestinal metaplasia or gastric ulcer bleeding. The second category involves PCR, NPCR, SAT, histology, and partial RUT, and relies strictly on cell abundance and the scale of their degradation. RUT and histology likely require bacterial loads of at least 10^4[10-64], an abundance that can be reached only in some biopsy samples[28]. For other methods, cell debris is sufficient for the identification of *H. pylori*, despite the threshold of occurrence in stool.

Generally, the fundamental problems in medical research are methods, their use, and their interpretation. The stumbling block is the common effort to detect various analytes (antigens, antibodies, pathogens) at levels around the detection limit, but the results are interpreted as data from an area of high confidence. The attempt to find the nature of the problem is then replaced by statistics and the comparison of inaccurate results. Its massive and improper use is the reason why half-true data are accumulated, complicating the solution to the problem[66]. Whether or not this is the case, medicine attempts to solve the problem *via* meta-analysis of the already published results (perhaps it is the only science to do so). The problem is significant in the identification of *H. pylori*.

The most common objection about the use of PCR is concerns about false-negatives that could be caused by polymorphism and the risk of false-positive results that could occur if non-specific primers are used[12,14,16].

One source of these misbeliefs is the phenomenon known as the 'gold standard'. This is a reliable method, which is usually histological examination together with urease or breath tests in the case of *H. pylori* detection. The gold standard implies

unanimously positive samples in all tests. Furthermore, the terms sensitivity and specificity are used, given as percentages[14]. The sensitivity expresses the percentage of samples in which the presence of *H. pylori* is detected compared to the gold standard, with 100% indicating that all samples identified as positive by the gold standard are identified as positive in the new test. The second concept, specificity, expresses the ability of the new test to accurately select samples in which *H. pylori* is absent. When a new test identifies samples as positive which were negative by the gold standard, they are considered false-positives. The fact that the new test might have a better detection threshold and is more sensitive, as is the case for NPCR, is disregarded. Samples are simply considered false-positives. However, the origin of the amplified DNA can be confirmed by DNA sequencing and comparison with databases such as GenBank. If the identity is > 97%, the isolates are considered to be the same bacterial species. This criterion is generally used in taxonomy and molecular biology [39,67], but for unknown reasons, it is ignored in medicine.

Apparently, the most promising *H. pylori* DNA detection method is the one-vial modification of short-amplicon NPCR. In addition to sensitivity and specificity, another advantage is that it can be used to verify the presence of *H. pylori* in questionable samples from patients that are SAT-negative but with achlorhydria, those receiving PPIs, antibiotics, or bismuth compounds, or in those with intestinal metaplasia or gastric ulcer bleeding, although all symptoms indicate *H. pylori* infection.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7113-7124

DOI: 10.3748/wjg.v27.i41.7113

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

MINIREVIEWS

Non-alcoholic fatty liver disease in patients with intestinal, pulmonary or skin diseases: Inflammatory cross-talk that needs a multidisciplinary approach

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Author contributions: Perez-Carreras M coordinated the review and wrote the majority of the article; Casis-Herce B and Martinez-Montiel P contributed to writing of the content on inflammatory bowel disease; Fernandez I contributed to writing of the content on non-alcoholic fatty liver disease; Rivera R contributed to writing of the content on psoriasis; Villena V contributed to writing of the content on obstructive apnea syndrome; and all authors have read and approve the final manuscript.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Country/Territory of origin: Spain

Specialty type: Gastroenterology and hepatology

Peer-review report's scientific

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is currently considered the most common cause of liver disease. Its prevalence is increasing in parallel with the obesity and type 2 diabetes mellitus (DM2) epidemics in developed countries. Several recent studies have suggested that NAFLD may be the hepatic manifestation of a systemic inflammatory metabolic disease that also affects other organs, such as intestine, lungs, skin and vascular endothelium. It appears that local and systemic proinflammatory/anti-inflammatory cytokine imbalance, together with insulin resistance and changes in the intestinal microbiota, are pathogenic mechanisms shared by NAFLD and other comorbidities. NAFLD is more common in patients with extrahepatic diseases such as inflammatory bowel disease (IBD), obstructive syndrome apnea (OSA) and psoriasis than in the general population. Furthermore, there is evidence that this association has a negative impact on the severity of liver lesions. Specific risk characteristics for NAFLD have been identified in populations with IBD (*i.e.* age, obesity, DM2, previous bowel surgery, IBD evolution time, methotrexate treatment), OSA (*i.e.* obesity, DM2, OSA severity, increased transaminases) and psoriasis (*i.e.* age,

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

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Received: May 20, 2021
Peer-review started: May 20, 2021
First decision: June 12, 2021
Revised: July 4, 2021
Accepted: September 16, 2021
Article in press: September 16, 2021
Published online: November 7, 2021

P-Reviewer: Ferraioli G, Grefhorst A
S-Editor: Wang JJ
L-Editor: A
P-Editor: Yuan YY



metabolic factors, severe psoriasis, arthropathy, elevated transaminases, methotrexate treatment). These specific phenotypes might be used by gastroenterologists, pneumologists and dermatologists to create screening algorithms for NAFLD. Such algorithms should include non-invasive markers of fibrosis used in NAFLD to select subjects for referral to the hepatologist. Prospective, controlled studies in NAFLD patients with extrahepatic comorbidities are required to demonstrate a causal relationship and also that appropriate multidisciplinary management improves these patients' prognosis and survival.

Key Words: Non-alcoholic fatty liver disease; Liver fibrosis; Psoriasis; Obstructive sleep apnea; Metabolic syndrome; Inflammatory bowel disease

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Core Tip: Non-alcoholic fatty liver disease (NAFLD) is currently considered the most common cause of liver disease. Its prevalence is increasing in parallel with the obesity and type 2 diabetes mellitus epidemics in developed countries. Several recent studies have suggested that NAFLD may be the hepatic manifestation of a systemic inflammatory metabolic disease that also affects other organs. This article reviews the currently available literature on issues relating to the co-existence of NAFLD and inflammatory bowel disease, obstructive syndrome apnea or psoriasis, with particular focus on the prevalence, risk factors and impact on clinical multidisciplinary management.

Citation: Perez-Carreras M, Casis-Herce B, Rivera R, Fernandez I, Martinez-Montiel P, Villena V. Non-alcoholic fatty liver disease in patients with intestinal, pulmonary or skin diseases: Inflammatory cross-talk that needs a multidisciplinary approach. *World J Gastroenterol* 2021; 27(41): 7113-7124
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7113.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7113

INTRODUCTION

Non-alcoholic liver disease (NAFLD) has become the leading cause of liver disease in western countries and has attracted researchers' and clinicians' interest in recent years. It affects 20%-30% of the adult population in developed countries and its prevalence rises to 95% among subjects with morbid obesity and 70% among those with type 2 diabetes mellitus (DM2)[1,2]. Close to two-thirds of NAFLD patients have some metabolic factor and one-third have metabolic syndrome (MetS). Although NAFLD has been proposed as the hepatic manifestation of MetS, in actual fact the relationship between these two conditions is complex and reciprocal, mediated by insulin resistance. NAFLD is known to be an independent risk factor for the development of DM2/prediabetes and cardiovascular mortality[2-4].

NAFLD is a dynamic disease that includes an evolving spectrum of anatomoclinical lesions, including hepatic steatosis (fat deposition in hepatocytes, usually in the form of macrovacuoles), steatohepatitis (NASH; which adds cell death and inflammatory infiltrates) and cirrhosis. Although most patients have a mild form of the disease, up to 20%-30% may have NASH lesions and one-third may have progressive fibrosis[1,5].

Excluding alcohol consumption has long been the key criterion to diagnosis of NAFLD[6,7]. However, its close association with metabolic factors has recently led some experts to propose replacing the term NAFLD with "MAFLD" (abbreviating for "metabolic dysfunction-associated fatty liver disease"), which would include patients with metabolic conditions, even if they consume alcohol and/or have other causes of fatty liver disease[8,9]. Likewise, "extra-hepatic manifestations" is probably not a suitable designation for the morbidities that coexist with NAFLD in metabolically unhealthy subjects, including inflammatory bowel disease (IBD), obstructive syndrome apnea (OSA) and psoriasis[7,10,11] (Figure 1).

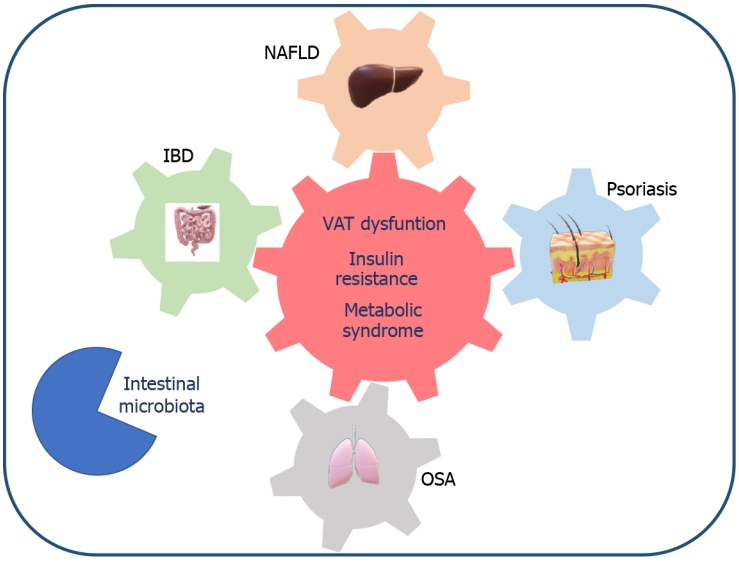


Figure 1 Potential pathogenic factors contributing to the co-existence of non-alcoholic fatty liver disease and extrahepatic comorbidities (*i.e.* inflammatory bowel disease, obstructive syndrome apnea and psoriasis). IBD: Inflammatory bowel disease; NAFLD: Non-alcoholic fatty liver disease; OSA: Obstructive syndrome apnea; VAT: Visceral adipose tissue.

The mechanisms that link NAFLD with these extrahepatic comorbidities are complex and multifactorial. It appears that the imbalance in favor of proinflammatory *vs* anti-inflammatory cytokines produced by the visceral adipose tissue (VAT) of patients with metabolic dysfunction not only plays a role in the onset of an insulin resistance state, hepatic lipotoxicity and NAFLD but also interacts synergistically with the local production of these same inflammatory mediators in the intestine or skin, as occurs in IBD and psoriasis, or is enhanced by the hypoxemia present in OSA[12-15]. Moreover, there is increasing evidence that changes in the intestinal microbiota and their metabolic interaction with the host also play a role in the multi-hit pathogenesis of NAFLD and related extra-hepatic diseases[12,16].

In this review, our aim was to update the available evidence on the link between NAFLD and three increasingly prevalent diseases: IBD, OSA and psoriasis. Ultimately, we aim to draw attention to the impact that NAFLD may have on the management and prognosis of patients with these extra-hepatic comorbidities in clinical practice.

NAFLD AND IBD

IBD is a complex and multifactorial gastrointestinal disease that usually presents in the form of acute outbreaks on a chronic immune-mediated inflammatory substrate. Although its main symptoms relate to the intestine, in both types of IBD — Crohn's disease and ulcerative colitis — extraintestinal symptoms can appear in organs such as the skin, joints, eyes and liver[15]. Hepatobiliary manifestations have been reported in 3%-50% of patients with IBD and elevated transaminases in up to one-third of them, often being transient and attributable to the immunomodulatory drugs used in IBD. NAFLD is now considered the leading cause of liver disease among IBD patients[17,18].

Since the link between ulcers in the intestine and fatty hepatomegaly was first described in 1873[19], multiple cases, series and observational cohort studies have been published that analyze the prevalence of NAFLD in patients with IBD and the factors that may link the two diseases, both metabolic (*e.g.*, DM2, obesity, arterial hypertension, dyslipemia, MetS) and relating to the IBD itself (*e.g.*, type, duration, inflammatory activity, extension, drugs, intestinal surgery)[20-30].

Zou *et al*[31] conducted a systematic review and meta-analysis covering 19 observational studies published up to 2018, involving a total of 5620 patients with IBD. Although NAFLD prevalence varied significantly (8%-40%), in the most recent studies they found an increase in the frequency of this liver disease among IBD patients, to the point that it now exceeds the level in the general population (33% *vs* 25%, respectively). Furthermore, the authors identify age, metabolic factors, methotrexate use, previous bowel surgery, and chronic kidney disease as risk factors for NAFLD. More

recent publications confirm most of these data and attribute them to the changed metabolic profile of IBD patients in recent years. Thanks to new biologic drugs, these patients have gone from being malnourished to being obesity/overweight in up to a third of cases[27,32,33].

The liver-intestine interaction in IBD and NAFLD patients could be explained by mechanisms such as the synergism between inflammatory mediators produced by hypertrophic adipocytes in the VAT and the increase of proinflammatory cytokines from the intestine[12,15]. It could also be explained by the involvement of intestinal microbiota, including changes in its diversity, interaction of metabolites produced by intestinal microorganisms with the host's lipogenesis and host hydrocarbon metabolism, or changes to intestinal permeability. All these mechanisms could favor the development of insulin resistance, MetS and NAFLD[34]. Animal experiments with drug delivery system (DDS)-induced colitis support this interrelationship between intestinal barrier disruption, endotoxemia, metabolic dysfunction in adipose tissue and NAFLD[35].

It has recently been recognized that patients with IBD, mainly those with Crohn's disease, have a specific type of mesenteric adipose tissue, located in the areas of inflamed bowel, called "creeping fat". This is an immunologically active tissue that behaves similarly to VAT, promoting inflammation of the intestinal mucosa and perhaps playing a role in the metabolic changes involved in the onset of NAFLD[36,37].

There are some practical issues of particular interest to IBD gastroenterologists, such as: (1) Identifying IBD patients with NAFLD risk; (2) The impact of a NAFLD diagnosis on the treatment and prognosis of patients with IBD; and (3) When to refer the patient with IBD to a hepatologist.

NAFLD should generally be suspected in older IBD patients with metabolic conditions, previous bowel surgery, or long-standing bowel disease[31,36]. However, comparing NAFLD patients with and without IBD, it appears that patients with NAFLD and IBD are younger and have less metabolic factors than those NAFLD patients without bowel disease. This is why some authors have proposed defining two phenotypes of patients with IBD and NAFLD: "classic or metabolic" and "IBD-specific". The first group included subjects > 45-years-old with elevated transaminases, obesity, DM2 or arterial hypertension, and a later onset of bowel disease, while the second group included younger individuals with normal transaminases and less metabolic factors[23,25] (Figure 2).

We do not have prospective studies analyzing whether there are differences in natural history and prognosis between these two phenotypes. Sartini *et al*[25] have associated NAFLD severity, measured by the degree of ultrasound steatosis, with fewer metabolic conditions and a "severe" IBD phenotype (*i.e.* more than one annual inflammatory bowel flare, more extensive IBD and previous intestinal surgery). These data suggest that in addition to metabolic conditions, other IBD-specific factors are likely to be involved in the onset and progression of NAFLD.

Based on animals and *in vitro* experimental studies, it has been speculated that tumor necrosis factor-alpha inhibitors may protect against developing NAFLD and also that glucocorticoids and immunomodulators increase the risk of liver disease progression[38,39]. However, few clinical studies have adequately collected the time and dose of treatments and thus conclusive information is not available at present. A synergistic effect of NAFLD and methotrexate treatment has been suggested, favoring liver toxicity and progression of NAFLD to more severe forms, especially in patients with obesity or DM2[40,41]. Lapumnuaypol *et al*[27] found a very high prevalence of NAFLD (54%) in their series of 80 patients with IBD treated only with biologics (*i.e.* infliximab, adalimumab, certolizumab or goligumab), most of whom were male and obese. In the multivariate analysis, they found an association between NAFLD and the clinical activity of IBD but not with drugs.

Considering that liver fibrosis is the main prognostic marker in NAFLD patients, it is important for gastroenterologists to use non-invasive markers of fibrosis in patients with IBD and NAFLD. These markers consist of mathematical algorithms that include clinical and analytical variables whose result enables the identification and stratification of patients with liver fibrosis. Among them, FIB-4 and NFS (non-alcoholic fatty liver disease score) have been validated in patients with NAFLD[4,6,7]. Retrospective and longitudinal studies that include the calculation of both serological markers of fibrosis found that only 2.2% of subjects with IBD and NAFLD have advanced fibrosis, and that it remains stable during a 3-5 year follow-up in most patients. Age, metabolic factors and duration of IBD appear to increase the risk of fibrosis progression[26,42,43].

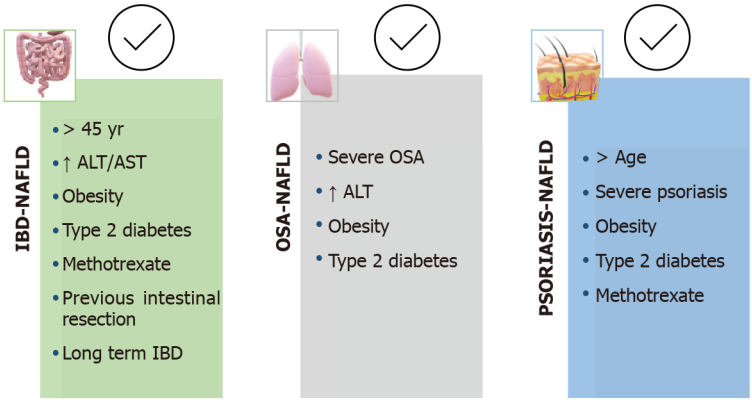


Figure 2 Non-alcoholic fatty liver disease and extrahepatic comorbidities (*i.e.* inflammatory bowel disease, obstructive syndrome apnea and psoriasis) risk phenotypes. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; IBD: Inflammatory bowel disease; NAFLD: Non-alcoholic fatty liver disease; OSA: Obstructive syndrome apnea.

Palumbo *et al*[42] were the authors of the only prospective study designed as part of a screening program for NAFLD and fibrosis involving 384 patients with IBD using control attenuation parameter (CAP) and hepatic elastography available on the FibroScan device probe (Echosens, France). They found any grade NAFLD in 32.8% of patients (CAP ≥ 248 dB/m), severe NAFLD (CAP > 300 dB/m), significant fibrosis in 24.6% (> 7 kPa) and advanced fibrosis in 18% (> 8.7 kPa). These NAFLD prevalence data should be taken with caution as there is evidence that higher CAP cutoffs than those used by these authors improve the diagnostic accuracy of this method[44,45]. The authors compared the presence of significant and advanced fibrosis in patients with NAFLD *vs* non-NAFLD and found a higher prevalence in the NAFLD group (24.6% *vs* 6.2% and 18.3% *vs* 3.1%, respectively; $P < 0.001$). Age, obesity, plasma triglycerides and methotrexate use were factors associated with liver fibrosis. In this way, they stratified the patients and referred a third of them to the hepatologist. In addition, they highlighted the presence of chronic kidney disease and cardiovascular disease among patients with IBD and NAFLD, in many cases related to subclinical atherosclerosis, information which supports the multidisciplinary approach to at-risk patients.

Although we now have scientific evidence to establish the suspicion of NAFLD in patients with IBD, prospective, longitudinal and control group studies are needed to identify those phenotypes at risk of developing fibrosis and advanced liver disease before we can establish an appropriate algorithm for screening, diagnosis and follow-up of patients with IBD and NAFLD.

NAFLD AND OSA

OSA is a clinical condition usually considered a respiratory disease, which has begun to be recognized as a multisystemic disease in the last two decades. It consists of recurrent nocturnal episodes of complete (apnea) and incomplete (hypopnea) obstruction of the upper airway, leading to hypoxemia and reoxygenation phenomena. It affects 1%-4% of the population, mostly males, with obesity and/or MetS, usually as a consequence of increased fat deposition in the upper airways and surrounding soft tissues[46,47]. Although, from a clinical perspective, OSA patients fundamentally consult due to sleep fragmentation and daytime sleepiness, the most important consequence is the chronic intermittent ischemia that occurs in different organs, such as the liver[46-48].

Over the last decade, at least 20 clinical studies have been published linking NAFLD and OSA, including systematic reviews and meta-analyses[49]. Although most include short series of patients and histological information is usually obtained from liver biopsies during morbid obesity surgery, OSA is considered to be an independent risk factor for the development and progression of NAFLD. In a recent meta-analysis and systematic review, Jin *et al*[50] found that patients with severe OSA (number of apnea-hypopnea episodes > 30/h) have higher aspartate aminotransferase values and a greater degree of steatosis, inflammation, ballooning degeneration and fibrosis. Trzepizur *et al*[51] found in their cohort of almost 1300 patients that those with severe

OSA had a 2.5-fold increased risk of liver fibrosis; although, this association was not independent of other factors when logistic regression analysis was performed. When determining the epidemiological and pathogenic relationship between NAFLD and OSA, it is difficult to avoid the impact of obesity and other metabolic conditions that are so frequent in both diseases. Evidence from experimental models shows that chronic intermittent ischemia and increased sympathetic tone triggered by nocturnal hypoxemia phenomena are the main causes of cardiometabolic manifestations linked to OSA (*i.e.* DM2, dyslipemia, arterial hypertension, atherosclerosis) and of the onset and progression of NAFLD lesions[13,46].

The decrease in oxygen tension that occurs during nocturnal apnea-hypopnea episodes primarily affects hepatocytes in zone 3 of the hepatic lobule, where NAFLD lesions predominate, and results in the release of ischemia-induced factors (commonly known as HIF)[52,53]. These HIF favor the expression of genes involved in lipogenesis, with the consequent excess of triglycerides in the hepatocytes (steatosis), free fatty acids and hepatokines. This state of liver lipotoxicity leads to inflammation, mitochondrial dysfunction, oxidative stress, lipoperoxidation, cell damage (steatohepatitis) and fibrosis. In addition to this direct hepatic mechanism, VAT ischemia and sympathetic nervous system stimulation secondary to chronic intermittent ischemia promote a state of insulin resistance, lipolysis and hepatic fatty acid overload, which contribute to the onset of DM2, atherogenic dyslipemia, arterial hypertension and NAFLD[46,51,54]. Finally, different authors have proposed that nocturnal hypoxemia may alter the integrity of the intestinal barrier and favor the hit attributed to dysbiosis and bacterial translocation in the pathogenesis of NAFLD[55,56].

At present, we do not have prospective studies that allow us to determine the prevalence of NAFLD in patients with OSA or *vice versa*. However, considering that chronic intermittent ischemia can promote and aggravate liver damage, some authors propose screening for OSA in patients with NAFLD[47,49]. Taking into account the available information, hepatologists should always ask NAFLD patients about OSA-related symptoms and consider referring to the pneumologist those with elevated transaminases and/or advanced liver lesions, and/or associated metabolic factors, especially DM2 and obesity (Figure 2).

The use of a continuous positive airway pressure (CPAP) device and lifestyle changes to diet and exercise, especially in obesity/overweight patients, comprise the standard treatment of OSA patients. CPAP increases pharyngeal intraluminal pneumatic pressure, prevents hypoxemic events related to airway collapse, improves quality of life and decreases cardiovascular mortality[57,58]. However, its impact on metabolic factors and NAFLD lesions is controversial. The available evidence in this regard is of low quality because it is based on observational uncontrolled studies in patients with OSA-NAFLD and had a follow-up of only 3 mo of CPAP treatment[59]. Prospective, controlled and randomized studies that assess patient adherence to CPAP for more than 12 mo, could establish whether this measure can improve or prevent the progression of NAFLD.

NAFLD AND PSORIASIS

Psoriasis is one of the diseases that dermatologists are most concerned about because of its prevalence (it affects 2%-3% of the adult population in developed countries), the gaps in our knowledge of its pathogenesis, and its relationship with extracutaneous pathologies[14]. Currently, psoriasis is considered a systemic immune-mediated chronic inflammatory disease, since psoriasis patients not only present skin lesions but also frequently have comorbidities that can condition their prognosis and treatment, such as MetS and its hepatic manifestation, NAFLD[60-63].

There is epidemiological evidence and pathogenic hypotheses linking psoriasis to NAFLD[64,65]. In the last 10 years, multiple controlled cross-sectional observational studies have been published, some in large populations, studying the prevalence of NAFLD in subjects with psoriasis and the specific characteristics of the subpopulation of patients with psoriasis and NAFLD[66-69]. Van der Voort *et al*[66] analyzed these data in a Dutch cohort of 2292 individuals > 55 years of age and found that the prevalence of NAFLD among the 118 psoriasis patients was significantly higher than among the 2174 healthy controls (46.2% *vs* 33.3%; $P < 0.005$). Furthermore, after adjusting for confounders, including MetS, they determined that elderly patients with psoriasis are 70% more likely to have NAFLD than those without psoriasis. Gisondi *et al*[67] found that 44% of the 124 psoriasis patients included in their study had NAFLD *vs* 26% of the 79 healthy controls ($P < 0.001$). Comparing patients with psoriasis and

NAFLD *vs* no-NAFLD, they found that those included in the first group were more frequently male and had a higher body mass index, transaminase values (Alanine aminotransferase and aspartate aminotransferase), and psoriasis severity [measured according to Psoriasis Area Severity Index (PASI) score. It combines the assessment of the severity of the lesion and affected area into a single figure between the values of 0-no disease to 72-maximum disease].

In 2015, Candia *et al*[68] published the first meta-analysis and systematic review of seven case-control studies evaluating the psoriasis-NAFLD association in populations from different continents (n = 267761). Their combined analysis indicated that psoriasis patients have twice the risk of developing NAFLD [odds ratio (OR): 2.15] and identified male sex (OR: 2.28), obesity (OR: 12.25), DM2 (OR: 2.63), arterial hypertension (OR: 2.7), MetS (OR: 9.03) and arthritis (OR: 2.25) as predisposing factors. They also found that patients with moderate to severe psoriasis had a 2-fold increased risk of NAFLD. These data have recently been confirmed in an updated systematic review and adjusted meta-analysis (n = 3019308 subjects)[69].

Approximately one-third of psoriasis patients have joint involvement and this has been linked to increased inflammatory burden, severity of skin lesions and risk of NAFLD[14,70]. However, not all authors found an association between arthropathy and liver disease[71]. The systematic review and meta-analysis by Candia *et al*[68] found that patients with psoriatic arthritis had double the risk of NAFLD when compared to those without arthropathy (OR: 2.25, 95%CI: 1.4-3.7; P < 0.05). Although this information would suggest considering patients with psoriasis and joint involvement as a special risk group for NAFLD, a recent meta-analysis raises doubts on the increased risk for NAFLD of such an association[69].

Although most studies found an independent link between psoriasis and NAFLD, those with metabolic factors appear to be particularly at risk[61,62]. The high prevalence of obesity (20%-40%), DM2 (12%) or MetS (20%-50%) among psoriasis patients, and the close relationship between these conditions and insulin resistance and NAFLD, means the same patient having both diseases cannot be seen as totally independent[72-74]. This clinical link has a common pathogenic substrate, the protagonist of which is the imbalance between overproduction of proinflammatory cytokines [*e.g.*, tumor necrosis factor-alpha, interleukin (IL)-1, IL-6, IL-17] and the decrease of anti-inflammatory ones, mainly adiponectin. These mediators act locally by promoting cell growth and differentiation of the epidermis and dermis, the proliferation of keratinocytes and the consequent appearance of psoriatic plaques[63,64]. However, they may also act systemically and contribute to insulin resistance and other inflammatory mediators implicated in the metabolic dysfunction involved in NAFLD [63,65]. Interestingly, it is speculated that modifications in the skin microbiota present in psoriasis and their relationship with gut microbes may play a role in the psoriasis-NAFLD association[75]. These pathogenic hypotheses may explain why patients with psoriasis and NAFLD have more advanced forms of liver disease and severe skin lesions.

Most studies have assessed NAFLD using ultrasound, while few have involved liver biopsy. Roberts *et al*[76] found NAFLD in half of psoriasis patients (n = 103) and NASH histological lesions in 22% (liver biopsy in 52/103), one-third of them with significant advanced fibrosis. Furthermore, they linked NASH lesions with higher PASI scores, obesity and higher transaminase levels. More authors have analyzed the severity of liver lesions in psoriasis using non-invasive markers of fibrosis, serological or technological (FibroScan®)[67,77,78]. Using these scores it was found that 7%-8% of psoriasis patients have advanced liver fibrosis. This risk is multiplied by 4 when NAFLD is associated with this skin disease. Although fibrosis is more frequent in older subjects with metabolic factors, psoriasis appears to be an independent risk factor for fibrosis when adjusting in the multivariate logistic regression analysis, including metabolic conditions and drugs used to treat psoriasis[78].

From a practical perspective, the psoriasis-NAFLD association not only has an impact on the severity of skin and liver lesions, but may also influence dermatologists' decisions when selecting systemic treatment for psoriasis[62,79].

Although some studies associate methotrexate liver toxicity with cumulative dose and treatment time, most find that the risk of liver damage is primarily related to the presence of risk conditions such as obesity, DM2, alcohol consumption, and NAFLD [80,81]. Cyclosporine and acitretin, a retinoid derived from vitamin A, are considered to be diabetogenic and to promote atherogenic dyslipemia and arterial hypertension [79,82].

It has been speculated that new biologic drugs could be beneficial in patients with psoriasis and NAFLD by acting on the proinflammatory cytokines involved in both diseases[63]. Although controlled studies with some tumor necrosis factor-alpha

inhibitors have demonstrated their ability to decrease insulin resistance and the risk of DM2 (etanercept), others appear to favor weight gain and dyslipemia (infliximab, adalimumab)[79]. Recent research studies in animals have shown that IL-17 is involved in the progression of hepatic steatosis to NASH. There is speculation that the use of anti-IL17 monoclonal antibodies (secukinumab) could be beneficial in patients with psoriasis and NAFLD[83,84].

Considering that being overweight or obesity significantly increases psoriasis risk and severity, recent clinical trials have shown that weight loss, both with a hypocaloric diet and physical exercise or with bariatric surgery, improves psoriasis activity and also favors the response to systemic treatment. These interventions could be of particular importance in patients with psoriasis and risk of NAFLD[61,74].

Aware of the impact of NAFLD on psoriasis patients, experts from the European and American academies of dermatology have recently published recommendations for the management of psoriasis comorbidities, including MetS and NAFLD[82,85]. According to the available evidence, they consider NAFLD screening to be indicated in patients with a risk phenotype (*i.e.* moderate-severe psoriasis and metabolic factors) by means of transaminase measures and ultrasound, and propose a monitoring and follow-up algorithm that includes evaluation by the hepatologist in patients with suspected liver disease (Figure 2). In addition, they recommend that a diagnosis of NAFLD be taken into account when selecting psoriasis treatment. However, these recommendations do not appear to be implemented universally in dermatologists' clinical practice, nor has a specific hepatologist referral protocol for patients with psoriasis and NAFLD been established in most hospitals.

CONCLUSION

NAFLD should be considered more than a liver disease and be taken into account not only by hepatologists but also by clinicians caring for patients with other related diseases, such as IBD, OSA and psoriasis. The scientific evidence shows that these comorbidities share an inflammatory background that synergistically impacts the severity and management of these patients. It is essential that screening and referral algorithms for NAFLD subjects are developed from a multidisciplinary perspective in which not only liver, intestinal, respiratory or skin lesions are analyzed but also the risk of morbidity and mortality from metabolic and cardiovascular causes.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7125-7133

DOI: 10.3748/wjg.v27.i41.7125

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

MINIREVIEWS

Current update on molecular cytogenetics, diagnosis and management of gastrointestinal stromal tumors

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Author contributions: Wang MX, Devine C and Segaran N contributed to the manuscript; all authors contributed to the design of the study; Ganeshan D designed the structure of the overall manuscript, made critical revisions related to important intellectual content of the manuscript; and all authors approved the final version of the manuscript.

Conflict-of-interest statement: The authors declare no conflict of interests for this article.

Country/Territory of origin: United States

Specialty type: Gastroenterology and hepatology

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 open-access article that was selected by an in-house editor and

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Abstract

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal (GI) tract and are thought to arise from precursors of the interstitial cells of Cajal. GISTs can arise anywhere in the GI tract, but most commonly originate from the stomach and small intestine. The majority of GISTs occur as a result of activating mutations in two receptor protein tyrosine kinases: KIT and/or platelet-derived growth factor receptor-α. Mutational analyses allow for predicting patient prognosis and treatment response. Clinical presentations can vary from no symptoms, typical in the case of small incidentally found tumors, to GI bleeding, abdominal discomfort, and ulcer-related symptoms when the tumor is enlarged. Imaging plays a critical role in the diagnosis and management of these tumors with multiphasic computed tomography serving as the imaging modality of choice. Magnetic resonance imaging and positron emission tomography-computed tomography can serve as imaging adjuncts in lesion characterization, especially with liver metastases, and subsequent staging and assessment for treatment response or recurrence. Surgical resection is the preferred management for small GISTs, while tyrosine kinase inhibitors − imatinib mesylate and sunitinib malate − serve as crucial molecular-targeted therapies for locally advanced and metastatic GISTs. This review article highlights the clinical presentation, pathology and molecular cytogenetics, imaging features, and current management of GISTs.

Key Words: Gastrointestinal stromal tumors; Cytogenetics; Diagnostic imaging; Computed tomography; Magnetic resonance imaging; Imatinib mesylate

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Received: June 7, 2021
Peer-review started: June 7, 2021
First decision: June 20, 2021
Revised: July 28, 2021
Accepted: September 15, 2021
Article in press: September 15, 2021
Published online: November 7, 2021

P-Reviewer: Kurokawa R
S-Editor: Wang JJ
L-Editor: A
P-Editor: Xing YX

Core Tip: Gastrointestinal stromal tumors (GISTs) often occur as a result of activating mutations in two receptor protein tyrosine kinases: KIT and/or platelet-derived growth factor receptor-α, allowing for effective molecular targeted therapies for these patients. Mutational analyses help predict patient prognosis and treatment response. Imaging plays a critical role in the diagnosis and management of GISTs. Multiphasic computed tomography serves as the imaging modality of choice in their diagnosis and follow-up. It is crucial to understand and identify the key imaging features of GISTs and their expected appearance with treatment response and disease recurrence.

Citation: Wang MX, Devine C, Segaran N, Ganeshan D. Current update on molecular cytogenetics, diagnosis and management of gastrointestinal stromal tumors. *World J Gastroenterol* 2021; 27(41): 7125-7133
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7125.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7125

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal (GI) tract and are thought to arise from the precursors of the interstitial cells of Cajal. GISTs can arise anywhere in the GI tract, most commonly from the stomach and small intestine[1,2]. The majority of GISTs occur as a result of activating mutations in two receptor protein tyrosine kinases: KIT and/or platelet-derived growth factor receptor-α (PDGFRA)[1]. Clinical presentations can vary from no symptoms, typical in the case of small incidentally found tumors, to GI bleeding, abdominal discomfort, and ulcer-related symptoms when the tumor is enlarged. Imaging plays a critical role in the diagnosis and management of these tumors with multiphasic computed tomography (CT) serving as the imaging modality of choice. Magnetic resonance imaging (MRI) and positron emission tomography-computed tomography (PET-CT) can serve as imaging adjuncts in lesion characterization, especially with liver metastases, and subsequent staging and assessment for treatment response or recurrence. Surgical resection is the preferred management for small GISTs, while tyrosine kinase inhibitors − imatinib mesylate and sunitinib malate − serve as crucial targeted therapies for locally advanced and metastatic GISTs[1].

This review article highlights the clinical presentation, pathology and molecular cytogenetics, imaging features, and current management of GISTs.

EPIDEMIOLOGY

The annual incidence of GISTs is estimated to be at least 3000 per year in the United States[1]. GISTs are often diagnosed in older adults ages 50-70 years with a median age at diagnosis ranging from 59 to 66 years[3-5]. GISTs can occur in all geographic and ethnic groups, and men and women are equally affected[6]. There are no known risk factors for developing GIST. A small subset of patients may present with the non-inherited Carney's triad, which is comprised of GIST, often with loss of function of succinate dehydrogenase (SDH), paragangliomas, and pulmonary chondromas[1]. While most GISTs occur sporadically, rare hereditary GISTs have been reported[5]. Familial GISTs are related to inherited germline mutations in either KIT or PDGFRA and also manifest with cutaneous hyperpigmentation, irritable bowel syndrome, dysphagia, and diverticular disease[1]. In Caryney-Stratakis syndrome, patients present with GIST and paragangliomas related to loss of function mutations within SDH genes. Small intestinal GISTs can also be associated with neurofibromatosis type 1, an autosomal dominant disorder in which patients more often present with café au lait spots, gliomas, and neurofibromas[1,5]. A very small group of GISTs (1%-2%) occur in the pediatric population.

CLINICAL FEATURES

Clinical presentations vary depending on the size and location of tumors. GISTs can arise anywhere along the GI tract. They most often arise from the stomach (60%), followed by the jejunum and ileum (30%), duodenum (5%), colorectum (4%), and esophagus or appendix (< 1%)[2]. Rarely, GISTs can develop outside the GI tract in the mesentery, omentum, or retroperitoneum. The majority of GISTs are benign (70%-80%). Patients are often asymptomatic, especially when the tumor size is small (less than 2 cm)[1]. When the tumor is enlarged, symptoms may include abdominal pain, bleeding, abdominal distension, early satiety, fatigue, and palpable mass[7]. Unfortunately, these nonspecific symptoms may result in delayed diagnosis and management of the disease. Infrequently, patients with advanced GISTs may present with severe hypoglycemia and hypothyroidism[8,9]. Laboratory work-up may reveal anemia, which may be related to bleeding or intratumoral hemorrhage. Metastases are uncommon (10%-20% of cases); however, when they do occur, they can occur *via* local or hematogenous spread. The most common metastatic sites include the liver, omentum, and peritoneal cavity[1,2]. Lymph node and extra-abdominal metastases are extremely rare[5]. In severe cases, patients may present with acute abdomen, melena, or hematemesis secondary to frank hemorrhage due to tumor invasion or rupture. Such emergent clinical presentation is more often seen in small intestine GISTs compared to gastric GISTs[5].

Pediatric GISTs occur in approximately 1%-2% of cases and are predominantly seen in girls presenting with multiple nodules in the stomach. These patients typically present with anemia, weakness, and syncope due to GI bleeding[1]. In addition to liver and peritoneal metastases, lymph node metastases uniquely occur in this group of patients.

PATHOLOGY AND MOLECULAR CYTOGENETICS

In gross pathology, GISTs can widely vary in size, ranging from 1-2 cm to more than 20 cm in diameter. The median size at presentation is approximately 5 cm[1]. They are well-circumscribed gray-white to red-brown masses in the bowel wall that can be submucosal, intramural, or subserosal in location[10]. They are generally unencapsulated but may have pseudocapsules. GISTs typically arise from the muscularis propria and exhibit an exophytic growth pattern. Intraluminal or mixed growth patterns may also be seen. There are three main histologic subtypes: (1) Spindle cell (60%-70%); (2) Epithelioid (30%-40%); and (3) Combination of both spindle cell and epithelioid in various proportions[10]. On microscopy, spindle cell subtypes demonstrate highly cellular, fascicular, whorled, storiform, or palisading architecture, while epithelioid tumors appear more fascicular or nested[10]. The mitotic rates can vary widely from virtually absent to high. Other findings may include areas of hemorrhage or necrosis.

The majority of GISTs (80%-90%) occur as a result of activating mutations in two receptor protein tyrosine kinases: KIT and/or PDGFRA. In 1998, Hirota *et al*[11] published the revolutionary finding that the majority of GISTs (94%) expressed activating mutations in KIT (CD117), now a key diagnostic immunohistochemical marker for GIST that distinguishes it from leiomyomas, leiomyosarcomas, or other mesenchymal tumors. KIT belongs to the type II transmembrane receptor tyrosine kinase family that includes PDGFRA and PDGFRB. The c-kit proto-oncogene encodes KIT; in combination with the stem cell factor extracellular ligand, this c-kit product normally plays an essential role in cellular survival, proliferation, and differentiation [10]. Activating mutations in KIT result in altered cell growth. In addition, Hirota *et al* [11] demonstrated that the interstitial cells of Cajal, which are the pacemaker cells involved in regulating the peristalsis located primarily in the muscularis propria, were the only cells that were double-positive for KIT and CD34 in the GI wall. Therefore, GISTs, which share morphological, structural, and immunohistochemical features with interstitial cells of Cajal, are thought to arise from them or their stem cell precursors [10-12]. Germline or sporadic gain of function mutations in c-kit result in both benign and malignant GIST tumorigenesis[10]. Aside from KIT mutation, PDFGFRA mutation can be an alternative cytogenetic change that can also result in similar downstream effects of tumor progression[13]. Hence, imatinib mesylate, a selective adenosine triphosphate-competitive inhibitor of KIT, PDGFRA and PDGFRB, serves as a groundbreaking therapy for GISTs[14]. Furthermore, DOG1, a calcium-dependent, receptor-activated chloride channel protein, has been found to be expressed in GISTs regardless

of mutation type; this marker can aid in the diagnosis of KIT-negative tumors, such as those with PDGFRA mutations that are KIT-negative[15].

Other markers for GISTs include CD34 antigen (70%), smooth muscle actin (30%-40%), desmin (< 5%), and S100 protein (approximately 5%)[16]. The expression of these markers varies depending on the location of the tumor. CD34 is often found in esophageal, gastric, and rectal tumors, while smooth muscle actin is seen in small intestine tumors. Prognostic predictors vary considerably in the literature. It has been suggested that mitotic activity and tumor size are potential prognostic predictors: A mitotic index of at least 5 per 50 high power fields (HPF) and a size greater than 5 cm are suggestive of malignant behavior, while a mitotic index of 5 or less per 50 HPF and a size less than 2 cm are suggestive of benign GIST[1,6,17]. Ki-67 can also be used to predict malignant potential[18]. Tumor location is another prognostic factor; intestinal GISTs demonstrate worse outcomes compared to gastric GISTs with regard to tumor size and mitotic rates[19]. GISTs that carry KIT exon 11 point mutations and insertions have a favorable prognosis, while those with KIT exon 9 mutations or KIT exon 11 deletions have a worse prognosis[19,20]. A small number of patients with GISTs may harbor concomitant *BRAF* gene mutations, which may portend poorer prognosis due to their primary resistance to imatinib mesylate therapy[21]; in such cases, patients may benefit from selective *BRAF* inhibitors. Further genotyping is advised for patients with KIT-negative GIST for management planning.

In the pediatric population, GISTs typically do not have KIT or PDGFRA mutations, and generally demonstrate the epithelioid subtype and express CD117[22]. Compared to adults, these pediatric GISTs uniquely overexpress fibroblast growth factor 4 (FGF4), brain and acute leukemia, cytoplasmic, insulin-like growth factor I receptor, NEL-like 1, cytokine receptor-like factor 1, pleomorphic adenoma gene 1, and FGF3)[23]. With KIT activation, these GISTs are similar to adult GISTs that carry KIT mutations. Although there is limited literature on the clinical benefits of tyrosine kinase inhibitors, sunitinib malate is suspected to be superior to imatinib mesylate in these pediatric cases[23].

IMAGING FEATURES

Fluoroscopic examination

Fluoroscopic examination is not routinely used for identifying GISTs. However, patients who undergo double-contrast barium studies may demonstrate a submucosal or well-circumscribed mass with smooth mucosal surface and obtuse angles at the margins[24]. With necrosis or ulceration, they may demonstrate irregular contours. Evaluation of extraluminal structures is limited with this approach. Further evaluation of the lesion and presence of metastatic disease with cross-sectional imaging is ultimately required.

Ultrasonography

Ultrasonography is not routinely used for imaging GISTs, especially since the tumor origin cannot be well-identified. When small, GIST may be homogeneously hypoechoic. When large, GIST may present as a heterogenous mass, which may reflect internal necrosis or hemorrhage; these findings suggest high malignant potential[25]. Hepatic metastases can be identified, although their sonographic appearance is nonspecific.

CT

CT serves as the imaging modality of choice in the diagnosis and follow-up of GISTs. Multiphasic protocol with noncontrast, arterial, and portal venous phases should be obtained. The noncontrast images help identify hemorrhage and provide a baseline for evaluating tumor enhancement. Adequate gastric distension is essential to help distinguish intramural mass; therefore, negative oral contrast agents can aid in the visualization of the enhancing mucosa[26]. The imaging appearance of GISTs depends on their size and aggressiveness. Classic CT features of GISTs include large, hypervascular, enhancing masses that may demonstrate heterogeneity due to hemorrhage, cystic degeneration, or necrosis[27]. These tumors typically displace adjacent structures and vessels, although they may exhibit direct invasion of adjacent structures resulting in ulceration and fistulization in the GI tract in advanced stages. When small, GISTs appear homogeneous and may be incidentally found on CT or endoscopy. Metastases are present in approximately 50% of patients, and metastases often involve the liver and mesentery; they demonstrate similar imaging features as primary GISTs

[24,27]. Lymph node metastases are extremely rare[27]. Features of high-grade GISTs include liver metastasis, GI wall infiltration, irregular surface, ill-defined margins, inhomogeneous enhancement, and peritoneal spread[28].

Regardless of tumor size, a change from a heterogeneously hyperattenuating mass to a homogeneously hypoattenuating mass with decreased enhancing tumor nodules and intratumoral vessels suggest response to imatinib mesylate[24,27]. The attenuation of treated lesions reaches approximately 20-25 Hounsfield units, which is close to simple density[29]. Although the tumors may enlarge during treatment as a result of intratumoral hemorrhage or myxoid degeneration, this does not suggest disease progression in the setting of decreased tumor enhancement[27]. The Response Evaluation Criteria in Solid Tumors has been found to be insensitive in evaluating treatment response as it does not account for tumor density, intratumoral vessels, or tumor metabolism[30]. Therefore, Choi *et al*[31] proposed a modified CT response evaluation criteria to account for such features on CT as tumor response to tyrosine kinase inhibitor therapy cannot be determined based on size alone (Table 1). Disease recurrence is signified by the development of enhancing tumor nodules within the treated hypoattenuating tumor[27]. A summary of key imaging features is highlighted in Table 2.

MRI

MRI serves as an imaging adjunct, especially for young patients for whom repeated ionizing radiation exposure should be minimized, for evaluating liver metastases, and for evaluating rectal tumors. MRI has been found to be superior in characterizing treated liver metastases compared to CT, especially with identifying foci of hypervascularity[32]. Conversely, MRI is less helpful in identifying mesenteric lesions due to the lack of oral contrast and respiratory gating[32]. Generally, the recommended MRI sequences include T1-weighted in and out of phase axial, T2-weighted coronal turbo spin echo, T2-weighted fat-saturated axial respiratory triggered turbo spin echo, and T1-weighted fat-saturated 3D volumetric acquisition in noncontrast, early arterial, portal venous, and hepatic venous phases[32].

MRI features of GISTs vary depending on the amount of hemorrhage, necrosis and cystic degeneration. Solid tumor components demonstrate low T1 signal, intermediate-to-high T2 signal, and enhancement with contrast[24]. The presence of intratumoral cystic change with low apparent diffusion coefficient (ADC) values are predictors of high malignant potential[33]. A negative correlation between mean ADC values and malignancy risk of GISTs has been demonstrated[33]. Upon treatment response, GIST metastases demonstrate increased T2 signal with increased cystic degeneration of solid tumoral components and increased ADC values[34]. With disease recurrence, new peripheral thickening and enhancement of cystic metastases can be seen[24].

PET-CT

18F-fluorodeoxyglucose (18F-FDG) PET-CT can aid in staging, detecting early response to treatment, and detecting early recurrence of GIST[34]. PET-CT can be helpful in distinguishing tumors from benign tissue given the expected increased glucose metabolism of viable tumor cells. PET-CT is more sensitive than CT in detecting treatment response due to detecting decreased 18F-FDG uptake, which is typically observed before a change in tumor size[35]. Such changes can be detected 24 h to 1 mo after therapy initiation[36]. For patients on imatinib mesylate, increased 18F-FDG uptake may signify treatment resistance or lack of medication compliance.

MANAGEMENT AND SURVEILLANCE

Surgical resection is the mainstay of treatment, especially for small-to-medium sized GISTs without metastasis. Obtaining preoperative biopsy is controversial due to the risk of tumoral hemorrhage and seeding; therefore, postoperative pathology is required for diagnosis[1]. During resection, the tumor should be handled carefully to avoid bleeding, rupture, and peritoneal seeding. Ideally, the tumor resection should include an intact pseudocapsule and negative microscopic margins. Follow-up imaging intervals depend on the GIST's risk group categorization: A very low-risk GIST is likely cured by surgery and does not require follow-up; a low-risk GIST may need annual CT or MRI follow-up for 5 years; an intermediate-risk GIST needs annual CT or MRI follow-up for 5 years with the first scan completed 6-8 mo after surgery; and a high-risk GIST should be followed every 6 mo for the first 5 years, then annually for the next 5 years[35].

Table 1 Modified computed tomography response evaluation criteria for gastrointestinal stromal tumors

Response	Definition
Complete response	Disappearance of all lesions; No new lesions
Partial response	A decrease in size[1] of ≥ 10% or decrease in tumor density (HU) ≥ 15% on CT; No new lesions; No obvious progression of nonmeasurable disease
Stable disease	Dose not meet criteria for complete response, partial response, or progressive disease; No symptomatic deterioration attributed to tumor progression
Progressive disease	An increase in tumor size of ≥ 10% and does not meet criteria of partial response by tumor density (HU) on CT; New lesions; New intratumoral nodules or increase in size of existing intratumoral nodules

[1]The sum of longest diameters of target lesions as defined in RECIST. CT: Computed tomography.

Table 2 Imaging features of gastrointestinal stromal tumors

	CT	MRI
Primary and metastatic GISTs	Small: Homogeneous mass; Large: Hypervascular, enhancing masses with heterogeneity due to hemorrhage, cystic degeneration, or necrosis	Depend on the amount of hemorrhage, necrosis and cystic degeneration; solid tumor components with low T1 signal, intermediate-to-high T2 signal, and enhancement; low mean ADC values may predict high malignancy potential
Treatment response	Homogeneously hypoattenuating mass with decreased enhancing tumor nodules and intratumoral vessels	Increased T2 signal, increased cystic degeneration of solid tumoral components, increased ADC values
Disease recurrence	Development of enhancing tumor nodules within the treated hypoattenuating tumor	New peripheral thickening and enhancement of cystic tumor

CT: Computed tomography; MRI: Magnetic resonance imaging; GISTs: Gastrointestinal stromal tumors; ADC: Apparent diffusion coefficient.

Prior to 2000, cytotoxic chemotherapy had not been found to be clinically effective in the management of GISTs[1]. However, following the Food and Drug Administration approval of imatinib mesylate for treating metastatic and locally advanced KIT-positive GISTs in 2002, the management of GISTs has rapidly expanded. As previously stated, imatinib mesylate is a potent tyrosine kinase inhibitor that acts on enzymes including KIT, leukemia-specific BCR-ABL chimera, and PDGFRA. Imatinib mesylate can be utilized preoperatively to downsize the tumor and/or as adjuvant therapy to prevent recurrence. Preoperative imatinib mesylate can be utilized for large and poorly positioned GISTs that may be marginally resectable; imatinib mesylate has been shown to induce tumor cell apoptosis and decrease tumor glucose metabolism on PET-CT[37]. Postoperatively, 1-year of adjuvant imatinib mesylate has been shown to prolong overall survival, although the optimal duration of postoperative treatment is unclear[38]. For unresectable or metastatic GISTs, a phase II trial of imatinib mesylate therapy demonstrated 68% objective response rate regardless of imatinib dosage, and the median time to at least partial response was 2.7 mo[39]. The median survival of patients with metastatic GISTs improved significantly from 19 mo as reported by DeMatteo *et al*[40] in the pre-imatinib era to 73 mo with imatinib mesylate as reported by Menge *et al*[5]. If there is imaging evidence of disease progression despite using standard-dose imatinib mesylate, dose escalation of imatinib mesylate or utilization of sunitinib malate, a second line tyrosine kinase inhibitor, may be considered[1]. Sunitinib malate acts as a less specific tyrosine kinase inhibitor on KIT, PDGFR, vascular endothelial growth factor receptors, Fms-related tyrosine kinase 3, colony-stimulating factor-1R, and RET; as a result, sunitinib malate demonstrates activity against angiogenesis in addition to tumor activity related to receptor tyrosine kinase inhibition[1]. For imatinib and sunitinib-resistant GISTs, investigational therapeutic options include second generation tyrosine kinase inhibitors, such as sorafenib, dasatinib, and nilotinib[1]. Follow-up CT should be obtained within 3 mo of initiating imatinib mesylate with surveillance scans completed every 3 to 6 mo for unresectable or metastatic GISTs; the follow-up interval can be less frequent for low-risk GISTs[1].

With the advancement of these molecular-targeted therapies, multiple associated adverse effects have been demonstrated, and some of these may be identified on follow-up imaging. Fluid retention is commonly seen with imatinib mesylate and can manifest with pleural effusions, pericardial effusion, ascites, or extensive

subcutaneous edema[27]. Imatinib mesylate can be associated with intratumoral hemorrhage, especially in patients with large bulky tumors[1,27]. Tyrosine kinase inhibitors are associated with pancreatic findings. For instance, imatinib mesylate is associated with asymptomatic pancreatic swelling; a ≥ 22% increase in pancreatic volume has been shown to be a poor prognostic indicator[41]. Conversely, sunitinib malate is associated with pancreatic atrophy, and this finding is associated with poor prognosis[42]. Moreover, there are several case reports of pancreatitis associated with sunitinib malate and sorafenib therapy[43]. It is important to identify these adverse effects on imaging, which would allow for dose reduction, dose interruption, or drug discontinuation in the appropriate setting.

CONCLUSION

GISTs are the most common mesenchymal tumors of the GI tract and often arise from the stomach or small intestine. The majority of GISTs occur as a result of activating mutations in two receptor protein tyrosine kinases, KIT and/or PDGFRA, leading to tumorigenesis. Mutational analyses allow for predicting patient prognosis and treatment response. Clinical presentations can vary from no symptoms to GI bleeding, abdominal discomfort, and ulcer-related symptoms. While most GISTs are benign, some cases can be aggressive with metastases. Imaging plays a key role in the diagnosis and follow-up of these tumors. It is crucial to understand and identify the key imaging features of GISTs and their expected appearance upon treatment response and disease recurrence. Surgical resection is the preferred management, especially for small tumors, while tyrosine kinase inhibitors, including imatinib mesylate and sunitinib malate, can serve as a neoadjuvant and/or adjuvant therapies.

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World J Gastroenterol 2021 November 7; 27(41): 7134-7143

DOI: 10.3748/wjg.v27.i41.7134

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

ORIGINAL ARTICLE

Basic Study

Circulating tumor DNA dynamics analysis in a xenograft mouse model with esophageal squamous cell carcinoma

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Author contributions: Kinugasa H designed the manuscript; Terasawa H drafted the manuscript; Kinugasa H, Terasawa H, Yamamoto S, Hirai M, Tanaka T and Takaki A were responsible for experiments; Kinugasa H, Nouso K and Okada H supervised the manuscript preparation; all authors approved the final manuscript.

Institutional review board statement: This study was reviewed approved by [the ethics committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Okayama University Hospital] Institutional Review Board.

Institutional animal care and use committee statement: Xenograft mouse experimental protocols were approved by the Ethical Committee of Okayama University Graduate School of Medicine,

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Abstract

BACKGROUND

It remains unclear which factors, such as tumor volume and tumor invasion, influence circulating tumor DNA (ctDNA), and the origin of ctDNA in liquid biopsy is always problematic. To use liquid biopsies clinically, it will be very important to address these questions.

AIM

To assess the origin of ctDNA, clarify the dynamics of ctDNA levels, assess ctDNA levels by using a xenograft mouse after treatment, and to determine whether tumor volume and invasion are related to ctDNA levels.

METHODS

Tumor xenotransplants were established by inoculating BALB/c-nu/nu mice with the TE11 cell line. Groups of mice were injected with xenografts at two or four sites and sacrificed at the appropriate time point after xenotransplantation for ctDNA analysis. Analysis of ctDNA was performed by droplet digital PCR, using the human telomerase reverse transcriptase (hTERT) gene.

RESULTS

Mice given two-site xenografts were sacrificed for ctDNA at week 4 and week 8. No hTERT was detected at week 4, but it was detected at week 8. However, in four-site xenograft mice, hTERT was detected both at week 4 and week 6. These experiments revealed that both tumor invasion and tumor volume were asso-

Dentistry and Pharmaceutical Sciences (OKU-2019276).

Conflict-of-interest statement: The authors declare that there are no conflicts of interest.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Supported by JSPS KAKENHI (19k17433).

Country/Territory of origin: Japan

Specialty type: Gastroenterology and Hepatology

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

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Received: May 14, 2021
Peer-review started: May 14, 2021
First decision: July 14, 2021
Revised: July 21, 2021
Accepted: August 30, 2021
Article in press: August 30, 2021
Published online: November 7, 2021

P-Reviewer: Norčič G
S-Editor: Wang LL
L-Editor: A

ciated with the detection of ctDNA. In resection experiments, hTERT was detected at resection, but had decreased by 6 h, and was no longer detected 1 and 3 d after resection.

CONCLUSION
We clarified the origin and dynamics of ctDNA, showing that tumor volume is an important factor. We also found that when the tumor was completely resected, ctDNA was absent after one or more days.

Key Words: Liquid biopsy; Circulating tumor DNA; Xenograft; Esophageal squamous cell carcinoma; Dynamics of circulating tumor DNA

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Core Tip: We clarified the origin and dynamics of circulating tumor DNA (ctDNA), showing that not only tumor invasion but also tumor volume was an important factor. The possibility of detecting ctDNA in early-stage cancers with shallow depth was demonstrated. Also, ctDNA could be measured at 1 d after tumor resection to evaluate the residuals, and the half-life of ctDNA was estimated to be 1.8-3.2 h.

Citation: Terasawa H, Kinugasa H, Nouso K, Yamamoto S, Hirai M, Tanaka T, Takaki A, Okada H. Circulating tumor DNA dynamics analysis in a xenograft mouse model with esophageal squamous cell carcinoma. *World J Gastroenterol* 2021; 27(41): 7134-7143
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7134.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7134

INTRODUCTION

Liquid biopsy, a molecular biological diagnostic method for blood and body fluids, has progressed dramatically in recent years. Circulating tumor DNA (ctDNA), one of the targets of liquid biopsy, is expected to be a useful method for screening and detection of cancer, monitoring therapy, prediction of prognosis, and personalized medicine[1-3]. Therefore, in addition to direct biopsy, which is the basis of conventional cancer diagnosis, a hybrid method, which includes non-invasive liquid biopsy, is becoming the mainstream.

Cell-free DNA (cfDNA), which includes ctDNA, is derived from apoptotic or necrotic cells[4,5]. Theoretically, it could be applied regardless of the stage. However, reports of its usefulness for early stages of cancer are controversial. Bettegowda *et al*[6] revealed that the rate of ctDNA detection is generally high in advanced stages of cancer, but ctDNA levels are generally lower in early stages of cancer. On the other hand, some reports indicated that ctDNA was useful for detecting early-stage cancers [6-9]. It remains unclear which factors, such as tumor volume and tumor invasion, influence ctDNA, and the origin of ctDNA in liquid biopsy is always problematic. To use liquid biopsies clinically, it will be very important to address these questions.

In this study, we used a xenograft mouse model to assess the origin of ctDNA, clarify the dynamics of ctDNA levels, assess ctDNA levels after treatment, and to determine whether tumor volume and invasion are related to ctDNA levels.

MATERIALS AND METHODS

Cell Line

The human esophageal squamous cell carcinoma cell line TE11 was used because we established an experimental system for TE11 previously[10] and used it to show that liquid biopsy is useful in esophageal cancer cells as well as other gastrointestinal cancers. Cells were grown in RPMI 1640 (Thermo Fisher Scientific, Tokyo, Japan) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma-Aldrich, Tokyo, Japan) at 37.0 °C in a 5% CO_2 atmosphere. Appropriate passages were made

P-Editor: Yuan YY

such that confluency did not exceed 70% prior to xenotransplantation. A Countess Automated Cell Counter (Thermo Fisher Scientific, Tokyo, Japan) was used to count cells, and 0.2% Trypan blue dye was used to exclude dead cells.

Xenograft mouse model

Xenograft mouse experimental protocols were approved by the Ethical Committee of Okayama University (OKU-2019276). Six-week-old female nude mice (BALB/c-nu/nu) (Charles River Laboratories, Japan) were used. Mice were raised in the animal facility of Okayama University and given food and water. The physical conditions of the mice, including the presence or absence of body movement or the availability of food and drink, were monitored daily. Mice were euthanized with isoflurane if mice stopped moving or eating.

Tumor xenotransplants were established in mice by inoculation in the shoulders or flanks with 1 × 10^6 TE11 cells suspended in 50 μL medium plus 50 μL Matrigel (Corning Product No. 356234). Inoculation was performed at two sites (*i.e.*, both shoulders, two-site xenograft mouse group, 28 mice) or at four sites (*i.e.*, both shoulders and both flanks, four-site xenograft mouse group, 28 mice) in order to determine the effect of tumor volume as well as the degree of invasion (Figure 1).

Tumor formation was confirmed in all xenograft mice; although, the changes in size varied. Differences in tumor volume were evaluated over time. Two-site and four-site xenograft mouse groups were sacrificed for ctDNA analysis at the appropriate time point after xenotransplantation. To minimize the effects of differences in tumor size, four mice were used for each ctDNA time point analysis.

A sample size calculation using power analysis determined 24 mice were needed in xenograft experiments and 32 mice were needed in resection experiments.

Xenograft experiments

Twelve mice received two-site xenografts, and 12 received four-site xenografts. Tumor size was measured every week after xenotransplantation, and ctDNA was evaluated at two time points: 4 wk and 8 wk after xenotransplantation (Figure 1).

Resection experiments

Sixteen mice received two-site xenografts, and 16 mice received four-site xenografts. All tumors were resected at week 7 after xenotransplantation in the two-site xenograft group or at week 5 in the four-site xenograft group. cfDNA and ctDNA were evaluated 6 h, 1 d, and 3 d after resection, or simultaneously with resection in the controls (Figure 1).

Blood and tumor tissue sample collection

For ctDNA analysis, whole blood was collected in BD Vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, NJ), and processed within 1 h after collection. The samples were centrifuged at 3000 × g at 4 °C to separate plasma from peripheral blood cells, and stored at -80 °C. DNA was extracted from 1000 μL of blood and the final solution was 25 μL of DNA. Plasma ctDNA was extracted (25 μl) with the QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, Calif), according to the manufacturer's instructions. At sacrifice, tumors were collected and divided into two fragments. One tumor fragment was snap-frozen in liquid nitrogen and used for preparation of genomic DNA. The other fragment was formalin-fixed and paraffin-embedded for histopathological diagnosis, morphological evaluation after hematoxylin/eosin staining, and immunohistochemistry. Four slides were made from the largest diameter section, where it was easy to obtain information on invasion.

Telomerase reverse transcriptase assay

The wild-type telomerase reverse transcriptase (TERT) gene was analyzed by a mouse TERT (mTERT) assay (Thermo Fisher Scientific, Tokyo, Japan) or human TERT (hTERT) assay (Bio-Rad Laboratories, Hercules, CA, United States of America) to take advantage of the differences between mTERT and hTERT genes. The verification experiments using a droplet digital PCR (QX200 system; Bio-Rad Laboratories, Hercules, CA, United States of America) was performed.

Droplet digital polymerase chain reaction and data analysis

To evaluate ctDNA, hTERT was detected *via* droplet digital polymerase chain reaction (PCR) according to the following protocol. DNA eluent (5 μL) from plasma was combined with Droplet PCR Supermix (10 μL; Bio-Rad Laboratories, Hercules, CA, United States of America), primer/probe mixture (1 μL), 5M Betaine (2 μL), 80

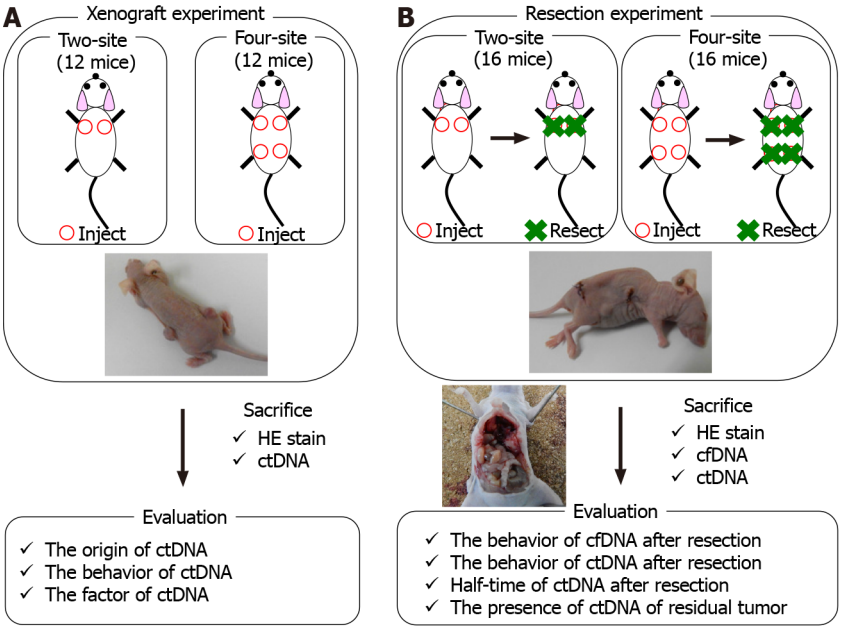


Figure 1 Xenograft mouse model with TE11 cell. A: In the xenograft experiment, groups of 12 mice each were given two-site xenografts or four-site xenografts; B: In the resection experiment, groups of 16 mice each were given two- or four-site xenografts. All tumors were resected at week 7 after xenotransplantation in two-site xenograft mice, or at week 5 in for-site xenograft mice.

mmol/L EDTA (0.25 μL), CviQl enzyme (0.25 μL), and sterile DNase- and RNase-free water (3.5 μL). The mixture (22 μL) was added to Droplet Generation Oil (70 μL; Bio-Rad Laboratories, Hercules, CA, United States of America) to produce droplets. Thermal cycling of the emulsion was as follows: an initial denaturation at 95 °C for 10 min, followed by 50 cycles of 96 °C for 30 s and 62 °C for 1 min. After a final enzyme deactivation step of 98 °C for 10 min, the reaction mixtures were analyzed using a droplet reader (Bio-Rad Laboratories, Hercules, CA, United States of America). For quantification, the fluorescence signal was acquired with QuantaSoft software (Bio-Rad Laboratories, Hercules, CA, United States of America). We set the threshold fluorescence intensity at 7500 (mTERT) or 2000 (hTERT), according to positive and negative controls in this study, i.e., plasma and tissue of healthy human, control mouse, or TE11 cell line.

Statistical analysis

We used JMP version 14.0 (SAS Institute, Cary, NC, United States of America) for statistical analysis and set the threshold of significance at $P < 0.05$. Continuous data were analyzed using the non-parametric Wilcoxon test, and categorical data were analyzed using a Chi-squared test.

RESULTS

Verification experiments

In verification experiments using a droplet digital PCR (QX200 system; Bio-Rad Laboratories, Hercules, CA, United States of America), we confirmed that the mTERT gene was detected in tissue and plasma of control mice, but not in TE11 genomic DNA, whereas the hTERT gene was detected in TE11 genomic DNA, but not in the tissue or plasma of control mice (Figure 2).

Xenograft experiments

Xenograft experiments were designed to reveal the origin of ctDNA and factors contributing to ctDNA increase. Average tumor sizes measured in the two-site xenograft group 1, 2, 3, 4, 5, 6, 7, and 8 wk after xenotransplantation were 1.8, 3.2, 4.6, 6.0, 6.8, 8.0, 8.5, and 12.5 mm, respectively. Two-site xenograft mice were sacrificed 4 or 8 wk after xenotransplantation to evaluate ctDNA. No hTERT was detected at week 4, but hTERT was detected at week 8 (Figure 3). These results indicated that ctDNA was associated with tumor growth.

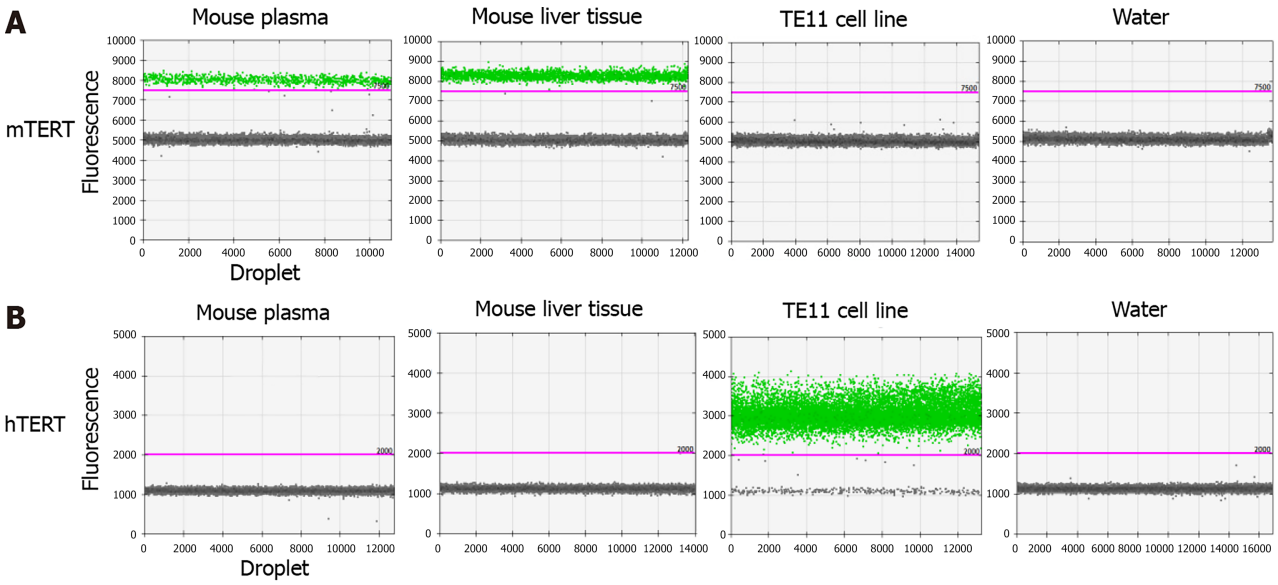


Figure 2 Telomerase reverse transcriptase assay by droplet digital polymerase chain reaction for mouse plasma, liver tissue, TE11 cell and water. The presence of mouse telomerase reverse transcriptase (mTERT) and human TERT (hTERT) forms of the wild type TERT was analyzed by droplet digital polymerase chain reaction. A: The assay correctly detected mTERT in mouse plasma and liver tissue; B: hTERT was detected in the TE11 cell line. Neither mTERT nor hTERT was detected in water.

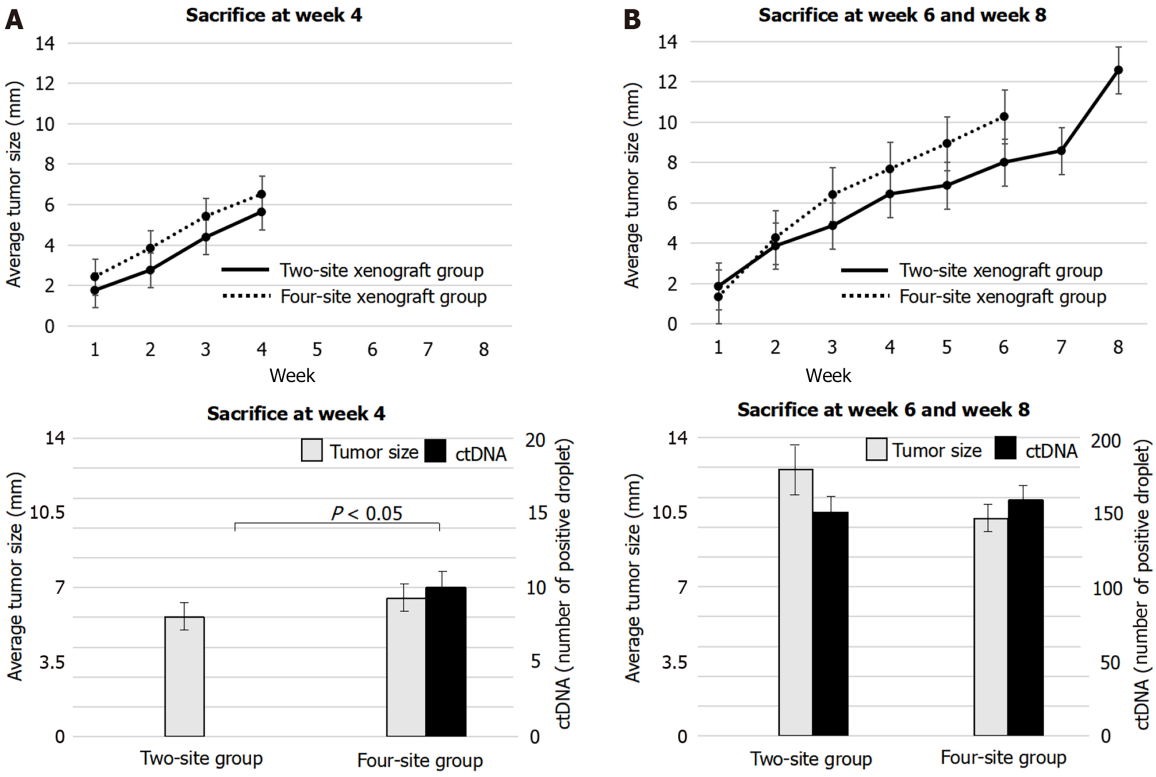


Figure 3 The dynamics of circulating tumor DNA in xenograft experiments. A: Two-site and four-site xenograft mice were sacrificed for circulating tumor DNA (ctDNA) at week 4. Human telomerase reverse transcriptase (hTERT) was detected only in four-site xenograft mice, not in two-site xenograft mice; B: In both two-site xenograft mice sacrificed for ctDNA at week 8 and four-site xenograft mice sacrificed at week 6, hTERT was detected.

In four-site xenograft mice, the average tumor sizes at week 1, 2, 3, 4, 5, and 6 after xenotransplantation were 1.8, 4.0, 5.9, 7.1, 8.9, and 10.2 mm. The 8 wk evaluation planned for this group was revised to occur at week 6, because the tumor in one mouse had grown rapidly to cause thoracic invasion, and it was unlikely to survive to week 8. Four-site xenograft mice were sacrificed for ctDNA at week 4 and week 6. hTERT was detected both at week 4 and at week 6 in this group (Figure 3). These results indicated that ctDNA was associated with tumor growth as well as those of

two-site xenograft mice. There were no other unexpected adverse events.

Histopathology of tumors at week 4 showed no invasion in either the two-site or four-site xenograft group, while tumors showed invasion into muscle both at week 8 in the two-site xenograft mice (P = 0.02) and at week 6 in the four-site xenograft mice (Figure 4; P = 0.03). These results indicated that ctDNA was associated with tumor invasion.

The rates of tumor size increase were similar between the two-site xenograft group and the four-site group. Interestingly, the two groups showed similar tumor diameters (P = 0.25) and invasion at week 4 (Figures 3 and 4), but a clear difference in the ctDNA detection rate (Figure 3; P = 0.02). These findings showed that not only invasion but also tumor volume might be related to the rate of ctDNA detection.

Resection experiments

Resection experiments were designed to clarify responses of ctDNA to tumor resection. Tumors in the two-site and four-site xenograft groups were resected when the diameter exceeded 10 mm. cfDNA and ctDNA were examined at sacrifice. In these resection experiments, two mice were excluded from the evaluation: one mouse with rapid tumor growth and a tendency toward paraplegia before resection, and another mouse with high invasion who died after tumor resection and before evaluation.

In two-site xenograft mice, tumor resection was performed at week 7. The average tumor size in the control group was 10.3 mm at the time of resection, and the average tumor sizes measured 6 h, 1 d, or 3 d at the time of resection were 10.1, 10.3, and 10.2 mm, respectively (P = 0.98). We detected hTERT at resection (control), but hTERT had decreased by 6 h, and was undetectable 1 d or 3 d after resection (Figure 5). The control cfDNA concentration was 1.1 μg/mL at the time of resection, and was 1.2, 1.3, and 1.4 μg/mL measured 6 h, 1 d, and 3 d after resection. Pathological autopsy confirmed the absence of macroscopic residual tumor at each evaluation in this experiment. Using data for the number of positive droplets measured 0 and 6 h after tumor resection in the two-site xenograft resection experiment, the half-life of ctDNA may be calculated from y = 155e - 0.368x. In our study, the half-life of ctDNA was estimated to be 1.8–3.2 h (Figure 6).

In four-site xenograft mice, tumor resection was performed at week 5. The average tumor size in the control group was 9.7 mm at the time of resection, while average tumor sizes measured 6 h, 1 d, or 3 d at the time of resection were 11.4, 10.6, and 10.2 mm, respectively (P = 0.34). In this experiment, hTERT was detected in all groups (Figure 5). The control cfDNA concentration was 1.3 μg/mL at resection and 1.2, 1.5, and 1.7 μg/mL measured 6 h, 1 d, and 3 d, respectively, after resection. Here, pathological autopsy revealed the presence of macroscopic residual tumor at each resection evaluation, with tumor invasion and intrathoracic metastasis in all mice. This experiment revealed that residual ctDNA was associated with incomplete resection and metastasis.

DISCUSSION

Because the TERT gene sequence differs between human and mouse, we were able to determine the origin and dynamics of ctDNA in a xenograft mouse model in which human-derived esophageal cancer cells were injected into the epidermis of mice. This model allowed assessment of ctDNA, which has traditionally been difficult to assess in the human body, due to tumor heterogeneity and the influence of other cells. In our experiment, tumor volume was involved in increases or decreases in ctDNA. In addition, if ctDNA was present over 1 d after resection, the presence of residual tumor is suspected.

Although studies of liquid biopsy using xenograft mouse model have been reported mainly in circulating tumor cells [11], we focused on ctDNA in this study. This model seems to be an ideal method because clinical samples contain a variety of cellular information as well as limitations such as ethical issues. Our report is also extremely valuable in providing direct evidence of the origin of plasma ctDNA, which we assessed in the xenograft mouse model by assaying mTERT and hTERT. Based on this ctDNA confirmation, other factors affecting ctDNA dynamics were examined. In our xenograft experiments, the average tumor sizes 4 wk after two-site and four-site xenografts were very similar (5.6 mm and 6.5 mm), and histology showed similar degrees of tumor invasion (Figure 4). However, ctDNA was detected in four-site xenograft mice but not in two-site xenograft mice. These findings revealed that tumor volume may influence ctDNA detection. In both groups, increasing ctDNA with tumor

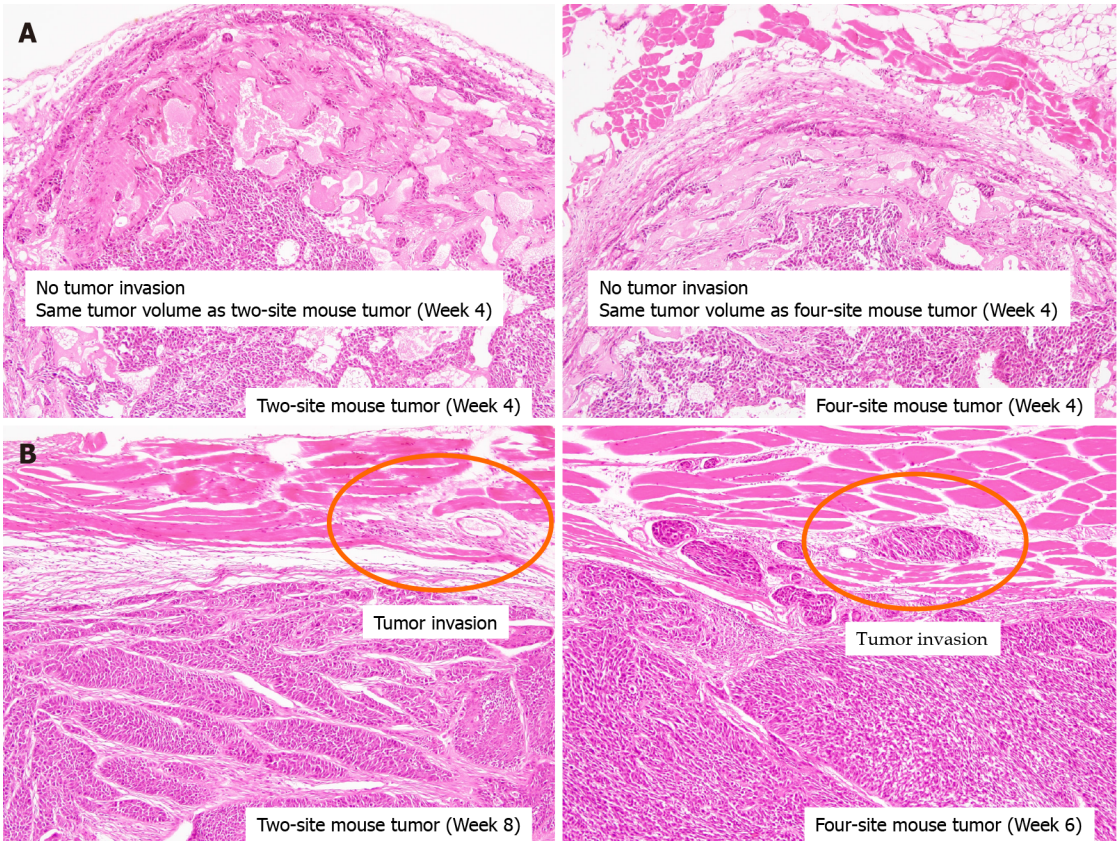


Figure 4 Histopathology of xenograft mouse with TE11. A: Histopathology showed absence of invasion in tumors at week 4 in mice with two-site or four-site xenografts; B: Muscle invasions were observed in tumors at week 8 in two-site xenograft mice, and at week 6 in four-site xenograft mice.

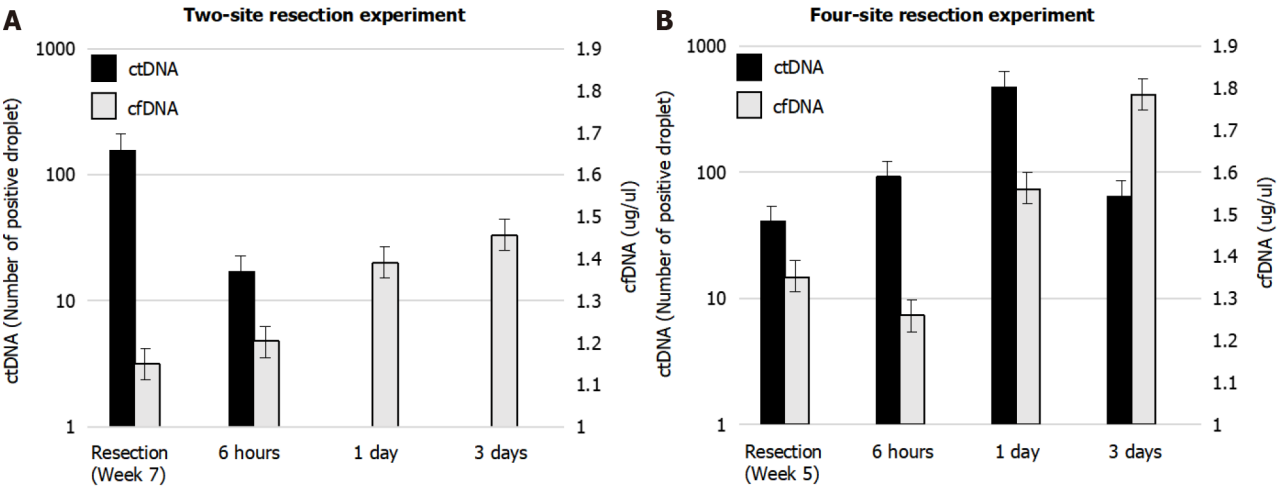


Figure 5 The dynamics of circulating tumor DNA in resection experiments. A: Tumor resection was performed when tumor diameter xenograft mice exceeded 10 mm, at week 7 in two-site xenograft mice, or at week 5 in four-site xenograft mice. Human telomerase reverse transcriptase (hTERT) circulating tumor DNA (ctDNA) was detected at resection (control), had decreased by 6 h, and was undetectable 1 d and 3 d after resection; B: On the other hand, in four-site xenograft mice, hTERT (ctDNA) was detected at resection (control), 6 h, 1 d, and 3 d after resection. cfDNA: Cell-free DNA.

progression was confirmed at week 8 and week 6. The amount and detection rate of ctDNA correlated with tumor progression in a previous clinical study[6], and our results may support that finding. Although detailed studies on the association between tumor volume or invasion and ctDNA have not been conducted, ctDNA is assumed to be detectable in early cancer once the tumor reaches a certain volume.

The presence of ctDNA after surgical resection is observed in clinical samples from cancer patients, and evaluation during the perioperative period is useful for prediction of prognosis[12-14]. Detection of ctDNA after surgery suggests some residual disease [15]. However, these clinical studies may inevitably detect circulating DNA from

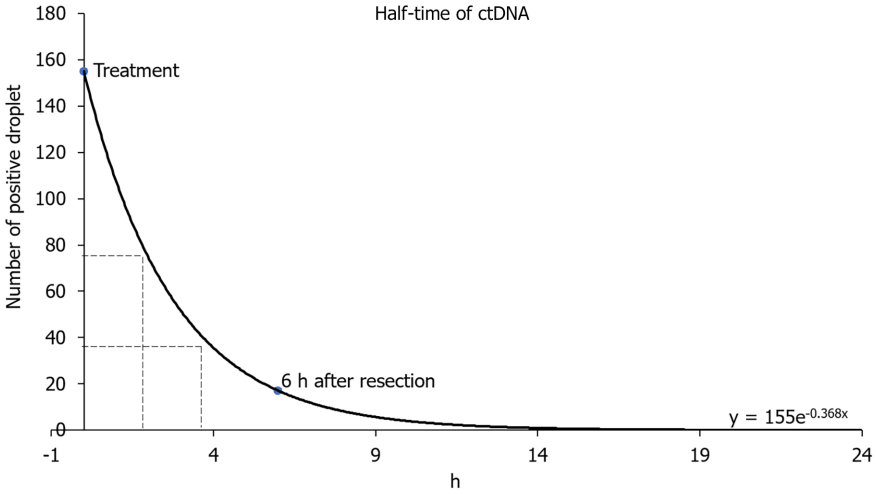


Figure 6 The half-life of circulating tumor DNA in resection experiments. To estimate half-life of circulating tumor DNA in two-site xenograft mice in the resection experiment, the number of positive droplets *vs* time after resection was fit to an exponential curve, y = 155e - 0.368x.

sources other than tumor cells, and there have been no reports to indicate when liquid biopsy should be used. Regarding this point, our resection experiments demonstrated reduced hTERT at 6 h and its absence 1 to 3 d after resection, indicating that ctDNA evaluation 1 d after resection might be useful to detect residual tumor in clinical cases. These experiments also revealed tumor volume was involved in the increase or decrease of ctDNA and that post-tumor resection evaluation requires an interval of one day or more after resection.

The half-life of ctDNA was reported as approximately 2 h in one study[16], but another study found the half-life to be 16 min[17]. The metabolism and excretion of cfDNA is affected by liver and kidney function[18], and ctDNA levels might be regulated by the same mechanism. In our study, we estimated the half-life of ctDNA 1.8–3.2 h, based on ctDNA levels measured 0 and 6 h after resection (Figure 6), which was similar to data from previous reports. Assuming a half-life of 3 h, ctDNA will decline by a factor of 28 after 1 d, and postoperative assessment of ctDNA should be evaluated after 1 d.

cfDNA is derived from apoptotic or necrotic cells[19,20], and its increase is considered to be caused by surgical manipulation, or perhaps cytokines, or cell proliferation in response to invasive therapy. Our results are consistent with these reports, indicating ctDNA decreased after complete resection, while cfDNA increased after resection.

Carcinoembryonic antigen (CEA) and squamous cell carcinoma antigen (SCC-Ag) are biomarkers for esophageal cancer. However, the usefulness of these biomarkers in the early diagnosis of esophageal cancer has not been established. Currently, upper endoscopy is the most useful examination to pick up early-stage esophageal cancer. However, since this examination is invasive, the development of non-invasive methods such as liquid biopsy is eagerly awaited. The combination of this method with conventional methods may lead to the next generation of diagnosis.

Our study had the following limitations. First, the artificial implantation of tumor under the skin in the xenograft model differs from the physiology of actual tumor development. Second, individual mice exhibit differences in tumor growth rates, and therefore, our comparative analyses in the present study used the average values for four animals per group. Third, regarding residual tumor, although pathological autopsies were performed on all mice, complete certainty with respect to residual disease is impossible. Forth, TE11 cell line alone is not necessarily sufficient, other cell lines should be examined as well. Fifth, comparison with conventional biomarkers such as CEA and SCC-Ag needs to be shown.

CONCLUSION

We clarified the origin and dynamics of ctDNA in the xenograft mouse model. We showed that tumor volume was an important factor in ctDNA, and that if the tumor volume was sufficiently large, ctDNA can be detected even in early-stage or superficial

cancers. We also found that, upon complete tumor resection, ctDNA disappeared after at least 1 d, unless residual tumor remained. These findings may indicate future clinical uses of liquid biopsy.

ARTICLE HIGHLIGHTS

Research background

The clinical application of liquid biopsy is becoming more widespread. However, it remains unclear which factors, such as tumor volume and tumor invasion, influence circulating tumor DNA (ctDNA), and the origin of ctDNA in liquid biopsy is always problematic.

Research motivation

It will be very important to address the origin and dynamics of ctDNA for further clinical application of liquid biopsy.

Research objectives

A xenograft mouse model was used to assess the origin of ctDNA, clarify the dynamics of ctDNA levels, assess ctDNA levels after treatment, and determine whether tumor volume and invasion are related to ctDNA levels.

Research methods

Tumor xenotransplants were established by inoculating BALB/c-nu/nu mice with the TE11 cell line (esophageal squamous cell carcinoma). Analysis of ctDNA was performed by droplet digital polymerase chain reaction, using the human telomerase reverse transcriptase (hTERT) gene.

Research results

Mice given two-site xenografts were sacrificed for ctDNA at week 4 and week 8. No hTERT was detected at week 4, but it was detected at week 8. However, in four-site xenograft mice, hTERT was detected both at week 4 and week 6. These experiments revealed that both tumor invasion and tumor volume were associated with the detection of ctDNA. In resection experiments, hTERT was detected at resection, but had decreased by 6 h, and was no longer detected 1 and 3 d after resection. The half-life of ctDNA was estimated to be 1.8-3.2 h.

Research conclusions

We clarified the origin and dynamics of ctDNA, showing that not only tumor invasion but also tumor volume was an important factor. Also, ctDNA could be measured at 1 d after tumor resection to evaluate the residuals.

Research perspectives

In the clinical application of liquid biopsy, early-stage cancers could be targeted, and post-treatment monitoring should be performed 1 d after treatment.

ACKNOWLEDGEMENTS

We thank all staff in the animal facility of Okayama University, Shinya Ohashi (MD, PhD; Department of Therapeutic Oncology, Kyoto University) and Hiroshi Nakagawa (MD, PhD; Department of Medicine, Columbia University).

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World Journal of Gastroenterology

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World J Gastroenterol 2021 November 7; 27(41): 7144-7158

DOI: 10.3748/wjg.v27.i41.7144

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

ORIGINAL ARTICLE

Basic Study

Cross-sectional evaluation of circulating hepatitis B virus RNA and DNA: Different quasispecies?

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Abstract

BACKGROUND

interpreted the results; Garcia-Garcia S and Tabernero D drafted the manuscript; Ferrer-Costa R, Rando-Segura A, Trejo-Zahínos J, Casis E, Riveiro-Barciela M, Buti M and Rodríguez-Frías F critically reviewed the manuscript.

Institutional review board statement: The study was approved by the Ethics Committee of the Vall d'Hebron Research Institute (PR(AG)146/2020) and all patients provided written informed consent for participation.

Conflict-of-interest statement: Authors have no conflict of interest for this manuscript.

Data sharing statement: Next-generation sequencing data were submitted to the GenBank SRA database (BioProject accession number PRJNA625435).

Supported by Instituto de Salud Carlos III, No. PI18/01436; and European Regional Development Fund (ERDF).

Country/Territory of origin: Spain

Specialty type: Gastroenterology and hepatology

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

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Received: July 8, 2021

Different forms of pregenomic and other hepatitis B virus (HBV) RNA have been detected in patients' sera. These circulating HBV-RNAs may be useful for monitoring covalently closed circular DNA activity, and predicting hepatitis B e-antigen seroconversion or viral rebound after nucleos(t)ide analog cessation. Data on serum HBV-RNA quasispecies, however, is scarce. It is therefore important to develop methodologies to thoroughly analyze this quasispecies, ensuring the elimination of any residual HBV-DNA. Studying circulating HBV-RNA quasispecies may facilitate achieving functional cure of HBV infection.

AIM

To establish a next-generation sequencing (NGS) methodology for analyzing serum HBV-RNA and comparing it with DNA quasispecies.

METHODS

Thirteen untreated chronic hepatitis B patients, showing different HBV-genotypes and degrees of severity of liver disease were enrolled in the study and a serum sample with HBV-DNA > 5 Log_{10} IU/mL and HBV-RNA > 4 Log_{10} copies/mL was taken from each patient. HBV-RNA was treated with DNAse I to remove any residual DNA, and the region between nucleotides (nt) 1255-1611 was amplified using a 3-nested polymerase chain reaction protocol, and analyzed with NGS. Variability/conservation and complexity was compared between HBV-DNA and RNA quasispecies.

RESULTS

No HBV-DNA contamination was detected in cDNA samples from HBV-RNA quasispecies. HBV quasispecies complexity showed heterogeneous behavior among patients. The Rare Haplotype Load at 1% was greater in DNA than in RNA quasispecies, with no statistically significant differences (P = 0.1641). Regarding conservation, information content was equal in RNA and DNA quasispecies in most nt positions [218/357 (61.06%)]. In 102 of the remaining 139 (73.38%), HBV-RNA showed slightly higher variability. Sliding window analysis identified 4 hyper-conserved sequence fragments in each quasispecies, 3 of them coincided between the 2 quasispecies: nts 1258-1286, 1545-1573 and 1575-1604. The 2 hyper-variable sequence fragments also coincided: nts 1311-1344 and 1461-1485. Sequences between nts 1519-1543 and 1559-1587 were only hyper-conserved in HBV-DNA and RNA, respectively.

CONCLUSION

Our methodology allowed analyzing HBV-RNA quasispecies complexity and conservation without interference from HBV-DNA. Thanks to this, we have been able to compare both quasispecies in the present study.

Key Words: Hepatitis B virus RNA; Hepatitis B X gene; Quasispecies; Next-generation sequencing; Quasispecies conservation; Quasispecies complexity

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Core Tip: Hepatitis B virus (HBV) quasispecies composition and its evolution are related to liver disease progression. HBV-RNA in serum is potentially useful for analyzing viral quasispecies, even in patients with low levels of or suppressed serum HBV-DNA. Few studies have analyzed circulating HBV-RNA quasispecies, and similarities and differences with DNA quasispecies should be assessed. We used next-generation sequencing to analyze RNA quasispecies variability/conservation and complexity, without interference of HBV-DNA, in untreated chronic hepatitis B patients. Comparison of both quasispecies showed similar results between them. DNA quasispecies tended toward greater complexity, while RNA quasispecies tended toward higher variability.

Citation: Garcia-Garcia S, Cortese MF, Tabernero D, Gregori J, Vila M, Pacín B, Quer J, Casillas R, Castillo-Ribelles L, Ferrer-Costa R, Rando-Segura A, Trejo-Zahínos J, Pumarola T,

Peer-review started: July 8, 2021
First decision: August 19, 2021
Revised: August 20, 2021
Accepted: October 18, 2021
Article in press: October 18, 2021
Published online: November 7, 2021

P-Reviewer: Wu W
S-Editor: Gao CC
L-Editor: A
P-Editor: Gao CC

Casis E, Esteban R, Riveiro-Barciela M, Buti M, Rodríguez-Frías F. Cross-sectional evaluation of circulating hepatitis B virus RNA and DNA: Different quasispecies? *World J Gastroenterol* 2021; 27(41): 7144-7158
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7144.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7144

INTRODUCTION

The hepatitis B virus (HBV) is the etiological agent of hepatitis B and can cause both acute and chronic liver disease. In 2015, roughly 257 million people worldwide were estimated to suffer from chronic hepatitis B (CHB) infection, the major complications of which are cirrhosis and hepatocellular carcinoma[1].

The HBV genome is a 3.2 kb-long partially double-stranded relaxed circular DNA (rcDNA), with a complete minus-strand and an incomplete plus-strand. After cytoplasmic release of the viral nucleocapsid containing the rcDNA, it is actively transported into the nucleoplasm, where it will be repaired and converted to covalently closed circular DNA (cccDNA). This form of viral genome remains as an episomal minichromosome in the hepatocyte nuclei for their entire life and serves as the template for the transcription of viral messenger RNAs by the cellular RNA polymerase II. One of them, the pregenomic RNA (pgRNA) is encapsidated and then reverse-transcribed to generate new rcDNA (or double stranded linear DNA), which is released as new infective DNA-containing virions[2]. In addition, full and spliced pgRNA, as well as X open reading frame (*HBX)* transcripts have been detected in patients' serum[3]. Serum HBV pgRNA has been reported to be useful for monitoring cccDNA activity in hepatitis B e-antigen (HBeAg)-positive (+ve) entecavir-treated patients[4] and to be predictive of HBeAg seroconversion during polymerase inhibitor treatment[5-7]. In addition, its detection in the absence of detectable serum HBV DNA in patients receiving nuclos(t)ide analogs (NA) therapy may allow inference in cccDNA transcription and has been shown to be a predictive biomarker for viral rebound after treatment cessation[8].

In a single HBV infection, viral genomes are present as a complex mutant spectrum constituted by closely related, but not identical, viral populations (genetic variants), termed viral quasispecies[9]. Those variants can occur through numerous host-virus interactions during the viral replication cycle, encompassing not only cellular factors but also viral factors, essentially the error-prone viral polymerase[10]. Serum circulating HBV-RNA is potentially useful for analyzing viral quasispecies, even in patients with low levels of or suppressed serum HBV-DNA. However, unlike DNA quasispecies, HBV-RNA quasispecies have not been subjected to reverse transcription, which is the main source of variability in the HBV genome[11]. Therefore, both quasispecies may be different in terms of nucleic acid variability/conservation and genetic variant complexity. Few studies to date have analyzed circulating HBV-RNA quasispecies[4,6] and similarities and differences with DNA quasispecies should therefore be assessed in more detail. In addition, serum HBV-RNA has been reported to be genetically homogenous to intrahepatic HBV-RNA[4], the main target for targeted gene therapy, which may favor achieving functional cure of HBV infection, the ideal end point of therapy based on sustained hepatitis B virus surface antigen (HBsAg) loss[12]. In order to interfere with the synthesis of all the viral proteins, these therapies should be directed to hyper-conserved sequence targets shared by all the HBV mRNAs. *HBX* thus provides an ideal target, as its sequence is located at the 3' end of all HBV mRNAs[13,14]. Taking this into account, analysis of serum HBV-RNA quasispecies may be an especially useful tool when looking for hyper-conserved targets for this kind of antiviral therapy. To confirm this, it is necessary to establish a reliable methodology to thoroughly analyze serum HBV-RNA quasispecies.

Taking this in mind, the aim of this study was to establish a methodology, based on next-generation sequencing (NGS), for thoroughly analyzing serum HBV-RNA quasispecies without interference from circulating HBV-DNA. This methodology has been tested in a group of untreated CHB patients to gain a preliminary insight into comparison of genetic variability/conservation and complexity between circulating HBV-RNA and DNA quasispecies.

MATERIALS AND METHODS

Patients and samples

The study was approved by the Ethics Committee of the Vall d'Hebron Research Institute (PR(AG)146/2020) and all patients provided written informed consent for participation. No animals were used.

In this cross-sectional study, we included a serum sample from 13 well-characterized untreated CHB patients attending the outpatient clinic of Vall d'Hebron University Hospital (Barcelona, Spain). All samples were selected taking into account HBV-DNA levels > 5 Log_{10} IU/mL and HBV-RNA levels > 4 Log_{10} copies/mL to ensure sufficient levels of both HBV-DNA and RNA to study their quasispecies. In addition, heterogeneity in terms of HBV genotypes and degrees of severity of liver disease were also taken into account in patient selection, in order to obtain an overall picture of differences between HBV-DNA and RNA quasispecies. Exclusion criteria were positive testing for hepatitis D virus, hepatitis C virus, and human immunodeficiency virus.

Serological and virological determinations

HBV serological markers such as HBsAg, HBeAg, anti-HBe antibodies were tested using commercial chemiluminescent immunoassays on a COBAS 8000 analyzer (Roche Diagnostics, Rotkreuz, Switzerland). HBV-DNA was quantified on a COBAS 6800 analyzer (Roche Diagnostics, Mannheim, Germany) with a lower detection limit of 10 IU/mL. HBV genotypes were determined as previously explained[13]. HBV-RNA was quantified using an in-house method. The standard RNA to create the standard curve for this quantification was obtained as follows: 1.1× HBV genome from pTRiEx1.1-HBV[15] was subcloned downstream of a T7 promoter in a pEF6/V5-His TOPO TA vector (Thermo Fisher Scientific-Life Technologies, Austin, TX, United States) following the manufacturer's instructions, to ensure its *in vitro* transcription (pEF6-HBV). Once cloned, the correct orientation and insertion of HBV genome was analyzed by Sanger Sequencing, and pEF6-HBV plasmid was isolated from bacteria using the NucleoBond Xtra Midi Kit (Macherey-Nagel, Dueren, Germany). The pEF6-HBV plasmid was then linearized by NotI restriction enzyme digestion (New England Biolabs, Beverly, MA, United States), and used as template for *in vitro* transcription reaction using the MEGAscript T7 Transcription Kit (Thermo Fisher Scientific-Life Technologies, Austin, TX, United States), by adding the plasmid pEF6-HBV concentration of 0.5 μg/μL diluted in water. At the same time, the *in vitro* transcription of pTRI-Xef positive control DNA provided with the kit was also performed. The RNA resulting from transcription was then purified using the MEGAclear Transcription Clean-up Kit (Thermo Fisher Scientific-Life Technologies, Austin, TX, United States) following the manufacturer's instructions. A DNase I treatment (Life Technologies, Austin, TX, United States) was then carried out for 15 min at room temperature, followed by heat-inactivation at 65 °C for 10 min and adding 2 μL of 25 mmol/L EDTA solution to the reaction mixture, in order to remove the template DNA. The purity of DNAse I-treated RNA was checked by measuring the absorbance of 1 μL of the sample at 260 nm using the NanoDrop spectrophotometer (Thermo Fisher Scientific-Life Technologies, Austin, TX, United States). The concentration of this RNA was quantified from 3 μL of the sample by Qubit fluorimeter using the Qubit RNA HS Assay Kit (Thermo Fisher Scientific-Life Technologies, Austin, TX, United States). The absence of DNA in DNAse I-treated RNA template was verified using the DNA Master PLUS HybProbe kit in a LightCycler 480 Instrument II system (Roche, Mannheim, Germany) using primers and probe specified in the Supplementary Table 1.

For absolute quantification analyses, the standard curve was defined using the quantified retrotranscribed RNA, taken to a concentration of 7.51 Log_{10} copies/mL, and serial 1:10 dilutions of this standard covering concentrations until 1.51 Log_{10} copies/mL. The points of the standard curve were defined as the mean Ct of a triplicate measurement of the original 7.51 Log_{10} copies/mL standard and its serial dilutions. The standard curve obtained was saved and imported as an external standard curve in each HBV-RNA quantification experiment. In those experiments, at least one dilution of standard RNA used to define one of the standard curve points must be included, in order to adjust the standard curve. Creation of the RNA standard curve and experiments to quantify HBV-RNA levels quantification were performed using one-step quantitative reverse transcription polymerase chain reaction (RT-qPCR) reaction, using the LightCycler 480 RNA Master Hydrolysis Probes kit (Roche, Mannheim, Germany). RT-qPCR program in the Light Cycler 480 instrument was

programmed as described in the Supplementary Table 1.

Amplification of HBV region of interest using NGS

In this study, we analyzed the region between nucleotides (nt) 1255 to 1611, located at the *HBX* 5′ end. In this region, we previously identified hyper-conserved sequence stretches in the circulating HBV-DNA quasispecies[13,14], which we aimed at analyzing at circulating HBV-RNA level.

For each serum sample, HBV-DNA was extracted from 200 μL of serum using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The region of interest was amplified through a 3-round polymerase chain reaction (PCR) protocol. The first-round PCR covered a 1338-bp region and was performed as previously described by our group[16] (Supplementary Table 1). The second-round and third-round PCRs, were also performed as previously detailed[13] (Supplementary Table 1). The second-round PCR amplified the region of interest between nt 1255 and 1611 of the HBV genome, yielding final products flanked by universal M13 sequences at both ends. In the third-round PCR, a specific pair of primers was used for each sample, consisting of the same M13 universal sequences (forward and reverse) at their 5′ ends and a multiplex identifier (MID) or barcode sequence at their 3′ ends. Each individual patient sample required a different MID. The PCR products obtained in this amplification, also known as amplicons, were visualized as single bands on a 1.5% agarose electrophoresis gel, stained with SybrSafe DNA gel Stain (Life Technologies, Carlsbad, CA, United States) with 1x TAE running buffer (Roche Diagnostics, Mannheim, Germany). PCR products were then purified from agarose gel using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Amplicon quality was verified using the Agilent 2200 TapeStation System and D1000 ScreenTape kit (Agilent Technologies, Waldbronn, Germany). Purified DNA from each sample was quantified by means of fluorescence, using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Carlsbad, CA, United States) adjusted to the same concentration, and pooled.

In the same serum samples, HBV-RNA was isolated from 140 μL serum using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. To remove any residual DNA present, isolated HBV-RNA was subsequently treated with DNAse I (Life Technologies, Austin, TX, United States) for 15 min at room temperature, followed by heat-inactivation at 65 °C for 10 min and adding 25 mmol/L EDTA solution to the reaction mixture, in keeping with manufacturer's instructions. After DNAse I treatment, RNA samples were retrotranscribed into cDNA in 2 steps. The first step involves denaturation of HBV-RNA and Oligo(dT)$_{17}$ primer, which binds to polyA sequence, by incubating at 65 °C for 5 min and 20 °C for 5 min. The second is reverse transcription itself involving Accuscript enzyme (Stratagene, Agilent Technologies, Santa Clara, United States) and RNAse OUT (Thermo Fisher Scientific-Life Technologies, Austin, United States). A 60-min incubation at 42 °C for the first-strand synthesis reaction is required, followed by a short incubation time of 15 min at 70 °C.

At the same time, elimination of any residual HBV-DNA after DNAse I treatment was verified by means of qPCR, as described for the DNAse I-treated RNA obtained from in vitro transcription of pEF6-HBV vector, described above. This qPCR experiment included the DNAse I-treated RNA samples and their respective cDNA obtained after reverse transcription. After obtaining the cDNA, the region of interest between nt 1255 and 1611 was amplified through the same 3-round PCR protocol as HBV-DNA samples, with some modifications according to Agilent's PfuUltra II Fusion HS DNA polymerase insert for cDNA targets.

Finally, amplicon pools obtained from both HBV-DNA and RNA were sequenced using the MiSeq platform (Illumina, San Diego, CA, United States). Those purified pools were processed using the DNA library preparation kit Kapa Hyper Prep kit (Kapa Biosystems-Roche, Cape Town, South Africa), with which amplicon ends were repaired and A-tailed. Each pool was then indexed using the SeqCap Adapter Kit A (Roche Sequencing, Pleasanton, CA, United States). After index ligation pools had been purified with KAPA Pure Beads (Kapa Biosystems-Roche, Cape Town, South Africa) and re-amplified to increase indexed amplicon concentration using the KAPA HiFi HotStart ReadyMix PCR Kit (Roche Sequencing, Pleasanton, CA, United States). PCR products were repurified with another round of KAPA Pure Beads and quantified using LightCycler 480 Instrument II with the Kapa Library Quantification kit (Kapa Biosystems-Roche, Cape Town, South Africa). Each DNA pool was then adjusted to a 4 nmol/L concentration and appropriate volumes of each pool were added to a final pool of all DNA pools, the DNA concentration of which was verified using LightCycler 480 Instrument II (Kapa Library Quantification kit). Finally, this

final pool was sequenced using MiSeq Reagent kit v3 (2 × 300 bp mode with the 600 cycle kit) (Illumina, San Diego, CA, United States).

NGS data analyses: Quasispecies complexity and conservation

Bioinformatics processing and haplotype-centric data analysis pipeline were performed as previously described by our group[16].

The HBV-DNA and RNA quasispecies complexities were evaluated by computation of the Rare Haplotype Load (RHL), a new diversity index that measures enrichment of quasispecies in minority genomes below a given threshold[17]. RHL was calculated using the haplotypes (unique sequences covering the full amplicon observed on the set of sequences) resulting from the intersection of forward and reverse strands without filtering by a minimum abundance. In this study, RHL was computed as the sum of the relative frequencies of all haplotypes obtained from HBV-DNA and RNA quasispecies whose abundance is equal to or less than 1%, as previously described[17].

Sequence conservation at nt level was determined by calculating the information content (IC, bits) of each position in a multiple alignment of all intersected haplotypes obtained in frequencies > 0.25%, as previously explained[13]. Nt IC ranges from 0 bits to 2 bits for a given position, where 2 bits represents the maximum IC value (100% conservation, *i.e.*, no nt changes in a given position in any of all haplotypes analyzed). The mean IC was calculated in windows of 25 nt, moved in steps of 1 nt through nt 1255-1611 (sliding window analysis), the 5% of those windows with the highest mean IC values (the most conserved) and the 5% with the lowest mean IC values (the most variable) were selected. Afterwards, those consecutive windows were connected and their sequences were represented as sequence logos using the R language package motifStack[18]. In each position of the logos, letters representing the nts identified are stacked. The relative sizes of letters indicate the relative frequencies of the nt represented at a given position in the multiple alignments of the haplotypes, and the total height of each stack represents the IC of each position.

Statistical analysis

All statistical analyses were performed in R[19]. Qualitative parameters were expressed as number of cases and percentage. Quantitative parameters were expressed as the median value and interquartile range (IQR) or as mean ± SD, where appropriate. Statistical comparisons between HBV-RNA and DNA quasispecies complexity were performed using the Kruskal-Wallis test, *t* test and two-proportions z-test with Yates continuity correction where appropriate. *P* values < 0.05 were considered significant. The bioinformatics and statistical methods used in this study were reviewed by Dr. Josep Gregori from the Liver Disease Viral Hepatitis Laboratory of Vall d'Hebron Hospital (Barcelona, Spain) and the CIBERehd research group.

RESULTS

Patient clinical and virological characteristics

Clinical, virological, and serological parameters from the 13 patients at the time of obtention of samples included are summarized in Table 1. Most of those patients were HBeAg +ve men, showing clinical characteristics compatible with chronic hepatitis stage of CHB infection[12]. In terms of severity of liver disease, 8 patients were classified as patients with nonsignificant fibrosis [Ishak fibrosis stage (F) < 3 or transient elastography < 7-8.5 kPa], and 5 patients were classified as patients with significant fibrosis (liver biopsy F ≥ 3 and < 5 or transient elastography > 7-8.5 and < 11-14 kPa, *n* = 3) or cirrhosis (liver biopsy F5-6 or transient elastography > 11-14 kPa, *n* = 2), according to World Health Organization Guidelines[20].

Comparison of HBV-DNA and RNA quasispecies using NGS

The qPCR verification of residual HBV-DNA elimination from HBV-RNA isolations confirmed the absence of HBV-DNA from DNAse I-treated RNA samples, while showing the presence of cDNA after their reverse transcription. An illustrative example is shown in Figure 1. No HBV-DNA contamination was therefore detected in cDNA samples derived from HBV-RNA quasispecies.

After NGS using the MiSeq platform, Fastq files obtained were filtered by our bioinformatics procedure to obtain the set of haplotypes common to forward and reverse strands. Before subsequent filtering by abundance, those haplotypes represented a total of 4.35×10^6 sequence reads with a median of 2.45×10^5 reads/sample

Table 1 Clinical and viral characteristics of chronic hepatitis B patients enrolled in the study

	Total, *n* = 13
Age, yr, median (IQR)	41.35 (31.7-47.2)
Male, *n* (%)	11 (84.6)
Genotype, *n* (%)	
A	3 (23.1)
C	3 (23.1)
D	2 (15.4)
E	2 (15.4)
F	3 (23.1)
ALT, IU/L, median (IQR)	124 (66-160)
HBeAg +ve, *n* (%)	11 (84.6)
HBV DNA, Log_{10} IU/mL, median (IQR)	8 (8-8.43)
HBV RNA, Log_{10} copies/mL, median (IQR)	5.18 (4.94-6.02)
Fibrosis, *n* (%)[1]	
Nonsignificant	8 (61.64)
Significant	3 (23.08)
Cirrhosis, *n* (%)	2 (15.39)

[1]Nonsignificant fibrosis indicates Ishak fibrosis stage < 3, significant fibrosis F ≥ 3 and < 5, and cirrhosis F5-6. Nonsignificant fibrosis by noninvasive markers indicates liver stiffness < 7-8.5 kPa, significant fibrosis > 7-8.5 and < 11-14 kPa and cirrhosis > 11-14 kPa by transient elastography according to World Health Organization Guidelines for the prevention, care and treatment of persons with chronic hepatitis B infection criteria[20].
F: Fibrosis; IQR: Interquartile range; ALT: Alanine aminotransferase; HBeAg +ve: Hepatitis B e-antigen positive; HBsAg: Hepatitis B virus surface antigen; HBV: Hepatitis B virus.

(IQR, 1.90 × 10^5-3.19 × 10^5) for HBV-RNA and 8.27 × 10^4 reads/sample (IQR, 6.03 × 10^4-1.01 × 10^5) for HBV-DNA and were included in the RHL analysis. By setting the threshold of abundance as equal to or less than 1% HBV quasispecies complexity showed a heterogeneous behavior between patients, and although mean RHL was greater in DNA (45.45 ± 4.80) than in RNA (42.50 ± 5.63), the difference was not significant (P = 0.1641, t test) (Figure 2). Similarly, no statistically significant differences were observed when comparing HBV-DNA and RNA quasispecies in the patients with nonsignificant fibrosis (46.57 ± 4.79 for HBV-DNA and 43.88 ± 6.32 for HBV-RNA, P = 0.4642, Kruskal-Wallis test), and in the patients with significant fibrosis or cirrhosis (43.66 ± 4.72 for HBV-DNA and 40.29 ± 3.92 for HBV-RNA, P = 0.3055, Kruskal-Wallis test).

After RHL calculation, we proceeded to eliminate all haplotypes with abundances below 0.25%, obtaining 2.91 × 10^6 sequence reads with a median of 1.58 × 10^5 reads/sample (IQR, 1.29 × 10^5-2.28 × 10^5) for HBV-RNA and 5.66 × 10^4 reads/sample (IQR, 3.2 × 10^4-5.76 × 10^4) for HBV-DNA. These haplotypes were the basis for conservation analysis through IC calculation position by position in both HBV-RNA and DNA quasispecies (Figure 3). Differences in sequence conservation between the 2 quasispecies were determined by subtracting IC DNA values from IC RNA of 357 nt positions analyzed; between nt 1255-1611. In 218 (61.06%) of these positions, IC values of both HBV-RNA and DNA quasispecies were coincident, while in the remaining 139 (38.93%) nt positions differences between IC of both quasispecies ranged from -0.26 to 0.23, with IC DNA > IC RNA in most of them (102/139, 73.38%) (Figure 4A). These differences were also studied by sliding window analysis of the mean IC RNA-IC DNA values (calculated in windows of 25 nt positions displaced in steps of 1 position between them) confirming that, in general, the HBV-RNA quasispecies was slightly less conserved than the DNA quasispecies (Figure 4B). In keeping with this analysis, HBV-RNA displayed more positions with some variability (IC < 2 bits), 135/357 (38%), than HBV-DNA, 85/357 (24%) (P < 0.01, two-proportions z-test).

The high degree of similarity between IC values in both HBV-DNA and HBV-RNA quasispecies was also observed in the sliding window analysis of IC in the multiple

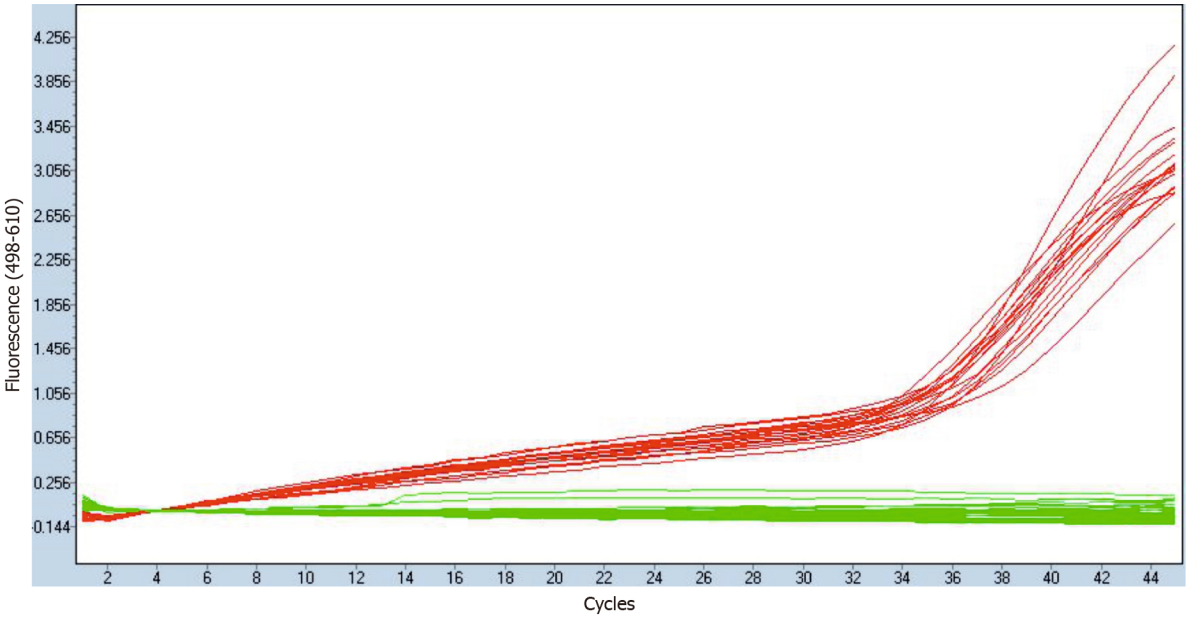


Figure 1 Quantitative polymerase chain reaction verification of residual hepatitis B virus-DNA elimination from hepatitis B virus-RNA isolations after DNAseI treatment. Fluorescence through quantitative polymerase chain reaction amplification samples is shown as green lines for DNAse I-treated RNA samples, and as red lines for their respective cDNA retrotranscribed samples.

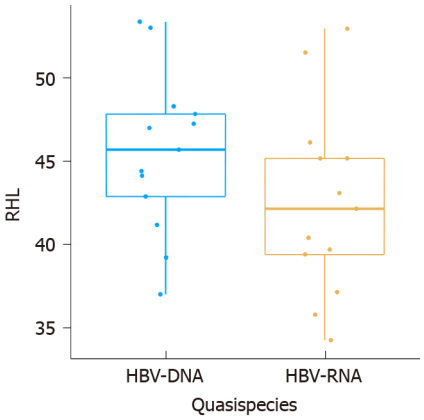


Figure 2 Comparison of mean hepatitis B virus quasispecies complexity. Hepatitis B virus (HBV) quasispecies complexity analyzed by Rare Haplotype Load of all 13 patients in HBV-DNA quasispecies (blue-framed boxes) and HBV-RNA quasispecies (yellow-framed boxes). Differences were statistically non-significant (P = 0.1641, t test). RHL: Rare Haplotype Load; HBV: Hepatitis B virus.

alignments of haplotypes obtained in both quasispecies. Concatenation of the 5% of windows with higher mean IC values (the most conserved), yielded 4 sequence logos in both quasispecies 3 (75%) of which coincided between both quasispecies: nts 1258-1286 (1283 in RNA quasispecies), 1545-1573 and 1575-1604 (Figure 5). In addition, sliding window analysis also showed the sequence stretch 1519-1543 to be hyper-conserved only in HBV-DNA quasispecies and 1559-1587 only in HBV-RNA quasispecies. Regarding the 5% of windows with lower IC values (the most variable), 2 sequence logos, which coincided in both quasispecies, were obtained: nts 1311-1344 and 1461-1485 (Figure 6). Thus, conservation was highly coincident between HBV-RNA and DNA quasispecies although, interestingly, HBV-RNA quasispecies showed slightly higher variability than HBV-DNA quasispecies.

DISCUSSION

Serum HBV RNA results from a mixture of different viral RNA species: Intact and spliced pgRNA, and truncated and polyA-free RNAs, with varying levels depending on the phase of HBV infection and antiviral treatment[21]. In addition, *HBX* transcripts

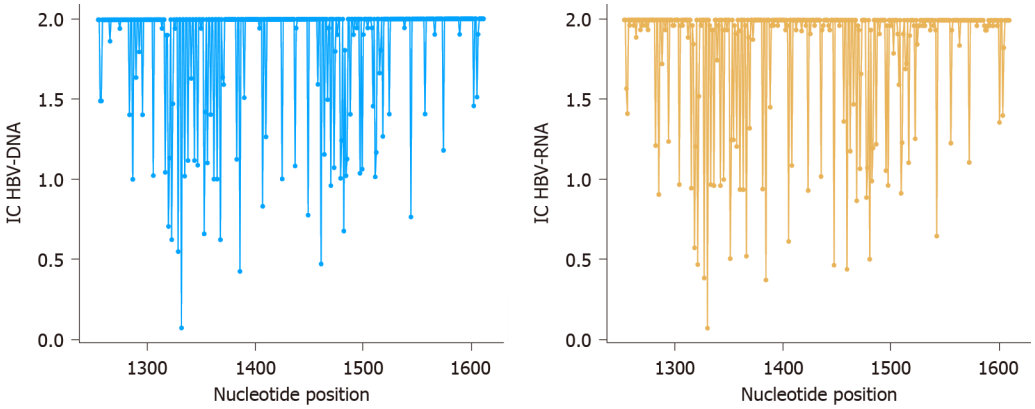


Figure 3 Conservation and variability of 357 nucleotide positions analyzed. Information content of nucleotide positions from 1255 to 1611 for hepatitis B virus (HBV)-DNA (blue lines) and HBV-RNA (orange lines) quasispecies. IC: Information content; HBV: Hepatitis B virus.

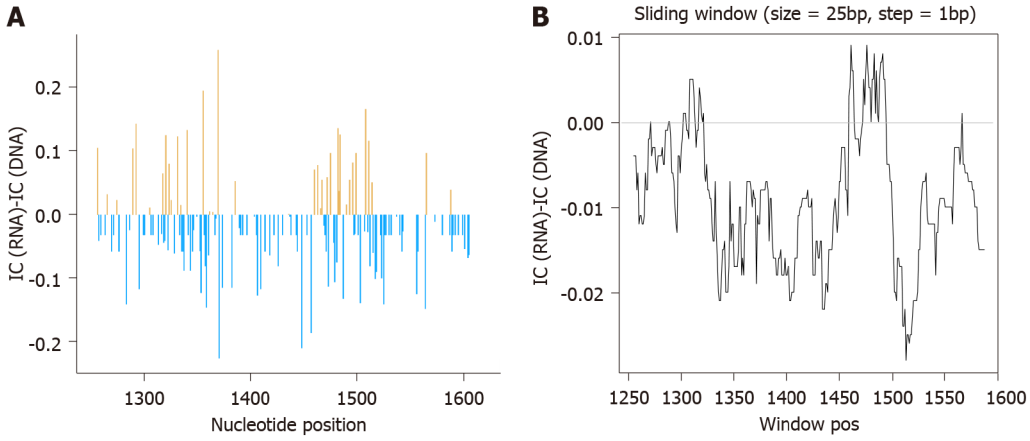


Figure 4 Differences between information content of hepatitis B virus-DNA and RNA quasispecies. A: Nucleotide positions in which information content (IC) hepatitis B virus (HBV)-RNA > HBV-DNA are depicted in orange while positions where IC HBV-DNA > HBV-RNA in blue; B: Sliding window analysis of the subtraction of mean IC RNA-IC DNA values, in windows of 25 nucleotide positions, displaced in steps of 1 position between them. IC: Information content.

have also been detected[3]. This serum HBV RNA has been suggested as a potential surrogate marker both to reflect intrahepatic cccDNA levels during the natural course of CHB infection[22,23] and under NA treatment[4], and to predict treatment outcome [4,21] such as HBeAg seroconversion[5-7]. During antiviral treatment, the kinetics of serum HBV-DNA and HBV-RNA seem to be dissociated. While serum HBV-DNA levels rapidly decline after starting treatment, HBV-RNA levels fall more slowly and the HBV-DNA/HBV-RNA ratio increases significantly. This allows inference in cccDNA transcription in the absence of detectable serum HBV DNA and may predict viral rebound after NA cessation[8]. In addition, the slower decline of serum HBV RNA levels may also make it possible to study circulating viral quasispecies, even when serum HBV-DNA levels are too low to do so. However, few studies to date have analyzed circulating HBV-RNA quasispecies[4,6] and little data is therefore available on their similarities and differences. Indeed, it is reasonable to think that both quasispecies may display significant differences between them, as they are subjected to different sources of genetic variability, due to the error-prone viral polymerase, contributing to an estimated error rate of approximately 1 per 10^4-10^5 bp for minus-strand DNA synthesis and approximately 1 per 3.6-15 × 10^4 bp for second-strand DNA synthesis[24]. Hence, reverse transcription becomes an outstanding source of variability in new HBV-DNA virion formation. However, HBV-RNA quasispecies are not affected by this source of variability.

The first aim of this study was to establish a reliable methodology to thoroughly analyze serum HBV-RNA quasispecies, without interference from HBV-DNA quasispecies. Previous studies have found RNA levels of between 0.8 and 2.8 Logs lower than DNA levels[5,25,26]. Although we measured HBV-DNA and RNA levels with different units (IU/mL for DNA and copies/mL for RNA) in this study, the results appear to be in line with those previous studies. Even if HBV-DNA is present at low

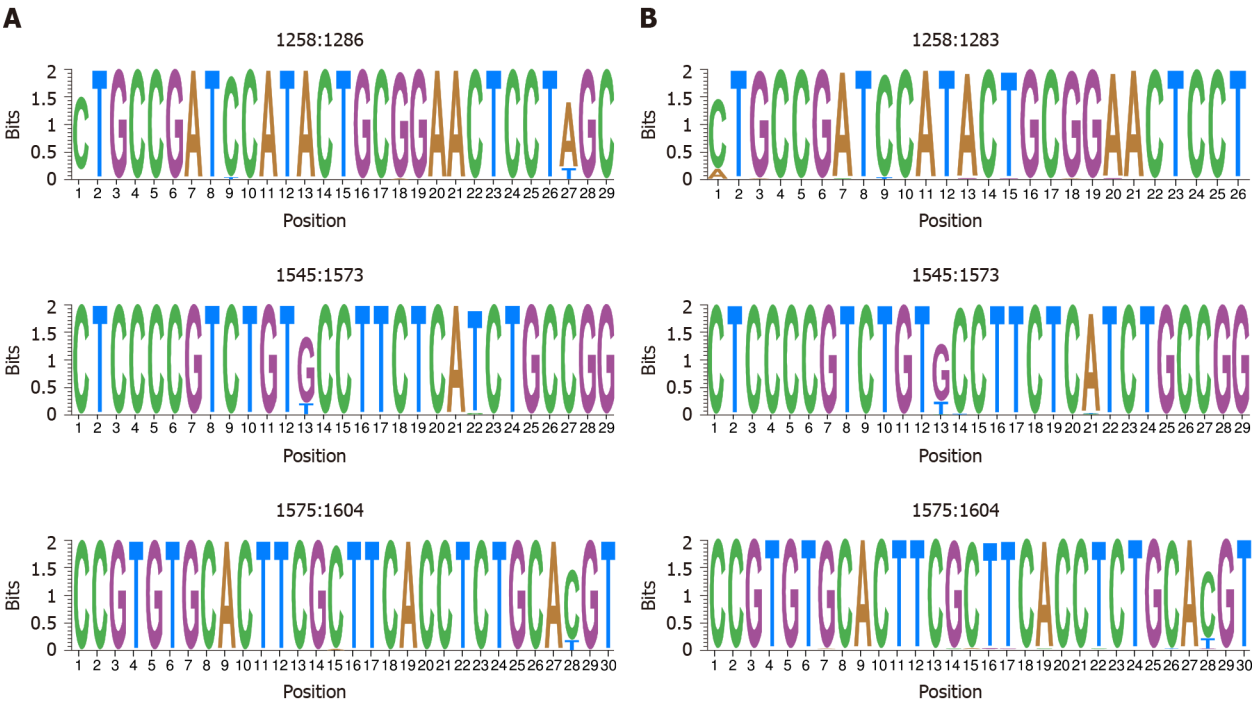


Figure 5 Representation by sequence logos of the information content of the most conserved regions in hepatitis B virus-DNA and hepatitis B virus-RNA quasispecies. The relative sizes of the letters in each stack, each of them representing a nucleotide (nt) position, indicate their relative frequencies at each position within the multiple alignments of nt haplotypes. The total height of each stack of letters depicts the IC of each nt position, measured in bits (Y-axis), therefore 0 bits is the minimum and 2 the maximum conservation. A: Hepatitis B virus-DNA; B: Hepatitis B virus-RNA.

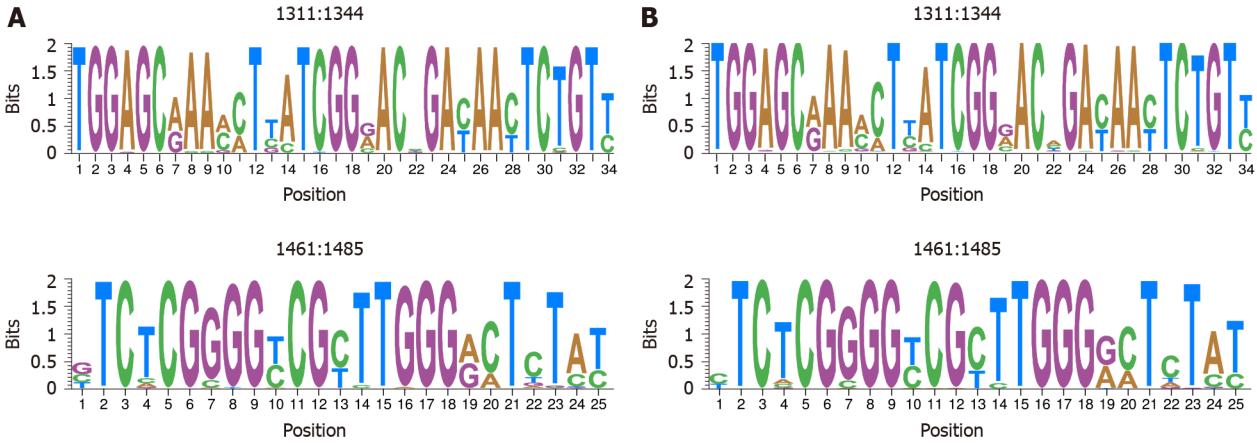


Figure 6 Representation by sequence logos of the information content of the most variable regions in hepatitis B virus-DNA and hepatitis B virus-RNA quasispecies. The relative sizes of the letters in each stack, each of them representing a nucleotide (nt) position, indicate their relative frequencies at each position within the multiple alignments of nt haplotypes. The total height of each stack of letters depicts the IC of each nt position, measured in bits (Y-axis), therefore 0 bits is the minimum and 2 the maximum conservation. A: Hepatitis B virus-DNA; B: Hepatitis B virus-RNA.

levels in patient serum, amplicons derived from it may bias NGS-obtained results to some extent, taking into account the high sensitivity of this technology. Thus, to ensure that results obtained from HBV-RNA quasispecies amplification procedure are free from HBV-DNA contamination, we pre-treated RNA isolates with DNase I, and verified the HBV-DNA elimination from these isolates by means of a qPCR assay. This verification step allowed us to confirm that no residual HBV-DNA was present in the HBV-RNA isolates, making it unlikely that reads obtained from HBV-RNA libraries were actually derived from serum HBV-DNA amplification.

Since viral polymerase errors are considered an important source of genetic variability for the HBV genome, we considered RHL to be a diversity index especially suitable for comparing complexities between HBV RNA and DNA quasispecies and assessing the effect of viral polymerase activity on the latter. This is because this

diversity index refers to the fraction of the quasispecies with the lowest fitness, indicative of the intensity with which replication errors accumulate. High viremia and long infection times would tend to result in high RHL values. High values may also be given to mutagen treatments[17]. In our study, HBV-DNA quasispecies showed a higher mean RHL than RNA quasispecies, which suggests an increased presence of low frequency HBV genomes in DNA quasispecies, probably due to the error-prone reverse-transcription origin of HBV-DNA. However, these differences were not statistically significant, either when including all patients or when separating them according to their degree of fibrosis (nonsignificant fibrosis and significant fibrosis or cirrhosis). The RHL shows high correlation with Shannon entropy, an abundance diversity index[17], which was used in a recent study by Yu *et al*[6] to compare HBV-DNA and RNA quasispecies in sequential serum samples in a group of HBeAg+ve patients receiving entecavir or pegylated interferon. Similarly to our results, no significant difference was found between HBV RNA and HBV DNA quasispecies complexity at baseline quasispecies. While the results were similar, we analyzed a different region of the HBV genome (the region of the *HBX* between nt 1255 to 1611) than the study by Yu *et al*[6] (a sequence stretch within the region encoding for the terminal protein domain of the viral polymerase), and our group of patients is more heterogeneous, especially in terms of viral genotype composition. Thus, results from the 2 studies are not directly comparable, and further studies in larger groups of patients are required to confirm the tendency of HBV DNA quasispecies toward higher quasispecies complexity, as shown by mean RHL values.

Comparison of both HBV-DNA and RNA quasispecies conservation yielded similar results, with HBV-DNA slightly more conserved than HBV-RNA despite the effect of reverse-transcription over DNA quasispecies. This observation may indicate that only a fraction of packaged pgRNA is reverse-transcribed and released. In addition, in this study, we identified 3 hyper-conserved regions which coincided in both HBV-RNA and HBV-DNA quasispecies. The first hyper-conserved region identified was between nt 1258-1286, while the second hyper-conserved region consisted of 2 nt fragments (1545-1573 and 1575-1604) spanning a region between nt 1545-1604. Interestingly, these 3 conserved sequence fragments almost overlapped with hyper-conserved sequence fragments described in a previous study carried out by our group at circulating DNA quasispecies level (1255-1286, 1545-1573 and 1575-1603)[13]. Likewise, a recently published study by our group[14] also performed in HBV-DNA quasispecies, reported some of these hyper-conserved regions (1258-1286 and 1575-1605). In addition, these 2 previous studies[13,14] also reported as hyper-conserved the sequence stretch between positions 1519-1543, which we found among the 5% most conserved windows at HBV-DNA quasispecies level but not at HBV-RNA quasispecies level. Conversely, we identified the sequence stretch 1559-1587 as hyper-conserved only in HBV-RNA quasispecies. This fragment was not identified in our previous studies, although it is included in the region between nt 1519 and 1603 defined by 3 conserved nt fragments (1519-1543, 1545-1573, and 1575-1603) described in one of them[13]. Thus, despite the high degree of similarity between sequence conservation in HBV-DNA and RNA quasispecies, this may follow different trends in some sequence stretches. Finally, assessment of the most variable windows (the 5% with the lowest mean IC values) identified the fragments between nt 1311-1344 and 1461-1485 as the most variable in both DNA and RNA quasispecies, similar to the most conserved stretches identified, which confirmed the similarities between both quasispecies.

Given the important and wide role of hepatitis B X protein (HBx), a multifunctional transactivator protein encoded by the *HBX* gene, in the replication of HBV, particularly in intrahepatic cccDNA stability and transcription, it is an interesting target for new therapies to treat CHB-infected patients[8]. Moreover, HBx is located next to the 3′ end of all the HBV mRNAs, which means that targeting its sequence may interfere in the synthesis of all the viral proteins[13]. This concept is in line with other strategies aiming for functional cure. Thus, identifying highly conserved regions may suggest valuable targets for pan-genotypic approaches with minimum likelihood of antiviral drug resistance. With this in mind, we analyzed a region of the *HBX* gene where we had previously identified hyper-conserved regions in the circulating HBV-DNA quasispecies[13,14], which we intended to confirm in circulating HBV-RNA. In fact, serum HBV-RNA was found to be genetically homogenous with intrahepatic HBV-RNA[4] the main target for this kind of antiviral therapies. Therefore, given that some sequence fragments may follow different conservation trends in both HBV-DNA and RNA quasispecies, when looking for targets for directed gene therapy, it may be worth verifying their conservation not only at serum HBV-DNA level but also at RNA level. This would make it possible to ensure their effect on their main target. In this study, we confirmed that most of the hyper-conserved regions identified by our group in

previous studies at DNA level[13,14] coincided with those identified in serum HBV-RNA, and may thus also be conserved at intrahepatic HBV-RNA level.

The broad range of HBV genotypes and degrees of severity of liver disease from all patients included in the present study enabled to get an overview of complexity and conservation of HBV-DNA and RNA quasispecies. Nevertheless, the main limitation of the present study was that the comparison with this small and heterogeneous group of patients just enabled to take a preliminary picture of HBV-DNA and RNA quasispecies, which needs to be complemented with further studies exploring both of them in larger and more homogeneous groups of patients in terms of severity of liver disease and CHB clinical stage, in order to verify whether the present results could be extrapolated to any stage of the disease. In addition, the implications of HBV quasispecies parameters analyzed in the context of both treatment and prognosis of CHB are still uncertain. Thus, longitudinal follow-up studies of circulating HBV-RNA quasispecies evolution are necessary to assess its adaptation to different evolutive pressures and to compare with that of HBV-DNA. In this way, much more robust conclusions regarding the comparison between both quasispecies could be drawn. Anyway, the results obtained demonstrate that the methodology established in the present study allows detailed analysis of serum HBV-RNA quasispecies, being a useful tool to perform those studies. Finally, *in vitro* functional studies should be performed to test the potential usefulness of the hyper-conserved regions described here as potential candidates for targeted gene therapy. Gene silencing could be a valuable strategy and *HBX* gene, thanks to its co-terminal localization, an optimal target. These hyper-conserved sequences were highly coincident with the findings of the present study[13,14]. Based on data obtained in those previous studies we have already tested several proposals about gene therapy based on antisense locked nucleic acid Gapmer and small interference RNA (siRNA)[27,28].

CONCLUSION

In summary, we developed a methodology for analyzing serum HBV-RNA quasispecies without HBV-DNA interference. By including patients with different clinical and virological characteristics we have been able to make a preliminary comparison of complexity and conservation of HBV-DNA and RNA quasispecies. Complexity analysis by the RHL index indicated an increased presence of low frequency HBV genomes in DNA quasispecies, which should be verified in larger groups of patients.

Interestingly, although HBV-DNA and RNA quasispecies showed a similar degree of conservation in the *HBX* 5′region, HBV-RNA quasispecies tended toward higher variability than HBV-DNA. Most hyper-conserved and hyper-variable regions identified were almost overlapped between HBV-DNA and RNA, and were highly coincident with our previous findings. However, we observed different conservation trends between both quasispecies in some sequence fragments, suggesting that serum HBV-RNA quasispecies should also be considered when looking for targets for directed gene therapy.

ARTICLE HIGHLIGHTS

Research background

Recent studies show that hepatitis B virus (HBV)-RNA detected in serum is mainly encapsidated pregenomic RNA (pgRNA), which could be a useful biomarker for monitoring covalently closed circular DNA activity. During nuclos(t)ide analogs (NA) therapy it is predictive of hepatitis B e-antigen seroconversion and for following viral rebound after treatment cessation. However, few studies have analyzed the serum HBV-RNA quasispecies, a complex mutant spectrum constituted by closely related, but not identical, viral populations. Analysis of serum circulating HBV-RNA quasispecies in those previous studies showed no significant difference between HBV-RNA and HBV-DNA quasispecies complexity before antiviral treatment and indicated that serum HBV-RNA is genetically homogenous with intrahepatic HBV-RNA, which is the main target for targeted gene therapy. Such therapies should be directed to hyper-conserved sequence targets, ideally located at hepatitis B X gene (*HBX*), located next to the 3′ end of all the HBV mRNAs.

Research motivation

Composition and evolution over time of HBV quasispecies is closely linked to liver disease progression, and serum circulating HBV-RNA is potentially useful for its analysis, even in situations with low or undetectable serum level of HBV-DNA. However, HBV-RNA has not been subjected to reverse transcription, which is the main source of variability in the HBV genome and it may therefore present significant differences with respect to the serum HBV-DNA quasiespecies. Nonetheless, little data is available on the comparison of genetic variability/conservation and complexity of both quasispecies. Moreover, analysis of serum HBV-RNA quasispecies may be a very useful tool when looking for hyper-conserved targets for targeted gene therapy, due to its similarities with intrahepatic HBV-RNA, the main target of this kind of antiviral therapy. Thus, in previous studies, we identified hyper-conserved sequence stretches in the circulating HBV-DNA quasispecies, which we aimed to analyze at the circulating HBV-RNA level. Studying circulating HBV-RNA quasispecies may thus favor achieving functional cure of HBV infection. However, it is necessary to establish a reliable methodology to thoroughly analyze this quasispecies, without interference from serum HBV-DNA.

Research objectives

This study aimed to establish a methodology to achieve an in-depth analysis serum HBV-RNA quasispecies without interference from circulating HBV-DNA, using next-generation sequencing (NGS). With this methodology, we aimed to compare both serum HBV-RNA and DNA quasispecies in a group of untreated chronic hepatitis B (CHB) patients. Considering the potential of HBV-RNA for analyzing HBV quasispecies, even in situations of low or undetectable serum HBV-DNA, this thorough analysis may serve as prognostic factor for clinical follow-up of CHB patients. Furthermore, it may be a useful tool for looking for hyper-conserved targets for targeted gene therapy.

Research methods

Serum samples were taken from 13 untreated CHB patients attending the outpatient clinic of Vall d'Hebron University Hospital (Barcelona, Spain). HBV-DNA levels > 5 $\log_{10}$ IU/mL, HBV-RNA levels > 4 Log_{10} copies/mL and heterogeneity in terms of HBV genotypes and degrees of severity of liver disease were considered as inclusion criteria. HBV-RNA and DNA were extracted differently using specific manual isolation protocols. In addition, HBV-RNA, which was quantified by an in-house method, was treated with DNAse I (Life Technologies, Austin, United Ststes) to remove any residual DNA present. The elimination of residual DNA after DNAse I digestion was verified by means of quantitative PCR (qPCR). In parallel, these samples were retrotranscribed into cDNA, and along with DNA isolates the 5' end region of *HBX*, between nucleotides 1255-1611 (the amplicon analysed), was amplified by a 3-nested PCR protocol and later sequenced using NGS (MiSeq, Illumina, United States). HBV-RNA and DNA quasispecies complexity was evaluated using Rare Haplotype Load (RHL) index, which measures enrichment of quasispecies in minority genomes. Sequence conservation and variability was determined by calculating the information content (IC) of each position by aligning all unique sequences covering the full amplicon (*i.e.*, haplotypes) in a multiple alignment. After this analysis, the most conserved and variable sequence stretches were represented as sequence logos, which were compared between both quasispecies.

Research results

After treatment of RNA isolates with DNase I, we confirmed that no residual HBV-DNA was present in the HBV-RNA isolates and that contamination by HBV-DNA in sequences obtained from HBV-RNA was therefore unlikely. HBV quasispecies complexity showed heterogeneous behavior among patients. While RHL was greater in DNA than in RNA quasispecies, differences were not statistically significant. This tendency of HBV DNA quasispecies toward higher quasispecies complexity than HBV-RNA needs to be studied further in larger groups of patients. In general, conservation was highly coincident between HBV-RNA and DNA quasispecies; the majority of nt positions showed the same IC value in both of them. Interestingly, HBV-RNA quasispecies was slightly less conserved than DNA and displayed more positions with some variability (IC < 2 bits). Sliding window analysis in HBV-DNA and RNA quasispecies showed 4 sequence fragments as the most conserved in each of them. Of those fragments 3 coincided between both quasispecies, but 1 was found to be among the most conserved only in HBV-DNA and 1 only in HBV-RNA. The most variable

sequence fragments coincided in both quasispecies.

Research conclusions

In this study, we describe a methodology for analyzing serum HBV-RNA quasispecies without HBV-DNA interference. This methodology allowed us to compare HBV-DNA and RNA quasispecies. For quasispecies complexity, we analyzed the RHL index, a new reliable diversity index to diagnose mutant spectrum expansions, such as may have occurred with the error-prone reverse transcription of HBV-RNA into DNA. However, although we detected a tendency to greater quasispecies complexity in HBV-DNA than in RNA, the differences were statistically non-significant, which may indicate an increased presence of low-frequency HBV genomes in DNA quasispecies. For this reason, we suggest that further studies in larger groups of patients be performed to confirm this observation. We also found a highly coincident conservation between HBV-RNA and DNA quasispecies in the *HBX* 5′ region. Interestingly, HBV-RNA quasispecies showed slightly higher variability than HBV-DNA quasispecies, which may indicate that only a fraction of packaged pgRNA is reverse-transcribed and released. The hyper-conserved sequences identified were highly coincident to what was observed in previous studies by our group in HBV-DNA quasispecies. Nevertheless, we observed that some sequence fragments were differently conserved between HBV-DNA and RNA quasispecies, which suggest that serum HBV-RNA quasispecies should also be considered when looking for targets for directed gene therapy, specially taking into account the similarities between serum and intrahepatic HBV-RNA.

Research perspectives

In this study, data were obtained from patients with a broad range of HBV genotypes and degrees of severity of liver disease. This allowed us to make a preliminary comparison of complexity and conservation of HBV-DNA and RNA quasispecies. However, further studies with a larger sample size are warranted to confirm our results. The main goal of the methodology for HBV-RNA quasispecies described here is to use it in groups of patients where HBV-DNA levels are usually too low for PCR amplification (*e.g.*, patients under NA treatment, since the generation of HBV-RNA is not inhibited by NA directly), in order to be able to monitor HBV quasispecies in those patients.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7159-7172

DOI: 10.3748/wjg.v27.i41.7159

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

ORIGINAL ARTICLE

Retrospective Cohort Study

Short-term and long-term outcomes of laparoscopic *vs* open ileocolic resection in patients with Crohn's disease: Propensity-score matching analysis

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Author contributions: Pak SJ and Kim YI contributed equally to this article; Pak SJ contributed to data acquisition, analysis, and interpretation, and wrote the paper; Yoon YS, Lee JL, Kim YI, and Yu CS generated the data; Lee JB analyzed the data; Yoon YS had the original idea for the paper and provided critical comments, Yoon YS and Kim YI revised the manuscript; and all authors approved the final version of the manuscript.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Asan Medical Center (IRB No. 2019-0972).

Informed consent statement: Informed consent was waived due to the retrospective nature of this study.

Conflict-of-interest statement: This study has no conflict of interest.

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Abstract

BACKGROUND

Laparoscopic ileocolic resection (LICR) is the preferred surgical approach for primary ileocolic Crohn's disease (CD) because it has greater recovery benefits than open ICR (OICR).

AIM

To compare short- and long-term outcomes in patients who underwent LICR and OICR.

METHODS

Patients who underwent ICR for primary CD from 2006 to 2017 at a single tertiary center specializing in CD were included. Patients who underwent LICR and OICR were subjected to propensity-score matching analysis. Patients were propensity-score matched 1:1 by factors potentially associated with 30-d perioperative morbidity. These included demographic characteristics and disease- and treatment-related variables. Factors were compared using univariate and multivariate analyses. Long-term surgical recurrence-free survival (SRFS) in the two groups was determined by the Kaplan-Meier method and compared by the log-rank test.

RESULTS

During the study period, 348 patients underwent ICR, 211 by the open approach and 137 laparoscopically. Propensity-score matching yielded 102 pairs of patients. The rate of postoperative complication was significantly lower (14% *versus* 32%, P = 0.003), postoperative hospital stay significantly shorter (8 d *versus* 13 d, P =

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

Country/Territory of origin: South Korea

Specialty type: Gastroenterology and hepatology

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

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Received: March 16, 2021
Peer-review started: March 16, 2021
First decision: May 1, 2021
Revised: May 8, 2021
Accepted: October 18, 2021
Article in press: October 18, 2021
Published online: November 7, 2021

P-Reviewer: Brisinda G, Shi Y
S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ



0.003), and postoperative pain on day 7 significantly lower (1.4 *versus* 2.3, $P < 0.001$) in propensity-score matched patients who underwent LICR than in those who underwent OICR. Multivariate analysis showed that postoperative complications were significantly associated with preoperative treatment with biologics [odds ratio (OR): 3.14, $P = 0.01$] and an open approach to surgery (OR: 2.86, $P = 0.005$). The 5- and 10-year SRFS rates in the matched pairs were 92.9% and 83.3%, respectively, with SRFS rates not differing significantly between the OICR and LICR groups. The performance of additional procedures was an independent risk factor for surgical recurrence [hazard ratio (HR): 3.28, $P = 0.02$].

CONCLUSION
LICR yielded better short-term outcomes and postoperative recovery than OICR, with no differences in long-term outcomes. LICR may provide greater benefits in selected patients with primary CD.

Key Words: Crohn's disease; Laparoscopic; Surgery; Postoperative complications; Recurrence; Propensity score; Retrospective study

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Core Tip: The laparoscopic approach to ileocolic resection can be safely performed in patients with primary Crohn's disease (CD), resulting in fewer postoperative complications, faster postoperative recovery, and non-inferior surgical recurrence rate when compared with open surgery. Postoperative complications were significantly associated with preoperative use of biologics and open ileocolic resection. Additional procedures were found to be independent risk factors for surgical recurrence in patients with CD.

Citation: Pak SJ, Kim YI, Yoon YS, Lee JL, Lee JB, Yu CS. Short-term and long-term outcomes of laparoscopic *vs* open ileocolic resection in patients with Crohn's disease: Propensity-score matching analysis. *World J Gastroenterol* 2021; 27(41): 7159-7172
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7159.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7159

INTRODUCTION

Ileocolic resection (ICR) is the most frequently performed operation for patients with abdominal Crohn's disease (CD) with involvement of the terminal ileum. Since the introduction of laparoscopic colectomy in 1991, experience with laparoscopic ICR (LICR) for CD has increased[1,2]. LICR has become the preferred surgical approach for primary ileocolic CD because it shows greater recovery benefits than open ICR (OICR). These benefits include reduced pain, lower rates of overall morbidity, shorter hospital stay, earlier return to full activity, lower costs, and improved quality of life and cosmesis compared with OICR[1-7].

Conventionally, patients with penetrating type or complex CD have not been candidates for laparoscopic surgery, with open surgery remaining the generally accepted approach for these patients. The laparoscopic approach is regarded as more technically challenging than open surgery in CD patients with complex features, including huge phlegmons, multiple enteric fistulas, and dense adhesions, as well as those requiring repeated surgery[8]. In addition, patients with complex CD have higher rates of host related risks, such as homeostasis disturbance, infection, and severe malnutrition prior to surgery caused by external or internal fistulas[9]. Therefore, utilization of LICR in patients with complex CD remains problematic.

In South Korea, the number of laparoscopic operations in CD patients has increased dramatically, from 11.6% in 2009 to more than 31% in 2015[10]. Although our institution is the highest volume center for CD in South Korea, laparoscopic surgery for CD was conservative, with performance increasing since 2014.

The present study compared the short- and long-term outcomes of LICR and OICR in patients with primary CD over a 12-year period. To overcome possible selection bias, patients in the two groups were analyzed after propensity-score matching.

MATERIALS AND METHODS

Patients and clinical variables

Patients who underwent LICR or OICR for primary CD at Asan Medical Center in Seoul, Korea, from January 2006 to December 2017, were retrospectively identified. Ileocolic resection included patients undergoing resection of the ileum and colon with ileocolic anastomosis within the right/transverse colon. Patients who underwent previous bowel resection for CD, those without anastomosis, those with ileocolic anastomosis distal to the transverse colon, and patients with missing data or loss to follow-up were excluded (Figure 1). Patients who initially underwent laparoscopic surgery but required conversion to open surgery were included in the intention-to-treat analysis. Factors recorded from patients' electronic medical records and charts included demographic characteristics, preoperative disease characteristics, operative details, and perioperative outcomes. Demographic characteristics included patient sex, age at time of surgery, body mass index (BMI), duration of disease from the time of diagnosis, smoking history, and comorbidities. Preoperative disease characteristics included Montreal classification at the time of surgery[11], extra-intestinal manifestations of disease, family history of CD, history of perianal CD, previous history of abdominal surgery, and specific history of intestinal resection for CD. Operative details included intraoperative findings from operation reports (adhesions, strictures, intestinal fistulas, abscesses, phlegmons), indications for surgical intervention, conversion to open surgery, diverting stoma, estimated blood loss, intraoperative red blood cell (RBC) transfusion, operation time, urgent surgery, and American Society of Anesthesiologists (ASA) score. Perioperative outcomes included intraoperative and postoperative morbidity and mortality within 30 d after surgery, readmission, reoperation, length of hospital stay, pain scale, and time to recovery of bowel function [12]. Additional variables included preoperative hemoglobin and albumin levels, preoperative CD medications, anastomosis configuration (side-to-side, end-to-side, side-to-end, or end-to-end), type of anastomosis (stapled or hand-sewn), and synchronous additional surgical procedures. Postoperative morbidity was graded according to the Clavien-Dindo classification. The study protocol was approved by the Institutional Review Board of Asan Medical Center (approval number: 2019-0972).

Definitions

Comorbidities included hypertension, diabetes mellitus, and others. Major intraoperative bleeding was defined as intraoperative hemorrhage reported in operation notes and requirement for transfusion of packed RBCs. However, postoperative transfusion without a need for surgical intervention was not regarded as a postoperative complication. Anemia was defined as hemoglobin concentrations < 11.5 g/dL in women and < 13 g/dL in men, and hypoalbuminemia was defined as albumin concentrations < 3.5 g/dL according to institutional guidelines. Details of additional, concomitant procedures were limited to intra-abdominal surgery, whereas perianal procedures were excluded. Recovery of bowel movement was defined as the day of first flatus.

Laparoscopic or open surgery was selected for each patient according to surgeon preference. A history of previous abdominal surgery with or without bowel resection was an important consideration when choosing the surgical method. Because patients with previous bowel resection were excluded, multiple factors such as age, general condition, and disease extent were taken into consideration. Anastomosis configurations were classified as side-to-side, end-to-side, side-to-end, and end-to-end. Anastomosis materials were categorized as stapled and hand-sewn. ICR involves the removal of the ileocecal valve and was categorized as right colectomy or ileocecal resection depending on the involvement of the hepatic flexure of the colon.

Preoperative CD medications were divided into four categories: Systemic steroids, biologics [infliximab (Remicade®, Janssen Biotech, Inc., Horsham, PA, United States) or adalimumab (Humira®, AbbVie Inc. Chicago, IL, United States)], immunomodulators (azathioprine, 6-mercaptopurine, or methotrexate), and anti-inflammatory agents (5-aminosalicylate acid or budesonide). Preoperative treatment with steroids and anti-inflammatory agents was defined as the administration of each medication within 1 mo before surgery. Treatment with biologics was defined as the administration of at least one infusion of anti-TNF agents within 3 mo before surgery, whereas treatment with immunomodulators was defined as administration within 2 mo before surgery [5]. Immunosuppressive medications included steroids, biologics, and immunomodulators, but not anti-inflammatory agents.

Complications within the first 30 postoperative days included inadvertent intraoperative injury, anastomosis leak, fistula formation, prolonged postoperative ileus,

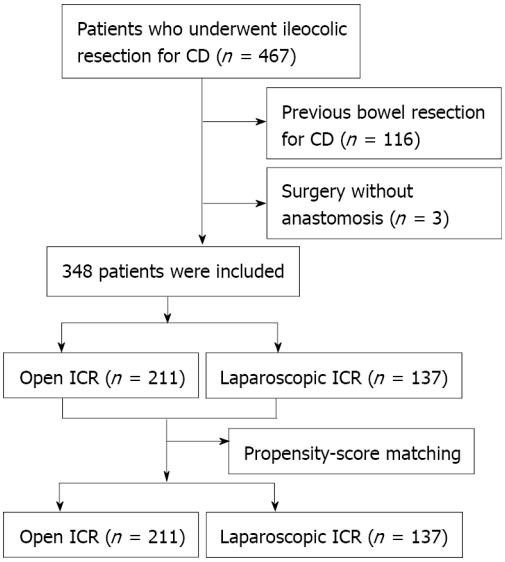


Figure 1 Flow chart of patient selection and propensity-score matching. CD: Crohn's disease; ICR: Ileocolic resection.

wound or intra-abdominal infection requiring antibiotics or drainage, readmission, or return to the operating room. Septic complications included anastomosis leakage, abdominal abscess, and resulting sepsis or septic shock. Ileus was defined as the absence of bowel function by postoperative day 5 and/or the need for nasogastric tube insertion due to abdominal distension, nausea, or vomiting, without evidence of mechanical bowel obstruction.

Surgical recurrence was defined as a repeat operation on any part of the bowel for pathologically confirmed CD or for pathologically confirmed anastomotic disease, including manifestations of the small bowel at the anastomosis or stoma site. Operations not performed to treat CD exacerbations (*e.g.*, adhesiolysis or stoma closure only) were not considered reoperations for surgical recurrence.

Propensity score matching analysis

To minimize the impact of selection bias for the surgical approach and potential confounding in this observational study, patients who underwent LICR and OICR were subjected to propensity-score matching, with rigorous adjustment for significant differences in patient characteristics. Propensity scores were estimated by multiple logistic regression analysis. All pre-specified covariates were included in the full non-parsimonious models. The covariates included demographic characteristics (age, gender, BMI, smoking history, previous history of abdominal surgery, previous history of comorbidity, and ASA score), disease-related variables (Montreal classification, disease duration, perianal CD, family history of CD and extra-intestinal CD manifestations), and treatment-related variables (preoperative hemoglobin and albumin concentrations, preoperative RBC transfusions, preoperative medications, and indications for surgery). These variables were selected because they can affect the choice of surgical approach and perioperative outcomes. The operative approach was entered into the regression model as a dependent variable. A 1:1 "nearest neighbor", case-control match without replacement was used. The discrimination and calibration abilities of the propensity-score model were 0.7332 by C-statistics and $P = 0.1219$ by Hosmer-Lemeshow statistics. Following propensity-score matching, short- and long-term results were compared in the two groups.

Statistical analysis

Continuous variables were reported as mean ± standard deviation (SD) or as median (min, max) and compared by *t*-tests or Wilcoxon rank sum tests, whereas categorical variables were reported as frequency (%) and compared by Pearson's χ^2 test or Fisher's exact test.

Univariate analyses were performed to assess the risk factors associated with postoperative complications and surgical recurrence. Multivariable models were created to identify factors independently associated with postoperative complications. Surgical recurrence-free survival (SRFS) was calculated using the Kaplan-Meier method and compared using log-rank tests. Multivariate analyses to assess the risk

Table 1 Patient demographic and clinical characteristics

	All patients			Propensity-score matched patients		
	Open (*n* = 211)	Laparoscopy (*n* = 137)	*P*	Open (*n* = 102)	Laparoscopy (*n* = 102)	SMD
Age (yr)	29.2 ± 9.7	29.2 ± 9.0	0.976	28.8 ± 9.0	28.6 ± 8.7	-0.026
Gender, male	153 (72.5)	95 (69.3)	0.546	78 (76.5)	72 (70.6)	-0.134
BMI (kg/m^2)	18.8 ± 3.2	18.9 ± 2.8	0.822	19.0 ± 3.3	18.9 ± 2.8	-0.041
Any smoking history	41 (19.4)	33 (24.1)	0.348	21 (20.6)	20 (19.6)	-0.025
Previous abdominal surgery	23 (10.9)	10 (7.3)	0.349	10 (9.8)	8 (7.8)	-0.069
Montreal classification						
Behavior			< 0.001			-0.044
Inflammatory (B1)	2 (0.1)	2 (1.5)		2 (2.0)	2 (2.0)	
Stricturing (B2)	38 (17.5)	52 (38.0)		30 (29.4)	26 (25.5)	
Penetrating (B3)	172 (82.0)	83 (60.6)		70 (68.6)	74 (72.5)	
Location			0.218			-0.060
Terminal ileal (L1)	68 (32.2)	50 (36.5)		36 (35.3)	37 (36.3)	
Colonic (L2)	10 (4.7)	2 (1.5)		6 (5.9)	2 (2.0)	
Ileocolic (L3)	133 (63.0)	85 (62.0)		60 (58.8)	63 (61.8)	
Disease duration (mo)	49.8 ± 50.9	68.1 ± 59.3	0.003	61.5 ± 57.4	67.0 ± 57.8	0.095
Perianal CD	103 (48.8)	57 (41.6)	0.226	45 (44.1)	45 (44.1)	0.000
Family history of CD	5 (2.4)	5 (3.6)	0.523	4 (3.9)	4 (3.9)	0.000
Extra-intestinal CD manifestation	31 (14.7)	18 (13.1)	0.754	13 (12.7)	10 (9.8)	-0.093
Comorbidity	21 (10.0)	5 (3.6)	0.036	2 (2.0)	4 (3.9)	0.116
Hypertension	4 (1.9)	0 (0.0)	0.157	1 (1.0)	0 (0.0)	
Diabetes mellitus	2 (0.9)	1 (0.7)	1.000	0 (0.0)	1 (1.0)	
Others	15 (7.1)	5 (3.6)	0.239	1 (1.0)	4 (3.9)	
ASA score, 3-4	7 (3.3)	2 (1.5)	0.397	3 (2.9)	2 (2.0)	[2]
Emergency	17 (8.1)	9 (6.6)	0.680	6 (5.9)	7 (6.9)	0.040
Preoperative data						
Hemoglobin (g/dL)	11.5 ± 1.5	11.5 ± 1.9	0.780	11.4 ± 1.4	11.4 ± 1.8	0.037
Albumin (g/dL)	3.1 ± 0.5	3.2 ± 0.5	0.140	3.1 ± 0.5	3.2 ± 0.5	0.075
Transfusion	40 (19.0)	26 (19.0)	1.000	22 (21.6)	20 (19.6)	[2]
Preoperative medications						
Steroids	44 (20.9)	25 (18.2)	0.584	22 (21.6)	15 (14.7)	-0.179
Immuno-modulators	96 (45.5)	67 (48.9)	0.583	48 (47.1)	50 (49.0)	0.039
Biologics	29 (13.7)	26 (19.0)	0.229	17 (16.7)	16 (15.7)	-0.027
Indication for surgery			< 0.001			
[1]Fistula *versus* others	83 (39.3)	34 (24.8)	0.022	33 (32.4)	32 (31.4)	0.021
[2]Obstruction *versus* others	39 (18.5)	54 (39.4)	< 0.001	31 (30.4)	26 (25.5)	-0.042

[1]Fistula and obstruction were both selected in some patients.
[2]These variables were excluded from the propensity-score matched set because of the small numbers.
Results are reported as mean ± SD or as number (%). SMD: Standardized mean difference; BMI: Body mass index; CD: Crohn's disease; ASA: American Society of Anesthesiologists.

factors associated with SRFS were performed using Cox proportional hazards model with 95% confidence interval (CI). All statistical analyses were performed using the Statistical Package for the Social Sciences, version 24.0 for Windows (SPSS, IBM Corp., Armonk, NY, United States), with *P* values < 0.05 considered statistically significant.

RESULTS

Patient characteristics, matching, and operative details

A total of 467 patients underwent ICR for CD during the study period. Of these, 119 were excluded, including three patients without anastomosis or with ileocolic anastomosis distal to the transverse colon and 116 patients who had previously undergone bowel resection for CD. Of the 348 eligible patients, 211 underwent OICR and 137 underwent LICR. Patient characteristics before and after propensity-score matching are summarized in Table 1. Compared with patients who underwent OICR for CD, those who underwent LICR had significantly lower rates of penetrating behavior ($P < 0.001$) and comorbidities ($P = 0.036$), longer disease duration ($P = 0.003$), a higher rate of surgical indication of obstruction ($P < 0.001$), and a lower rate of fistula as a surgical indication ($P = 0.022$). Crohn's Disease Activity Index (CDAI) scores were compared between the two groups. LICR and OICR groups had moderate CDAI scores (230.8 ± 9.5 and 269.1 ± 10.8, respectively). Because the demographic data differed between the OICR and LICR groups, these patients were subjected to 1:1 propensity-score matching to reduce selection bias. A total of 102 pairs was therefore included in the propensity-score matched population.

A comparison of the propensity-score matched groups showed that the rate of intraoperative transfusion was significantly lower ($P = 0.017$), and the length of small bowel resection significantly shorter ($P < 0.001$), in the LICR than in the OICR group. Three patients (2.9%) in the OICR group, but none in the LICR group, underwent a diverting ileostomy. Although most patients in both groups underwent side-to-side anastomosis, end-to-side anastomosis was more frequently performed in the LICR than in the OICR group ($P = 0.014$). The open conversion rate in the LICR group was 4.9%. The reasons for open conversion were adhesions in two patients, huge phlegmons in two, and an abscess in one (Table 2).

Short-term outcomes

Of the 348 CD patients who underwent ICR, 75 (21.6%) experienced complications within 30 d, but none died. Of the 204 matched patients, 47 (23%) experienced postoperative complications, with 15 cases being classified as class III Clavien-Dindo complications. All six patients who required reoperation had undergone OICR, including three for anastomotic leakage and one each for an intra-abdominal abscess, luminal bleeding, and a surgical wound complication.

The overall complication rate was significantly lower in the LICR than in the OICR group (13.7% *versus* 32.4%, $P = 0.003$). The rates of septic complications, including intra-abdominal abscess ($P = 0.035$) and/or anastomosis leakage ($P = 0.014$), were also lower in the LICR group. In addition, the rates of reoperation ($P = 0.029$) and blood transfusion ($P = 0.021$) were significantly lower, hospital stay ($P = 0.003$) significantly shorter, and postoperative pain on postoperative day 7 significantly lower ($P < 0.001$) in the LICR than in the OICR group (Table 3).

Univariate analysis showed that an open approach ($P = 0.003$), previous abdominal surgery ($P = 0.037$), preoperative use of biologics ($P = 0.023$), additional procedures ($P = 0.050$), and longer operation time (> 135 min) ($P = 0.016$) were associated with postoperative complications. In multivariate analysis, an open approach [odds ratio (OR): 2.86; 95%CI: 0.17-0.73; $P = 0.005$], previous abdominal surgery (OR: 3.61; 95%CI: 1.23-10.60, $P = 0.020$), preoperative use of biologics (OR: 3.14; 95%CI: 1.33-7.40; $P = 0.009$), and longer operation time (> 135 min) (OR: 2.38; 95%CI: 1.17-4.84; $P = 0.017$) were found to be independent risk factors for postoperative complications (Table 4).

Sixteen patients experienced septic complications, 15 in the OICR and one in the LICR group. Preoperative use of steroids (OR: 4.19; 95%CI: 1.19-14.71; $P = 0.025$) and fistula as a surgical indication (OR: 4.03; 95%CI: 1.23-13.22; $P = 0.021$) were significantly associated with septic complications, whereas preoperative use of biologics was not. The laparoscopic approach was also associated with a lower risk of septic complications (OR: 0.06; 95%CI: 0.01-0.45; $P = 0.007$).

Table 2 Operative details of propensity-score matched patients

	Open (*n* = 102)	Laparoscopy (*n* = 102)	*P*
Operation time (min)	136.6 ± 4.5	130.4 ± 2.7	0.241
Estimated blood loss (mL)	201.8 ± 19.2	145.7 ± 22.1	0.057
Intraoperative transfusion	13 (12.7)	3 (2.9)	0.017
Diverting ileostomy	3 (2.9)	0 (0.0)	0.246
Anastomosis configuration			0.014
Side-to-side	89 (87.3)	74 (72.5)	
End-to-side	9 (8.8)	26 (25.5)	
Side-to-end	3 (2.9)	1 (1.0)	
End-to-end	1 (1.0)	1 (1.0)	
Stapled anastomosis	100 (98.0)	101 (99.0)	1.000
Operation type			0.322
Right colectomy	48 (47.1)	40 (39.2)	
Ileocecal resection	54 (52.9)	62 (60.8)	
Additional procedure	40 (39.2)	30 (29.4)	0.184
Strictureplasty	16 (15.7)	11 (10.8)	
Small bowel resection	20 (19.6)	19 (18.6)	
Colon resection	4 (3.9)	0 (0.0)	
Length of small bowel resected (cm)	60.6 ± 4.2	43.9 ± 2.8	0.001

Results are reported as mean ± SD or as number (%).

Long-term outcomes

The median follow-up duration was 74.8 mo for all patients, 88.13 mo in the OICR group, and 61.45 mo in the LICR group. By the end of the study period, 21 patients (10.3%) in the propensity-score matched cohort had experienced surgical recurrence of CD, including 14 (13.7%) who underwent OICR and seven (6.9%) who underwent LICR; these included 12 patients with fistula/abscess, four with perforation, and five with stricture. The median time to surgical recurrence was 70.3 mo for all patients, 81.7 mo in the OICR group, and 58.9 mo in the LICR group.

The overall 5- and 10-year SRFS rates were 92.9% and 83.3%, respectively, in the 204 propensity-score matched patients, 92.6% and 82.4%, respectively, in the OICR group, and 92.8% and 84.3%, respectively, in the LICR group. Kaplan-Meier analysis showed that the difference between the two groups was not statistically significant (P = 0.407, Figure 2). Univariate analysis showed that preoperative treatment with biologics (P = 0.024), side-to-side anastomosis (P = 0.042), and additional procedures (P = 0.049) were associated with surgical recurrence. Multivariate analysis revealed that the performance of simultaneous additional procedures was a risk factor for surgical recurrence [hazard ratio (HR): 3.28; 95%CI: 1.20-8.91; P = 0.020; Table 5].

DISCUSSION

This propensity-score matched case-control study compared short and long-term outcomes of LICR and OICR for primary CD at a single tertiary center specializing in CD. The results confirmed that LICR can be safely performed in these patients, resulting in a lower rate of postoperative complications, faster postoperative recovery, and non-inferior surgical recurrence rate compared with open surgery.

The laparoscopic approach is the preferred surgical approach for simple CD, as it is associated with a lower postoperative morbidity rate, a shorter hospital stay, earlier return to full activity, and improved quality of life[1-4]. Laparoscopic surgery is being performed more frequently in patients with complex or recurrent CD, with ran-

Table 3 Short-term outcomes of the propensity-score matched patients

	Open (*n* = 102)	Laparoscop (*n* = 102)	*P*	OR	95%CI	*P*
Total complications	33 (32.4)	14 (13.7)	0.003	0.379	0.189-0.759	0.006
Intra-abdominal abscess	8 (7.8)	1 (1.0)	0.035			
Anastomotic leakage	7 (6.9)	0 (0.0)	0.014			
Wound complication	12 (11.8)	4 (3.9)	0.065			
Ileus	2 (2.0)	7 (6.9)	0.170			
Bleeding	7 (6.9)	2 (2.0)	0.170			
Other	0 (0.0)	2 (2.0)	0.498			
Septic complications[1]	15 (14.7)	1 (1.0)	< 0.001	0.091	0.012-0.704	0.006[a]
Reoperation	6 (5.9)	0 (0.0)	0.029	0.107	0.006-2.039	0.121[a]
Readmission	4 (3.9)	2 (2.0)	0.689	0.250	0.053-1.177	0.109[a]
Recovery of bowel movement (d)	2.97 ± 1.0	2.82 ± 1.0	0.295	0.971	0.694-1.359	0.864
Duration of NPO (d)	4.2 ± 1.9	3.5 ± 2.2	0.026	0.573	0.400-0.820	0.002
Total hospital stay (d)	20.7 ± 17.3	16.1 ± 8.3	0.016	0.594	0.404-0.875	0.008
Postoperative hospital stay (d)	13.1 ± 16.3	8.2 ± 3.3	0.003	0.443	0.298-0.660	< 0.001
Pain scale (NRS)						
POD#1	5.1 ± 2.0	4.9 ± 1.9	0.458	0.386	-0.183-0.956	0.181
POD#2	3.9 ± 1.8	3.9 ± 2.1	0.756	0.159	-0.392-0.710	0.567
POD#3	3.8 ± 2.1	3.3 ± 1.8	0.057	0.602	0.00-1.198	0.048
POD#7	2.3 ± 1.9	1.4 ± 1.4	< 0.001	0.628	0.156-1.100	0.010
Postoperative transfusion	23 (22.5)	10 (9.8)	0.021	0.278	0.103-0.748	0.011

[1]Septic complications, including intra-abdominal abscess and anastomosis leakage.
[a]Exact test. Results are reported as mean ± SD or as number (%).
NRS: Numeric Pain Rating Scale; NPO: Nothing Per Oral; POD: Postoperative day; OR: Odds ratio; CI: Confidence interval.

domized-controlled studies and meta-analyses providing evidence that LICR is safe and effective, even in patients with severe CD[5-7]. The present study showed that LICR was associated with a lower rate of postoperative complications, a shorter postoperative hospital stay, and reduced postoperative pain, with no difference in the behavior or severity of CD. These results support recent trends showing that LICR may be feasible in selected patients with CD.

Operation time was comparable in the LICR and OICR groups. Although several studies have reported that operation time is longer for laparoscopic than for open surgery[1,13,14], other studies have found that operation time is shorter for LICR than for OICR[15,16]. Our finding, of no difference in operation time between laparoscopic and open surgery, may have been due to lack of calibration of selection, even after propensity-score matching. For example, the resected length of the small bowel differed between the LICR and OICR groups, and additional procedures were more frequent in patients who underwent open surgery.

The present study found that preoperative use of corticosteroids was associated with higher rates of intra-abdominal abscess and anastomosis leakage. Moreover, preoperative treatment with biologics was associated with a higher risk of short-term complications. These results are similar to those of the large observational TREAT registry (n = 6273), in which use of infliximab and corticosteroid increased the risks of serious infection[17-19]. In addition, our study found that a history of previous abdominal surgery was associated with postoperative complications. The specific impact of previous intestinal resection on postoperative complications in patients with CD has not been determined. One study reported that 47.6% of postoperative complications occurred in patients with a history of previous abdominal surgery (n = 10, P = 0.310), although the difference was not statistically significant[20]. Other studies have found that previous abdominal surgery is a significant risk factor for postoperative

Table 4 Univariate and multivariate regression analyses of risk factors associated with postoperative complications in propensity-score matched patients

	Univariate analysis			Multivariate analysis		
	No complication (*n* = 157, %)	Complication (*n* = 47, %)	*P*	OR	95%CI	*P*
Demographics						
Male	115 (73.2)	35 (74.5)	1.000			
Family history	6 (3.8)	2 (4.3)	1.000			
Smoking history	32 (20.4)	9 (19.1)	1.000			
Comorbidity	6 (3.8)	0 (0.0)	0.340			
Fistula-in-ano	73 (46.5)	17 (36.2)	0.243			
Previous abdominal surgery	10 (6.4)	8 (17.0)	0.037	3.61	1.23-10.60	0.020
Penetrating type	112 (71.3)	34 (72.3)	1.000			
Open approach	69 (43.9)	14 (70.2)	0.003	2.86	0.17-0.73	0.005
Preoperative medications						
Biologics	20 (12.7)	13 (27.7)	0.023	3.14	1.33-7.40	0.009
Steroid	24 (15.3)	13 (27.7)	0.082			
Immunomodulators	77 (49.0)	21 (44.7)	0.622			
Operation details						
Indications						
Fistula	53 (33.8)	20 (42.6)	0.300			
Obstruction	52 (33.1)	16 (34.0)	1.000			
Anastomosis configuration						
Side-to-side	126 (80.3)	37 (78.7)	0.837			
End-to-side	29 (18.5)	6 (12.8)	0.508			
Stapled anastomosis	155 (99.7)	46 (2.1)	0.546			
Additional procedures	45 (28.7)	21 (44.7)	0.050	1.38	0.61-3.14	0.444
Operation time[1] > 135 min	52 (33.1)	25 (53.2)	0.016	2.38	1.17-4.84	0.017

[1]Average operation time for CD was 135.6 min.

Results are reported as number (%). OR: Odds ratio; CI: Confidence interval.

complications[21,22]. Moreover, patients who had previous surgery were more inclined to develop postoperative complications (P = 0.047), particularly anastomotic leak (P = 0.021) and severe (Clavien-Dindo grade III/IV) complications (P = 0.038)[23]. A previous abdominal surgery history may result in prolonged dissection during surgery because of the atypical planes of disrupted normal anatomy, increasing the risks of accidental enterotomy and additional bowel devascularization[23].

In our study, the rate of conversion from laparoscopic to open surgery was about 5%. Pooled conversion rates have been found to range from 0% to 21.5%[13,24]. Recurrent disease with dense adhesions, pelvic sepsis with fistulizing disease, large inflammatory mass, and thickened mesentery are all conditions predisposing to conversion open surgery[24]. The low conversion rate in the present study may have been due to the relatively mild complexities and complications in patients who underwent laparoscopic surgery. Laparoscopy can be attempted in patients with CD, even if they have risk factors for open conversion. For safety reasons, however, patients should be converted to open surgery without delay.

Prevention of long-term postoperative recurrence in CD patients is a major challenge, especially as 10-15 year post-surgical recurrence rates are approximately 45% to 50%[25]. The long-term effects of laparoscopy have not been determined, as few studies have compared long-term outcomes, especially surgical recurrence, in CD patients who have undergone laparoscopic and open surgery. A meta-analysis

Table 5 Univariate and multivariate regression analyses of risk factors associated with surgical recurrence in propensity-score matched patients

	Univariate analysis			Multivariate analysis		
	No recurrence (*n* = 183)	Recurrence (*n* = 21)	*P*	HR	95%CI	*P*
Demographics						
Male	134 (73.2)	16 (76.2)	1.000			
Family history	8 (4.4)	0 (0.0)	1.000			
Smoking history	38 (20.8)	3 (14.3)	0.579			
Comorbidity	6 (3.3)	0 (0.0)	1.000			
Fistula-in-ano	81 (44.3)	9 (42.9)	1.000			
Previous abdominal surgery	15 (8.2)	3 (14.3)	0.407			
Penetrating type	129 (70.5)	17 (81.0)	0.445			
Open approach	95 (48.1)	14 (66.7)	0.166			
Preoperative medications						
Biologics	26 (14.2)	7 (33.3)	0.024	2.64	0.89-7.86	0.081
Steroid	31 (16.9)	6 (28.6)	0.229			
Immunomodulators	88 (48.1)	10 (47.6)	1.000			
Operation details						
Indication						
Fistula	69 (37.7)	4 (19.0)	0.099			
Obstruction	62 (33.9)	6 (28.6)	0.808			
Anastomosis configuration						
Side-to-side	150 (82.0)	13 (61.9)	0.042	3.82	1.24-11.78	0.078
End-to-side	30 (16.4)	5 (23.8)	0.370			
Stapled anastomosis	180 (98.4)	21 (100.0)	1.000			
Additional procedures	55 (30.1)	11 (52.4)	0.049	3.28	1.20-8.91	0.020
Operation time > 135 min	66 (36.1)	11 (52.4)	0.159			

Results are reported as number (%). HR: Hazard ratio; CI: Confidence interval.

reported that laparoscopic surgery is associated with a lower rate of late reoperations for CD recurrence (OR: 0.46; 95%CI: 0.27-0.80)[14], and laparoscopy was found to protect against surgical recurrence (HR: 0.24; 95%CI: 0.10-0.53; $P = 0.04$)[26]. Another study, however, reported no difference in surgical recurrence rates between surgical techniques (OR: 0.78; 95%CI: 0.54-1.11; $P = 0.17$)[27]. The present study also showed no difference in long-term outcomes between the LICR and OICR groups. Because this study was a retrospective analysis of a small number of patients, future large randomized-controlled trials are needed to assess the impact of laparoscopy on surgical recurrence.

Simultaneous surgery at the time of ICR was also associated with a higher risk of surgical recurrence. The extent of disease at diagnosis had an impact on recurrence, with higher recurrence rates in patients with small bowel and continuous ileocolonic CD than in patients with ileocecal and colorectal disease[28]. Disease extent > 50 cm is considered a risk factor for postoperative recurrence of CD[29]. An additional surgical procedure may be related to surgical recurrence because these patients may have more severe disease. Prospective clinical studies in larger numbers of patients are needed to further evaluate the results of the present study.

Since the introduction of the first anti-TNF agent in the late 1990s, biologic therapy has revolutionized the medical treatment of patients with CD[30]. Although administering biologics prior to surgery has been reported to reduce clinical/endoscopic recurrence rates[31-33], most of these trials had small sample sizes and limited follow-

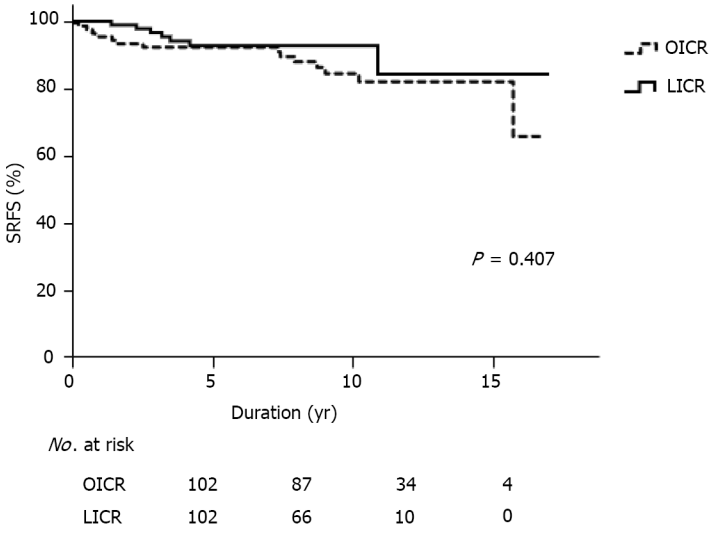


Figure 2 Kaplan-Meier analysis of surgical recurrence-free survival according to type of surgery in patients with Crohn's disease. SRFS: Surgical recurrence-free survival; LICR: Laparoscopic ileocolic resection; OICR: Open ileocolic resection.

up, and focused on endoscopic findings and clinical scores rather than repeat operations[30,34]. In our study, preoperative treatment with biologics was related to higher risk of surgical recurrence. South Korea strictly regulates the use of biologics due to health insurance policies, and prophylactic treatment with biologics is not available[35]. Therefore, the correlation between administration of biologics and higher risk of recurrence may be due to the greater severity of disease in patients selected for treatment with these agents. Large randomized-controlled trials are needed to determine the ability of routine prophylactic biologics after surgery to prevent surgical recurrence.

Stapled side-to-side anastomosis has been associated with lower rates of leakage and surgical recurrence than other types of anastomosis[36-39]. Side-to-side anastomoses maintain better lateral blood flow and a wide lumen, which prevents luminal stenosis and fecal pooling, thereby preventing early disease recurrence[40]. By contrast, another study reported that side-to-side anastomoses (both hand-sewn and stapled) did not reduce short-term complications and postoperative recurrence[41]. Moreover, anastomotic configuration or the material used was not significantly related to reoperations or complications[40]. The present study found that the type of anastomosis did not significantly affect short-term complications. Although the surgical recurrence rate tended to be higher in patients who underwent side-to-side than other types of anastomosis, the difference was not statistically significant (HR: 3.82; $P = 0.078$). However, most patients in the present study underwent side-to-side anastomosis, with only a few receiving end-to-side or end-to-end anastomosis. Surgeons in our center prefer side-to-side anastomosis in CD patients with extensive inflammation. Thus, to evaluate the role of anastomosis configuration, a more controlled study, adjusting for time period and surgeon, is needed.

The present study had several limitations. First, this study was a retrospective evaluation of patients at a single center. Randomized-controlled trials are required to specifically evaluate the ability of a laparoscopic approach to minimize postoperative complications. Although propensity-score matching can reduce selection bias, resulting in a situation similar to a randomized-controlled trial, our propensity-score matching models could not eliminate all selection biases. For example, the most frequent reasons for conversion to open surgery, such as adhesions and huge phlegmons, could not be calibrated by propensity-score matching analysis. Also, although the CDAI scores for both groups were moderate, the laparoscopic group had a significantly lower CDAI score (230.8) than the open group (269.1) ($P = 0.008$)[42]. Inevitably, a randomized controlled trial will be required to evaluate the role of the laparoscopic approach with more reliable evidence. Second, the study included only East Asian patients from a single country, thus not representing a global population. Korean and western CD patients differ in gender distribution, disease location, and perianal fistula occurrence[43]. Third, the use of biologics in this study was less frequent than in western studies because the health insurance reimbursement policy of the Korean government was strict during the study period. This study period was dominated by 'step-up treatment'. Although 'top-down treatment' has been more

frequent in recent years, its use in Korea is limited.

CONCLUSION

LICR yielded better short-term outcomes, including more rapid postoperative recovery, than open surgery. Long-term outcomes, however, did not differ between the two groups. Laparoscopic surgery might be a better surgical option in selected patients with CD.

ARTICLE HIGHLIGHTS

Research background

Ileocolic resection (ICR) is the most frequently performed operation for patients with abdominal Crohn's disease (CD) with involvement of the terminal ileum. Laparoscopic ICR (LICR) has become the preferred surgical approach for primary ileocolic CD because it has greater recovery benefits than open ICR (OICR).

Research motivation

The laparoscopic approach is regarded as more technically challenging than open surgery in CD patients with complex features, including huge phlegmons, multiple enteric fistulas, and dense adhesions, as well as those requiring repeated surgery. Utilization of LICR in patients with complex CD remains problematic.

Research objectives

This study aimed to compare the short- and long-term outcomes of LICR and OICR in patients with primary CD.

Research methods

A total of 348 eligible patients who underwent LICR or OICR for primary CD at Asan Medical Center in Seoul, Korea, from January 2006 to December 2017, were retrospectively analyzed. Data on demographic characteristics, preoperative disease characteristics, operative details, perioperative outcomes, and long-term surgical recurrence were collected. Patients were propensity-score matched 1:1 by factors potentially associated with 30 d perioperative morbidity.

Research results

During the study period, 348 patients underwent ICR, 211 by the open approach and 137 by the laparoscopic approach. Propensity-score matching yielded 102 pairs of patients. The rate of postoperative complications was significantly lower, postoperative hospital stay significantly shorter, and postoperative pain on day 7 significantly lower in patients who underwent laparoscopic than OICR. Surgical recurrence free survival (SRFS) rates in the OICR and LICR groups were not significantly different.

Research conclusions

LICR yielded better short-term outcomes and postoperative recovery than OICR, with no differences in long-term outcomes. LICR may provide greater benefits in selected patients with primary CD.

Research perspectives

The laparoscopic approach to ileocolic resection can be safely performed in patients with primary CD, resulting in fewer postoperative complications, faster postoperative recovery, and non-inferior surgical recurrence rate when compared with open surgery.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7173-7189

DOI: 10.3748/wjg.v27.i41.7173

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

ORIGINAL ARTICLE

Retrospective Study

Comprehensive radiomics nomogram for predicting survival of patients with combined hepatocellular carcinoma and cholangiocarcinoma

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Author contributions: Tang YY and Zhao YN provided the study concept and designed this study; Tang YY acquired the data; Zhang T and Zhao YN carried out data analysis and interpretation; Tang YY and Zhang T were responsible for drafting and preliminarily revising the manuscript; Ma XL and Chen ZY performed study supervision and final approval.

Institutional review board statement: This retrospective study was approved by the West China Hospital Ethics Committee (Approval No. 2019903).

Conflict-of-interest statement: The authors declare that they have no competing interests as defined by Nature Research, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

Data sharing statement: The clinical data and radiomics data were available from the corresponding author at

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Abstract

BACKGROUND

Combined hepatocellular carcinoma (HCC) and cholangiocarcinoma (cHCC-CCA) is defined as a single nodule showing differentiation into HCC and intrahepatic cholangiocarcinoma and has a poor prognosis.

AIM

To develop a radiomics nomogram for predicting post-resection survival of patients with cHCC-CCA.

METHODS

Patients with pathologically diagnosed cHCC-CCA were randomly divided into training and validation sets. Radiomics features were extracted from portal venous phase computed tomography (CT) images using the least absolute shrinkage and selection operator Cox regression and random forest analysis. A nomogram integrating the radiomics score and clinical factors was developed using univariate analysis and multivariate Cox regression. Nomogram performance was assessed in terms of the C-index as well as calibration, decision, and survival curves.

RESULTS

Chenzheyu@scu.edu.cn. And no additional data are available.

Country/Territory of origin: China

Specialty type: Gastroenterology and hepatology

Peer-review report's scientific quality classification

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

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Received: April 2, 2021
Peer-review started: April 2, 2021
First decision: June 24, 2021
Revised: June 26, 2021
Accepted: September 3, 2021
Article in press: September 3, 2021
Published online: November 7, 2021

P-Reviewer: Antwi SO, Farid K
S-Editor: Ma YJ
L-Editor: Wang TQ
P-Editor: Yuan YY

CT and clinical data of 118 patients were included in the study. The radiomics score, vascular invasion, anatomical resection, total bilirubin level, and satellite lesions were found to be independent predictors of overall survival (OS) and were therefore included in an integrative nomogram. The nomogram was more strongly associated with OS (hazard ratio: 8.155, 95% confidence interval: 4.498-14.785, $P < 0.001$) than a model based on the radiomics score or only clinical factors. The area under the curve values for 1-year and 3-year OS in the training set were 0.878 and 0.875, respectively. Patients stratified as being at high risk of poor prognosis showed a significantly shorter median OS than those stratified as being at low risk (6.1 *vs* 81.6 mo, $P < 0.001$).

CONCLUSION

This nomogram may predict survival of cHCC-CCA patients after hepatectomy and therefore help identify those more likely to benefit from surgery.

Key Words: Radiomics; Nomogram; Combined hepatocellular carcinoma and cholangiocarcinoma; Risk strata; Prognosis

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Core Tip: Combined hepatocellular carcinoma (HCC) and cholangiocarcinoma (cHCC-CCA) is defined as a single nodule showing differentiation into HCC and intrahepatic cholangiocarcinoma. Studies vary regarding the prognosis of cHCC-CCA patients after potentially curative hepatectomy, with 5-year postoperative overall survival rates ranging from 8% to 63%. A reliable method to predict prognosis after resection may help select cHCC-CCA patients more likely to benefit from surgery. We established an integrative nomogram based on radiomics features and clinical variables to predict the survival of cHCC-CCA patients after potentially curative resection. The nomogram showed good predictive potential and may help guide treatment decisions.

Citation: Tang YY, Zhao YN, Zhang T, Chen ZY, Ma XL. Comprehensive radiomics nomogram for predicting survival of patients with combined hepatocellular carcinoma and cholangiocarcinoma. *World J Gastroenterol* 2021; 27(41): 7173-7189
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7173.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7173

INTRODUCTION

Combined hepatocellular carcinoma (HCC) and cholangiocarcinoma (cHCC-CCA), which arises in hepatic progenitor cells, accounts for 0.8%-6.5% of primary liver carcinoma cases[1-5]. The World Health Organization defines the condition as the presence of a single nodule showing differentiation into HCC and intrahepatic cholangiocarcinoma (ICC)[6,7]. There is disagreement in the literature on whether the prognosis of cHCC-CCA patients is worse or similar to that of patients with only HCC. Several studies concur that the prognosis of cHCC-CCA patients is comparable to that of patients with only ICC[8-11]. Studies vary regarding the prognosis of cHCC-CCA patients after potentially curative hepatectomy, with 5-year postoperative overall survival (OS) rates ranging from 8% to 63%[12-15]. A reliable method to predict prognosis after resection may help select cHCC-CCA patients more likely to benefit from surgery.

Radiomics is a promising comprehensive analysis to predict the prognosis of liver cancer patients after hepatectomy, which is a post-processing method to quantitatively evaluate imaging features in order to assess cancer heterogeneity non-invasively and objectively[16,17]. Radiomics features have proven effective in predicting the survival of patients with HCC or ICC alone[18-21]. Radiomics can also differentiate cHCC-CCA from common HCC or ICC[18,22], although no radiomics models have been established for predicting long-term survival of cHCC-CCA patients after resection.

The predictive performance of radiomics features may improve when combined with clinical factors, as demonstrated for patients with ICC[23-25]. Therefore, the current study aimed to construct and validate a nomogram based on radiomics and clinical features for predicting postoperative survival of cHCC-CCA patients. This prognostic model may help guide treatment decisions for these patients.

MATERIALS AND METHODS

Study design and patient selection

This retrospective study was approved by the West China Hospital Ethics Committee, and the requirement for informed consent was waived. All patients agreed to undergo medical examination and were informed that their anonymized medical data would be analyzed and published for the purposes of medical research. We retrospectively reviewed the data of all patients: (1) Who were diagnosed with cHCC-CCA based on the 2019 guidelines of the World Health Organization which defined cHCC-CCA as a single nodule showing differentiation into HCC and ICC; (2) Who underwent hepatectomy with curative intent at West China Hospital between February 2012 and May 2017; and (3) For whom complete medical records were available during hospitalization and during follow-up, as well as computed tomography (CT) data within 2 wk before surgery.

Patients were excluded if they were diagnosed with morphologically typical HCC or ICC based on the expression of markers for cholangiocytes, hepatocytes, or progenitor cells (*e.g.*, keratins 7 and 19 based on immunostaining). Patients were considered to have common HCC if they showed trabecular growth (often accompanied by bile production), hyaline bodies, prominent nucleoli, immunoreactivity against HepPar1 or alpha-fetoprotein, and expression of keratin 19[26,27]. Patients presenting typical adenocarcinoma together with abundant stroma and mucin production were considered to have ICC only. Patients diagnosed with cholangiolocellular carcinoma were excluded from this study as the latest guidelines[7] no longer consider this condition a subtype of cHCC-CCA.

Patients were also excluded if they had received transcatheter arterial chemoembolization or any other type of chemotherapy before CT, or if they had other malignancies simultaneously with cHCC-CCA. The primary endpoint of this study was OS, defined as the time from the date of surgery until the date of all-cause death or last follow-up. Patients were routinely followed at 1 mo after surgery and then every 3-6 mo thereafter, until April 30, 2020.

Computed tomography examination

Enhanced CT of the abdomen was performed with a single 64-detector row scanner (Brilliance 64, Philips Medical Systems, Eindhoven, The Netherlands) in all the patients. The scan parameters were as follows: Beam pitch, 0.891; tube voltage, 120 kV; tube current, 200 mA; detector collimation, 0.75 mm; slice thickness, 1.0 mm; reconstruction increment, 5.0 mm; and rotation time, 0.42 s. Arterial phase scanning began at 25 s and portal venous phase scanning began at 60 s[22].

Extraction of radiomics features

All patients were randomly divided into a training set and validation set at a ratio of 7:3. All CT images from portal venous phase scanning were loaded into LIFEx software (version 3.74; CEA-SHFJ, Orsay, France)[28]. Working independently, two radiologists manually drew regions of interest for each patient within the hepatic neoplasm in all portal venous phase CT images. Radiomics features in the CT images were screened using the Least Absolute Shrinkage and Selection Operator (LASSO) and Cox regression, followed by random forest analysis[29]. The selected radiomics features were linearly combined with their own weighting coefficients, generating a radiomics score for each patient.

Selection of clinical factors

All clinical variables in the training set were subjected to univariate analysis followed by multivariate Cox analysis with step-wise selection in order to identify independent predictors of OS. In these analyses, total bilirubin level was converted into a categorical variable.

Development and validation of an integrative nomogram

To develop the nomogram, radiomics scores were categorized as "high" or "low" based on whether they were greater or smaller than the median score. Then the nomogram was constructed based on the radiomics score and the clinical risk factors identified in multivariate Cox regression. Within the nomogram, each variable was scored ranging from 0 to 100, and the variable associated with the greatest hazard ratio (HR) was assigned 100 points[30]. Using the nomogram, we classified patients as being at high or low risk based on the maximum Youden index[31].

The performance of the nomogram was assessed in terms of a calibration curve related to the predicted and observed OS, the C-index used to assess model discrimination, and receiver operating characteristic (ROC) curve[32]. The clinical usefulness of the nomogram was assessed using decision curve analysis[33].

Statistical analysis

Differences in continuous variables were assessed for significance using the Wilcoxon rank-sum test if the data were skewed, or Student's *t* test if the data showed a normal distribution. Differences in categorical variables were assessed using the χ^2 or Fisher's exact test. OS was plotted using the Kaplan-Meier method, and groups were compared using the log-rank test. All statistical analyses were performed with EmpowerStats (version 2.20; 2011 X&Y Solutions) and R software (version 4.0.0; The R Foundation). The following packages in R were used: glmnet, cmprsk, rms, survival, rmda, and devtools. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Patients

A total of 118 eligible patients (86.4% men) were enrolled (Table 1). Their mean age was 51.6 years, and 90 patients had been diagnosed when they were younger than 60 years. Follow-up data were complete for 110 patients, who were followed for a median of 25.1 mo (95% confidence interval [CI]: 17.3-59.7 mo). Median OS was 21.6 mo, and OS rates were 61.0% at 1 year, 48.3% at 3 years, and 37.4% at 5 years.

Patients were randomly assigned to either the training or validation set, and the two sets did not differ significantly in terms of clinical features, except for tumor size, American Joint Committee on Cancer stage and T stage. OS rates at 1 and 3 years were 58.3% and 46.4% in the training set, compared to 67.7% and 52.9% in the validation set.

Feature selection and construction of radiomics score

The integrative nomogram flow chart is depicted in Figures 1 and 2. For each patient, data on 49 radiomics features were extracted from portal venous phase CT images. Among these 49 features, LASSO regression selected nine with non-zero coefficients, of which random forest analysis selected three (MeanValue, NGLDM Busyness and GLZLM HGZE) (Supplementary Table 1) that showed the highest prediction values (variable importance > 0.01, Figure 3A). Radiomics scores were calculated based on these three features, and scores were subsequently categorized into "high" or "low" based on whether they were lower or higher than the median score (Figure 3).

Selection of prognostic clinical factors

In total, 31 clinical variables were initially considered in the univariate analysis; and seven variables with $P < 0.1$ were then entered into the multivariate Cox analysis (Table 2). The multivariate analysis identified four predictors of OS: Vascular invasion, anatomical resection, total bilirubin level, and satellite lesions. Total bilirubin level (> 17.1 μmol/L) resulted in a larger HR (13.94) than the other three risk factors. Nevertheless, all four factors were subsequently included in the nomogram.

Construction and validation of a radiomics nomogram model

Based on the above-mentioned four clinical factors and the radiomics score, we developed a comprehensive integrative nomogram to predict 1-year and 3-year OS of cHCC-CCA patients after surgical resection with curative intent (Figure 4A). The area under the ROC curve (AUC) for 1-year OS was 0.878 in the training set and 0.937 in the validation set (Figure 4B). The calibration curve of 1-year OS showed good agreement between predicted and observed values in both the training and validation sets (Figure 4C). The AUC for 3-year OS was 0.875 in the training set and 0.866 in the validation set. The C-index was 0.807 (95%CI: 0.756-0.858) in the training set and 0.820

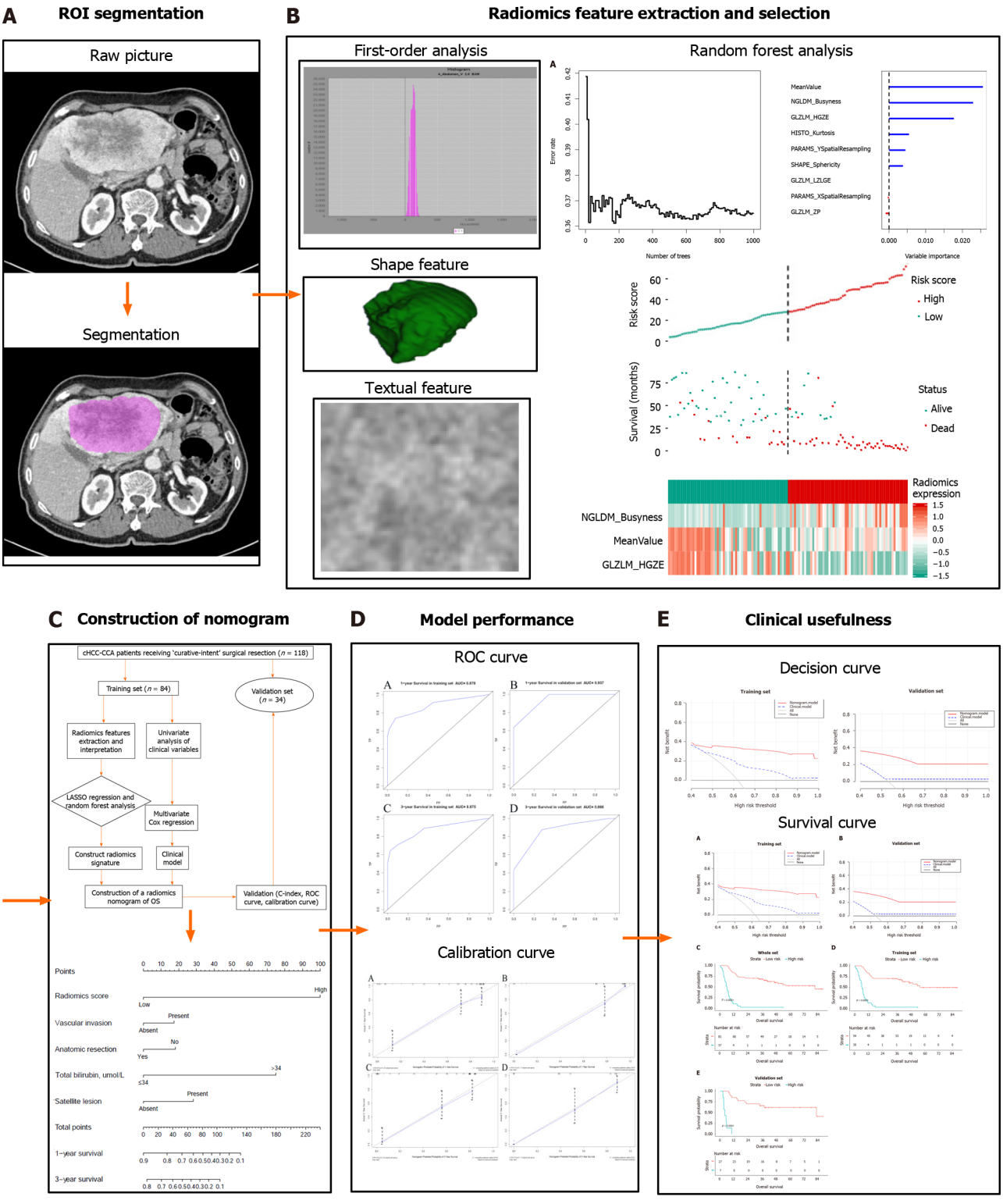


Figure 1 Study workflow. A: Segmentation of the region of interest; B: Extraction and selection of radiomics features; C: Construction of nomogram; D: Comparison of model performance; E: Decision curve analysis and overall survival comparisons between the training and validation sets. ROI: Region of interest; cHCC-CCA: Combined hepatocellular carcinoma and cholangiocarcinoma; LASSO: Least absolute shrinkage and selection operator; OS: Overall survival; ROC: Receiver operating characteristic.

(95%CI: 0.723-0.917) in the validation set. An example of predicting 1- and 3-year OS using the nomogram is shown in Figure 5.

In decision curve analysis, the nomogram showed higher "net benefit" than a model based only on the four clinical factors or models based on "treat-all-patients" or "treat-no-patients" approaches. These results were observed at nearly all threshold probabilities in the training set (Figure 6A) and validation set (Figure 6B).

Table 1 Baseline characteristics of patients with combined hepatocellular carcinoma and cholangiocarcinoma in the training and validation sets

Variable	Entire cohort (*n* = 118)	Training set (*n* = 84)	Validation set (*n* = 34)	*P* value
Male sex	102 (86.4)	73 (86.9)	29 (85.3)	0.817
Age, yr	51.6 ± 10.5	51.2 ± 10.5	52.7 ± 10.6	0.484
Hypertension	11 (9.3)	7 (8.3)	4 (11.8)	0.561
Diabetes mellitus	7 (5.9)	6 (7.1)	1 (2.9)	0.382
Hepatitis B/C	61 (51.7)	40 (47.6)	21 (61.8)	0.164
Child-Pugh, A/B	116/2	83/1	33/1	0.495
Liver cirrhosis	47 (39.8)	35 (41.7)	12 (35.3)	0.522
Hypersplenia	15 (12.7)	11 (13.1)	4 (11.8)	0.844
ALT (U/L)	55.2 ± 100.4	46.1 ± 29.3	77.6 ± 181.1	0.807
AST (U/L)	59.8 ± 136.6	48.1 ± 28.8	88.6 ± 250.7	0.513
ALB (g/L)	42.1 ± 4.6	42.3 ± 4.0	41.5 ± 5.7	0.643
TB (mmol/L)	15.9 ± 10.1	15.7 ± 10.1	16.5 ± 10.0	0.597
AFP (ng/mL)	285.2 ± 475.1	256.3 ± 454.6	356.5 ± 522.4	0.156
CA19-9 (U/mL)	106.8 ± 251.2	109.7 ± 258.3	99.6 ± 236.2	0.184
CA125 (U/mL)	117.0 ± 624.6	152.9 ± 727.5	18.3 ± 11.9	0.541
CEA (ng/mL)	6.4 ± 30.3	7.5 ± 35.5	3.4 ± 3.2	0.444
Liver fibrosis				0.871
No significant fibrosis	15 (13.8)	11 (13.8)	4 (13.8)	
Significant fibrosis	37 (33.9)	26 (32.5)	11 (37.9)	
Advanced fibrosis	57 (52.3)	43 (53.8)	14 (48.3)	
Not mentioned	8 (6.8)	3 (3.6)	5 (14.7)	
Tumor size, ≤ 5 cm	38 (32.2)	20 (23.8)	18 (52.9)	0.002
Tumor number, ≥ 2	67 (56.8)	52 (61.9)	15 (44.1)	0.077
Satellite lesions	42 (35.6)	29 (34.5)	13 (38.2)	0.703
Vascular invasion	46 (39.0)	35 (41.7)	11 (32.4)	0.347
Lymph node infiltration	15 (12.7)	10 (11.9)	5 (14.7)	0.679
Differentiation				0.578
Well	44 (37.3)	30 (35.7)	14 (41.2)	
Moderate	22 (18.6)	18 (21.4)	4 (11.8)	
Poor	1 (0.8)	1 (1.2)	0 (0.0)	
Undifferentiated	51 (43.2)	35 (41.7)	16 (47.1)	
8th AJCC stage				0.027
I	9 (7.6)	7 (8.3)	2 (5.9)	
II	28 (23.7)	14 (16.7)	14 (41.2)	
III	66 (55.9)	53 (63.1)	13 (38.2)	
IV	15 (12.7)	10 (11.9)	5 (14.7)	
T stage				0.042
T1	13 (11.0)	9 (10.7)	4 (11.8)	
T2	29 (24.6)	15 (17.9)	14 (41.2)	
T3	45 (38.1)	37 (44.0)	8 (23.5)	

T4	31 (26.3)	23 (27.4)	8 (23.5)	
N stage				0.762
N0	103 (87.3)	74 (88.1)	29 (85.3)	
N1	15 (12.7)	10 (11.9)	5 (14.7)	
Transfusion	17 (14.4)	14 (16.7)	3 (8.8)	0.388
Blood loss ≤ 400 mL	71 (60.2)	49 (58.3)	22 (64.7)	0.522
Margin, R1	13 (11.0)	9 (10.7)	4 (11.8)	0.869
Surgical method				0.285
Major resection	57 (48.3)	44 (52.4)	13 (38.2)	
Minor resection	50 (42.4)	32 (38.1)	18 (52.9)	
Resection + ablation	11 (9.3)	8 (9.5)	3 (8.8)	
Anatomical resection	50 (43.9)	39 (48.1)	11 (33.3)	0.148
Postoperative TACE	35 (29.7)	28 (33.3)	7 (20.6)	0.17
Hospital stay (d)	12.2 ± 4.5	12.3 ± 4.4	11.9 ± 5.0	0.608
Overall survival (mo)	30.8 ± 26.3	29.6 ± 26.2	33.6 ± 26.9	0.462

[1]Values are *n*, *n* (%), or mean ± SD, unless otherwise noted.

AFP: Alpha fetoprotein; AJCC: American Joint Committee on Cancer; ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CEA: Carcinoembryonic antigen; TACE: Transhepatic arterial chemotherapy and embolization; TB: Total bilirubin.

Risk stratification using the nomogram

A total risk score was calculated for each patient by summing the scores for each variable in the nomogram. The maximum Youden index of 105 points in the nomogram led us to determine a cut-off value of 39.66, and patients were categorized as being at "high" or "low" risk based on whether their risk score was above or below this cut-off. Kaplan-Meier curves showed that OS was significantly longer for low-risk patients than for high-risk patients, regardless of whether the analysis included all patients (Figure 6C) or only the training set (Figure 6D) or validation set (Figure 6E). Across all patients, OS rates at 1 year were 10.8% for the high-risk group and 84.0% for the low-risk group ($P < 0.001$), while the corresponding OS rates at 3 years were 2.7% and 69.1%, respectively ($P < 0.001$).

Table 3 compares HRs obtained with the integrated nomogram, the radiomics score alone, or a model based only on clinical factors. The model based only on the four clinical risk factors resulted in an HR of 2.65 (95%CI: 1.53-4.60), even though total bilirubin level resulted in an HR of 13.94 (95%CI: 3.56-54.60) in multivariate analysis. The nomogram HR was higher than that provided by models based on the radiomics score or on clinical factors alone.

DISCUSSION

In the present study, we developed a comprehensive integrative nomogram that takes into account CT radiomics scores and four clinical risk factors that independently predict OS (vascular invasion, anatomical resection, total bilirubin, and satellite lesions), and we showed that this nomogram can predict OS in cHCC-CCA patients following potentially curative hepatectomy. The AUC for 1-year OS was 0.878 in the training set and 0.937 in the validation set. To our knowledge, this is the first CT-based radiomics model to predict postoperative survival of cHCC-CCA patients.

Our results extend the number of situations in which radiomics has shown potential in predicting the survival of patients with liver tumors[34,35]. The patients in our study who were assigned a high radiomics score had a 5.91-fold higher risk of death than those with a low score, consistent with a previously reported association between high radiomics score and risk of recurrence in patients with HCC or ICC[24,36]. These findings imply that radiomics scores may be able to identify patients preoperatively who are more likely to benefit from surgical resection.

Table 2 Univariate analysis and multivariate Cox regression to identify clinical factors associated with overall survival after curative hepatectomy

Variable	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Male sex	0.470 (0.203-1.088)	0.078	1.767 (0.244-1.316)	0.186
Age, yr				
≤ 60	Ref.			
> 60	1.173 (0.644-2.139)	0.602		
Liver cirrhosis				
Absent	Ref.			
Present	1.370 (0.852-2.203)	0.194		
AFP (ng/mL)	0.990 (0.597-1.643)	0.970		
CA 19-9 (U/mL)	0.987 (0.586-1.662)	0.960		
Albumin (g/L)	2.496 (0.997-6.244)	0.051	1.025 (0.968-1.085)	0.403
TB (μmol/L)				
≤ 34	Ref.		Ref.	
> 34	17.994 (4.726-68.509)	< 0.001	13.943 (3.561-54.602)	< 0.001
Tumor number, multiple	0.766 (0.473-1.240)	0.277		
Satellite lesions				
Absent	Ref.		Ref.	
Present	2.037 (1.267-3.268)	0.003	1.762 (1.079-2.877)	0.024
Vascular invasion				
Absent	Ref.		Ref.	
Present	2.009 (1.247-3.239)	0.004	1.725 (1.049-2.834)	0.032
T stage				
T1	Ref.			
T2	1.171 (0.705-1.942)	0.542		
T3	2.424 (0.704-8.348)	0.161		
T4	3.823 (1.158-12.615)	0.028		
Anatomy resection				
Yes	Ref.		Ref.	
No	2.011 (1.344-3.006)	0.006	1.731 (1.083-2.767)	0.028
Margin				
R0	Ref.			
R1	1.032 (0.446-2.387)	0.941		
Postoperative TACE				
Yes	Ref.			
No	1.597 (0.924-2.759)	0.093	1.6051 (0.3546-1.0947)	0.100

AFP: Alpha-fetoprotein; CI: Confidence interval; HR: Hazard ratio; Ref.: Reference; TB: Total bilirubin; TACE: Transhepatic arterial chemotherapy and embolization.

Our results further support previous work indicating that combining clinical variables with radiomics features may predict prognosis better than either the variables or the features separately[37,38]. Combining the radiomics score with clinical variables allowed us to classify patients into a high-risk group that had an 8.16-fold

Table 3 Comparison of hazard ratios describing risk for different predictive models

Model	HR (95%CI)	*P* value
Radiomics score		< 0.001
Low risk	Ref.	
High risk	5.908 (3.285-10.626)	
Clinical model		< 0.001
Low risk	Ref.	
High risk	2.653 (1.532-4.595)	
Radiomics nomogram		< 0.001
Low risk	Ref.	
High risk	8.155 (4.498-14.785)	

CI: Confidence interval; HR: Hazard ratio; Ref.: Reference.

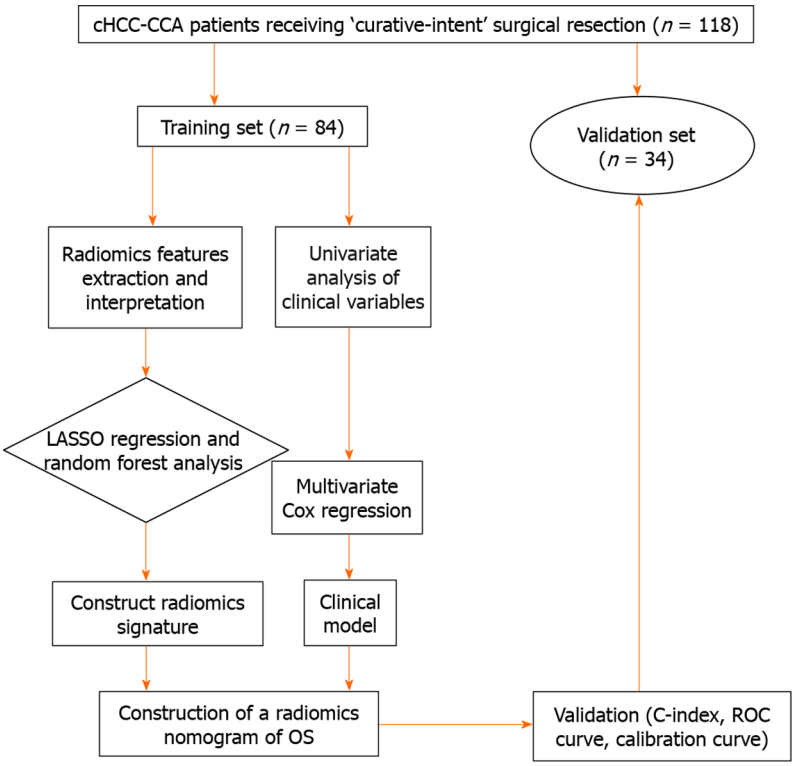


Figure 2 Flow diagram of patient selection. cHCC-CCA: Combined hepatocellular carcinoma and cholangiocarcinoma; LASSO: Least absolute shrinkage and selection operator; OS: Overall survival; ROC: Receiver operating characteristic.

higher risk of death than the low-risk group, with the two groups showing a median OS of 6.1 and 81.6 mo, respectively ($P < 0.001$). This integrative nomogram may help identify cHCC-CCA patients who are more likely to benefit from resection.

The rate of vascular invasion in our patients was 39.0%, similar to previous studies and within the prevalence of 9%-89.5% reported for cHCC-CCA[3,39,40]. As shown in Supplementary Figure 1, the OS rate at 3 years was 56.8% among our patients without vascular invasion, compared to only 36.8% among those with invasion, consistent with the association between vascular invasion and worse postoperative prognosis[2,13,41]. Indeed, vascular invasion has been shown to be an independent predictor of postoperative survival in patients with combined hepatocellular-cholangiocarcinoma and it increases the risk of death in these patients by 1.6- fold to 5.2-fold[42,43].

In addition, elevated total bilirubin level (> 34 μmol/L) and no anatomic surgical resection were considered to be independent risk factors related to the poor prognosis of cHCC-CCA patients. Total bilirubin level is one element of the Child-Pugh classi-

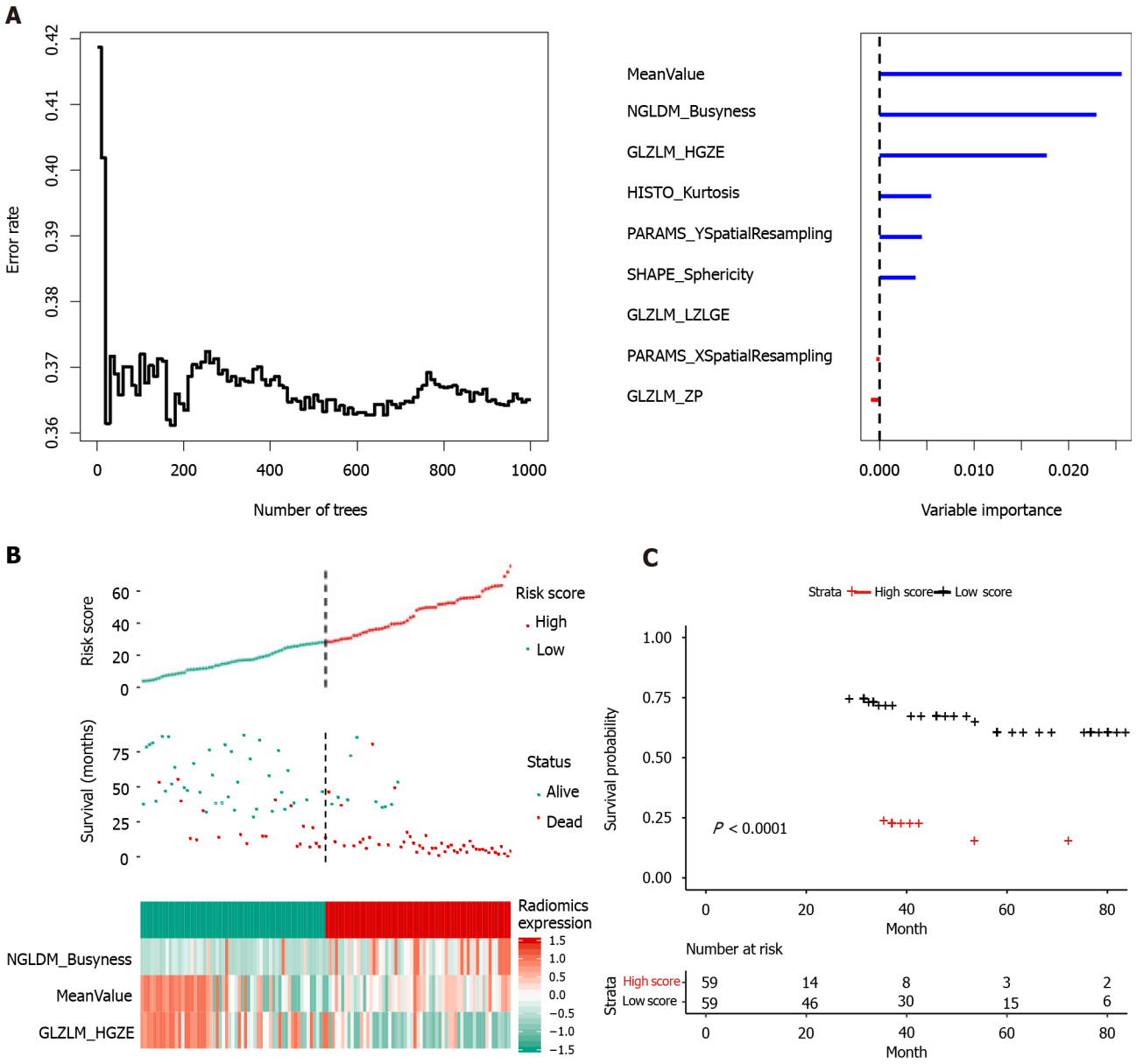


Figure 3 Radiomics feature selection. A: Random forest analysis. Least absolute shrinkage and selection operator regression selected nine radiomics features, of which three were chosen by random forest analysis; B: Weights of MeanValue, NGLDM Busyness, and GLZLM HGZE in each patient; C: Overall survival curves for the entire cohort of patients, stratified by low or high radiomics score.

fication which plays a remarkable role in survival prediction of liver malignancy. In a previous study, Chen *et al*[44] revealed that elevated total bilirubin level (> 17.1 μmol/L) was an independent risk factor resulting in poor prognosis in advanced HCC patients. Peak postoperative bilirubin > 7.0 mg/dL was significantly related to liver-related death and worse outcomes after major hepatectomy. The group of patients with a total bilirubin level higher than the cut-off value (22.7 μmol/L) was also associated with a poorer OS in another study[45]. Moreover, Chantajitr *et al*[46] found that dilation of the intrahepatic bile duct was related to a poor prognosis in cHCC-CCA patients, and Lee *et al*[47] suggested that an increased Child-Pugh score (mean score: 5.8) was related to early death in cHCC-CCA patients. The role of anatomical hepatectomy in the prognosis of cHCC-CCA patients has rarely been evaluated, and some studies have reported that anatomical hepatectomy can prolong the survival time of HCC, but had no benefit in ICC patients[48,49]. These findings imply that the impact of anatomical hepatectomy on OS in cHCC-CCA is unclear and further large scale studies with a prospective design should be conducted to verify the results of this study.

Studies have suggested that anatomical hepatectomy can prolong survival in HCC but not ICC patients[48,49]; however, we are unaware of studies that have examined this issue in cHCC-CCA patients. The impact of anatomical hepatectomy on OS of

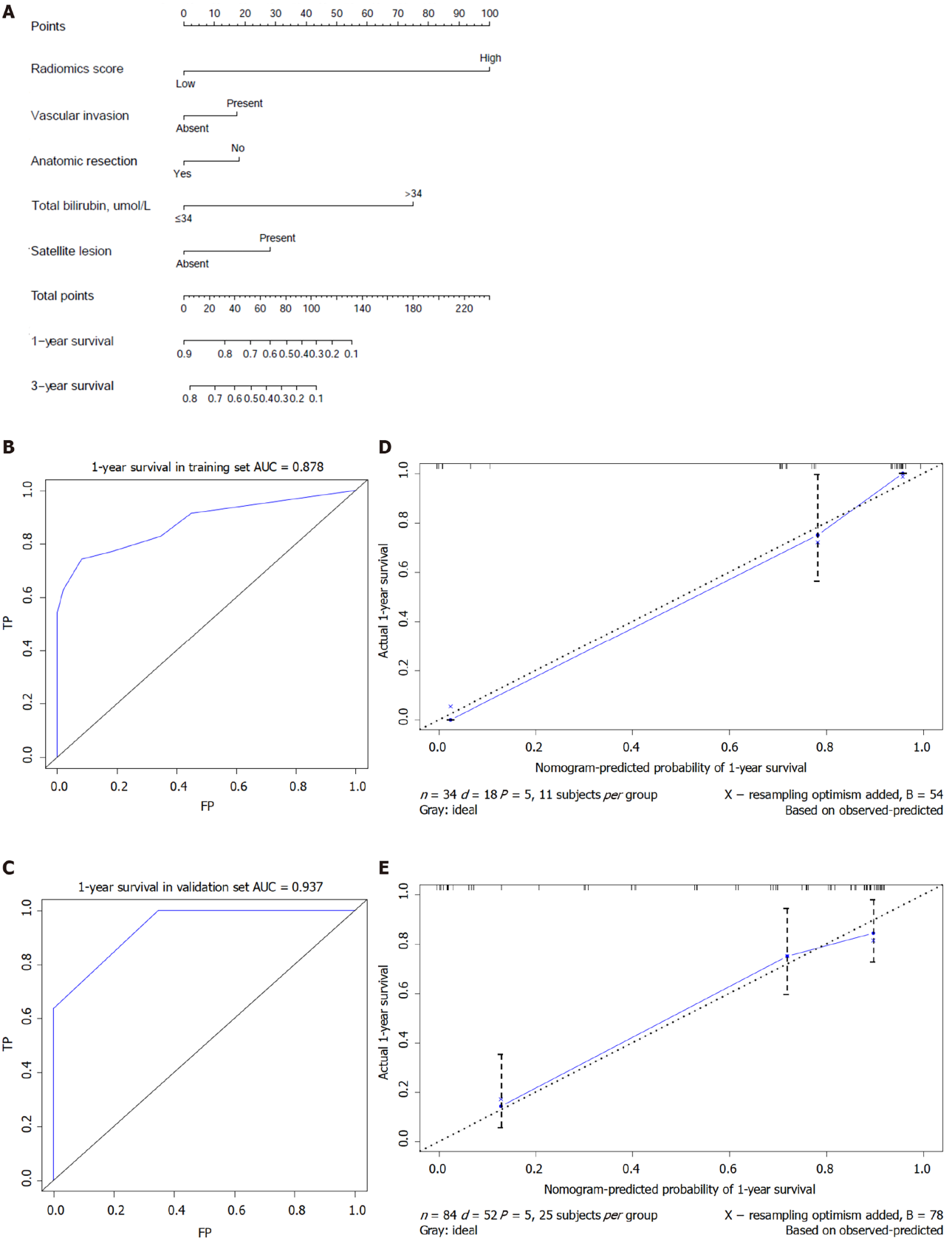


Figure 4 Construction and validation of a radiomics nomogram to predict overall survival of combined hepatocellular carcinoma and cholangiocarcinoma patients after surgical resection. A: Radiomics nomogram to predict overall survival (OS) at 1 and 3 years; B and C: Receiver operating characteristic curves for predicting 1-year OS in the training or validation set. The area under the curve in both cases was > 0.85; D and E: Calibration curves for 1-year OS in the training and validation sets. The horizontal axis is the survival rate predicted by the nomogram, and the vertical axis is the actual survival rate. The black dashed line indicates the case of perfect agreement between the two rates.

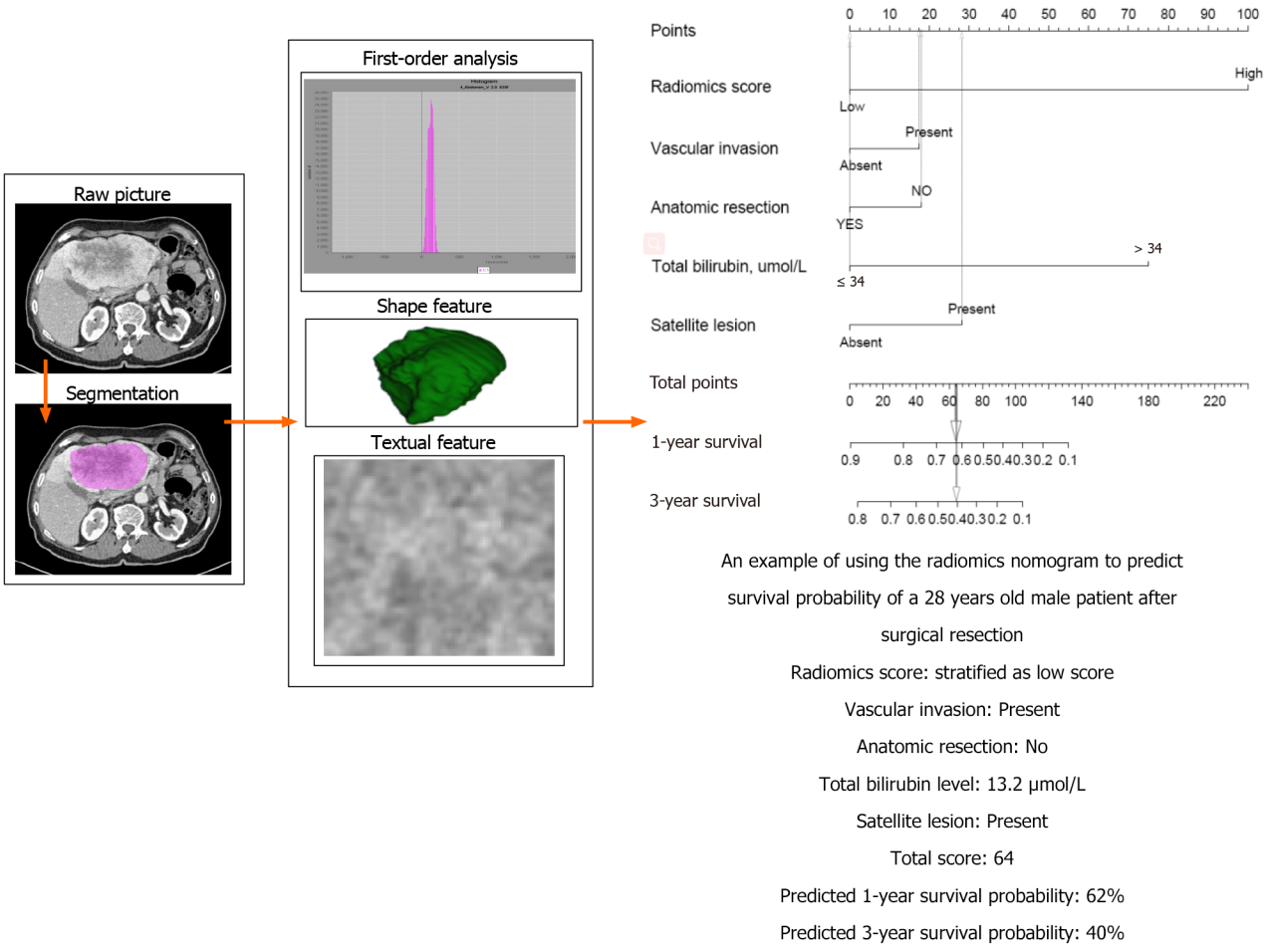


Figure 5 Example of using the radiomics nomogram to predict the overall survival of a 28-year-old man with combined hepatocellular carcinoma and cholangiocarcinoma.

cHCC-CCA patients after resection should be explored in large, prospective studies.

The present study has some limitations. First, its retrospective nature may be associated with a greater risk of selection bias and loss to follow-up, although only eight (6.8%) patients were lost to follow-up. Second, we validated the nomogram internally, not externally; nevertheless, AUCs were > 0.85 for both training and validation sets. Third, the study involved a small sample; thus, the nomogram described here should be validated and optimized using larger samples.

CONCLUSION

This study established a nomogram which combined the CT radiomics score with clinical risk factors to predict OS in patients with cHCC-CCA after resection with curative intent. The radiomics score was strongly associated with postoperative prognosis, and the integrative nomogram predicted OS well: High-risk patients showed a significantly shorter OS than low-risk patients. This integrative nomogram may aid in predicting the prognosis of cHCC-CCA patients after resection, and may support clinical decision-making.

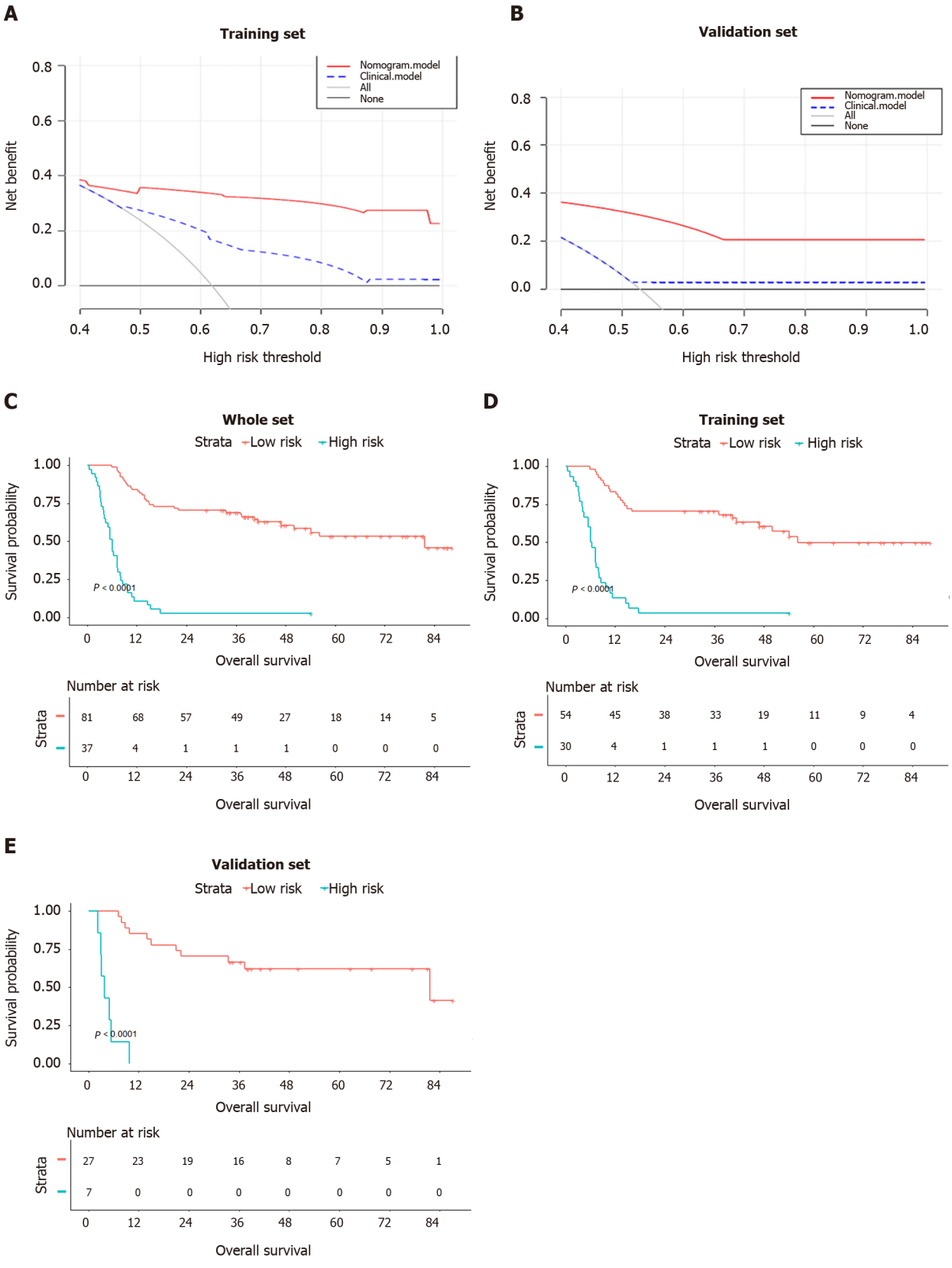


Figure 6 Clinical usefulness of the radiomics nomogram. A and B: Decision curve analysis assessing the ability of the radiomics nomogram or a model based on four clinical factors to predict overall survival (OS) in the training and validation sets. The y-axis indicates "net benefit"; the red line, the radiomics nomogram; the blue dotted line, the model based on clinical factors; the gray dotted line, the result in the event that all patients died; and the black dotted line, the result in the event that no patient died; C-E: OS comparison between patients classified by the radiomics nomogram as at "low risk" or "high risk" of poor OS; C: All patients; D: The training set; and E: The validation set.

ARTICLE HIGHLIGHTS

Research background

Combined hepatocellular carcinoma (HCC) and cholangiocarcinoma (cHCC-CCA) arises in hepatic progenitor cells and are defined as a single nodule showing differentiation into HCC and intrahepatic cholangiocarcinoma (ICC) with 5-year postoperative overall survival (OS) rates ranging from 8% to 63%. There are different opinions in the literature on whether the prognosis of patients with cHCC-CCA is worse than that of patients with simple HCC or similar ICC.

Research motivation

Due to the poor prognosis of cHCC-CCA and absence of a promising way to predict prognosis of cHCC-CCA, the authors aimed to construct a radiomics nomogram for predicting postoperative survival of cHCC-CCA patients. This prognostic model may help guide treatment decisions for these patients.

Research objectives

The purpose of this study was to construct and validate a nomogram based on radiomics and clinical characteristics to predict the postoperative survival rate of patients with cHCC-CCA.

Research methods

We collected the clinical data and computed tomography (CT) imaging data of patients with cHCC-CCA. Radiomics features were extracted from portal venous phase CT images using the least absolute shrinkage and selection operator Cox regression and random forest analysis. A nomogram integrating radiomics score and clinical factors was developed using multivariate Cox regression and each patient got a risk score. And patients were categorized as being at "high" or "low" risk based on their risk scores.

Research results

A total of five factors, which were Radiomics score, vascular invasion, anatomical resection, total bilirubin level, and satellite lesions, were independent predictors of prognosis and the nomogram was associated with OS more strongly than a model based on radiomics score or only clinical factors. Patients stratified as being at high risk showed a significantly shorter median OS than those stratified as being at low risk (6.1 *vs* 81.6 mo, $P < 0.001$).

Research conclusions

This nomogram have potential usefulness in predicting postoperative survival of cHCC-CCA patients and may therefore help identify those more likely to benefit from it, which may facilitate clinical decision-making.

Research perspectives

Considering the high AUC of this radiomics nomogram in predicting prognosis of cHCC-CCA, this prognostic model may help guide treatment decisions for these patients.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 7; 27(41): 7190-7206

DOI: 10.3748/wjg.v27.i41.7190

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

ORIGINAL ARTICLE

Retrospective Study

Clinical characteristics of gastrointestinal immune-related adverse events of immune checkpoint inhibitors and their association with survival

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Author contributions: Yamada K and Sawada T contributed to the conception and design of the study; Yamada K, Sawada T, Yamamura T, and Nakamura M contributed to the analysis and

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Abstract

BACKGROUND

Despite the popularity of immune checkpoint inhibitors (ICIs) in the treatment of advanced cancer, patients often develop gastrointestinal (GI) and non-GI immune-related adverse events (irAEs). The clinical characteristics and survival outcomes of GI-irAEs have not been fully elucidated in previous reports. This necessitates the evaluation of the impact of GI-irAEs on patients receiving ICI treatment.

interpretation of the data; Yamada K drafted the article; Ishikawa E, Maeda K, Kakushima N, Furukawa K, Honda T, Iida T, Mizutani Y, Ishikawa T, Ohno E, Kawashima H, Ishigami M, Furune S, Hase T, Yokota K, Maeda O, Hashimoto N, Akiyama M, and Ando Y critically revised the article for important intellectual content; Yamamura T performed the statistical analysis; Fujishiro M approved the final version of the article to be published; All authors have read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Nagoya University Hospital (No. 2018-0438, 15006).

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: Hase T received personal fees from AstraZeneca, Chugai Pharmaceutical Co. Ltd., Ono Pharmaceutical Co. Ltd., and Bristol-Myers Squibb Co. and grants from AstraZeneca and Chugai Pharmaceutical Co. Ltd., outside the submitted work. Hashimoto N received a grant from Boehringer Ingelheim, outside the submitted work. Ando Y received grants from Mochida Pharmaceutical Co. Ltd.; grants and personal fees from Chugai Pharmaceutical Co. Ltd., Kyowa Kirin Co. Ltd., Nippon Kayaku Co. Ltd., Yakult Honsha Co. Ltd., Ono Pharmaceutical Co. Ltd., Taiho Pharmaceutical Co. Ltd., Daiichi Sankyo Company Ltd., Eisai Co. Ltd.; and personal fees from Eli Lilly Japan K.K., Novartis Pharma K.K., Bayer Holding Ltd., Bristol-Myers Squibb, Sawai Pharmaceutical Co. Ltd., Tsumura & Co., Otsuka Holdings Co. Ltd., Roche Diagnostics K.K., AstraZeneca K.K., and MSD K.K, outside the submitted work. We

AIM

To evaluate the clinical characteristics of GI-irAEs and their impact on survival in patients treated with ICIs.

METHODS

In this single-center, retrospective, observational study, we reviewed the records of 661 patients who received ICIs for various cancers at Nagoya University Hospital from September 2014 to August 2020. We analyzed the clinical characteristics of patients who received ICI treatment. We also evaluated the correlation between GI-irAE development and prognosis in non-small cell lung cancer (LC) and malignant melanoma (MM). Kaplan-Meier analysis was used to compare the median overall survival (OS). Multivariate Cox proportional hazards models were used to identify prognostic factors. A P value < 0.05 was considered statistically significant.

RESULTS

GI-irAEs occurred in 34 of 605 patients (5.6%) treated with an anti-programmed cell death-1/programmed death-ligand 1 (anti-PD-1/PD-L1) antibody alone and in nine of 56 patients (16.1%) treated with an anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) antibody alone or a combination of anti-PD-1 and anti-CTLA-4 antibodies. The cumulative incidence and median daily diarrhea frequency were significantly higher in patients receiving anti-CTLA-4 antibodies (P < 0.05). In 130 patients with MM, OS was significantly prolonged in the group that continued ICI treatment despite the development of GI-irAEs compared to the group that did not experience GI-irAEs (P = 0.035). In contrast, in 209 patients with non-small cell LC, there was no significant difference in OS between the groups. The multivariate analyses showed that a performance status of 2-3 (hazard ratio: 2.406; 95% confidence interval: 1.125–5.147; P = 0.024) was an independent predictive factor for OS in patients with MM.

CONCLUSION

Patients receiving anti-CTLA-4 antibodies develop GI-irAEs more frequently and with higher severity than those receiving anti-PD-1/PD-L1 antibodies. Continuing ICI treatment in patients with MM with GI-irAEs have better OS.

Key Words: Colitis; Cytotoxic T-lymphocyte antigen 4; Diarrhea; Drug-related side effects and adverse reactions; Immune checkpoint inhibitors; Prognosis

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Core Tip: We compared the clinical characteristics of gastrointestinal immune-related adverse events (GI-irAEs) in patients receiving anti-programmed cell death-1/programmed death-ligand 1 (anti-PD-1/PD-L1), anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4), or a combination of anti-PD-1 and anti-CTLA-4 antibodies. We also examined the correlation between GI-irAE development and the prognosis of patients with non-small cell lung cancer (LC) and malignant melanoma (MM). GI-irAEs occurred more frequently and with higher severity in patients receiving anti-CTLA-4 antibodies than in those receiving anti-PD-1/PD-L1 antibodies. Patients with MM, but not non-small cell LC, who continued immune checkpoint inhibitor treatment after developing GI-irAEs had better overall survival.

Citation: Yamada K, Sawada T, Nakamura M, Yamamura T, Maeda K, Ishikawa E, Iida T, Mizutani Y, Kakushima N, Ishikawa T, Furukawa K, Ohno E, Honda T, Kawashima H, Ishigami M, Furune S, Hase T, Yokota K, Maeda O, Hashimoto N, Akiyama M, Ando Y, Fujishiro M. Clinical characteristics of gastrointestinal immune-related adverse events of immune checkpoint inhibitors and their association with survival. *World J Gastroenterol* 2021; 27(41): 7190-7206

URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7190.htm

DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7190

declare that there are no conflict of interest.

Data sharing statement: No additional data are available.

Country/Territory of origin: Japan

Specialty type: Gastroenterology and hepatology

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

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Received: July 4, 2021
Peer-review started: July 4, 2021
First decision: July 14, 2021
Revised: July 27, 2021
Accepted: August 31, 2021
Article in press: August 31, 2021
Published online: November 7, 2021

P-Reviewer: Soldera J, Yu F
S-Editor: Yan JP
L-Editor: Filipodia
P-Editor: Yuan YY

INTRODUCTION

In recent years, the prognosis of various malignancies has been improved with the introduction of immune checkpoint inhibitors (ICIs)[1-4] Monoclonal antibodies targeting programmed cell death-1 (PD-1), programmed death-ligand 1 (PD-L1), and cytotoxic T-lymphocyte antigen-4 (CTLA-4) exhibit antitumor activity by specifically activating T-cells and suppressing the inhibition of the immune system.

Adverse events (AEs) specific to ICIs, which are different from those of conventional chemotherapy, have been observed in patients receiving ICIs. These AEs are called immune-related AEs (irAEs) and include skin disorders, gastrointestinal (GI) disorders, liver disorders, interstitial lung disorders, and endocrine disorders. Among these, GI-irAEs are the most frequent and substantial. GI-irAEs occur between 0.6 and 120 wk after the initiation of therapy and are characterized by symptoms such as diarrhea, abdominal pain, and bloody stools. Inflammatory sites are found throughout the GI tract[5], and endoscopic findings have been reported to range from normal to ulcerative colitis-like findings[6].

These clinical features reportedly differ between anti-CTLA-4 antibodies, anti-PD-1/anti-PD-L1 antibodies, and their combinations[7-10]. However, there are only a few coherent reports, and the clinical features of GI-irAEs for each ICI have not yet been fully clarified.

The development of irAEs is associated with an improved prognosis in different malignancies[11-14]. In contrast, a previous study[15] showed a poor prognosis with the development of irAEs; hence, a consensus has not yet been reached. In addition, few studies observed prolongation of overall survival (OS) with GI-irAE development [16-18].

Therefore, this study aimed to investigate differences in the clinical characteristics of GI-irAEs associated with anti-PD-1/PD-L1 antibodies, anti-CTLA-4 antibodies, and combination therapy with anti-PD-1 and anti-CTLA-4 antibodies. Further, we examined the correlation between the development of GI-irAEs and patient prognosis.

MATERIALS AND METHODS

Subjects and study design

This single-center, retrospective, observational study included consecutive patients who received ICIs (*i.e.* nivolumab, pembrolizumab, atezolizumab, avelumab, durvalumab, or ipilimumab) for various cancers [lung cancer (LC), malignant melanoma (MM), gastric cancer (GC), renal cell carcinoma (RCC), head and neck cancer, urothelial cancer, gynecological cancer, breast cancer, or colorectal cancer] at the Nagoya University Hospital from September 2014 to August 2020. Patients who received ICIs as maintenance therapy after curative chemoradiation for non-small cell lung (NSCLC) or postoperative adjuvant therapy for MM were excluded from the prognostic analysis. The background of the patients, presence or absence of irAEs, time of onset, clinical characteristics at the time of onset, and laboratory findings were collected and reviewed using medical records.

Eligible patients were those treated with ICIs at standard doses. Nivolumab was administered at 3 mg/kg or 240 mg/body every 2 wk. However, some MM patients were administered 2 mg/kg nivolumab every 3 wk. Pembrolizumab was administered at 2 mg/kg or 200 mg/body every 3 wk. Atezolizumab was administered at 1200 mg/body every 3 wk. Avelumab was administered at 10 mg/kg every 2 wk. Durvalumab was administered at 10 mg/kg every 2 wk. Ipilimumab was administered at 3 mg/kg every 3 wk for four cycles. Combination therapy consisted of nivolumab (3 mg/kg or 240 mg/body) and ipilimumab (3 mg/kg) every 3 wk for four cycles.

GI-irAEs were defined as diarrhea or bloody stools after ICI administration in patients in whom infectious enteritis could be excluded. Infectious enteritis (*Clostridium difficile*, other bacterial infections, or viral pathogens, such as cytomegalovirus) was ruled out using blood tests and stool samples. The National Cancer Institute Common Terminology Criteria for AEs (version 5.0) was used to assess the severity of enterocolitis and diarrhea.

Treatment of GI-irAEs was based on the strategy of the attending physician, but in most cases was based on the American Society of Clinical Oncology clinical practice guideline[19]. In the case of Grade 1 GI-irAEs, ICIs were continued or were stopped temporarily and resumed if toxicity did not exceed Grade 1. In the case of Grade 2 or higher GI-irAEs, ICIs were discontinued until the patient's symptoms recovered to Grade 1 or lower, at which point restarting ICIs was considered depending on the

patient's condition.

Outcome measures

We divided the patients into those who received an anti-PD-1/PD-L1 antibody alone (PD-1/PD-L1 group) and those who received an anti-CTLA-4 antibody alone or a combination of anti-PD-1 and anti-CTLA-4 antibodies (CTLA-4 group). The clinical characteristics of GI-irAEs [incidence, cumulative incidence, severity, computed tomography (CT) findings, endoscopic findings, and treatments] were compared between the PD-1/PD-L1 and CTLA-4 groups.

We examined the correlation between the incidence of GI-irAEs and the prognosis of patients with NSCLC and MM, which included more cases of ICI administration than other cancers. Patients who developed GI-irAEs were categorized into two groups: The ICI continuation group in which ICI administration was continued or resumed after improvement of GI-irAEs and the ICI discontinuation group in which ICI administration was permanently discontinued after the development of GI-irAEs. Prognosis was assessed in three groups: Non-GI-irAE, ICI continuation, and ICI discontinuation. Prognosis was also examined by cancer stage (stage III or IV).

Statistical analysis

To compare each group, the Mann–Whitney *U* test was used for continuous variables and Fisher's exact test was used for categorical variables. The Kaplan–Meier method and log-rank tests were used to compare the cumulative incidence and median OS among the groups. Univariate and multivariate Cox proportional hazards models were used to identify prognostic factors associated with GI-irAEs. SPSS Statistics software (version 27.0; IBM Corp., Armonk, NY, United States) was used for analysis. For all analyses, a P value < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics

Overall, there were 605 patients in the PD-1/PD-L1 group and 56 patients in the CTLA-4 group. The clinical characteristics of the PD-1/PD-L1 and CTLA-4 groups are described in Table 1. The median ages were 69 (range: 22–87) and 65 (range: 21–85) years in the PD-1/PD-L1 and CTLA-4 groups, respectively. Patients in the PD-1/PD-L1 group were significantly older than those in the CTLA-4 group (P = 0.039). There were no significant differences in sex, body mass index (BMI), or Eastern Cooperative Oncology Group performance status (ECOG PS) between the groups. The PD-1/PD-L1 group included patients with LC, MM, RCC, GC, and other cancers (head and neck cancer, urothelial cancer, gynecological cancer, breast cancer, and colorectal cancer); the CTLA-4 group included patients with MM and RCC.

Incidence of GI-irAEs

Among 47 patients who developed diarrhea or bloody stools after ICI administration, 39 had diarrhea, one had bloody stools, and seven had diarrhea and bloody stools. Forty-three patients (excluding four patients diagnosed with infectious enteritis) were included in the analysis. GI-irAEs occurred in 34 of 605 patients (5.6%) in the PD-1/PD-L1 group and 9 of 56 patients (16.1%) in the CTLA-4 group. The incidence of GI-irAEs in the CTLA-4 group was significantly higher than that in the PD-1/PD-L1 group (P = 0.008). We compared the cumulative incidence of GI-irAEs in the PD-1/PD-L1 group with that in the CTLA-4 group for all cancers (Figure 1). The cumulative incidence was significantly higher in the CTLA-4 group than in the PD-1/PD-L1 group (P = 0.003). The median observation periods until the development of GI-irAEs were 1683.1 ± 28.3 d [95% confidence interval (CI): 1627.6-1738.5] in the PD-1/PD-L1 group and 1299.7 ± 77.7 d (95%CI: 1147.5-1452.0) in the CTLA-4 group.

Severity of GI-irAEs

The clinical characteristics of patients with GI-irAEs in the PD-1/PD-L1 and CTLA-4 groups are shown in Table 2. There were no differences in age, sex, or median ICI duration before the development of GI-irAEs between the groups. In the PD-1/PD-L1 group, nine patients (26%) had Grade 1 GI-irAEs, 18 (53%) had Grade 2 GI-irAEs, and seven (21%) had Grade 3 GI-irAEs. In the CTLA-4 group, no patients had Grade 1 GI-irAEs, five (55.6%) developed Grade 2 GI-irAEs, and four (44.4%) had Grade 3 GI-irAEs.

Table 1 Clinical characteristics of all patients in the programmed cell death-1/programmed death-ligand 1 and cytotoxic T-lymphocyte antigen 4 groups

Characteristic	PD-1/PD-L1, *n* = 605	CTLA-4, *n* = 56	*P* value
Age, yr, median (range)	69 (22-87)	65 (21-85)	0.039
Sex, *n* (%)			0.228
Male	419 (69.3)	34 (60.7)	
Female	186 (30.7)	22 (39.3)	
BMI, kg/m^2	21.3 (12.0-37.0)	21.6 (13.9-43.0)	0.532
ECOG PS, *n* (%)			0.073
0-1	534 (88.3)	54 (96.4)	
2-3	71 (11.7)	2 (3.6)	
Cancer type, *n* (%)			
NSCLC	241 (39.8)	0 (0.0)	
MM	110 (18.2)	39 (69.6)	
RCC	52 (8.6)	17 (30.4)	
GC	49 (8.1)	0 (0.0)	
Others	153(25.3)	0 (0.0)	
Drugs, *n* (%)			
Nivolumab	317 (52.4)	0 (0.0)	
Pembrolizumab	180 (29.8)	0 (0.0)	
Atezolizumab	74 (12.2)	0 (0.0)	
Durvalumab	32 (5.3)	0 (0.0)	
Avelumab	2 (0.3)	0 (0.0)	
Ipilimumab	0 (0.0)	28 (50.0)	
Nivolumab + ipilimumab	0 (0.0)	28 (50.0)	

BMI: Body mass index; CTLA-4: Cytotoxic T-lymphocyte antigen 4; ECOG PS: Eastern Cooperative Oncology Group performance status; GC: Gastric cancer; MM: Malignant melanoma; NSCLC: Non-small cell lung cancer; PD-1/PD-L1: Programmed cell death-1/programmed death-ligand 1; RCC: Renal cell carcinoma.

Grade 2 or 3 GI-irAEs were more frequent in the CTLA-4 group than in the PD-1/PD-L1 group, but there was no statistically significant difference (P = 0.166). The median daily diarrhea frequencies were 5.0 (range: 0-10) times and 6.5 (range: 4-15) times in the PD-1/PD-L1 and CTLA-4 groups, respectively. The median daily diarrhea frequency was significantly higher in the CTLA-4 group than in the PD-1/PD-L1 group (P = 0.03).

Treatment

Thirteen patients (38.2%) in the PD-1/PD-L1 group and one (11.1%) in the CTLA-4 group improved without medication (Table 2). More patients in the CTLA-4 group than in the PD-1/PD-L1 group improved without medication (P = 0.017). Corticosteroids were required in 11 patients (32.4%) in the PD-1/PD-L1 group and eight (88.9%) in the CTLA-4 group. More patients in the CTLA-4 group than in the PD-1/PD-L1 group required steroid therapy (P = 0.006). All patients in this study improved with observation or treatment with steroids, and no patient was treated with biological agents or surgery.

CT and endoscopic findings

Twenty-three patients in the PD-1/PD-L1 group underwent abdominal CT, which showed many inflammation sites, especially between the descending colon and rectum. Eight patients in the CTLA-4 group had diffused CT findings between the jejunum and rectum (Table 3). There was no statistically significant difference between

Table 2 Clinical characteristics of patients who developed gastrointestinal-immune-related adverse events in the programmed cell death-1/programmed death-ligand 1 and cytotoxic T-lymphocyte antigen 4 groups

Characteristic	PD-1/PD-L1, *n* = 34	CTLA-4, *n* = 9	*P* value
Age, yr, median (range)	69 (37-86)	56 (46-80)	0.187
Sex, *n* (%)			0.427
Male	29 (85.3)	7 (77.8)	
Female	5 (14.7)	2 (22.2)	
Drugs, *n* (%)			
Nivolumab	12 (35.3)	0 (0.0)	
Pembrolizumab	16 (47.0)	0 (0.0)	
Atezolizumab	2 (5.9)	0 (0.0)	
Durvalumab	4 (11.8)	0 (0.0)	
Ipilimumab	0 (0.0)	6 (66.7)	
Nivolumab + ipilimumab	0 (0.0)	3 (33.3)	
Median ICI duration before GI-irAE onset (d), median (range)	77 (4-733)	42 (11-92)	0.127
Diarrhea frequency per day, times (range)	5.0 (0-10)	6.5 (4-15)	0.031
CTCAE Grade, *n* (%)			0.288
1	9 (26.5)	0 (0.0)	
2-3	25 (73.5)	9 (100)	
GI-irAE treatment, *n* (%)			
Improvement without medication	13 (38.2)	1 (11.1)	0.017
Corticosteroids	11 (32.4)	8 (88.9)	0.006
Loperamide	8 (23.5)	1 (11.1)	0.657

CTCAE: Common Terminology Criteria for Adverse Events; CTLA-4: Cytotoxic T-lymphocyte antigen 4; GI-irAE: Gastrointestinal-immune-related adverse event; ICI: Immune checkpoint inhibitor; PD-1/PD-L1: Programmed cell death-1/programmed death-ligand 1.

the PD-1/PD-L1 group and the CTLA-4 group in each segment from the jejunum to the rectum.

Lower GI endoscopy was performed in 16 patients (12 in the PD-1/PD-L1 group and four in the CTLA-4 group). Endoscopic findings were classified as follows, according to a previous report[6]: (1) Large deep ulcer [PD-1/PD-L1 group (*n* = 1)]; (2) Diffuse erythema with exudate [PD-1/PD-L1 group (*n* = 2); CTLA-4 group (*n* = 1)]; (3) Patchy erythema [CTLA-4 group (*n* = 1)]; (4) Aphtha [PD-1/PD-L1 group (*n* = 2)]; (5) Edema [PD-1/PD-L1 group (*n* = 4); CTLA-4 group (*n* = 2)]; and (6) Normal [PD-1/PD-L1 group (*n* = 2)]. There was no specific trend in endoscopic findings based on treatment group. There was an ischemic change [PD-1/PD-L1 group (*n* = 1)] that could not be classified using the classifications above (Figure 2).

Development of GI-irAEs and prognosis

The study included 209 patients with NSCLC and 130 patients with MM, excluding 30 patients who received durvalumab as maintenance therapy after curative chemoradiation for unresectable advanced NSCLC and 19 patients with MM who received ICIs as postoperative adjuvant therapy.

Twelve patients with NSCLC and 13 with MM developed GI-irAEs. The clinical characteristics of all patients and those who developed GI-irAEs are shown in Tables 4 and 5, respectively.

There were eight and four patients with NSCLC in the ICI continuation and ICI discontinuation groups, respectively, and 10 and three patients with MM in the ICI continuation and ICI discontinuation groups, respectively. Among patients with NSCLC, the median OS was 488.0 ± 20.6 d (95%CI: 447.6-528.4) in the ICI continuation group, 829.0 ± 558.3 d (95%CI: 0-1923.3) in the ICI discontinuation group, and 521.0 ±

Table 3 Site of inflammation on abdominal computed tomography in the programmed cell death-1/programmed death-ligand 1 and cytotoxic T-lymphocyte antigen 4 groups

Site of inflammation	PD-1/PD-L1, *n* = 23	CTLA-4, *n* = 8
Jejunum, *n* (%)	1 (4.3)	2 (25.0)
Ileum, *n* (%)	2 (8.7)	2 (25.0)
Cecum, *n* (%)	2 (8.7)	2 (25.0)
Ascending colon, *n* (%)	3 (13.0)	4 (50.0)
Transverse colon, *n* (%)	3 (13.0)	3 (37.5)
Descending colon, *n* (%)	6 (26.1)	3 (37.5)
Sigmoid colon, *n* (%)	7 (30.4)	2 (25.0)
Rectum, *n* (%)	8 (34.8)	2 (25.0)
No findings, *n* (%)	9 (39.1)	2 (25.0)

CT: Computed tomography; CTLA-4: Cytotoxic T-lymphocyte antigen 4; PD-1/PD-L1: Programmed cell death-1/programmed death-ligand 1.

102.4 d (95%CI: 320.2-721.8) in the non-GI-irAE group. There was no significant difference in OS among the three groups. The results were similar when patients were stratified by disease stage (stage III or IV). Among patients with MM, there was a significant prolongation of OS in the ICI continuation group compared to the non-GI-irAE group [not reached *vs* 481.0 d (95%CI: 329.1-632.9); P = 0.035]. There was no significant difference in OS based on the development of GI-irAEs among patients with stage III disease. Among patients with stage IV disease, there was a significant prolongation of OS in the ICI continuation group compared to the non-GI-irAE group [not reached *vs* 427.0 d (95%CI: 248.5-605.5); P = 0.017] (Figure 3).

Prognostic factors for OS in NSCLC and MM were examined using Cox proportional hazards models (Tables 6 and 7). The following factors were considered: Age (< 75 *vs* ≥ 75 years), sex, BMI [underweight (< 18.5 kg/m^2) *vs* normal weight (18.5−24.9 kg/m^2) *vs* overweight (≥ 25.0 kg/m^2)], stage (III *vs* IV), ECOG PS (0-1 *vs* 2-3), ICIs (PD-1/PD-L1 *vs* CTLA-4), and GI-irAEs (ICI continuation *vs* ICI discontinuation *vs* non-GI-irAE groups). In NSCLC, the univariate analysis showed significant associations of OS with age, BMI, stage, and ECOG PS. In the multivariate analysis of these factors, stage IV disease [hazard ratio (HR): 2.182; 95%CI: 1.085–4.387; P = 0.029] and a ECOG PS of 2-3 (HR: 12.772; 95%CI: 7.067-23.085; P < 0.001) were identified as independent predictors of OS. Similarly, in MM, the univariate analysis showed significant associations of OS with age, sex, ECOG PS, and GI-irAEs. In the multivariate analysis of these factors, only ECOG PS (HR: 2.406; 95%CI: 1.125-5.147; P = 0.024) was identified as an independent predictor of OS.

DISCUSSION

In this study, patients in the CTLA-4 group had a higher incidence of GI-irAEs and a higher frequency of diarrhea than those in the PD-1/PD-L1 group. In addition, treatment of GI-irAEs required more steroids in the CTLA-4 group than in the PD-1/PD-L1 group. In a previous report[7], the onset of GI-irAEs has been reported to occur 6-7 wk after the initiation of ipilimumab, an anti-CTLA-4 antibody. Patients who received anti-PD-1 monotherapy developed GI-irAEs at a median of 25.4 wk (range: 0.6-119.9), whereas those who received combination therapy with anti-PD-1 and anti-CTLA-4 antibodies developed GI-irAEs earlier, at a median of 7.2 wk (range: 0.7-51) [8]. Regarding incidence, a meta-analysis by Wang *et al*[20] reported overall incidences of colitis of 9.1% among patients treated with anti-CTLA-4 antibody monotherapy, 1.3% among those treated with anti-PD-1/PD-L1 antibody monotherapy, and 13.6% among those treated with a combination of anti-PD-1 and anti-CTLA-4 antibodies. Similarly, our study showed that the incidence of GI-irAEs in the CTLA-4 group was indeed higher than that in the PD-1/PD-L1 group.

There are several possible explanations for the higher incidence, severity, and use of steroids among patients treated with anti-CTLA-4 antibodies compared to those treated with anti-PD-1/PD-L1 antibodies. CTLA-4 is required for the accumulation of

Table 4 Clinical characteristics and frequency of each type of immune-related adverse event in patients with non-small cell lung carcinoma and malignant melanoma

Characteristic	NSCLC, *n* = 209	MM, *n* = 130
Age, yr	66 ± 11	66 ± 13
Sex, *n* (%)		
Male	143 (68.4)	75 (57.7)
Female	66 (31.6)	55 (42.3)
BMI, kg/m^2	21.7 ± 3.4	22.4 ± 4.3
ECOG PS, *n*		
0-1	184	119
2-3	25	11
Drugs, *n* (%)		
Nivolumab	61 (29.2)	58 (44.6)
Pembrolizumab	87 (41.6)	35 (26.9)
Atezolizumab	61 (29.2)	0 (0.0)
Ipilimumab	0 (0.0)	27 (20.8)
Nivolumab + ipilimumab	0 (0.0)	10 (7.7)
History of ICI use, *n* (%)	11 (5.3)	34 (26.2)
Follow-up, d	365 ± 335	466 ± 419
Total irAEs, *n* (%)		
GI-irAEs	9 (4.3)	13 (10.0)
Liver-irAEs	7 (3.3)	13 (10.0)
Lung-irAEs	10 (4.8)	11 (8.5)
Skin-irAEs	9 (4.3)	9 (6.9)
Thyroid-irAEs	12 (5.7)	9 (6.9)

BMI: Body mass index; ECOG PS: Eastern Cooperative Oncology Group performance status; GI: Gastrointestinal; irAE: Immune-related adverse event; ICI: Immune checkpoint inhibitor; MM: Malignant melanoma; NSCLC: Non-small cell lung carcinoma.

regulatory T cells in the bowel[21], and is thought to play an essential role in the maintenance of bowel homeostasis, even in the absence of inflammation. In contrast, PD-1 depends on PD-L1/PD-L2 expression to regulate T-cell activation, and PD-L1 and PD-L2 are upregulated by inflammation, inflammatory cytokines, and chemokines [22-25]. When this mechanism is blocked by anti-PD-1/PD-L1 antibodies, it may be difficult to control inflammation, given that blocking with ICIs leads to a partial block of the mechanisms that trigger inflammation. Some reports suggest that these mechanistic differences may account for the difference in the frequency of enteritis caused by anti-CTLA-4 and anti-PD-1/PD-L1 antibodies[9].

There were no reported differences in the CT or endoscopic findings of enteritis caused by anti-CTLA-4 and anti-PD-1/PD-L1 antibodies. There was no specific trend, and a variety of findings were found in this study. In one case in the PD-1/PD-L1 group, an ischemic colitis-like endoscopic finding, which is rarely reported as a GI-irAE, was observed. Since necrotizing gastritis has been previously reported as an AE in the upper GI tract[9], it was included as a GI-irAE in this study. When GI-irAEs are suspected, CT and endoscopy should be considered, as they are useful for confirming the extent of the lesion and excluding other diseases[19].

In this study, OS was prolonged in the ICI continuation group compared to the non-GI-irAE group among patients with MM, but not among those with NSCLC. According to the multivariate Cox regression analysis, continued administration of ICIs after the development of GI-irAEs was not an independent factor that affected the survival of patients with MM (HR: 3.081; P = 0.058). However, a statistically significant difference may be expected with the accumulation of future cases. Many studies have

Table 5 Clinical characteristics of patients with non-small cell lung carcinoma and malignant melanoma who developed gastrointestinal-immune-related adverse event

Characteristic	NSCLC, *n* = 12	MM, *n* = 13
Age, yr	67 ± 11	67 ± 12
Sex, *n*		
Male	10	9
Female	2	4
BMI, kg/m^2	22.2 ± 3.7	22.1 ± 4.4
ECOG PS, *n*		
0-1	12	13
2-3	0	0
Stage, *n*		
III	1	2
IV	11	11
Latest ICI, *n*		
Nivolumab	2	5
Pembrolizumab	8	3
Atezolizumab	2	0
Ipilimumab	0	5
Nivolumab + ipilimumab	0	0
Diarrhea frequency	4.3 ± 1.8	5.5 ± 2.5
CTCAE Grade, *n*		
1	4	3
2	7	8
3	1	2
Median ICI duration before GI-irAE onset (d), median (range)	60 (7-567)	75 (24-733)
Treatment with ICIs after the onset of GI-irAEs		
Continued or resumed	8	10
Discontinued	4	3

BMI: Body mass index; CTCAE: Common Terminology Criteria for Adverse Events; ECOG PS: Eastern Cooperative Oncology Group performance status; GI-irAE: Gastrointestinal-immune-related adverse event; ICI: Immune checkpoint inhibitor; MM: Malignant melanoma; NSCLC: Non-small cell lung carcinoma.

reported a correlation between the development of irAEs and a favorable prognosis [26-28]. A shared antigen is one promising hypothesis for the mechanism, which was reported in a case study of fatal myocarditis after ipilimumab and nivolumab treatment for MM[29]. Autopsy of the patient revealed infiltration of myocardial T cells and macrophages. Receptor sequencing of the infiltrating T cells revealed that the selective clonal T-cell population infiltrating the myocardium was identical to that in tumors and skeletal muscle. According to another report[30], in patients with NSCLC and MM under PD-1 blockade, lung irAE lesions were infiltrated by T cells with similar specificity to tumor-infiltrating T cells. These reports suggest that specific autoimmune T cell clones recognize shared antigens between normal and tumor tissues, triggering an immune response that results in irAEs. IrAEs may represent a strong immune response to both tumor and healthy tissues, leading to stronger antitumor effects and improved prognosis. However, GI-irAEs may develop due to excessive activation of intestinal immunity by T cells as described above. There may be a common underlying factor between the susceptibility to ICIs due to enhanced activation of tumor immunity and the susceptibility to GI-irAEs due to activation of

Table 6 Univariate and multivariate analyses of clinical factors related to overall survival in non-small cell lung carcinoma

Factor	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age, yr						
< 75	1			1		
≥ 75	0.520	0.277-0.976	0.042	0.658	0.345-1.253	0.203
Sex						
Male	1					
Female	1.301	0.845-2.003	0.233			
BMI, kg/m^2						
Underweight (< 18.5)	1			1		
Normal (18.5-24.9)	0.527	0.316-0.878	0.014	0.635	0.377-1.067	0.086
Overweight (> 25.0)	0.394	0.195-0.795	0.009	0.506	0.250-1.040	0.064
Stage						
III	1			1		
IV	2.447	1.227-4.881	0.011	2.182	1.085-4.387	0.029
ECOG PS						
0-1	1			1		
2-3	15.197	8.486-27.214	< 0.001	12.772	7.067-23.085	< 0.001
GI-irAE						
Continued administration of ICIs	1					
Discontinued administration of ICIs	0.904	0.165-4.945	0.907			
Non-GI-irAEs	1.334	0.489-3.642	0.574			

BMI: Body mass index; CI: Confidence interval; ECOG PS: Eastern Cooperative Oncology Group performance status; GI-irAE: Gastrointestinal-immune-related adverse event; HR: Hazard ratio; ICI: Immune checkpoint inhibitor.

intestinal immunity.

Conversely, patients who discontinued ICIs after developing GI-irAEs did not have prolonged OS compared to those who did not develop GI-irAEs in this study. We consider this result to be caused by the difficulty in continuing ICIs in patients with no antitumor effects at the time of developing GI-irAEs. We also consider the possibility that OS may have been shortened by not administering ICIs for a sufficient period.

ICI administration often cannot be continued depending on the extent of irAEs. According to consensus guidelines[19], careful follow-up after the onset of irAEs or the administration of steroids and continuation of ICIs after improvement of irAEs to Grade 1 can be considered. However, severe irAEs (Grade 3 or higher) may result in permanent discontinuation of ICIs, necessitating hospitalization or surgery, and may be fatal in some cases.

As irAEs are life-threatening, depending on severity, it is difficult to determine whether to continue ICIs in patients with Grade 1 irAEs or to resume ICIs after recovery from Grade 2 or higher irAEs. However, it is expected that the number of cases in which resuming ICIs is considered will increase in the future when irAEs occur and no other alternative treatments are available. IrAEs that occur during ICI reintroduction are less severe than initial irAEs and can be tolerated if monitored appropriately[31,32]. GI-irAE recurrence in cases of ICI re-administration after GI-irAE onset has been observed in about one-third of patients[33], and GI-irAEs occurring at re-administration were less frequent and less severe than the initial GI-irAEs[34].

This study showed that the continuation of ICI administration after GI-irAEs in MM may contribute to enhanced patient prognosis. However, which patients with severe GI-irAEs (Grade ≥ 3) should continue ICIs is a topic for future studies. In addition, we believe that the decision to reinitiate ICI treatment after irAEs should be based on the clinical judgment of the treating physician, considering the physical health of the

Table 7 Univariate and multivariate analyses of clinical factors related to overall survival in malignant melanoma

Factor	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age, yr						
< 75	1			1		
≥ 75	1.717	1.067-2.761	0.026	1.474	0.816-2.663	0.199
Sex						
Male	1			1		
Female	0.593	0.354-0.993	0.047	0.793	0.418-1.506	0.479
BMI, kg/m^2						
Underweight (< 18.5)	1					
Normal (18.5-24.9)	1.252	0.671-2.336	0.48			
Overweight (> 25.0)	1.044	0.510-2.137	0.906			
Stage						
III	1					
IV	1.758	0.838-3.686	0.135			
ECOG PS						
0-1	1			1		
2-3	3.014	1.427-6.366	0.004	2.406	1.125-5.147	0.024
GI-irAE						
Continued administration of ICIs	1			1		
Discontinued administration of ICIs	3.818	0.767-18.996	0.102	4.079	0.779-21.368	0.096
Non-GI-irAEs	3.25	1.020-10.360	0.046	3.081	0.963-9.861	0.058
ICI						
Anti PD-1/PD-L1 antibody	1					
Anti CTLA-4 antibody	1.366	0.837-2.228	0.212			

BMI: Body mass index; CI: Confidence interval; CTLA-4: Cytotoxic T-lymphocyte antigen 4; GI-irAE: Gastrointestinal-immune-related adverse event; HR: Hazard ratio; ICI: Immune checkpoint inhibitor; PD-1: Programmed cell death-1.

patient, and appropriate informed consent.

Once the mechanism of irAE development and predictive markers for irAE development are identified, it will be possible to continue ICI treatment for a longer period while monitoring patients prone to irAE development and preventing irAEs. Since this may improve the prognosis of patients, further research to clarify the pathogenesis is desirable.

There are several limitations to this study. First, it is a single-center, retrospective study. Second, abdominal CT scans and lower GI endoscopy were not performed in all patients in this study, and the number of patients who had abdominal CT scans and lower GI endoscopy was not enough. More cases are expected in the future. Third, the OS analysis for each ICI regimen was not statistically significant, probably due to the small sample size. Therefore, patients who received ICI monotherapy and those who received multiple ICIs sequentially were included in the analysis.

CONCLUSION

GI-irAEs tended to occur more frequently and with higher severity in patients treated with anti-CTLA-4 antibodies or combination therapy than in those treated with anti-PD-1/PD-L1 antibodies. Abdominal CT and lower GI endoscopy did not show any characteristic findings or trends. We found evidence that the clinical characteristics

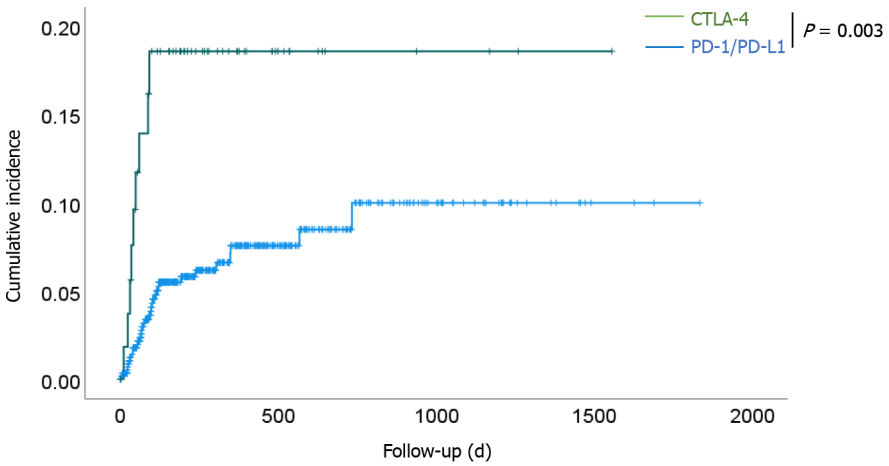


Figure 1 Kaplan–Meier curves of the cumulative incidence of gastrointestinal- immune-related adverse events for all patients in the programmed cell death-1/programmed death-ligand 1 and cytotoxic T-lymphocyte antigen 4 groups. The cumulative incidence was significantly higher in the cytotoxic T-lymphocyte antigen 4 group than in the programmed cell death-1/programmed death-ligand 1 group (P = 0.003). CTLA-4: Cytotoxic T-lymphocyte antigen 4; PD-1: Programmed cell death-1; PD-L1: Programmed death-ligand 1.

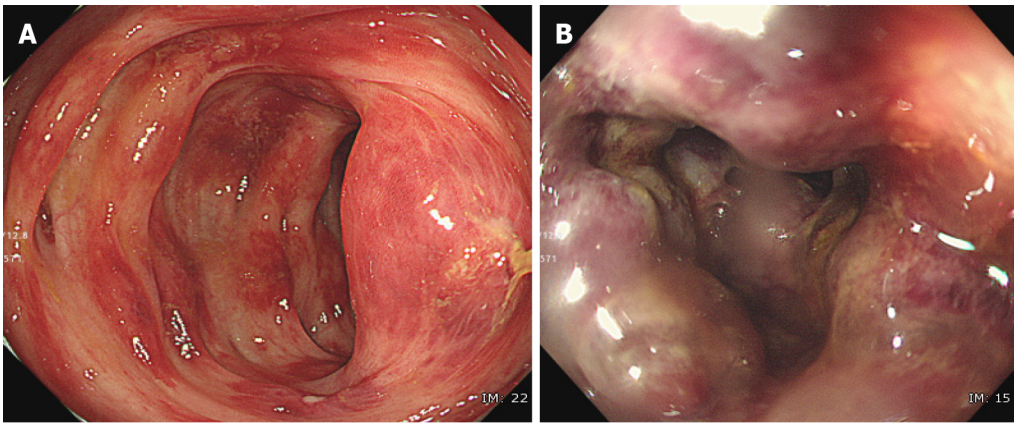
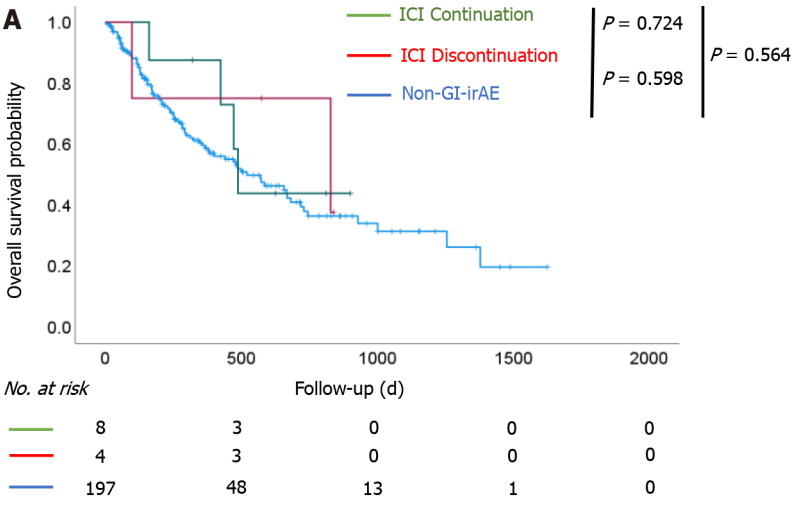
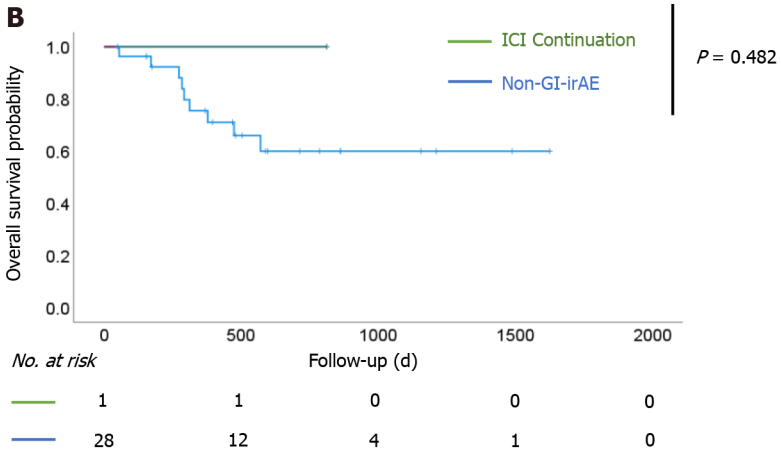
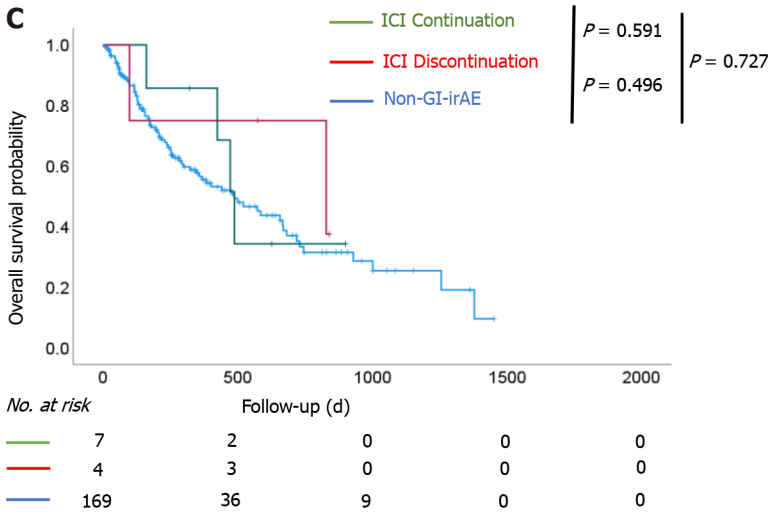
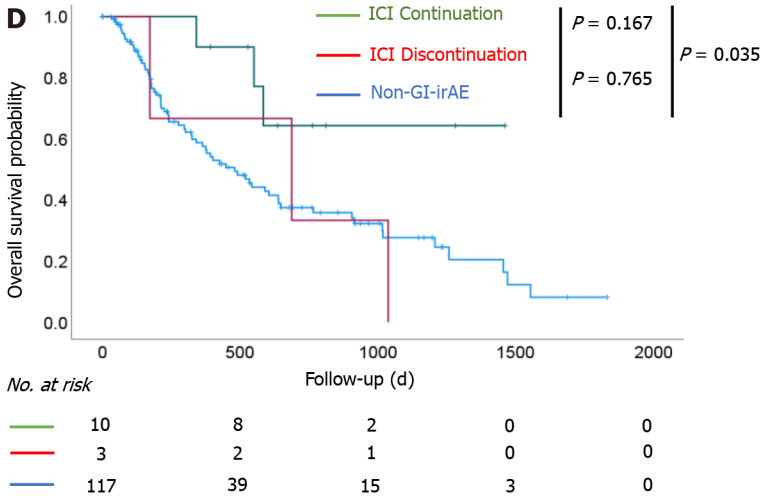


Figure 2 Ischemic change caused by an immune checkpoint inhibitor. Endoscopic images of gastrointestinal-immune-related adverse events in the sigmoid colon of a patient. A: The mucosa of the sigmoid colon shows redness, erosion, hemorrhage, and edema; B: Some of the mucosa is pale, with submucosal edema and bleeding.




B
ICI Continuation
Non-GI-irAE
P = 0.482
Overall survival probability
1.0
0.8
0.6
0.4
0.2
0.0
0
500
1000
1500
2000
Follow-up (d)
No. at risk
1 1 0 0 0
28 12 4 1 0


C
ICI Continuation
ICI Discontinuation
Non-GI-irAE
P = 0.591
P = 0.496
P = 0.727
Overall survival probability
1.0
0.8
0.6
0.4
0.2
0.0
0
500
1000
1500
2000
Follow-up (d)
No. at risk
7 2 0 0 0
4 3 0 0 0
169 36 9 0 0


D
ICI Continuation
ICI Discontinuation
Non-GI-irAE
P = 0.167
P = 0.765
P = 0.035
Overall survival probability
1.0
0.8
0.6
0.4
0.2
0.0
0
500
1000
1500
2000
Follow-up (d)
No. at risk
10 8 2 0 0
3 2 1 0 0
117 39 15 3 0

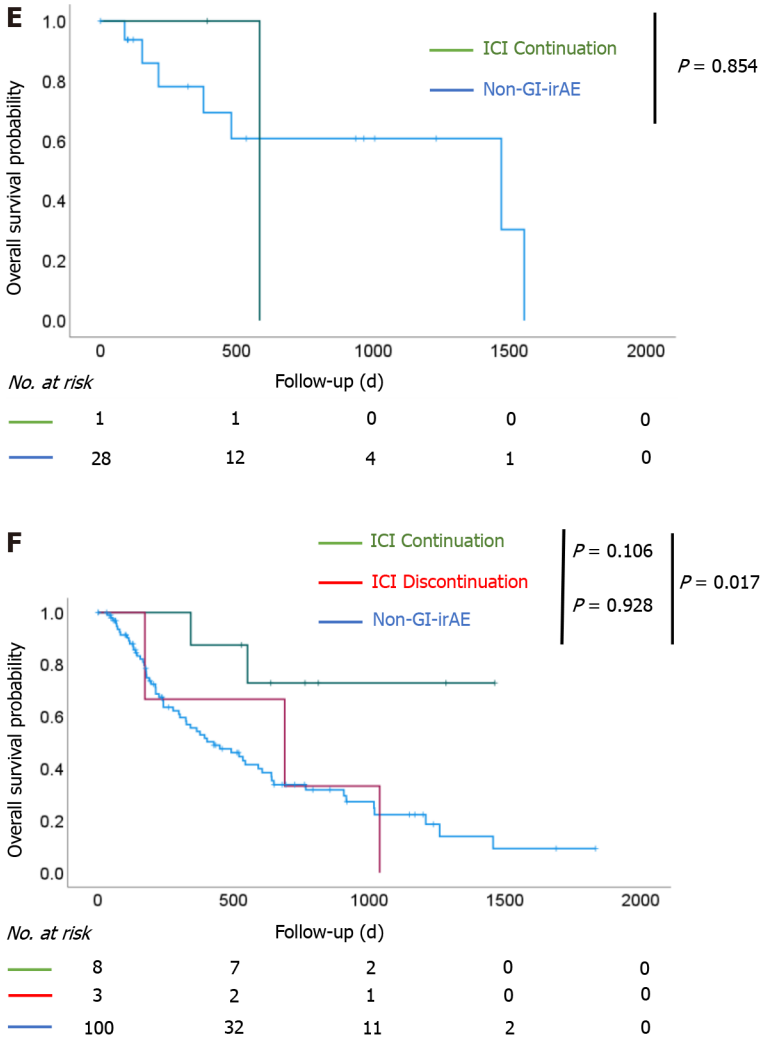


Figure 3 Overall survival after initiation of immune checkpoint inhibitor treatment. Overall survival (OS) of patients with non-small cell lung cancer (NSCLC) and OS of patients with malignant melanoma (MM). A: In patients with NSCLC, there was no significant difference in OS among the three groups; B: The results were similar when stratified in stage III; C: The results were similar when stratified in stage IV; D: In patients with MM, there was a significant prolongation of OS in the immune checkpoint inhibitor (ICI) continuation group compared to the non-gastrointestinal immune-related adverse event (non-GI-irAE) group (P = 0.035); E: There was no significant difference in OS between the ICI continuation group and the non-GI-irAE group among patients with stage III disease; F: Among patients with stage IV disease, there was a significant prolongation of OS in the ICI continuation group compared to the non-GI-irAE group (P = 0.017). ICI continuation group: Patients who continued ICI treatment after developing GI-irAEs; ICI discontinuation group: Patients who discontinued ICI treatment after developing GI-irAEs; non-GI-irAE group: Patients with no GI-irAEs.

and pathologies may be different between the groups, but a further investigation with a larger sample size is necessary.

In terms of prognosis, OS was not prolonged in NSCLC patients with or without GI-irAEs; however, in MM patients, OS was significantly prolonged in patients who developed GI-irAEs and continued ICI treatment compared with that in other patients.

ARTICLE HIGHLIGHTS

Research background

Immune checkpoint inhibitors (ICIs) are gaining popularity as a treatment for advanced cancer. However, among immune-related adverse events (irAEs), gastrointestinal-related immune AEs (GI-irAEs) have limited their use.

Research motivation

There are only a few coherent reports on the clinical characteristics of GI-irAEs for each ICI. In addition, the correlation between the development of GI-irAEs and patient prognosis has not been fully elucidated.

Research objectives
We aimed to evaluate the clinical characteristics of GI-irAEs and its influence on survival in patients treated with ICI.

Research methods
We retrospectively reviewed the records of 661 patients who received ICIs at Nagoya University Hospital from September 2014 to August 2020. We analyzed the clinical characteristics of patients who received an anti-programmed cell death-1/programmed death-ligand 1 (anti-PD-1/PD-L1) antibody, anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) antibody, or combination therapy with anti-PD-1 and anti-CTLA-4 antibodies. We also evaluated the correlation between GI-irAEs development and the prognoses.

Research results
GI-irAEs occurred in 34 of 605 patients (5.6%) treated with an anti-PD-1/PD-L1 antibody alone and in nine of 56 patients (16.1%) treated with an CTLA-4 antibody alone or a combination of anti-PD-1 and anti-CTLA-4 antibodies. The cumulative incidence and median daily diarrhea frequency were significantly higher in patients receiving anti-CTLA-4 antibodies ($P < 0.05$). In 130 patients with malignant melanoma (MM), overall survival was significantly prolonged in the group that continued ICI treatment despite the development of GI-irAEs compared to the group that did not experience GI-irAEs ($P = 0.035$).

Research conclusions
GI-irAEs occurred more frequently and with a higher severity in patients using anti-CTLA-4 antibodies than in those using anti-PD-1/PD-L1 antibodies. In patients with MM who developed GI-irAEs and continued treatment with ICIs, overall survival was significantly prolonged.

Research perspectives
Multicenter studies with large samples are expected to evaluate clinical characteristics of GI-irAEs and their association to long-term survival outcomes.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 November 7; 27(41): 7207-7209

DOI: 10.3748/wjg.v27.i41.7207

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

LETTER TO THE EDITOR

Pancreatic cyst dilemma: Between physical and biochemical markers

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Author contributions: Khamaysi I designed and wrote the letter; and Eyal Zussman revised the letter; the manuscript has been read and approved by all the authors.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Country/Territory of origin: Israel

Specialty type: Gastroenterology and Hepatology

Peer-review report's scientific quality classification

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

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Abstract

Physical analysis of the pancreatic cystic lesions (PCLs) fluid as expressed by the rheological behavior ("string sign") can improve the diagnostic yield and should be integrated in every multimodal PCLs workup.

Key Words: Pancreatic cyst; Fluid analysis; String sign; Rheology

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Core Tip: No single optimal test reliably determines the pancreatic cyst subtype including all imaging modalities and biochemical fluid analysis. Physical analysis of the fluid as expressed by the string sign can improve the diagnostic yield and should be integrated in every multimodal pancreatic cystic lesions workup. The string sign as it is currently performed, suffers from significant shortcoming due to its subjective nature. Rheological (physical) properties, instead, can overcome the disadvantages of the standard string sign and replace it in clinical practice.

Citation: Khamaysi I, Zussman E. Pancreatic cyst dilemma: Between physical and biochemical markers. *World J Gastroenterol* 2021; 27(41): 7207-7209
URL: https://www.wjgnet.com/1007-9327/full/v27/i41/7207.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i41.7207

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Received: June 6, 2021
Peer-review started: June 6, 2021
First decision: June 30, 2021
Revised: July 1, 2021
Accepted: September 30, 2021
Article in press: September 30, 2021
Published online: November 7, 2021

P-Reviewer: Kikuyama M, Masuda A
S-Editor: Wang JL
L-Editor: A
P-Editor: Wang JL

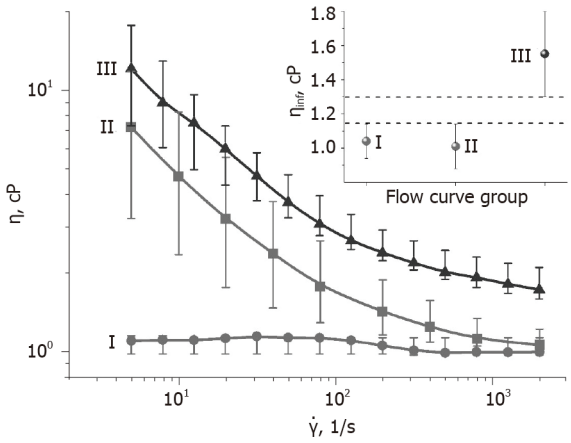


Figure 1 Representative types I, II and III flow curves. The graph inset shows the values of infinite viscosity, ηc, depicting the difference between the minimal value of type III and the maximum value of types I and II.

TO THE EDITOR

We read with great interest the frontier by Okasha *et al*[1] regarding the diagnosis of pancreatic cystic lesions (PCLs) and the benefits of various diagnostic models.

The authors described a vast array of available diagnostic test and concluded that the combination of both endoscopic ultrasound-fine needle aspiration (EUS-FNA) findings with cystic fluid tumor markers analysis, along with clinical, radiologic, histologic, genetic, and molecular characteristics, enhances the diagnostic accuracy and helps to construct a novel model in the era of PCLs[1].

Unfortunately, the authors did not mention the viscosity of the cystic fluid as an important marker for differentiation between PCLs subtypes (mucinous and non-mucinous cysts).

The string sign, as a surrogate marker of fluid viscosity, is a useful and reliable test that can be used to improve the diagnostic accuracy of other pancreatic cyst fluid studies when used in combination[2], however, the sting sign suffers from, relatively, high interobserver variability regarding its positivity and should be interpreted with caution and not used as a single test but in combination with other tests to differentiate mucinous from non-mucinous cysts[3].

String sign is inherently a subjective test and lacks a theoretical framework for predicting the viscoelastic nature of the fluid, which can be objectively characterized by the viscous and elastic response of a fluid under deformation (rheological behavior).

In order to overcome the subjective nature of the string sign, we developed a new rheological assay in which (using a rheometer) a wide array of viscoelastic properties (rheological curves) can be generated and recorded.

Use of a rotational viscometer supports simulation of true rheological conditions (the stepping change of either the shear stress or the shear rate is programmed but the parameter remains constant during each step). The viscosity of the samples was measured with a DHR-2 Rheometer (TA Instruments, USA) at 25 °C. The preferred geometry was cone-and-plate, with a cone diameter of 40 mm and a surface-plate angle of 1°. The rheometer was operated in shear rate control mode. Several time sweep tests at different constant shear rates (5-2000 1/s) were performed. The measured steady-state shear viscosity values (when the viscosity was constant in time) were used to construct flow curves of the fluids

In our study[4], we found that the cutoff value of pancreatic cyst fluid viscosity, ηc, can serve as an independent marker to distinguish between mucinous and non-mucinous cysts. It was found that ηc > 1.3 cP characterizes mucinous cysts, whereas ηc > 1.3 cP is typical for non-mucinous cysts. Moreover, we could differentiate between three distinct flow curves of the rheological behavior of pancreatic cyst fluids according to dynamic viscoelastic properties. Types I and II hypothesized to correlate with non-mucinous cysts, and type III with mucinous cysts (Figure 1). This simple and rapid diagnostic tool can be immediately implemented after EUS-FNA sampling, and provides for a low variability rate compared to the commonly used, subjective string sign technique. Although the findings are promising, they must be further confirmed in a large-scale study.

In conclusion, no single optimal test reliably determines the pancreatic cyst subtype including all imaging modalities and biochemical fluid analysis. Physical analysis of the fluid as expressed by the string sign can improve the diagnostic yield and should be integrated in every multimodal PCLs workup.

The string sign as it is currently performed, suffers from significant shortcoming due to its subjective nature. Rheological (physical) properties, instead, can overcome the disadvantages of the standard string sign and replace it in clinical practice.

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

World Journal of *Gastroenterology*

World J Gastroenterol 2021 November 14; 27(42): 7210-7401



Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

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Production Editor: *Ji-Hong Liu*; **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, Subrata Ghosh

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

PUBLICATION DATE
November 14, 2021

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World Journal of Gastroenterology

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World J Gastroenterol 2021 November 14; 27(42): 7210-7232

DOI: 10.3748/wjg.v27.i42.7210

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

FRONTIER

Frontiers in antibiotic alternatives for *Clostridioides difficile* infection

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Author contributions: Phanchana M and Janvilisri T conceptualized and conceived the idea; all authors have been involved equally and have read and approved the final manuscript; Janvilisri T supervised the project.

Supported by Research Cluster (Multi-generation Researchers) Grant, Mahidol University, No. MRC-MGR 02/2563.

Conflict-of-interest statement: The authors declare no conflict of interest.

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Abstract

Clostridioides difficile (*C. difficile*) is a gram-positive, anaerobic spore-forming bacterium and a major cause of antibiotic-associated diarrhea. Humans are naturally resistant to *C. difficile* infection (CDI) owing to the protection provided by healthy gut microbiota. When the gut microbiota is disturbed, *C. difficile* can colonize, produce toxins, and manifest clinical symptoms, ranging from asymptomatic diarrhea and colitis to death. Despite the steady-if not rising-prevalence of CDI, it will certainly become more problematic in a world of antibiotic overuse and the post-antibiotic era. *C. difficile* is naturally resistant to most of the currently used antibiotics as it uses multiple resistance mechanisms. Therefore, current CDI treatment regimens are extremely limited to only a few antibiotics, which include vancomycin, fidaxomicin, and metronidazole. Therefore, one of the main challenges experienced by the scientific community is the development of alternative approaches to control and treat CDI. In this Frontier article, we collectively summarize recent advances in alternative treatment approaches for CDI. Over the past few years, several studies have reported on natural product-derived compounds, drug repurposing, high-throughput library screening, phage therapy, and fecal microbiota transplantation. We also include an update on vaccine development, pre- and probiotics for CDI, and toxin antidote approaches. These measures tackle CDI at every stage of disease pathology *via* multiple mechanisms. We also discuss the

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Specialty type: Microbiology

Country/Territory of origin: Thailand

Peer-review report's scientific quality classification

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

Received: March 21, 2021
Peer-review started: March 21, 2021
First decision: April 30, 2021
Revised: May 12, 2021
Accepted: October 25, 2021
Article in press: October 25, 2021
Published online: November 14, 2021

P-Reviewer: Ong LT, Sales-Campos H
S-Editor: Ma YJ
L-Editor: A
P-Editor: Ma YJ

gaps and concerns in these developments. The next epidemic of CDI is not a matter of if but a matter of when. Therefore, being well-equipped with a collection of alternative therapeutics is necessary and should be prioritized.

Key Words: Bacteriophage; Pharmaceutical; *Clostridioides difficile*; Alternative therapy; Drug resistance; Fecal microbiota

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Core Tip: *Clostridioides difficile* is considered a threat to public health owing to increases in treatment failure over the past few years. Current antibiotic treatment options are highly limited. Therefore, alternative strategies are critical. Herein, we review recent advances in alternative therapeutics, including the development of new chemical entities, fecal microbiota transplantation, pre- and pro-biotic, antitoxin antibodies, use of bacteriophages, and vaccines. We also highlight the concerns, limitations, and directions for each of these developments.

Citation: Phanchana M, Harnvoravongchai P, Wongkuna S, Phetruen T, Phothichaisri W, Panturat S, Pipatthana M, Charoensutthivarakul S, Chankhamhaengdecha S, Janvilisri T. Frontiers in antibiotic alternatives for *Clostridioides difficile* infection. *World J Gastroenterol* 2021; 27(42): 7210-7232
URL: https://www.wjgnet.com/1007-9327/full/v27/i42/7210.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i42.7210

INTRODUCTION

Clostridioides difficile (*C. difficile*) is a gram-positive, spore-forming, toxin-producing, rod-shaped anaerobic bacterium. *C. difficile* infection (CDI) is a leading cause of nosocomial infections in several countries. The source of *C. difficile* still remains debatable. Studies have proposed that *C. difficile* was part of the human gut commensal bacterial community, as the bacterium could often be isolated from neonates[1,2]. The pathogenicity of *C. difficile* was believed to be attributed to its toxin-production properties; however, recent developments have suggested that outgrowth and colonization are also a pivotal feature of pathogenicity. Under normal conditions, when the gut microbiota is in balance, in a stage called "eubiosis", *C. difficile* can neither multiply nor colonize the gut, thus preventing it from causing disease. On the other hand, when the composition of the gut microbiota is altered from its normal state, so-called "dysbiosis" occurs, which allows *C. difficile* to multiply and colonize[3,4]. Once colonized, *C. difficile* can produce up to 3 toxins, *i.e.*, toxin A (TcdA), toxin B (TcdB), and binary toxin (CDT). The first two are prominent virulent factors, whilst the latter is controversial[5-7]. The binary toxin is believed to enhance the toxicity and pathogenicity of the primary toxins; however, only a few reports have shown that PCR-negative, but CDT-positive, *TcdA* and *TcdB* can cause CDI[8,9]. Details of the pathogenesis of *C. difficile* can be found in reviews elsewhere[5,7,10,11].

CDI is one of the most prominent causes of healthcare-associated infections (HAI). In the United States and European countries, *C. difficile* has been ranked among the top 10 causes of HAI[12]. The challenges of CDI control and prevention are intrinsic drug resistance and environmental resistant spore of *C. difficile*. To date, CDI treatment with most antibiotics has generally resulted in failure. Use of certain antibiotics, such as clindamycin, cephalosporins, quinolones, and penicillins reportedly increased the risk of CDI[13]. Since these antibiotics are broad-spectrum and deplete other gut microbiota that inhibit *C. difficile* multiplication[13], the use of such antibiotics increases CDI risk. To the best of our knowledge, some classes of antibiotics do not increase the risk of CDI, including tetracyclines and aminoglycosides[14]. The antibiotics currently used to treat CDI are vancomycin and fidaxomicin, whereas metronidazole is the antibiotic of choice only when the first two are not available[15]. Along with treatment, prevention is a good control strategy for CDI in most settings. Multiple strategies are being implemented to reduce the rate of CDI, *e.g.*, case management, infrastructure, and antibiotic stewardship, in hospitals[16]. To make all

these control measures meaningful, monitoring of *C. difficile* situations is necessary to ensure that *C. difficile* does not develop resistance to current antibiotics, which would threaten the control and treatment of CDI. There has been an increase in the number of fatal cases of CDI in the past decade[17]. In most countries, a national surveillance system for CDI is still lacking, resulting in under-represented cases of CDI.

Current antibiotic options seem to have reduced efficacy. Recent CDI treatment guidelines have moved away from metronidazole because its efficacy has reduced from 95% pre-2000 to 75% since[15]. A similar reduction has been observed for vancomycin, from 98% to 85%[15]. The increases in recurrent episodes of CDI have reflected the failure of current antibiotics to sustain positive treatment outcomes[18]. Therefore, there is a critical need to develop alternative treatment approaches for CDI (Figure 1). In fact, several alternative approaches are under development, and some are more advanced than others. The development of new antibiotics is the most active research field. Fecal microbiota transplantation (FMT), including pre/probiotic approaches, has also gained extensive research interest in the past few years, as evidenced by several clinical trial registrations. Phage therapy, antitoxin antibody, and vaccines are trying to catch up with others in the league. Notably, there is one antitoxin antibody, bezlotoxumab, approved for preventing the recurrence of *C. difficile* by the United States Food and Drug Administration (FDA), in 2016.

We explored the developments that are currently in the pipeline for alternative therapies by examining data from the ClinicalTrials website. Several trials are investigating current antibiotics for CDI but in various aspects, most commonly in conjunction with FMT or pre/probiotic or antitoxin antibodies. Several molecular entities, such as nitazoxanide, are under investigation. Based on this insight, we extrapolated that the developing treatment options aim to treat/prevent recurrent CDI rather than the initial episode of CDI. Interestingly, currently no trials have been reported on bacteriophage therapy, despite this technology having been active since the early 1990s[19], and its therapeutic application having been demonstrated in other pathogenic bacteria[20]. This indicates that some complexities underline the technology.

SMALL MOLECULES TAKE THE LEAD IN CDI TREATMENT DEVELOPMENT

In the realm of drug discovery and development, small molecules dominate the pharmacy shelf, irrespective of the sources of the small molecules, *e.g.*, natural product-derived/inspired or (semi-)synthetics. According to the United States FDA database, small molecules account for most approved drugs. Regarding the nature and pathogenesis of *C. difficile*, the drugs used to treat CDI have a specific set of requirements, although no target product profiles have been proposed. We believe that the community consensus for the expected properties of the CDI drugs is that they could effectively kill vegetative and spore stages with low systemic absorption (high colonic concentration)[21,22]. Currently in clinical trials, several candidate compounds are being investigated for the development of CDI drugs. Petrosillo *et al*[23] have published a comprehensive review on the development of small molecules for CDI drugs.

Cadazolid is a synthetic oxazolidinone, a derivative of linezolid, which was developed for CDI and also exhibited potency against other enterococci[24]. Cadazolid exhibits good clearance of *C. difficile* with sub-mM MIC and limited effects on other gut microorganisms[25]. Its mechanism of action is protein synthesis inhibition by binding to peptidyl transferase center (PTC). Although cadazolid shares similar mechanism to linezolid, cadazolid is active against linezolid-resistant *C. difficile*[24]. The results from phase 3 clinical trials of cadazolid demonstrated a comparable clinical cure to vancomycin, good safety profile and well tolerated. However, it did not show non-inferiority to vancomycin, which subsequently resulted in the discontinuation of the development by the developer.

Nitazoxanide is an FDA-approved drug with an indication for treatments of cryptosporidiosis and giardiasis. At the millimolar range, this drug inhibited multiple gram-negative and gram-positive anaerobic bacteria, *e.g.*, *Bacteroides* spp., *Prevotella* spp., *C. difficile*, *C. perfringens* and *Mycobacterium tuberculosis*[26]. The mechanism of action of nitazoxanide has been suggested to involve the inhibition of pyruvate ferredoxin oxidoreductase (PFOR) in anaerobic energy metabolism[27] and the disruption of membrane potential in aerobic bacteria[28]. In addition, studies have shown that nitazoxanide is effective against various RNA and DNA viruses including coronavirus

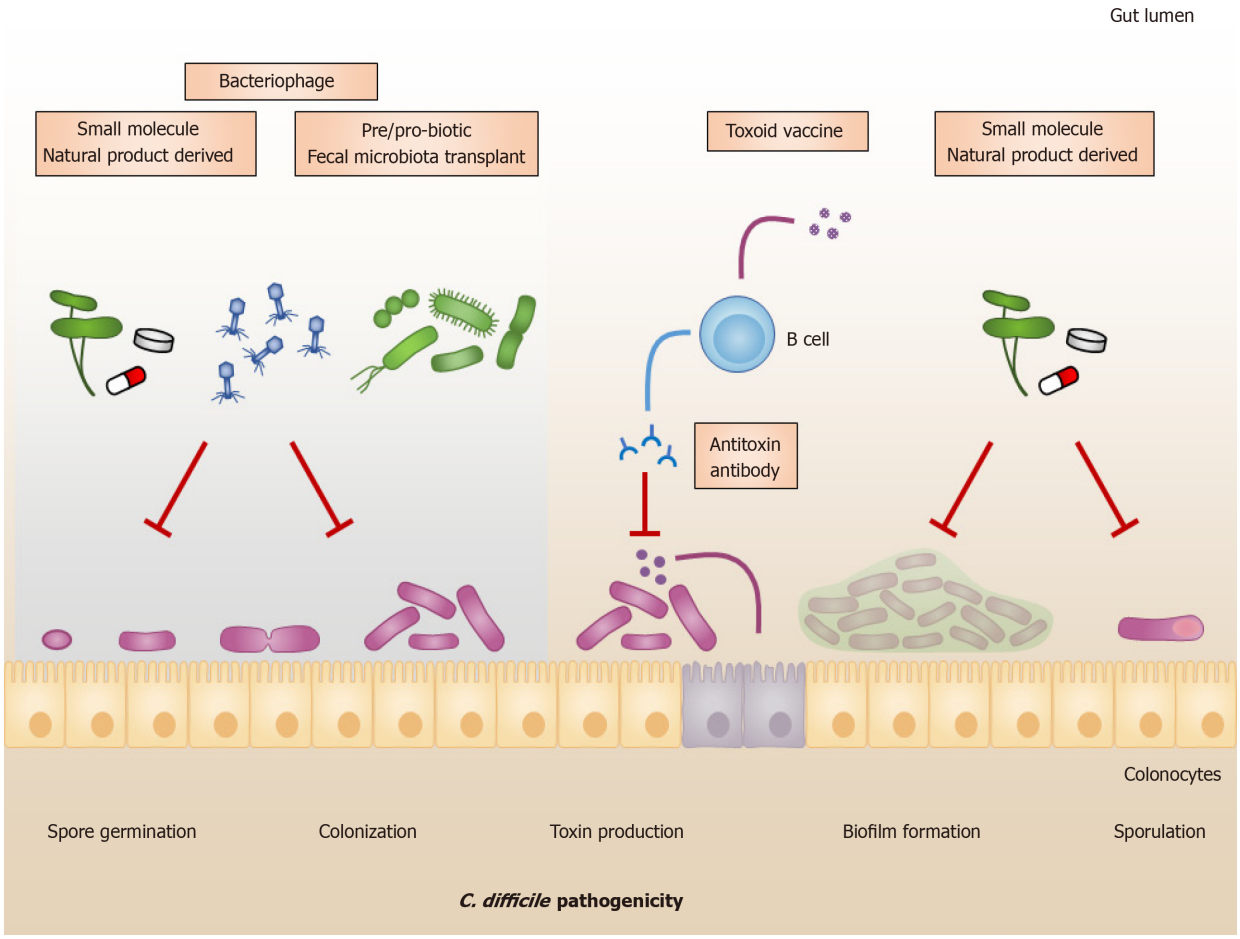


Figure 1 Alternative approaches for *Clostridioides difficile* infection treatment under development. Different approaches are aiming at the different pathological stages of *Clostridioides difficile* (*C. difficile*) infection. Small molecule and natural product derived have broad range activity from vegetative cell inhibition to biofilm and spore effects. Bacteriophage therapy affects mostly the vegetative stage of *C. difficile*. Fecal microbiota transplantation and pre- and pro-biotics aim to restore the balance of the gut microbiota mitigating the chance of *C. difficile* population and production of toxins. Vaccine and antitoxin antibody are targeting toxin neutralization.

[29] and SARS-CoV-2[30]. The result of an early phase-3 trial have suggested that the effectiveness of nitazoxanide is comparable to that of vancomycin, although noninferiority to vancomycin is inconclusive owing to small sample size and early termination[31]. Hence, the mechanism of action of nitazoxanide compared with current CDI treatments is anticipated from other ongoing trials because of the high expectations for nitazoxanide in the treatment of recurrent CDI[32].

Ridinilazole (SMT19969) is an antibiotic developed specifically for CDI. It exhibits good anticlostridial activity, with different magnitude, against multiple *Clostridium* spp. and also *C. difficile* with minimal effect to other gut microbiome[33,34] and bile acid profiles[35]. The mechanism of action of ridinilazole is believed to be unique from that of other drugs. It does not interfere with cell division by inhibiting cell wall synthesis; it does so by decreasing septum formation[36]. Ridinilazole has a low systemic absorption and, therefore, has a high colonic concentration, making it a good agent for CDI treatment[34]. The results obtained from a phase-2 clinical trial demonstrated sustained clinical response with ridinilazole treatment, noninferiority over vancomycin, and good tolerance[37]. These results altogether support further development of ridinilazole for clinical use. Phase-3 clinical trials are ongoing.

Advancements in technology and compound libraries has helped considerably in screening thousands of compounds, and has been made possible within a much shorter time. In the past few decades, several FDA-approved drugs were initially screened from large compound libraries through phenotypic screening[38]. A recent analysis showed that more than 16 million compounds can be purchased from various chemical suppliers[39]. This extends our opportunity to discover even more hits for drug discovery and development.

Apart from a rationally designed drug approach or large library screening, one recent popular drug development approach is drug repurposing or repositioning. The approach is regarded as a shortcut for a lengthy conventional drug development pipeline, as safety and pharmacokinetic data are readily available, which allows the compounds to proceed directly into clinical trials. Many works have demonstrated approved drugs can be repurposed for *C. difficile*[40,41]. The screening of both random compound libraries and approved drugs has become a very intriguing approach for drug development in the past years. Several studies conducted using this approach have been published[30,40,41]. However, if the route of administration is different from the approved indication, preclinical stages are necessary to evaluate the safety profile[42].

We examined the molecular similarity among the FDA-approved drugs from screening campaigns mentioned in this paper[40,43]. Molecular clustering of these drugs will help us predict a key biomolecular pathway or target the hits interact with or act upon. Molecular analysis was performed using the KNIME Analytics Platform, free and open-source software, aiming to solve large-data analysis problems[44]. KNIME offers an extensive toolset for data pre-processing and transformation as well as visualization and it can be equipped with extensions and nodes that are suitable for pharmaceutical research. We employed KNIME 4.1.2 equipped with various extensions, including RDKit KNIME integration, KNIME Distance Matrix Extension, and KNIME-CDK to create our workflow[45,46]. The workflow of the molecular analysis of the hits is shown in Scheme 1 of the supplement file.

The results shown in Table 1 and Supplementary material revealed that those hits can be categorized structurally into two large clusters and six smaller clusters based on molecular clustering analysis (> 3 hits per cluster). The largest group of clusters belongs to the β-lactam antibiotics (clusters 88–97, Supplementary material in SI) and the second largest is the tetracycline and its derivatives (clusters 98–99, Supplementary material in SI). Although these drugs are well-known antibiotics and are expected to exert some biological activity against *C. difficile*, the clinical use of these materials might not be of best interest since they are broad-spectrum antibiotics that could cause concern in gut microbiota dysbiosis and may drive an undesired side-effect of antibiotic resistance elsewhere in the body[47]. Other known antibiotics, such as aminoglycosides (cluster 123) and benzalkoniums cationic surfactant (cluster 61) which could be used topically as antiseptic were also identified.

Interestingly, metronidazole, one of the current treatments for *C. difficile*, and its derivatives (cluster 42) also show up as one cluster. Further exploration in this chemical moiety seems a way forward to avoid cross-resistance with current antibiotics as the drugs within this class exert their biological activity through the formation of nitroso radical. The mechanism that causes microbial DNA damage is not related to a certain enzyme and, therefore, makes it more challenging for the bacteria to develop resistance. Antifungal imidazoles were identified in another interesting cluster (cluster 21). These drugs act as an inhibitor of lanosterol 14α-demethylase, which catalyzes the formation of ergosterol, an important sterol found in eukaryotic cell membranes[48]. Although the mechanism of this class of drugs in *C. difficile* is not known, the result may prompt scientists to investigate the potential of these materials further, since these azoles are not currently in use as antibiotics, and it could avoid unwanted antibiotic cross-resistance with other current drugs[40,43]. Furthermore, most of the drugs identified from the FDA-approved panels resulted in drugs with anthelmintic (parasitic) indication[40]. It is possible that these parasites share a similar anaerobic/microaerophilic environment, and to some extent anaerobic metabolism, to that of *C. difficile*, thereby allowing those drugs to be active against *C. difficile*. It would be very interesting to observe whether this hypothesis remains valid when more screenings are performed. Although several drugs and lead compounds have been identified in the literature, only a few have been put forward into the development pipeline. This is largely due to the lack of academic and industrial partnerships. We have seen what the scientific community has achieved, in an unprecedented time scale, with the development of COVID-19 vaccines with the leading role of big pharmaceutical companies and their academic partners. This emphasizes the importance of academic-industrial partnerships in drug and vaccine developments, as this field of research is resource-intensive and requires considerable funding and workforce. Nevertheless, non-profit organizations can play an important role in drug discovery and development as well evidenced by the approval of anti-tubercular pretomanid, which was led by the TB Alliance[49].

Table 1 Representatives of anti-*Clostridioides difficile* chemical clusters from reported literature

Cluster No.	Compound class
21	Antifungal imidazoles
42	Metronidazole and its derivatives
61	Benzalkonium cationic surfactants
88–97	β-lactams
98–99	Tetracycline and its derivatives
123	Aminoglycosides

NATURAL PRODUCTS SERVE AS A GOOD SOURCE OF ANTI-CLOSTRIDIAL ACTIVITIES

Humans have been using natural products as traditional medicine for treating several illnesses for centuries. It has been estimated that approximately 500000 plant species exist on the planet, but only 1% of them have been explored for bioactive compounds [50]. A detailed analysis of FDA-approved drugs from 1931 to 2013 revealed that natural products and their derivatives represented over 30% of new drugs[51]. Due to the increasing prevalence of antibiotic resistance in several pathogenic bacteria, the prescribed antibiotics may no longer be effective. Therefore, exploration of plant-derived compounds could provide us a tremendous opportunity to discover novel bioactive agents.

Several plant extracts and plant-derived compounds possess antibacterial activity against *C. difficile* and their action has been investigated previously. Roshan *et al*[52] reported the anti-*C. difficile* activities of natural products that are commercially available as both processed and unprocessed products. Some processed products such as aloe vera gel, peppermint oil, artichoke capsules, and garlic tablets demonstrated antimicrobial activity against *C. difficile* with MICs of 16% (v/v), 8% (v/v), 75–150 mg/mL, and 37.5–75 mg/mL, respectively[52]. Regarding the unprocessed products, allicin (derived from garlic) and cinnamon powder demonstrated antimicrobial activity against *C. difficile* with MICs of 2.3–4.7 and 75 mg/mL, respectively. Zingerone (derived from ginger) and menthol (derived from peppermint) inhibited *C. difficile* at the same inhibition concentration of 9.4 mg/mL, whereas trans-cinnamaldehyde, an active constituent found in cinnamon bark, exhibited strong inhibitory activity with an MIC of 0.2 mg/mL[52]. δ-3-Carene, a monoterpene derived from the root of *Asarum heterotropoides*, exhibited anticlostridial activity with an MIC of 0.7 mg/mL with a less potential of suppressing beneficial intestinal bacteria[53]. Moreover, an essential oil extract that contains 16.5% of δ-3-carene exhibited antimicrobial activity against *C. difficile* at a concentration of 0.25% (v/v)[54]. Xanthohumol, derived from *Humulus lupulus* L., exhibited anti-*C. difficile* activity with MICs of 0.032–0.107 mg/mL against 28 different *C. difficile* isolates[55]. Crude methanol extract of the bark of *Mammea africana* demonstrated anti-*C. difficile* activity with an MIC of 2 μg/mL. The identified active flavonic compound in *M. africana*, mammeisin or mammea A/AA, exhibited strong inhibitory activity with an MIC of 0.25 μg/mL[56]. Curcumin (phenol), an active agent derived from *Curcuma longa*, is reportedly active against 27 *C. difficile* strains, with MICs ranging between 16 and 32 μg/mL. The inhibition was specific to *C. difficile* as it did not affect beneficial gut microbiota[57]. Curcumin was also able to reduce sporulation and toxin production in *C. difficile*[57].

Several studies have reported that the anticlostridial activity of crude plant extracts contain unidentified active compounds. For instance, pomegranate extract was shown to exhibit specific inhibitory activity against 23 tested isolates of *C. difficile* with MICs of 12.5–25 μg/mL, but no inhibitory effect was observed on the tested normal intestinal bacteria (MIC > 400 μg/mL)[58]. Investigation of a commercial animal supplement, BIOCITRO, a citrus fruit extract, revealed an inhibitory effect on *C. difficile* with MICs of 16–32 μg/mL with a possible mode of action of disrupting polysaccharides and carbohydrates of the cell wall[59]. In addition, methanolic extract of the leaf and rhizomes of *Aristolochia paucinervis* Pomel demonstrated an inhibitory effect on *C. difficile*, with concentrations ranging from 8 to 64 μg/mL[60].

Several compounds are known to kill *C. difficile* through changes in cell permeability. The ability of membrane disruption was suggested for zingerone, menthol, and trans-cinnamaldehyde[61]. Asiatic acid, an active triterpenoid derived

from *Centella asiatica*, exhibited significant antimicrobial activity against *C. difficile* strains isolated from different sources by disrupting the cytoplasmic membrane with MICs of 10–20 μg/mL[62]. Lauric acid, an active component found in virgin coconut oil, exhibited anticlostridial activity at a 250-μmol/L concentration through the membrane disruption mechanism[63]. Cannabidiol, an active ingredient derived from cannabis, could inhibit *C. difficile* at concentrations of 2–4 μg/mL, possibly by disrupting the bacterial membrane[64]. Another study of cannabidiol in caco-2 cells infected with *C. difficile* reported its potential to inhibit toxin A-induced cytotoxicity [65]. Interestingly, data analysis of hospitalization from the Healthcare Cost and Utilization Project in 2014 suggested that patients associated with cannabis usage had potentially lower risk for CDI by 28%[66].

The dormant spores of *C. difficile* contribute to transmission and link with the pathogenesis of CDI. *C. difficile* spores can persist in harsh conditions. They can then translocate to the intestinal tract, germinate in response to specific bile salts, and initiate infection. Most antibiotics are inactive against *C. difficile* spores because of the spores' intrinsic durability. In addition to vegetative cell activity, natural products are active against several spore stages of *C. difficile*. Peppermint oil and trans-cinnamaldehyde have been shown to exhibit sporicidal activity, which reduces the number of spores by up to 200 times when spores were exposed for 7 d[67]. Several compounds such as allicin and carvacrol, essential oils found in oregano, and fresh onion bulb extracts could inhibit spore outgrowth[67,68]. As *C. difficile* spores play an essential role in CDI, the inhibition of sporulation is an attractive strategy to reduce infection. A study conducted by Roshan *et al*[67] showed that the subinhibitory concentration of coconut oil, fresh onion bulb, and fresh ginger can reduce the number of spores production by 90%. Another study conducted on Manuka honey or Leptospermum honey also reported the inhibition of sporulation in *C. difficile*[69]. Baicalin, a flavonoid derivative present in the root of *Scutellaria baicalensis*, could inhibit both sporulation and outgrowth of *C. difficile* at a concentration of 1.6 mmol/L. It also reduced toxin production by downregulating *tcdA* and *tcdB* gene expression[70]. Toxin A and toxin B produced by *C. difficile* are cytotoxic and cause colitis. Leptospermum honey, fresh onion bulb, and trans-cinnamaldehyde were able to reduce the cytotoxicity of *C. difficile* toxins in Vero cells by 70% and toxin production by 40%[71].

In an *in vivo* mouse model, it was observed that berberine, a compound derived from the genus *Berberis*, has the potential to prevent recurrent CDI and restore gut community, either alone or in combination with vancomycin[72]. It is noteworthy that successful therapy by endoscopic lavage with Manuka honey was reported in the patient with vancomycin treatment failure[73].

ANTITOXIN ANTIBODIES WITH ANTIBIOTICS IMPROVE TREATMENT SUSTAINABILITY

Toxin production has long been associated with CDI pathogenicity. Classical antibiotic treatments effectively eliminate the pathogen, although they inevitably affect the normal gut microbiota to some extent. Antitoxin antibody treatment aims to neutralize the toxicity of the toxin, rather than interfere directly with the bacterium. Therefore, it can reduce clinical severity, in conjunction with antibiotic treatment, and reduce recurrent CDI. To date, the only FDA-approved antitoxin antibody for CDI treatment is bezlotoxumab, a human monoclonal antibody against toxin B. A detailed study demonstrated that bezlotoxumab binds to carbohydrate-binding pockets on toxin B, which directly prevents the interaction between the toxin and host cells[74]. This hypothesis was supported by a mutant antibody that does not bind to Fc receptors of host immune cells, which provides similar toxin neutralization and protection effects as wild-type antibody[75]. Its toxin A counterpart, actoxumab, did not demonstrate improvement in clinical efficacy, and therefore it was discontinued during the phase 3 trial[76]. These results support the hypothesis that TcdB is a more prominent virulent factor than other toxins and that the interplay between these toxins is a complex process.

IM-01 is an experimental polyclonal antibody for inflammatory bowel disease, including Crohn's disease, and CDI. IM-01 is produced using a chicken egg technique by immunizing hens with toxin A, toxin B, and *C. difficile* spores. Currently, detailed information on this intervention is lacking, but its phase 2 clinical trial is being recruited. The results from patent filing showed good antibody production, toxin neutralization, reduction of spore burden, and inhibition of vegetative cell growth[77]. These results indicate that IM-01 is a promising development for CDI treatment;

however, it is too early to state whether it would work effectively independently or in combination with antibiotics.

Colostrum is a fluid produced by the mammary glands immediately after the birth of a newborn. It has been demonstrated that cows persistently injected with antigens will produce antibodies in the colostrum, which are collectively referred to as hyperimmune bovine colostrum (HBC). A preclinical piglet model study showed that HBC can reduce the severity of CDI[78]. A limitation of using colostrum is the scale of production, which is limited to a few hours after birth. Therefore, some studies have examined the use of milk or whey protein isolate (WPI) instead of colostrum for CDI treatment. WPI exhibited superior and sustained treatment outcome compared with vancomycin in a hamster model[79]. Considering its clinical use, we found only one terminated phase 2 trial, which was completed, but there were no results, of the whey protein concentrate MucoMilk.

Altogether, antitoxin antibodies in any form appear to be a significant candidate for alternative treatment of CDI, since preclinical studies have demonstrated good inhibition of vegetative cells and spores and neutralization effect of toxins in initial and recurrent episodes of CDI. Nonetheless, it will take some time to observe this group of treatments for clinical use, as production is more complicated and potentially expensive.

FECAL MICROBIOTA TRANSPLANT IS A VERY ATTRACTIVE APPROACH FOR CDI

Although antibiotic administration is the first-line treatment for CDI, severe outbreaks and recurrent episodes remain excessive due to a significant increase in resistant strains[80]. Treatment options for CDI, especially recurrent CDI, become limited, due to which the development of alternative therapeutics is highly required. In particular, CDI develops when the indigenous microbiota is dysbiotic, typically *via* exposure to antibiotics, thereby allowing *C. difficile* to acquire nutrient niches in the gut and cause disease[13,81]. Based on this concept, restoration of the healthy microbial community, eubiosis, can help eliminate and prevent this growing problem. To date, researchers have expressed interest in biotherapeutic strategies that confer curative effects on recurrences. A burgeoning alternative treatment approach for recurrent CDI is FMT, which refers to the administration of feces collected from a healthy individual to the intestinal tract of a patient[82,83]. In general, FMT is applied to a patient with refractory CDI who had failed standard antibiotic treatments in an initial episode of CDI[84]. This approach emerged due to its unique feature, restoring the balance of the gut microbiota, which is unlikely to be achieved by other approaches. FMT provides multiple mechanisms of colonization resistance, such as competition for nutrients, production of antimicrobial peptides, and production of short-chain fatty acids (SCFAs) to inhibit vegetative growth and spore germination of *C. difficile*[85,86].

The first clinical trial of FMT, whose efficacy was significantly greater than the use of antibiotics for recurrent CDI treatment, was performed by van Nood *et al*[87] and published in 2013. Among the 16 patients, 13 with FMT recovered from CDI after the first infusion and the symptoms of 2 patients resolved after a second infusion, whereas only 3 of 13 patients resolved with vancomycin treatment alone. Other clinical studies have demonstrated that FMT provides better benefits than standard antibiotics for treating recurrent CDI by replenishing the balance of the gut microbiota. Currently, clinical studies are ongoing with the recruitment of patients with relapsing CDI to evaluate the safety and efficacy of FMT (Table 2). The upper and lower route of administration has been used in delivering the transplant. Several methods, including nasoduodenal, enema, upper endoscopy, colonoscopy, and oral capsules, are commonly used for administering FMT, with diverse outcomes[88]. A study using colonoscopy demonstrated the resolution of CDI in 18 of 20 patients treated with FMT compared with 5 of 19 patients treated with vancomycin[89]. Conversely, FMT administered by enema was found to have a low success rate in treating acute episodes of recurrent CDI[90]. To simplify administration, oral capsules of fecal filtrates have been developed and widely used. Patients with relapsing CDI had a decent response to FMT administered by oral capsules, wherein the resolution rate of recurrent CDI was similar to that observed with colonoscopy[91]. These findings demonstrated no definite preference of the administration route for FMT.

Another factor questioned in FMT is the preparation of fecal material. Initially, fresh stools from donors were used for infusion. As the process of collecting fresh stools is difficult, frozen and lyophilized stools were then considered for transplant delivery.

Table 2 Recent clinical trials examining potential fecal microbiota transplantation for treatment of *Clostridioides difficile* infection

Current phase	Title	ClinicalTrials.gov Identifier	First posted
NA	Fecal microbiota transplantation for *C. difficile* infection	NCT01905709	July 23, 2013
	Immune response to FMT for *C. difficile*	NCT02797288	June 13, 2016
	Outcomes and data collection for fecal microbiota transplantation for the treatment of recurrent *C. difficile*	NCT03562741	June 19, 2018
	Fecal microbiota transplantation (FMT) for *C. difficile* (CEFTA)	NCT03712722	October 1, 2018
	Rescue fecal microbiota transplantation for national refractory intestinal infection	NCT03895593	March 29, 2019
	Safety and efficacy of fecal microbiota transplantation	NCT04014413	July 10, 2019
1	Fecal transplant for pediatric patients who have recurrent *C. difficile* infection (FMT)	NCT02134392	May 9, 2014
2	Stool transplants to treat refractory *C. difficile*	NCT02127398	April 30, 2014
	FMT versus antimicrobials for initial treatment of recurrent CDI	NCT02255305	October 2, 2014
	Fecal microbiota therapy for recurrent *C. difficile* infection	NCT02686645	February 19, 2016
	Phase II trial of fecal microbiota transplant (FMT) for VRE and CRE patients	NCT03643887	August 23, 2018
	Fecal microbiota transplantation (FMT) plus fidaxomicin for severe of fulminant *C. difficile*	NCT03760484	November 30, 2018
	Multicentre blinded comparison of lyophilized sterile fecal filtrate to lyophilized fecal microbiota transplant in recurrent *C. difficile* infection	NCT03806803	January 16, 2019
	FMT and bezltoxumab compared to FMT and placebo for patients with IBD and CDI (ICON-2)	NCT03829475	February 4, 2019
	PMT for severe-CDI	NCT03970200	May 31, 2019
	Penn microbiome therapy (PMT) for recurrent *C. difficile* infection	NCT03973697	June 4, 2019
3	Fecal transplantation for primary *C. difficile* infection (COLONIZE)	NCT03796650	January 8, 2019
	Microbiota restoration therapy for recurrent *C. difficile* infection (PUNCH CD3-OLS) (CD3-OLS)	NCT03931941	April 30, 2019
	Fecal microbiota transplantation for primary *C. difficile* diarrhea	NCT02801656	June 16, 2016

FMT: Fecal microbiota transplantation; *C. difficile*: *Clostridioides difficile*; PMT: Penn microbiome therapy.

Several clinical trials claimed that frozen stool was comparable to fresh stool with no loss of effectiveness[92,93]. In contrast, lyophilization reduced the efficacy of the transplant material compared with fresh and frozen materials[94]. In particular, frozen fecal material is becoming available for commercial purchase. In addition to its use for treating multiple episodes of recurrent CDI, FMT has been proposed as a promising treatment approach for severe and complicated CDI. Evidence obtained from clinical trials has shown that FMT improved survival in severe cases, including immunocompromised patients[95,96].

Although FMT is a favorable therapeutic option for recurrent CDI, a major concern is that there is no universal or industrial standard such as a defined bacterial formula; moreover, there is no information on the mechanism associated with FMT-screening methods for adverse transmission events and the period of treatment[97,98]. Such limitations of FMT led to the discovery of beneficial microorganisms in fecal materials that activate colonization resistance and the determination of their roles in inhibiting *C. difficile*. Correspondingly, the refined stool-derived microbial suspension RBX2660 has been successfully used to treat relapsing CDI, and is currently under a phase 2b study[99]; furthermore, SER-109 and SER-262, defined microbial preparations containing spore-forming bacteria purified from human feces and formulated as a capsule, are currently being investigated in a phase 3 and 1b trial[100,101]. These data conclude that FMT continues to be a recommended therapy for recurrent CDI, and in the meantime, a number of studies continue identifying essential bacteria species from the feces of healthy donors to formulate standard microbial preparations approved by the FDA.

Another point of concern for FMT is the unintentional transfer of drug-resistant bacteria, which could possibly lead to complicated infection and death. There have been multiple bacteremia incidents in patients who received FMT[102], some of which were severe and life-threatening. This indicates the need for a standardized protocol for stool preparation, to minimize potential drug-resistant bacterial infection from FMT, including, but not limited to, extended-spectrum beta-lactamase (EBSL)-producing *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *etc.*[103]. Recently, a caution has been issued regarding the transmission of SARS-CoV-2 in FMT[104].

PROBIOTICS AND PROBIOTIC PROMOTE GUT BALANCING

Probiotics are live microorganisms that confer health benefits on the host when administered in adequate amounts[105,106]. They can improve host immunity by producing beneficial metabolites, such as SCFAs, as well as preventing enteric infections *via* colonization resistance mechanisms, such as competition for nutrients, inhibition of bile acid conversion, and production of antimicrobial peptides[107,108]. Numerous probiotic strains and probiotic mixtures have been evaluated to combat CDI in *in vitro* studies; however, there are limitations due to the lack of evidence from human clinical trials.

Lactobacillus rhamnosus GG and *Saccharomyces boulardii*, the most common probiotics, have provided promising applications against CDI and have been widely investigated in clinical trials. The efficacy of *S. boulardii* in CDI management was first examined by McFarland *et al*[109], who reported a significant reduction in recurrence rates after the administration of *S. boulardii* twice a day for 4 wk during and after antibiotic treatment. Their study demonstrated a significant reduction in the CDI recurrence rate in the *S. boulardii* treatment group compared with the placebo group. Moreover, patients with relapsing CDI had a statistically significant response to *S. boulardii* compared with placebo. The probiotic effect of *L. rhamnosus* GG in a clinical study was first verified by Gorbach *et al*[110], in 1987. They successfully used the organism to treat 5 patients with multiple recurrent CDI episodes. In addition to its beneficial effects in treating refractory CDI, *L. rhamnosus* GG displayed a protective potential in healthy individuals[111]. Further meta-analytical studies have been performed to confirm the usefulness of *S. boulardii* and *L. rhamnosus* GG in the prevention of CDI [112,113]. In addition to single-probiotic strains, probiotic mixtures have been developed for quite some time. The probiotic mixture BioK+ containing *L. acidophilus* CL1285, *L. rhamnosus* CL2, and *L. casei* LBC80R was able successfully to reduce the CDI rate from 18.0 to 2.3 cases per 10000 patients. Furthermore, new mixtures have been developed and are in the process of clinical trials[114]. The probiotic mixture of *L. casei* DN-114 001, *S. thermophilus*, and *L. bulgaricus* was randomly administered to hospital inpatients twice-daily during, and for 1 wk after, antibiotic treatment[115]. This probiotic mixture showed a positive result, wherein no individual in the probiotic group developed CDI compared with 9 of 53 individuals who contracted CDI in the placebo group. Another study evaluated the efficacy of the probiotic mixture of *L. acidophilus* and *L. casei*[116]. The probiotic doses were varied and administered within 1.5 d of initial antibiotic therapy and then continued for 5 d after the final antibiotic dose. Both higher dose and lower dose probiotic groups showed significantly reduced CDI incidence rates, at 1.2% and 9.4%, respectively, compared with 23.8% in the placebo group. The most recent clinical trial of a multi-strain probiotic consisting of *L. acidophilus* NCFM, ATCC700396, *L. paracasei* Lcp-37, ATCC SD5275, *Bifidobacterium lactis* Bi-07, ATCC SC5220, and *B. lactis* B1-04, ATCC SD5219 has been conducted[117]. A phase 2 study evaluated the potential benefits of this probiotic mixture by administration daily for 4 wk. The results revealed a shorter duration of diarrhea in patients with an initial episode of mild-to-moderate CDI compared with the placebo group. Based on the available evidence, probiotics are strongly advocated as an alternative for preventing and treating CDI. However, the high heterogeneity in existing studies indicates that the beneficial effects of probiotics are rather subjective[118]. Therefore, discovering novel probiotics and understanding their functions and interactions have been continuing to improve the probiotic effects in a clinical setting.

It is known that diet has a key influence on the composition and functions of the gut microbiota[119]. Several types of fiber, particularly, galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), have been found to increase the abundance of common probiotic bacteria, such as *Bifidobacterium* and *Lactobacillus* species[120]. Hence, plant-based foods containing dietary fiber are generally accepted as favorable for gut health. Certain fiber types, including FOS, GOS, and inulin, are considered to

be prebiotics, which are defined as substrates that are selectively used by host microorganisms conferring a health benefit[121]. The combination of probiotics and prebiotics has been proposed as an alternative, known as synbiotics, to prevent and treat refractory CDI. A recent study investigated the effects of a synbiotic, *L. plantarum* DSM 21379 and xylitol, on the germination of *C. difficile* spores[122]. *In vitro* experiments demonstrated that the synbiotic completely inhibited the germination of *C. difficile* spores. Moreover, the administration of this synbiotic for 5–6 d before ampicillin and *C. difficile* challenge in mice reduced the CDI incidence from 44% to 22% mortality. Another *in vitro* study examined the inhibitory capability of four different *Bifidobacterium* sp. strains combined with various prebiotics against *C. difficile* growth. Using oligo-fructosaccharides as a carbon source, it was observed that *B. longum* and *B. breve* rescued the survival of a cell line exposed to *C. difficile* cell-free supernatant. These findings indicate that a probiotic strain requires a specific prebiotic substrate. To produce more effective synbiotics to control CDI, it is necessary to determine the optimal prebiotic for each probiotic.

BACTERIOPHAGE AND ITS PRODUCTS SPECIFICALLY ELIMINATE *C. DIFFICILE*

As bacteria-infecting viruses, bacteriophages or phages have received attention for potential use as an alternative treatment for several bacterial infections. The high specificity to their bacterial hosts and the self-replicating mechanism of phages are claimed to have advantages over other approaches. Although *C. difficile* phages have been discovered and investigated since 1983[123], the use of phages for human infection is restricted due to some limitations. The key bacteriophage studies of *C. difficile* are summarized in Table 3.

C. difficile phages were first described for bacterial typing purposes[123]. Morphological analysis showed that most of them are either *Myoviridae* or *Siphoviridae*, belonging to the order *Caudovirales*. They possess dsDNA as their genetic materials [124,125]. Currently, all 26 complete genomes of *C. difficile* phages are characterized as temperate; they can alternate their life cycles between lytic and lysogenic cycles[126, 127]. This is a major constraint, as most therapeutic phage applications require a virulent phage, *i.e.*, a phage with a strictly lytic life cycle. Therefore, current research has been focusing on the use of temperate phage and phage-derived proteins to combat CDI[128-131]. At the very beginning, phage therapy was performed as a single-phage treatment to ensure its capacity. It has been demonstrated that phiCD27 significantly reduced the growth of *C. difficile* cells, as well as the production of toxin A and toxin B in an *in vitro* batch fermentation and artificial gut model. Furthermore, phiCD27 treatment did not affect commensal microbiota in both models, suggesting high specificity on the bacterial target[132]. However, using only one phage has some limitations due to narrow host range and lysogenic capacity. Therefore, a combination of different phages or phage cocktails has become more intriguing[133-135].

A phage cocktail was successfully developed both *in vitro* and *in vivo*, as observed in the study conducted by Nale *et al*[134]. Optimized cocktails of phiCDHM1 to phiCDHM6 and phiCDHS1 were tested against *C. difficile* ribotypes 076, 014/020, and 027 strains. The best combination included phiCDHM 1, 2, 4, and 6, which could completely kill *C. difficile* without regrowth. The CDI hamster model showed a significantly lower number of spores in the cecum and colon with the combination of phiCDHM 1, 2, 5, and 6. The same phage mixture was used in the wax moth larva *Galleria mellonella*. The efficiency of using phage combined with antibiotics, including vancomycin and clindamycin, was evaluated. The results suggested that phage could be used as a supplement to antibiotic treatment and prevent the onset of CDI. Prophylaxis was the most effective therapy with 100% protection, and efficiency was reduced when used as a remedial treatment. Moreover, the authors found that the phage could penetrate and prevent biofilm formation in the *C. difficile* ribotype 014/020[136]. Phage treatment in an artificial gut model was further investigated using these sets of phage combinations. A six-log reduction in *C. difficile* growth was observed after 5 h in the prophylaxis group, and the vegetative cells were completely removed within 24 h[136]. Furthermore, metagenomic analysis was conducted using fecal samples of volunteers to observe the impact of phage therapy on the total gut microbiome. Another *in vitro* study conducted on the human colonic cell line HT-29, which is the CDI site, reported that phiCDHS1 preferentially adsorbed onto HT-29 cells, thereby promoting the interaction between the phage and bacterial cells[137]. It has also been shown that either bacterial lysis by phage or the phage itself was

Table 3 Key experiments in bacteriophage for *Clostridioides difficile* infection treatment

Phage	Experiment	Finding	Ref.
phiCD140	A single dose of phage treatment for *C. difficile* infection in hamsters	Surviving of phage treated hamster	[135]
phiCD27	Phage treatment of CDI in an *in vitro* batch fermentation and human colon model	(1) Reduction of both vegetative cell and toxin A and toxin B productions from *C. difficile*; and (2) No impact on others gut microbes	[135]
phiCDHM1 to phiCDHM6, and phiCDHS1	(1) Investigation for an effective phage combination; and (2) Phage delivered orally in hamster model every 8 h after *C. difficile* challenge	(1) Discovery of phage-resistant colonies after a single phage treatment; and (2) Reduction of *C. difficile* amount and colonization using phage combination *in vivo*	[124]
phiCDHM1, 2, 5, and 6	(1) Phage treatment before and after the biofilm formation; (2) First time using *Galleria mellonella* (wax moth) model for *C. difficile* phage; and (3) Using phage in combination with antibiotics (vancomycin)	(1) Reduction and prevention of the biofilm establishment *in vitro*; and (2) Disease prevention in the prophylaxis group and increasing the wax moth survival rates	[130]
phiCDHM1, 2, 5, and 6	(1) Optimized temperate phage cocktail to treat in batch fermentation model; and (2) First metagenomic analysis of phage treatment on gut microbiome	(1) *C. difficile* elimination after 24 h in prophylactic condition while maintain other microbiota components; and (2) No significant impact on other bacterial groups in human gut	[127]
phiCDHS1	Measurement of planktonic and adhered *C. difficile* cells and free phage to human colon tumorigenic cell line HT-29	(1) Reduction of planktonic and adhered *C. difficile*; and (2) No cytotoxicity to human cells	[129]
phiCD24-2	(1) Using engineered phage delivered Type 1-B CRISPR system as antimicrobial agent *in vitro* and *in vivo*; and (2) Mutation of phage lysogenic gene by the cI repressor and integrase gene deletion	(1) *C. difficile* eradication effectively in engineered phage comparing with wild-type phage; and (2) Detection of lysogen due to potentially functional complements from *C. difficile* prophage	[125]

C. difficile: *Clostridioides difficile*.

nontoxic to the colonic cells.

Recently, phiCD24-2 has been engineered to contain a genome targeting CRISPR-Cas3, which is commonly found in the genome of *C. difficile*[135]. The major characteristics of the engineered phage or CRISPR-enhanced phage (crPhage) have been investigated. There was no difference in phage morphology or host range compared with the wild-type phage (wtPhage). The efficiency of phage treatment for *C. difficile* was determined both *in vitro* and *in vivo*. crPhage demonstrated a higher efficiency to reduce the growth of vegetative cells than wtPhage in both models. However, the bacterial number rebounded by 24 h, suggesting lysogination into the host genome rather than bacterial lysis. The mouse model exhibited a significant difference in the number of *C. difficile* cells recovered from mouse feces between wtPhage and crPhage treatments, indicating the superiority of crPhage treatment *in vivo*. The CRISPR approach enhances phage efficiency during the lytic cycle as bacterial lysis can occur *via* two independent mechanisms, including genome damage and phage lytic activity *via* endolysin and holin expression. The challenge of engineered phages remains due to lysogenic conversion. Therefore, the removal of cI repressor and integrase genes was performed to generate lysogenic phage mutants[135]. Although lysogen from *in vitro* culture was not detected, it was detectable in mouse feces. These findings suggested the functional complement in the *C. difficile* genome for those removed genes.

An alternative to using temperate phages for therapeutic purposes is to utilize their products. Endolysin, a peptidoglycan hydrolase enzyme, is encoded by the phage genome. Endolysin is required to disrupt the bacterial cell wall to release phage progeny at the final step of viral infection. The endolysin CD27L is derived from the phage CD27, which is the first phage endolysin characterized in *C. difficile*. The specificity test demonstrated that CD27L was active against a panel of 30 *C. difficile* strains, including a hypervirulent ribotype 027[125]. The N-terminal truncated CD27L, $CD27L_{1–179}$, improved the lytic activity when tested against *C. difficile*. $CD27L_{1–179}$ exhibited a slightly broader lytic range than the full-length phage. It was also active against *Listeria* spp. However, both CD27L and $CD27L_{1–179}$ did not harm the selected gut commensal bacteria[130]. Another endolysin retrieved from *C. difficile* 630 prophage has been described. The lytic activity of full-length PlyCD, and that of a truncated N-terminal containing the catalytic domain $PlyCD_{1–174}$, were evaluated. Similar to CD27L, the truncated $PlyCD_{1–174}$ exhibited greater lytic activity than its full-length counterpart and also displayed a broader activity range against *C. difficile* strains than the full-length PlyCD. The bactericidal assay demonstrated that $PlyCD_{1–174}$

reduced more than 4-log growth of the *C. difficile* hypervirulent MLST2 strains 217B, 615H, and UK1 (027). This result highlighted the potency of $PlyCD_{1-174}$ against the crucial clinical strains. Interestingly, $PlyCD_{1-174}$ exhibited a synergistic effect with vancomycin pretreatment. The combination treatment between vancomycin and $PlyCD_{1-174}$ *in vivo* demonstrated significant inhibition of *C. difficile* growth (> 2-log). Unfortunately, the *in vivo* study of $PlyCD_{1-174}$ was unsuccessful due to inconsistent results. *Ex vivo* experiments were conducted using infected mouse cecum and anus as an infecting area. $PlyCD_{1-174}$ exhibited 2-log reduction of *C. difficile* cell growth, indicating the activity of $PlyCD_{1-174}$ in the gastrointestinal environment[131]. The mechanism by which truncated endolysins exhibit higher efficiency than the wild-type counterpart remains to be explored.

Phage-derived proteins with high specificity to *C. difficile* strains could be developed for targeted therapy. Diffiocin, a contractile R-type phage tail-like bacteriocin, was originally derived from the *C. difficile* strain CD4 (Diffiocin-4). This protein exhibits a higher efficiency when fused with the receptor-binding protein (RBP) of prophage phi027 in the genome of strain R20291 (Av-CD291.1 and Av-CD291.2). Interestingly, avidocin is stably active throughout the mouse gastrointestinal tract when supplied in drinking water containing 4% sucrose and 1% sodium bicarbonate. The specificity of avidocin-CDs was evaluated across *C. difficile* strains, based on which the relationship between RBPs in avidocin-CDs construct and *slpA* allele was concluded[129]. Despite the advances in the knowledge of bacteriophage biology, the use of phage for therapeutic purposes remains much of a challenge. The first challenge is lysogenic conversion, which results in ineffective treatment outcomes. Therefore, the remaining challenge is to identify or engineer the phage to obtain a strictly lytic phage. The subsequent concern is the battle between the host and the phage that occurs during phage infection. Some bacteria naturally mutate and become resistant to phage infection using different mechanisms, *e.g.*, extracellular modification or intracellular modification, as mentioned in a review elsewhere[138]. Still, there is room for lytic phage hunters and phage modification, which are desperately needed. However, to apply this technology effectively in clinical practice, further research is warranted to overcome these limitations.

ILEOSTOMY AND COLONIC LAVAGE ARE RESERVED FOR FULMINANT CDI

At the other end of the spectrum, one alternative treatment is the removal of part of the gut, *i.e.*, colectomy, ileostomy, loop ileostomy, and colonic lavage. This treatment approach is largely reserved for severe and complicated (fulminant) CDI as it is the most invasive treatment. Earlier, colectomy was opted, but it did not improve the clinical outcome, resulting in some mortality[139]. Diverting loop ileostomy and colonic lavage were developed at the Pittsburgh School of Medicine and therefore referred to as the Pittsburgh protocol. This protocol exhibited benefits over conventional colectomy and end ileostomy, which decreased the mortality rate and preserved the colon[140]. We found 2 clinical trials that were terminated due to slow recruitment of participants; hence, there is limited insight into how this method confers benefit to patients with CDI. This indicates to some degree that the current treatment regimen is sufficient to save patients' lives without involving this invasive intervention.

VACCINES ARE THE HOPE FOR CDI CONTROL AND PREVENTION

Current treatment approaches for CDI concentrate primarily on antibiotics. Although antibiotic administration commonly serves as the first-line therapy, prolonged disruption of the normal microbiota can result in recurrent CDI, with incidences of up to 45% after antibiotic therapy[141]. The incidence of healthcare-associated CDI has reduced over time, although there have been recent reports on the growing number of cases of community-associated CDI[142]. Therefore, there exists an urgent need to control and prevent CDI transmission. To date, several preventive remedies for recurrent CDI have been proposed, including antibiotics and FMT. Although their efficacy has been proven, none are recommended by the Infectious Disease Society of America (IDSA), and the protracted efficacy is still questionable. Therefore, vaccines could be a valuable option to serve as long-term prophylaxis for CDI due to their memory function.

Several vaccine candidates have been developed and launched to animal and clinical trials. Currently, a total of 21 clinical trials on *C. difficile* vaccines have been reported, of which 3 were terminated. Most of the vaccine studies have been developed based on 3 formulations, *viz.*, toxoid, recombinant peptide, and surface-associated antigen. In this section, we shall briefly describe each vaccine formulation regarding its development and efficacy in preventing CDI.

Because toxin A and toxin B are key virulence determinants of CDI and associated with the severity of colon damage, they become a remarkable target for vaccine development. The alteration of toxin structure by chemical treatment, thereby inactivating toxicity while preserving its immunogenicity—termed as toxoid—has been developed for over 3 decades. Toxoid-based vaccine use in animal models has demonstrated a satisfactory production level of serum antibody responses against both toxins and a preventive efficacy against a lethal outcome of CDI[143]. The first clinical trial on toxoid-based vaccine in healthy individuals was introduced 20 years ago. It was observed that the majority of study subjects developed a massive level of specific antibodies for both toxins. Although some adverse reactions were documented, the vaccine was demonstrated to be safe and immunogenic in healthy adults[144]. This information paves the way for further development of the next generation of formulated vaccine candidates that are currently under investigation.

The use of a recombinant protein-based design is another approach for vaccine development to avoid residual toxins, which are not completely inactivated by chemical, and also maximizes the protective effect using only the toxin domain responsible for immunogenicity. Furthermore, this genetic modification allows uncomplicated manufacture of vaccine preparations compared with toxoid production. Three functional domains of both toxins, including glucosyl-transferase (GT) and cysteine protease (CP), central translocation (T), and C-terminal receptor-binding domain (RBD), were tested for immunogenic validation. The first study conducted by Lyerly *et al*[145] on the use of the RBD of toxin A reported partial protection against CDI and death in a hamster model. The genetic modifications of toxin A RBD were widely investigated by several research groups. These modifications included the use of RBD subdomain, recombinant fusion with other immunogenic proteins, and combination with a mucosal adjuvant[146]. These constructions exhibited strong action by evoking a complete seroconversion for toxin A and preventing fluid secretion and histological changes. Although seroconversion against toxin A was found to be greater than that against toxin B, an optimal vaccine needs to include both toxin A and toxin B fragments to achieve maximum protective efficacy. Therefore, later developments focused on the combination of recombinant peptides with/without immune adjuvants. The fusion peptide, containing fragments of toxin A and toxin B, generates an immune response to both toxins. Complete protection against toxin A was observed at all doses, whereas less immunogenicity toward toxin B was noticed[147]. Co-administration of the combined recombinant protein with the adjuvant also demonstrated satisfactory positive protection, hence this vaccine formulation is currently in phase 1 clinical trial.

To overcome the limitations of previous vaccine candidates related to the prevention of CDI, surface protein antigens have emerged as an alternative target for vaccine development. Several studies have confirmed the induction of immune response during infection against surface layer protein (SLP), including flagella components, adhesin, fibronectin-binding protein, and cysteine protease[148,149]. However, none of the vaccinations provided significant protection in animal models. Furthermore, glycans, the polysaccharide coat on the surface of the bacterium, serve as another target for eliciting specific antibodies. It has been documented that all 3 glycan structures, PSI, PSII and PSIII, conjugated with other immune inducers stimulated specific antibodies, including IgA and IgG, in animal models[150,151]. It was also shown that immune cells can recognize both native and synthesized glycans, supporting the importance of this molecule to be developed as a vaccine and/or a vaccine additive.

There have been several attempts to develop vaccines for CDI. Unfortunately, there is still no approved vaccine to prevent initial and/or recurrent CDI. Nevertheless, there are 2 potential vaccine candidates reaching the final stage of development[152-154]. VLA84, a chimeric protein containing truncated toxin A and toxin B, developed by Valneva Austria GmbH, demonstrated good efficacy in a phase 1 trial[155,156]. It is currently under phase 2 clinical trial (NCT02316470). This clinical phase is a randomized placebo-controlled study of a total of 500 healthy subjects aged > 50 years. The subjects were separated into 4 groups, which individually received different vaccine doses, including VLA84 75 μg without alum, VLA84 200 μg with and without alum, and phosphate-buffered saline as the placebo group. All subjects received 3

doses of vaccination on days 0, 7, and 28. The immunogenicity and safety of VLA84 were evaluated after the last vaccination for up to 6 mo. Based on the primary outcome, it was reported that seroconversion for IgG was ≥ 4-fold increase for toxin A and toxin B on day 56. However, efficacy and data analysis are yet to be reported.

Pfizer's vaccine has been developed using genetically modified full-length toxin A and toxin B, using a novel detoxification process that preserves the critical epitopes responsible for immunogenicity for maximizing the production of neutralizing antibodies[157,158]. This vaccine entered clinical trials in 2012 and received Fast Track designation from the United States FDA in 2014. The phase 2 clinical trial was completed on patients aged 50–85 years (NCT02117570). The vaccine induced robust immune responses and exerted protective effects in preclinical models. The Pfizer vaccine is currently undergoing phase 3 clinical trials with ≥ 17000 subjects in 23 countries. Subjects aged ≥ 50 years will receive 3-dose vaccinations at months 0, 1, and 6. Volunteers will be followed up for 3 years after the last vaccination. As the trial is ongoing, the results of data analysis from this vaccine are expected in the near future.

Although several vaccines have been developed for decades, aiming to serve as a prophylaxis for CDI, there are concerns and limitations for rapid, long-lasting, and protective immunity. Further efforts are still required to identify optimal dose, dosing schedule, and vaccine formulation, and also to determine potential application for high-risk healthy populations and immunocompromised individuals. Regarding the promising efficacy of a CDI vaccine, if approved, it will provide the primary prevention and reduction of CDI cases worldwide.

CONCLUSION

CDI is a serious healthcare concern as most countries move towards aging societies [159]; *C. difficile* can have maximum impact on this age group. CDI treatment is also threatened by treatment failures, especially recurrent CDI. Therefore, an urgent need exists to develop alternative treatment approaches. Small molecules and natural products have been subjected to the most advanced progress compared with other approaches, followed by an erupting trend of FMT. Both single and combinational therapies appear to be the way forward, such as antibiotic/FMT, antibiotic/antitoxin antibody, and antibiotic/synbiotic. Vaccine development is highly anticipated as the results are extremely promising and would provide a significant tool for CDI prevention and control in community and healthcare settings. Bacteriophage therapy has to overcome the grand challenge before it can be used in clinical practice. These developments are the future of CDI treatment; they require a huge amount of effort and capital; meanwhile, the management of antibiotic use, hygiene precautions and education, and monitoring systems, must be implemented to reduce the incidence of CDI.

ACKNOWLEDGEMENTS

Authors would like to thank Paul Adams for kindly proofreading the final manuscript and the Faculty of Tropical Medicine for subsidizing the English editing service on the first manuscript.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 November 14; 27(42): 7233-7239

DOI: 10.3748/wjg.v27.i42.7233

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

OPINION REVIEW

Serologic diagnosis of celiac disease: May it be suitable for adults?

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Conflict-of-interest statement: The authors declare no conflict of interest for this manuscript.

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Abstract

The diagnosis of coeliac disease (CD) in adult patients requires the simultaneous assessment of clinical presentation, serology, and typical histological picture of villous atrophy. However, several years ago, the European Society of Pediatric Gastroenterology, Hepatology, and Nutrition guidelines approved new criteria for the diagnosis in children: Biopsy could be avoided when anti-transglutaminase antibody (TGA) values exceed the cut-off of × 10 upper limit of normal (ULN) and anti-endomysium antibodies are positive, independently from value. This "no biopsy" approach is a decisive need for pediatric population, allowing to avoid stressful endoscopic procedures in children, if unnecessary. This approach relies on the correlation existing in children between TGA levels and assessment of mucosal atrophy according to Marsh's classification. Several lines of evidence have shown that patients with villous atrophy have markedly elevated TGA levels. Therefore, we aim to perform a narrative review on the topic in adults. Despite that some studies confirmed that the × 10 ULN threshold value has a very good diagnostic performance, several lines of evidence in adults suggest that TGA cut off should be different from that of pediatric population for reaching a good correlation with histological picture. In conclusion, the heterogeneity of study reports as well as some conditions, which may hamper the serological diagnosis of CD (such as seronegative CD and non-celiac villous atrophy) and are much more common in adults than in children, could represent a limitation for the "no biopsy" approach to CD diagnosis in patients outside the pediatric age.

Key Words: Celiac disease; Villous atrophy; Serology; Biopsy; Anti-transglutaminase antibody

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Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Italy

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

Received: March 21, 2021
Peer-review started: March 21, 2021
First decision: April 29, 2021
Revised: May 2, 2021
Accepted: October 25, 2021
Article in press: October 25, 2021
Published online: November 14, 2021

P-Reviewer: Balaban YH, Jin SY, Sabelnikova EA
S-Editor: Ma YJ
L-Editor: Wang TQ
P-Editor: Ma YJ



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Core Tip: A "no biopsy" approach to celiac disease diagnosis, based only on anti-transglutaminase antibody titer, is a well-established strategy in children and an appealing matter of debate in adults. Indeed, the same strategy is recommended by pediatric guidelines, since it allows to avoid about one third of upper endoscopy procedures. In adults, literature on the topic is flourishing even if the topic is still under-investigated, results are heterogeneous, and some conditions may be relevant limiting factors.

Citation: Losurdo G, Di Leo M, Santamato E, Arena M, Rendina M, Luigiano C, Ierardi E, Di Leo A. Serologic diagnosis of celiac disease: May it be suitable for adults? *World J Gastroenterol* 2021; 27(42): 7233-7239
URL: https://www.wjgnet.com/1007-9327/full/v27/i42/7233.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i42.7233

INTRODUCTION

Celiac disease (CD) is the most common immune-mediated enteropathy. It affects subjects with a genetic predisposition based on the presence of a human leukocyte antigen (HLA) DQ2/DQ8 haplotype and polymorphisms of several other inflammatory genes. The same genes are frequently involved in several other autoimmune conditions, and this explains why a high rate of coeliac patients suffer from at least another immune-mediated disease[1,2].

CD is the only autoimmune disease certainly triggered by an exogenous factor, *i.e.*, the ingestion of gluten. Gluten is a complex of alcohol soluble proteins such as gliadins, avenins, and secalins. These rich-in-proline and glutamine peptides are difficultly hydrolyzed by humans for the absence of an enzyme called prolyl-endopeptidase on the brush border of enterocytes[3].

The global prevalence of CD is about 1%[4]. However, considerable differences exist among various countries. Additionally, it is more frequent in females (2:1-3:1), like other autoimmune diseases. Diagnosis may occur in every moment of life. In the past, CD was considered a disease of the childhood[4], but nowadays the trend is changing because the 50% of new diagnoses occur in people over 50 years old. The most important difference between pediatric and adult patients concerns symptoms at onset: In children the intestinal signs are more frequent, while in adults the extra-intestinal manifestations are more typical[5].

Clinical manifestations may include both intestinal and extra-intestinal symptoms. Intestinal manifestations are diarrhea, dyspepsia, bloating, and abdominal pain. Extraintestinal findings are weight loss, iron deficiency anemia, microcytic or megaloblastic anemia, and osteopenia[6].

Malabsorption is the consequence of the mucosal injury caused by humoral and cell-mediated autoimmunity. In fact, tissue transglutaminase 2 (TTG2), an intestinal enzyme, makes gluten peptides toxic by reactions of transamidation and deamidation [7]. Plasma cells release IgA antibodies against both self-components of the mucosal layer and deamidated gluten peptides. IgA molecules pass into the bloodstream as antibodies against transglutaminase 2 (TGA), endomysium (EMA), and deamidated gliadin peptides (DGPs) and their detection is useful for the diagnosis of CD[4,8].

On the other side, an immune response mediated by CD3+ T cells takes place. These CD3+ T lymphocytes are called intraepithelial lymphocytes (IELs). IELs infiltrate the mucosal layer, thus damaging enterocytes. In CD, IELs are usually more than 25/100 enterocytes and lose their normal pattern of distribution in the villous area, which is called base-tip pattern and is characterized by a few number of IELs located at the base of the villi. Conversely, in CD, IELs are abnormally distributed in the whole surface of the villi[9].

The number of IELs is one of the two main histological criteria used for assessment of mucosal damage according to Marsh classification; the other one is the reduction of the villous-crypt ratio. In the normal duodenum, the villi are 3-fold longer than Lieberkhun crypt depth; in CD, the flattening of villi causes an inversion of the normal ratio from 3:1 to 1:1 until to 1:3.

Table 1 Sensitivity and specificity of serologic tests

Antibody	Sensitivity (range)	Specificity (range)
IgA TGA	98% (78%-100%)	98% (90%-100%)
EMA	95% (86%-100%)	99% (97%-100%)
IgA DGP	88% (74%-100%)	95% (90%-99%)
IgG TGA	70% (45%-95%)	95% (94%-100%)
IgG DGP	80% (63%-95%)	98% (90%-99%)

DGP: Deamidated gliadin antibodies; EMA: Anti-endomysium antibodies; TGA: Anti-transglutaminase antibodies.

These histological findings are assessed on biopsy samples taken from the duodenum. At least two samples from the bulb and four from the second part of the duodenum should be taken in order to obtain an adequate sample[10,11].

DIAGNOSIS

Currently, a combination of clinical presentation, serology, and histology is required to diagnose CD in adults.

A patient with suggestive intestinal or extraintestinal symptoms/signs should undergo a serological analysis to assess the IgA levels: IgA-class TGA are the most sensitive and specific antibodies for CD even if they do not allow to diagnose CD alone. The IgA-class TGA test is performed by enzyme-linked immunosorbent assay. It is reliable and inexpensive, and represents the most sensitive test for CD (98%), with a very low percentage of false positive when the titer is more than 5-fold the upper limit of normal value[12]. The hypothesis of CD should be confirmed by IgA-class EMA positivity. Indeed, IgA-class EMA measurement is the most specific test (near to 100%) but the test is immunofluorescence–based, so it is operator dependent for its difficult interpretation[13].

In patients with an IgA deficiency (a frequent condition in celiac patients), IgG levels should be assessed[3]. A summary of diagnostic performance of serologic tests in CD is reported in Table 1[14].

Antibodies against DPGs are not very useful in diagnosis, except if the patient is less than 2 years old; they could be considered in the follow-up, because their variations are very rapid after the starting of a gluten free diet (GFD)[15,16].

In adult population, endoscopy with duodenal biopsy samples is considered the gold standard for CD diagnosis. Several endoscopic findings may suggest CD with a high sensitivity and specificity. However, more than 33% of CD patients have a normal endoscopic appearance, so biopsy samples should be collected in all patients with suspected CD irrespectively of endoscopic appearance. During upper GI endoscopy, at least 4-6 specimens should be collected, including samples from the duodenal bulb, in order to increase the diagnostic yield[17]. In each pass of biopsy forceps, the endoscopist should take only a single biopsy specimen[18]. However, at least 10% of specimens may not have an acceptable quality, due to insufficient size or lack of orientation and, sometimes, endoscopy should be repeated. Moreover, endoscopy is an invasive procedure with risk of complications and expensive, and the sedation is often required due to the duration of the procedure.

A level 3 in Marsh assessment corresponds to a complete villous atrophy and is required to diagnose CD.

However, the European Society of Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) guidelines in these last years stated that a different diagnostic algorithm could be used for children.

Then, guidelines stated that, if clinical features are present, TGA level overcomes the threshold of 10 × UNL, and EMAs are positive, histology and genetics could not be carried out. This conclusion relies on the strong association between TGA and Marsh's grade of atrophy[19].

This approach, despite being not applicable to all children, has changed the clinical practice since, at least in children, upper endoscopy is not easily performed. It has been estimated that the cited cut-off may avoid endoscopy in 18% of celiac children, with a sensitivity of 96.3 and specificity of 98.6%[20]. In another study, 29% of children

could have avoided biopsy *as per* the 2020 ESPGHAN guidelines, and levels of TGA ≥ 60 U/mL or DGP ≥ 28 U/mL had a 100% specificity and 100% positive predictive value (PPV) for CD. HLA typing and EMA did not improve the PPV in patients with a TGA level ≥ 60 U/mL, but addition of DGP ≥ 28 U/mL improved the diagnostic sensitivity albeit maintaining the 100% specificity[21].

The promising data found in pediatric literature have, therefore, pushed researchers to investigate whether a pure serologic approach could be used in adults with suspicion of CD. Therefore, we aimed to perform a narrative review on the topic in adults.

A NO-BIOPSY, SEROLOGY-BASED APPROACH IN ADULTS: CURRENT EVIDENCE

Several studies supported the "no-biopsy strategy" in adult population. Sugai *et al*[22], in a prospective study, evaluated the diagnostic accuracy of duodenal biopsy and serology for CD diagnosis (TGA and DGP), in two cohorts of subjects with different pre-test probabilities. In the high-risk group (161 enrolled patients), the prevalence of CD was 39.1%, while in the low-risk group (518 enrolled patients), the CD prevalence was 3.3%. Using assay combinations, it would be possible to confirm or rule out a diagnosis of CD without biopsy in 92% of cases in both pre-test populations. Salmi *et al*[23] compared histological examination to serum and intestinal celiac autoantibodies in untreated CD. They corroborated a high sensitivity and specificity of autoantibodies TGAs for detection of CD with villous atrophy. In 2008, Hill *et al*[24] found that an IgA-class TGA level of × 10 ULN could be used as a diagnostic cut-off with a positive predictive value of 100% for CD in adults. A similar cut-off of TGA antibody level (× 10) was suggested by Beltran *et al*[25] for CD diagnosis, with a 100% specificity. However, the authors emphasized the necessity of local validation for the cut-off value. The study of Penny *et al*[26] confirmed that an IgA-class TGA titer of × 10 ULN had a 100% specificity as a cut-off value for detection of Marsh type 3 lesions.

Other cut-off values of TGA levels have also been suggested. In a retrospective study, Holmes *et al*[27] enrolled 270 CD adults with IgA-TGA levels measured and small bowel biopsy samples. The authors found that a cut-off greater than 45 U/mL (> × 8 ULN + 2SDs) had a PPV of 100% for CD. The same value was suggested by Tortora *et al*[28]. In their study, a cut-off value of TGA of 45 U/mL had a sensitivity of 70% and specificity of 100% for predicting Marsh ≥ 2 lesions. Moreover, the authors found that the best cut-off for predicting villous atrophy was 62.4 U/mL (sensitivity 69%, specificity 100%). A lower cut-off value of TGA was found in the retrospective study of Zanini *et al*[29]: They demonstrated a 100% specificity for duodenal atrophy with a cut-off value of five times higher than the ULN. The application of this diagnostic approach could avoid upper GI endoscopy in one out of three patients. In a multicenter retrospective analysis enrolling both pediatric and adult patients who underwent small-bowel biopsy for suspicion of CD and positivity for both TGA and EMA, Alessio *et al*[30] demonstrated that a TGA level ≥ × 7 ULN was able to diagnose CD with a specificity and PPV close to 100%. On the other hand, Di Tola *et al*[31] determined that the best TGA serum level/cut-off ratio was > 3.6 with a sensitivity of 76.8 % and PPV of 97.2 %. The use of threshold value for CD diagnosis could avoid endoscopy with biopsy in 75% of the patients. The authors also found a strong correlation between TGA serum levels/cut-off ratio and the degree of duodenal lesions.

The combination of serology for IgA-TGA and IgA-EMA for CD diagnosis was retrospectively evaluated by Wakim-Fleming *et al*[32]. In their cohort, a value of serum IgA-class TGA greater than 118 U had only a 2% false-positive rate. While, if the value of serum IgA TGA was between 21 and 118, the value of EMA at least 1:60 had a PPV of 83% for CD. IgA-class TGA level less than 20 U, in combination with an EMA dilution titer less than 1:10, had a negative predictive value of 92% for CD.

Oyaert *et al*[33] evaluated the use of IgA-class TGA value associated with IgG-DGP antibody for CD diagnosis, in both pediatric and adult populations. Patients with double positivity and high antibody levels (> 3 times and > 10 times ULN) had a high probability of having CD (likelihood ratio ≥ 649 for > 3 times ULN and ∞ for > 10 times ULN). However, the sensitivity was significantly higher for all test combinations in the group aged younger than 16 years compared to the adult group.

The study by Efthymakis *et al*[34] found that the optimal cut-off anti-TGA value was ≥ × 16 ULN. In this study, 11 different assays were used for TGA titer determination. Analyzing the two more prevalent, the authors found different optimal cut-off values

(14.3 × ULN *vs* 3.7 × ULN), even after standardization (-0.14 *vs* -1.2).

SEROLOGY AND PERSISTENT ATROPHY IN FOLLOW-UP

Key endpoints in the follow-up of CD patients are the absence of symptoms and the achievement of mucosal healing, *i.e.*, regression of atrophy. After 6-12 mo of adhering to a GFD, serology becomes negative in 80% of the patients and in 90% after 5 years.

Unfortunately, a normal TGA level at follow-up does not predict recovery of villous atrophy. Really, the lack of declining values and/or persistently positive serology 1 year after starting a GFD strongly suggest gluten contamination. Indeed, a recent meta-analysis demonstrated that IgA-class TGA and IgA-class EMA detected persistent villous atrophy with a high specificity (83%) but low sensitivity (50%).

Of interest, this study emphasized a presumable different CD diagnostic tool pattern between pediatric and adult ages. Indeed, the area under the curve for villous atrophy prediction was higher for children than for adults (0.879 *vs* 0.781)[16].

CONCLUSION

Biopsy-free strategy is a promising approach for the diagnosis of CD in adult population, with a sensitivity and specificity close to 100%. However, it should be highlighted that in adults the diagnosis of CD may be more challenging than in children, since villous atrophy and increased IELs might be related not only to CD, but even to other pathologic conditions, including drug damage, infections, or functional gastrointestinal disorders[35-41].

On the other hand, seronegative CD is a rare condition that may be found in adults. It should be always kept into account when clinical symptoms are highly suggestive of the disorder despite the absence of serological markers and, in this case, histological examination is the mandatory diagnostic tool[42,43].

Moreover, the possibility of false positivity of TGA has been described, especially after viral respiratory infections[44].

In conclusion, despite that the results show that biopsy-free strategy may be promising in adults, some cautions should be taken into account before performing a fully serologic diagnosis of CD. Indeed, the topic is still under-investigated, the results of the studies are heterogeneous, and some conditions, such as seronegative CD or intestinal damage due to causes other than gluten, may be relevant limiting factors. Furthermore, since most of studies are retrospective, the real possibility of avoiding endoscopic examination for diagnosing CD in adults is still a matter of debate and requires further research. Therefore, further studies with a standardized approach are still required to evaluate this strategy and determine the best cut-off.

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World J Gastroenterol 2021 November 14; 27(42): 7240-7246

DOI: 10.3748/wjg.v27.i42.7240

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

OPINION REVIEW

Digital surgery for gastroenterological diseases

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Author contributions: Hardy NP and Cahill RA were involved in the ideation, collation and drafting of this work.

Supported by Disruptive Technologies and Innovation Fund, Enterprise Ireland, Ireland.

Conflict-of-interest statement: Cahill RA receives speaker fees from Stryker Corp, Johnson and Johnson/Ethicon and Olympus, consultancy fees from Touch Surgery and DistalMotion, and research funding from Intuitive Surgery. Cahill RA also holds research funding from EU Horizon 2020 with Palliare and the Irish Government in collaboration with IBM Research in Ireland and Deciphex. Hardy NP is employed as a researcher in this collaboration.

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Abstract

Advances in machine learning, computer vision and artificial intelligence methods, in combination with those in processing and cloud computing capability, portend the advent of true decision support during interventions in real-time and soon perhaps in automated surgical steps. Such capability, deployed alongside technology intraoperatively, is termed digital surgery and can be delivered without the need for high-end capital robotic investment. An area close to clinical usefulness right now harnesses advances in near infrared endolaparoscopy and fluorescence guidance for tissue characterisation through the use of biophysics-inspired algorithms. This represents a potential synergistic methodology for the deep learning methods currently advancing in ophthalmology, radiology, and recently gastroenterology *via* colonoscopy. As databanks of more general surgical videos are created, greater analytic insights can be derived across the operative spectrum of gastroenterological disease and operations (including instrumentation and operative step sequencing and recognition, followed over time by surgeon and instrument performance assessment) and linked to value-based outcomes. However, issues of legality, ethics and even morality need consideration, as do the limiting effects of monopolies, cartels and isolated data silos. Furthermore, the role of the surgeon, surgical societies and healthcare institutions in this evolving field needs active deliberation, as the default risks relegation to bystander or passive recipient. This editorial provides insight into this accelerating field by illuminating the near-future and next decade evolutionary steps towards widespread clinical integration for patient and societal benefit.

Key Words: Digital surgery; Artificial intelligence; Gastrointestinal disease; Biophysics; Deep learning; Fluorescence-guided surgery

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Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Ireland

Peer-review report's scientific quality classification

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

Received: April 30, 2021
Peer-review started: April 30, 2021
First decision: June 13, 2021
Revised: June 27, 2021
Accepted: October 20, 2021
Article in press: October 20, 2021
Published online: November 14, 2021

P-Reviewer: Hanada E, Qiang Y, Sharma J
S-Editor: Fan JR
L-Editor: Kerr C
P-Editor: Fan JR



Core Tip: Here, we introduce the concept of digital surgery and why it is important for everyone involved in the area of gastroenterological disease management. The current state and near-future of the art in this area are discussed, including the use of artificial intelligence methods to provide intraoperative real-time augmented decision making. Moral, ethical and legal challenges pertinent to digital surgery are explored, including the concerns relating to big data in health care and the transitioning role of industry in surgical development, as well as the implications such profound and imminent changes may have on the role of the clinician.

Citation: Hardy NP, Cahill RA. Digital surgery for gastroenterological diseases. *World J Gastroenterol* 2021; 27(42): 7240-7246
URL: https://www.wjgnet.com/1007-9327/full/v27/i42/7240.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i42.7240

INTRODUCTION

Since the mid-20th century, our analog electromechanical world has increasingly transitioned to a digital and even automated one, leading to our current epoch being named the "Digital Age" or "Information Age". While many sectors have been rapid converts to this new order (notably retail and finance), adoption in the healthcare domain has been slower. In particular, in surgery, the focus has been on incremental iterations of existing technologies (such as upgraded displays and tweaked surgical instruments) over radical reformatting of existing methods. The reasons behind this are multifactorial, including a natural and understandable professional conservatism regarding clinical care, along with privacy concerns (patient and practitioner), ethical, moral and liability uncertainties, as well as a potential reluctance on behalf of system developers to involve themselves in intraprocedural decision making (preferring instead to leave this responsibility firmly in the hands of the human operator, for now at least). However, the evolution of surgical practice has plateaued.

Even the most sophisticated of new surgical machinery in general surgery, such as present-day robotic platforms, depend fully on the practitioner for their value and have led only to marginal benefit over the past 20 years. This is partly because of inequity in access but mostly because the fundamental distinguishing characteristic of best operative care relates to the intraoperative decision-making rather than dexterity of the practitioner. The new great hope is that the digitalisation of surgery, including the integration of artificial intelligence (AI) to the surgical workflow, will lead to better outcomes broadly; although, how exactly this will happen remains the challenge. Specifically, "digital surgery" exploits real-time analytics with technology during operations, and this editorial provides perspectives of its impact now and in the near future to all those looking after patients with digestive diseases.

AI DECISION-PROMPTING IN GASTROENTEROLOGY

This month's landmark publication of the European Commission's "Laying Down Harmonised Rules on Artificial Intelligence" attempts to shape the future of AI's incorporation across society[1]. In this document, the enormity of the socioeconomic potential of AI is stressed, and it emphasizes that the same elements that drive benefits will bring about new risks of potential great consequence. Also this month, the first commercial AI system for endoscopy, "GI Genius" by Medtronic (Dublin, Ireland), has been approved for clinical use by the United States Food and Drug Administration (FDA)[2]. This system and others like it (*e.g.*, Olympus ENDO-AID) act as a "second pair of eyes", constantly watching the screen and alerting the user to any potential anomaly by highlighting the detected area and leaving the human to decide on its importance[3]. Unlike the endoscopist, the computer aid does not have a single point of focus on the screen and so provides accurate full field of view observation at all times, a digital safety net. While impressive results are reported regarding increased polyp detection rates by this combination (14% absolute increase in adenoma detection rate over standard endoscopy), intra-procedural polyp characterisation remains

unsupported. Detailed descriptions of AI advances in endoscopic systems have been covered elsewhere[4-6].

When assessing such applications, the FDA stratifies the risk associated with the AI tool by the device's intended use[7]. Technologies designed to treat and diagnose or to drive clinical management receive higher risk ratings than those that aim to inform clinical management. Furthermore, to date, the FDA has only approved AI or machine learning systems that are "locked" prior to marketing. In locked systems, the algorithms used must provide the same output each time the same input is provided (*i.e.*, these systems cannot "learn" or adapt as they accrue more data, which really should be their hallmark). However, it is understandable that these early models should function within the safety of a locked domain while the appropriate regulatory and surveillance frameworks for unlocked AI are put in place. Such oversight would need to ensure that the performance and effectiveness of an unlocked system in clinical use was maintained as it evolved through learning over time. Furthermore, locking helps to ensure that the functionality of a device can be understood by clinicians and its abilities not overstated.

As more advanced systems come online, however, it is likely such "unlocked" systems will become approved for use. Therefore, the endoscopic support systems currently approved for use only represent a first pass at clinical entry, allowing sequential iterations to evolve serially with increasing degrees of data recording, analytics and self-learning, and therefore automatism.

AI IN SURGERY

While other areas of surgical practice, such as orthopedics, neurosurgery and maxillo-facial surgery, have embraced surgical preplanning through computer-generated models and intraoperative navigational aids, the incorporation of AI methods for gastrointestinal surgery is only in its infancy[8-10]. The lack of fixed, easily identifiable landmarks and the tendency for the operator to completely shift the anatomical field intraoperatively (from the preoperatively recorded imagery) has proven difficult to accommodate in the same way as is possible with interventions in osseous lattices. Furthermore, the most valuable asset in the advancement of AI methods has been the rapid ability to accumulate, store, annotate and distribute data streams. As a result, it has been most applicable to nonsurgical uses, where enormous pre-existing archives of categorised images exist (with data points in the millions), such as breast mammography and retinal screening[11,12]. For similar impact in surgery, computer methods need integration with surgical video, which is inherently more complex and, for this reason, harder to extract meaningful datapoints from.

Indeed, the necessary technology is only now proving sufficiently capable for such analysis to be envisaged, and the main rush right now relates to the accumulation of videos for future exploitation. The major companies are, therefore, looking at placing systems alongside their core imaging technology that allow uploading of material at the operator's discretion rather than automatically. This is because, with some exceptions, the prevailing business model has been to sell imaging systems with the imagery belonging fully to the purchaser. With the current realisation that such data have real value on an aggregated basis, it is likely that the next full-service subscription models will include data sharing as a contractual clause. Therefore, the technical obstacles of video set-up, storage and distribution have already diminished in this era of big data, leaving then annotation, curation and linking to metadata to be addressed.

IMAGE-GUIDED SURGERY

Surgical video contains more information than still images do, which means greater value but also complexity. Superimposition of more easily interpreted signals to these videos greatly enables data extraction. One such method successfully used in this manner utilizes near infrared imaging and indocyanine green, a fluorescent dye, to disclose information regarding the perfusion status of tissues in surgical videos and, hence, characterise their nature[13-15]. Indocyanine green binds to albumin within the vasculature, and real-time tracking of fluorescence intensity as it circulates can be used to create perfusion signatures of tissues. Alongside prediction of healing–nonhealing risk, this technique has been successfully piloted for the interrogation and classification of neoplasia of the colorectum using computer vision and AI with high levels

of accuracy intraoperatively based on biophysics-inspired principles (*i.e.*, perfusion within malignant tissue is fundamentally altered when compared to adjacent healthy regions in the same field of view[16]) (Figure 1).

BEYOND FLUORESCENCE

Accurate computerised identification and labelling of structures at operation, through fluorescence or otherwise, could soon lead to better, safer surgery. Automated localisation with operator-prompting for important structures, such as the inferior mesenteric artery, ureter or neurological bundles, during left-sided colonic surgery would assist experienced surgeons in difficult cases or normalise for experience in those at the beginning of their career or with low volume practices. Deep learning models have even been demonstrated to accurately identify when the critical view of safety has been demonstrated during laparoscopic cholecystectomies[17]. Once a tissue can be accurately characterised, the next logical progression from on-screen display is the integration of such data into smart instruments to guide operative steps. This may be by way of haptic or auditory feedback or even automatic shutoff in the case of destructive instruments, such as diathermy encountering structures that require safeguarding (this already exists with some orthopaedic instrumentation).

More rudimentary systems of instrument tracking and operative step identification are already available that allow retrospective auditing of performed operations and instrument efficiency[18,19]. Parsing of operations into component steps linked with metadata including operative costs and standard (*e.g.* hospital stay and complications requiring intervention) as well as evolved patient metrics (*e.g.*, patient-reported outcome measures) could greatly facilitate our move toward value-based health care. Early-stage iterations are now emerging, with automated operative phase identification in peroral endoscopic myotomy recently being described with the hope that further technological refinement may facilitate intraoperative decision support[20].

SURGICAL VIDEO DATA BANKS AT SCALE

Collation of surgical outcomes and surgical performance, either by the individuals wishing to gain insight into their own practice or as part of professional regulatory oversight, still presents potential controversies. The centralised deposition of surgical video followed by anonymised analysis of technique and outcomes, likely by other specialty practitioners initially and followed by AI when permissible, would provide a means for impartial appraisal and maintenance of standards (both of practitioners and indeed surgical instruments). However, there are risks to use of the data to promote commercial interests rather than the public good (this also applies to practitioners and/or institutions); as such, great transparency is needed and trust-boards should be considered. In addition, practitioners have privacy rights, as do patients, and the special status of the doctor-patient relationship prevents exploitation of this privilege, which is an important consideration alongside legality alone (*e.g.*, respect of frameworks such as General Data Protection Regulation). Furthermore, aggregation of surgical video in siloed collections, however individually large, risks lack of proper representation. Closed datasets also limit progress if others with relevant interest are excluded (frustrating the principle of reusability as a findability, accessibility, interoperability, and reusability principle), which may precipitate monopolistic practices.

AI PITFALLS AND CONCERNS

The relatively delayed adoption of AI within surgery permits a glimpse into potential pitfalls by observing the experience of other sectors. In October 2020, the United States House Antitrust Subcommittee Report on Competition in the Digital Market noted that, in many digital domains, few dominant corporations held singular control of channels of mass distribution in a fashion that allowed them to maintain power and absorb or remove competitors with ease[21]. This is a real concern, considering the enormous financial incentives associated with success in healthcare provision. Potential safeguards include an open/shared data repository (with appropriate regulatory compliance) upon which clinicians, academics and companies could work, as well as the standardisation of policies for the acquisition and storage of digital data,

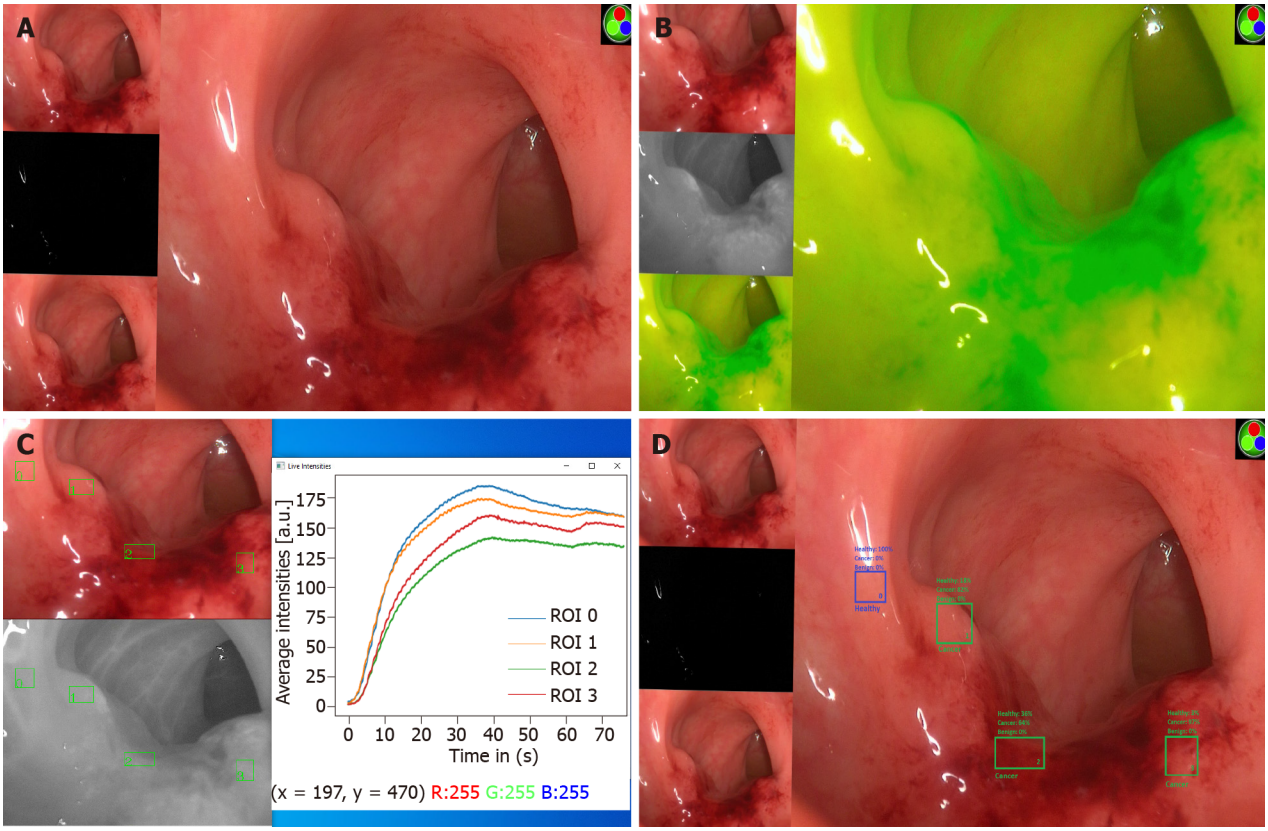


Figure 1 Digital surgery in action–real-time discrimination of tissue nature using fluorescence imaging and artificial intelligence. A: Transanal imaging of a rectal lesion using a Pinpoint (Novadaq, Mississauga, Canada; Stryker, Kalamazoo, MI, United States) near infrared imaging system; B: Following intravenous administration of indocyanine green (ICG) (0.25 mg/kg), fluorescence is observed within the lesion and surrounding tissue; C: In tandem with the ICG administration, regions of interest within healthy and unhealthy tissue (regions 0–3) are chosen by the surgeon for real-time assessment. Image tracking is performed using the visible light mode and light intensity readings extracted from the corresponding regions within the infrared spectrum video for each of these regions over time; D: Intensity profiles are subsequently fitted to biophysics-inspired artificial intelligence models of fluid movement in tissue to predict tissue nature with a binary outcome (healthy *vs* cancer) and a probability score (%).

to ensure universal accessibility. Additionally, the history of AI is replete with "boom and bust" cycles, and it is possible that its promised integration into surgery could prove another false dawn, especially with regard to more complex unsupervised machine learning and deep learning methods[22]. This may be a result of the complexity required to fine tune these systems to achieve acceptable results for use in humans. Controversies surrounding explainability (describing the settings used in the machine), interpretability (the degree to which a human can understand the decision) and bias within data training sets, as well as possible societal rejection of this form of technology in healthcare (akin to the criticisms of AI-based facial recognition software and recidivism predictors or any perception of data harvesting for surveillance capitalism), are other concerns.

CONCLUSION

Nevertheless, the formative years of digital surgery have begun. As a consequence, the landscape of surgical practice as it currently stands is likely to undergo rapid and most probably frequent evolutions. Such changes will have far reaching implications on the role of professional bodies, societies and institutions and, most fundamentally, clinicians. Moore's law cautions that the limits of such changes will be neither technological nor computational. The role of implementation of digital surgery remains, for now, with the clinician in their duty as patient caregiver. Whether this role will, in time, increasingly become one of the clinician as early enabler and later bystander or clinician as co-developer and collaborator remains yet to be determined. For the latter, co-ordinated, purposeful cross-collegiate action is needed urgently.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 14; 27(42): 7247-7270

DOI: 10.3748/wjg.v27.i42.7247

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

REVIEW

Gossip in the gut: Quorum sensing, a new player in the host-microbiota interactions

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Author contributions: Coquant G and Seksik P designed the outline of the review; Coquant G, Grill JP and Seksik P wrote part I; Coquant G, Aguanno D, Thenet S, Carrière V and Seksik P wrote part II; Aguanno D, Coquant G, Pham S and Grellier N wrote part III; Aguanno D and Coquant G made the figures; Coquant G and Aguanno D harmonized and formatted the different sections; all authors have reviewed the entire manuscript.

Supported by Fondation pour la Recherche Médicale, No. EQU202003010171; Association François Aupetit, No. AHLs 2019 and No. AHLs 2021; Fondation pour la Recherche Médical FRM, No. ECO201806006843 (to Coquant G); and CORDDIM, Ile-de-France Region (to Aguanno D).

Conflict-of-interest statement: Seksik P reports consulting fees from Abbvie, Takeda, Merck-MSD,

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Abstract

Bacteria are known to communicate with each other and regulate their activities in social networks by secreting and sensing signaling molecules called autoinducers, a process known as quorum sensing (QS). This is a growing area of research in which we are expanding our understanding of how bacteria collectively modify their behavior but are also involved in the crosstalk between the host and gut microbiome. This is particularly relevant in the case of pathologies associated with dysbiosis or disorders of the intestinal ecosystem. This review will examine the different QS systems and the evidence for their presence in the intestinal ecosystem. We will also provide clues on the role of QS molecules that may exert, directly or indirectly through their bacterial gossip, an influence on intestinal epithelial barrier function, intestinal inflammation, and intestinal carcinogenesis. This review aims to provide evidence on the role of QS molecules in gut physiology and the potential shared by this new player. Better understanding the impact of intestinal bacterial social networks and ultimately developing new therapeutic strategies to control intestinal disorders remains a challenge that needs to be addressed in the future.

Key Words: Inflammatory bowel disease; Quorum sensing; Gut microbiota; Dysbiosis; Inflammation; Intestinal barrier

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Pfizer, Astellas, Janssen and Biocodex and grants from Biocodex and Janssen. These COIs are unrelated to the current presentation. All other authors declare no conflict of interests for this article.

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Specialty type: Gastroenterology and hepatology

Country/Territory of origin: France

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 16, 2021
Peer-review started: March 16, 2021
First decision: August 9, 2021
Revised: August 17, 2021
Accepted: October 27, 2021
Article in press: October 27, 2021
Published online: November 14, 2021

P-Reviewer: Dai YC
S-Editor: Gao CC
L-Editor: A
P-Editor: Gao CC

Core Tip: Host-microbiota interactions play a crucial role in the pathophysiology of many intestinal diseases. While biological components have been repeatedly described, a largely overlooked component is quorum sensing (QS), a density-dependent system able to coordinate bacterial responses and interact with host cells constantly exposed to bacteria. This review intends to describe the different QS systems to show evidence that QS is part of the intestinal ecosystem and highlight its impact on intestinal epithelial barrier function, inflammation, and intestinal carcinogenesis. From this report, we open up a new area of intestinal physiology.

Citation: Coquant G, Aguanno D, Pham S, Grellier N, Thenet S, Carrière V, Grill JP, Seksik P. Gossip in the gut: Quorum sensing, a new player in the host-microbiota interactions. *World J Gastroenterol* 2021; 27(42): 7247-7270
URL: https://www.wjgnet.com/1007-9327/full/v27/i42/7247.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i42.7247

INTRODUCTION

Gut microbiota mutually interacts with coevolved host epithelial and immune cells in a beneficial reciprocal relationship[1]. The advent of multi-omics sequencing in the past decade has allowed researchers to investigate the complexity of the intestinal microbiota in various human disorders[2]. Many lines of evidence support a role for alteration of gut microbiota (dysbiosis) in the development or perpetuation of inflammatory and metabolic disorders; recent data pointed out the consequences of dysbiosis on host-microbiota interactions in this setting[3,4]. Currently, gut microbiota metabolites recognized as the main drivers of the impact of gut microbiota on hosts are short-chain fatty acids (SFCAs), branched-chain amino acids, trimethylamine N-oxide, bile acids, tryptophan (Trp), and indole derivatives[3,5]. A largely overlooked component is diffusible signaling molecules, which modulate the physiological response in the three domains of life[6]. A particular class of these signaling compounds is represented by bacterial quorum sensing (QS) molecules called autoinducers (AIs). QS is a density-dependent mechanism allowing bacterial populations to coordinate gene expression and physiology by modulating, for example, metabolic pathways, secretion of virulence factors, or biofilm formation in response to AIs[7]. Drawing on its density-dependent nature, it can be hypothesized that the production of bacterial signaling molecules is abundant in the highly densely populated environment of the mammalian intestinal tract.

Moreover, since several eukaryotic systems from fungi to plants and animals are known to recognize and respond to bacterial signaling compounds, it seems likely that human intestinal cells constantly exposed to bacterial compounds might also have developed response mechanisms to AIs with consequences on intestinal physiology [8]. The purpose of this current review is to provide clues to consider bacterial QS as a new actor of host-microbiota interactions. We will start by presenting the bacterial QS systems and evidence of QS in the gut. We will then provide an overview of the impact of QS molecules on host cell functions within the gut. Finally, we will investigate how modulation of the QS could be thought of as a therapeutic option, determine the key challenges, and suggest directions for future QS research.

QS: GOSSIP IN A BACTERIAL WORLD

In the conventional view of prokaryotic existence, bacteria live as unicellular organisms, with responses to external stimuli limited to detecting chemical and physical signals of environmental origin. This view of bacteriology is now recognized as overly simplistic because bacteria communicate through small 'hormone-like' organic compounds. QS is a bacterial cell-cell communication process that involves the production, detection, and response to extracellular signaling molecules called AIs. AIs enable bacteria to perceive and respond to temporal and contiguous environments and coordinate the behavior of colonies by altering gene expression. QS controls genes that direct beneficial activities when performed by groups of bacteria acting in

synchrony. Processes controlled by QS include bioluminescence, sporulation, competence, antibiotic production, biofilm formation, and virulence factor secretion (Figure 1A). Similar to languages between humans, these signals vary between species. Some bacterial species can interpret many different signals, while others respond to a few. The first such system was described in 1979 in *Vibrio fischeri*[9], a symbiotic species that provides marine eukaryotic hosts with light. Light emission depends on transcription of the luciferase operon, which occurs when the cell population density is sufficient to produce a threshold accumulation of a secreted AI, a specific N-acyl Homoserine Lactone (AHL). It was only in 1994 that the term QS was first used to introduce the idea of a minimal population (quorum) that was needed to trigger a group behavior thanks to a signal[10]. The science of studying group behavior in microorganisms has even been named "sociomicrobiology"[11].

Different ways of talking

Bacterial QS is highly complex and mediates communications thanks to the diversity of its different systems. QS systems can be divided into systems specific to species and mediating communication between Gram-positive bacteria, Gram-negative bacteria, and interspecies systems (Table 1 and Figure 1).

QS in Gram-positive species is driven in most cases by 5-17 amino acid oligopeptides (AIPs for AutoInducer Peptides), which are detected by membrane receptors belonging to the histidine kinase family and are involved in virulence or competence[12]. In addition, γ-butyrolactones are produced and integrated by *Streptomyces sp.* as signals controlling antibiotic production[13] or metabolism[14].

QS in Gram-negative bacteria relies on a high diversity of different systems, with some bacteria, such as *Pseudomonas aeruginosa (P. aeruginosa)*, possessing several QS systems (Table 1). The expression of over 300 genes is regulated by QS. The most common system is driven by AI-1 molecules belonging to the AHL family, which are constituted by a homoserine lactone ring carrying a 4-18 carbon acyl chain. The lengths and modifications of the acyl chain give each AHL its species specificity[15]. The first described model is the *Vibrio fischeri* system, in which N-3-oxohexanoyl-homoserine lactone (3-oxo-C6) is synthesized by LuxI synthase, passively diffuses out of the cell and enters another bacterium in which it binds its receptor LuxR[16] (Figure 1B). Above a threshold, the AHL-receptor complex binds a consensus DNA sequence, thus triggering luciferase expression[17]. This model applies to all AHL systems (Table 1 and Figure 1B). The system involves a positive feedback loop, thus promoting QS activation at the population scale (Figure 1B). To date, numerous homologous systems (*i.e.*, genes coding synthases and receptors) have been described in many Gram-negative bacteria, including over 70 Proteobacteria species[18] (Table 1).

Other Gram-negative QS systems involve the AI-3 molecule, initially identified in enterohemorrhagic *Escherichia coli* (*E. coli*) (EHEC) serotype O157:H7[19]. AI-3 regulates flagellar genes and pathogenicity[20,21] and is thought to be present in other enteropathogens (Table 1). A recent study[22] uncovered the structure of AI-3 and its natural analogs, including the prominent analog in mouse feces *in vivo*, which belongs to the pyrazinone family. The authors showed that various gram-negative and Gram-positive bacteria produce AI-3 analogs, thus redefining the specificity of AI-3 molecules.

Last, the third type of QS system has been identified in Gram-negative bacteria such as EHEC or *Vibrio* species and Gram-positive bacteria such as *Salmonella enterica*[23,24]. It relies on AI-2 molecules such as S-THMF-borate [for (2S,4S)-2-methyl-2,3,3',4-tetrahydroxy-tetrahydrofurane-borate][25] and R-THMF [for (2R,4S)-2-methyl-2,3,3',4-tetrahydroxy-tetrahydro furane][26]. AI-2 has now been found in various bacterial species in which it regulates many processes[27] and is proposed to mediate poly-species communication (Figure 1C).

In addition, indole is produced from Trp by Gram-negative and Gram-positive commensal and pathogenic bacteria displaying tryptophanase activity[28-31]. As the source of Trp is supplied by the diet and cannot be synthesized endogenously, either by bacteria or by the host, indole is a bacterial byproduct of Trp metabolism. However, in recent years, some authors have considered indole to be a QS molecule, as it is produced in a density-dependent manner and regulates several bacterial physiological processes, such as the formation of spores or biofilms, virulence traits, bacterial motility, and drug resistance[29,32,33].

The versatility of QS systems and their AI molecules highlights the complexity of communication and thus emphasizes the key role QS could play in a diverse ecosystem: the intestinal microbiota.

Table 1 Examples of bacterial quorum sensing autoinducer and corresponding systems

	AI	Example of producing bacteria	QS system	Bacterial QS-regulated processes	Ref.
Gram +	AI peptide	*Staphylococcus aureus*	*agr*	Virulence	Novick *et al*[155]
		Listeria monocytogenes	*agr*	Virulence	Autret *et al*[156]
		Clostridium perfringens	*agr*	Virulence	Ohtani *et al*[157]
		Enterococcus faecalis	*FsR*	Virulence	Sifri *et al*[158]
		Bacillus subtilis	*com*	Competence	Magnuson *et al*[159]
	γ-butyrolactone	*Streptomyces* genus	*scb*	Antibiotics	Takano *et al*[13]
			scg	Metabolism	Du *et al*[14]
Gram -	AI-1 (acyl-homoserine lactones)	*Vibrio fischeri*	LuxI/LuxR	Luminescence	Engebrecht *et al*[16]
		Vibrio harveyi	LuxLM/LuxN	Luminescence	Mok *et al*[160]
				Virulence	Waters and Bassler[161]
		Pseudomonas aeruginosa	LasI/LasR	Virulence and biofilm	Gambello and Iglewski[162], Gambello *et al*[163], Winson *et al*[164], and Chapon-Hervé *et al*[165]
			RhlI/RhlR		
	PQS	*Pseudomonas aeruginosa*	PqsABCD/PqsR	QS regulation	Pesci *et al*[166]
				Pyocyanin	Gallagher *et al*[167]
				Iron homeostasis	Bredenbruch *et al*[168] and Diggle *et al*[169]
				Virulence	Gallagher *et al*[167] and Cao *et al*[170]
				Biofilm	Diggle *et al*[171]
	IQS	*Pseudomonas aeruginosa*	AmbBCDE/IqsR	Response to stress	Lee *et al*[172]
	CAI	*Vibrio (cholerae)*	CqsA/CqsS	Virulence	Ng *et al*[173]
	AI-3	EHEC O157:H7	Qse/QseBC	Attachment-effacement	Sperandio *et al*[19], Walters *et al*[21], and Kim *et al*[22]
		EPEC O26:H11	Qse/unknown	Unknown	Kim *et al*[22], and Kaper and Sperandio[40]
		AIEC LF82	Qse/unknown	Unknown	Kim *et al*[22]
		Escherichia coli MG1655	Unknown	Unknown	Kim *et al*[22]
		Escherichia coli BW25113	Unknown	Unknown	Kim *et al*[22]
		Salmonella enterica	Qse/unknown	Unknown	Kim *et al*[22], Kaper and Sperandio[40], and Walters and Sperandio[174]
		Shigella flexneri	Qse/unknown	Unknown	Kim *et al*[22], Kaper and Sperandio[40], and Walters and Sperandio[174]
		Yersinia sp.	Qse/unknown	Unknown	Kim *et al*[22], Kaper and Sperandio[40], and Walters and Sperandio[174]
Gram + and -	AI-2	*Vibrio harveyi*	LuxS/LuxPQ	Bioluminescence, TSS, protease	Surette *et al*[24], Mok *et al*[160], and Schauder *et al*[175]
		Vibrio cholerae	LuxS/LuxPQ	Virulence and Biofilm	Schauder *et al*[175], Zhu *et al*[176], and Hammer and Bassler[177]
		Enterococcus faecalis	LuxS/LuxPQ	Unknown	Surette *et al*[24], and Schauder *et al*[175]
		EHEC	LuxS/LsrB (?)	Attachment-effacement	Schauder *et al*[175], and Bansal *et al*[178]
		Salmonella enterica	LuxS/LsrB	Pathogenicity and invasion	Miller *et al*[26], Schauder *et al*[175], and Choi *et al*[179]

AI: Autoinducer; AIEC: Adherent-invasive *Escherichia coli*; AIP: AutoInducer peptides; CAI: Cholera autoinducer-1; EHEC: Enterohemorrhagic *Escherichia coli*; EPEC: Enteropathogenic *Escherichia coli*; IQS: Integrated quorum sensing; PQS: Pseudomonas quinolone signal; QS: Quorum sensing.

QS in the gut

The study of QS in the gut is still a relatively recent matter of interest, as QS is generally addressed from the pathogenic bacterium *P. aeruginosa* point of view in the lung ecosystem. However, many arguments suggest that QS is a new player in the gut ecosystem.

AHLs in the gut: As part of the eavesdropping mechanism, some bacteria from the human gut can sense AHLs from other species (Figure 1A). Gram-negative bacilli such as *E. coli*, *Enterobacter*, or *Klebsiella* express the receptor SdiA, which can sense AHL, without producing such a signal[34]. The opportunistic pathogen *P. aeruginosa,* which targets the digestive tract in severely immune-compromised patients[35,36], and the more common enteropathogen *Yersinia enterolitica* are known to produce AHLs[37]. Analyses from sequencing databases have shown the presence of LuxI/LuxR homologs in a few commensals: *Hafnia alvei, Edwardsiella tarda*, and *Ralstonia sp.* strain 5_7_47FAA[37]. However, the latter article did not demonstrate the presence of AHLs but only homologs of the genes encoding the synthase complex and the receptor. A cohort low sample size pediatric study (n = 4) demonstrated, thanks to bacterial reporter systems, the presence of AHLs in the feces of patients without identifying them[38]. Our team investigated the question of AHLs in the human gut in the context of inflammatory bowel disease (IBD). With high-resolution mass spectrometry, we identified approximately ten AHLs in the feces of healthy patients, IBD patients in remission, and flare[39]. We also found a never-described AHL, 3-oxo-C12:2-HSL, that was less represented in IBD patients, especially in flares, than in healthy subjects[39].

AI-2 in the gut: AI-2 presence in the gut has been reported by several articles[19,40,41] but is mainly linked to pathogenic bacteria such as enterohemorrhagic *E. coli*[19]. As AI-2 is considered a "universal language", it is not surprising to find this AI in an ecosystem as diverse as the gut microbiota.

Thompson *et al*[42] showed that most Firmicutes contain LuxS protein orthologs, an important enzyme that allows AI-2 production. In contrast, its presence is less represented in Bacteroidetes. Mutant *E. coli* engineered to regulate AI-2 levels in the mouse gut counteract antibiotic-induced dysbiosis[42]. AI-2 produced by *E. coli* benefits Firmicutes while restraining Bacteroidetes representation[42], suggesting an important role of AI-2 in the gut ecosystem.

Other QS signals: Concerning other QS signals, there is less evidence of their implications in gut microbiota. A recent study showed a correlation between indole and *Clostridioides difficile* (*C. difficile*) infection (CDI) with higher indole concentrations for patients with CDI than for CDI-negative patients with diarrhea[43]. *C. difficile* induces indole production through overexpression of the tryptophanase gene tnaA in enterotoxigenic *E. coli* and other indole-producing anaerobes. This increased indole level has been shown to be detrimental to some of the beneficial bacteria and favors *C. difficile* colonization.

These mechanisms collectively suggest a complex bacteria-bacteria QS network in the gut ecosystem. The key issue is now to decode every language to fully understand its potential in host-microbiota interactions.

Gossip in the gut: Direct dialog with the host and indirect effects through bacterial behavior

In an ecosystem as networked and complex as that in the gut, bacterial communication has to be seen from a large perspective, with multiple bacterial populations crosstalking to each other through eavesdropping or crosstalking between species (Figure 1A). Therefore, the question of how QS affects the host can be addressed in two ways (Figure 2).

QS modulates microorganism metabolism, which in turn can affect the host's physiology; one could consider this to be an indirect effect of QS molecules on the host (Figure 2). Bacterial metabolism modifies beneficial byproducts such as SFCAs and bile acids[42]. By modulating intestinal microbiota composition, QS can indirectly influence gut physiology by promoting deleterious or beneficial bacteria (Figure 2). Thompson *et al*[42] demonstrated that AI-2 modulates dysbiotic microbiota composition by enhancing Firmicutes growth. Several reports *in vitro* and *in vivo* describe how enteropathogens can signal through QS to commensals and trigger the expression of toxins, virulence factors, and biofilm formation[44-46]. In addition, AI-3 controls the genes that enable enterohemorrhagic *Escherichia coli* to cause lesions by the attachment and effacing process[19].

The question addressed in this review is how quorum-sensing molecules can directly affect the host, independent of the producing bacteria (Figure 2).

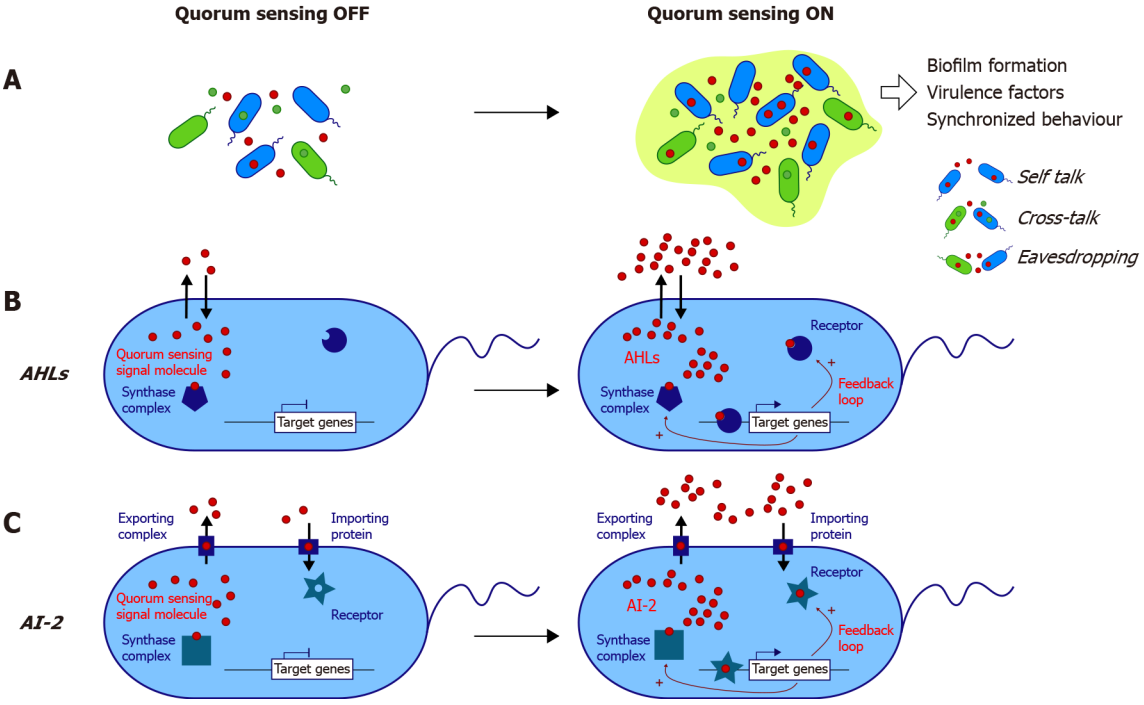


Figure 1 Main known mechanisms of quorum sensing activation in bacteria. A: Quorum sensing (QS) signaling depends on the release of autoinducers (AIs) in the environment. Above a threshold concentration depending on bacterial density, QS is activated and triggers gene expression. QS can be classified into three categories: self-talk (*i.e.*, one species "talking" to itself), crosstalk (*i.e.*, different species communicating using common AI), and eavesdropping, which refers to "listening" by species unable to produce AI by itself; B: The acyl-homoserine lactone (AHL) used by Gram-negative bacteria is produced by the synthase complex, and AHL can freely diffuse through the membrane. AHL is recognized by its intracellular receptor, and the complex binds to target gene regulatory elements; C: The AI-2 system is used by both Gram-negative and Gram-positive bacteria. AI-2 needs a transporter protein to exit and enter the cell. For both AHLs and AI-2, there is a positive feedback loop, allowing the expression of the synthase complex and receptor of AIs. AHL: Acyl-homoserine lactone; AI: Autoinducer.

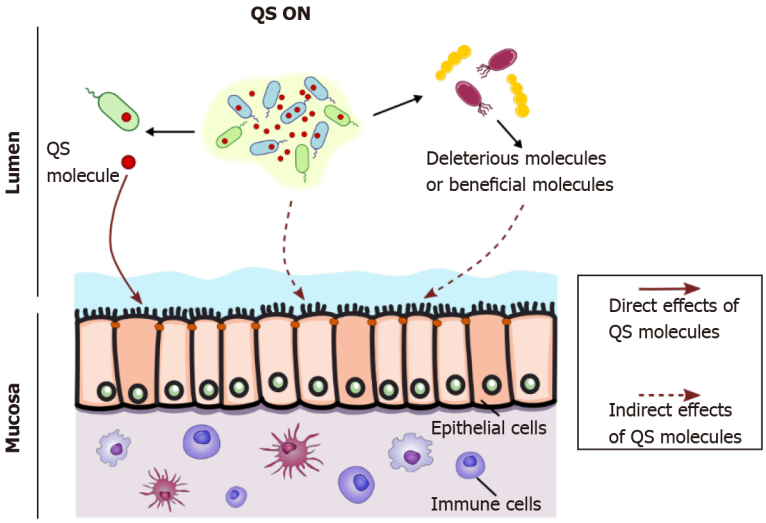


Figure 2 Interkingdom dialog between bacteria and the host through quorum sensing molecules. When reaching a threshold concentration within a bacterial community, quorum sensing (QS) autoinducers synchronize group behaviors such as virulence and attachment-effacement strategies as in enterohemorrhagic *Escherichia coli*, thus indirectly affecting the host (dotted line arrow, middle). QS molecules can impact the host through direct contacts (full arrow, left) with host cells, such as epithelial or immune cells, as has been extensively shown for the *Pseudomonas aeruginosa* QS molecule 3-oxo-C12-HSL, which freely enters mammalian cells. In addition, QS molecules can indirectly modify the host (dotted line arrow, right) through effects on other bacterial populations with different metabolic properties. QS: Quorum sensing.

HOST: "YOU'VE GOT MAIL"

As discussed above, it remains largely unknown how the microbial communities hosted in the gut lumen use QS communication systems. However, there is evidence that at least several bacterial species commonly found in the gastrointestinal tract have

the capacity to synthesize QS molecules[6,8,39].

Studies on the impact of QS molecules on the biology of intestinal host cells have focused on key actors of barrier function and the immune response. Intestinal barrier function includes the ability of epithelial cells to form: (1) A selective barrier whose permeability is controlled by cell-cell junctions[47], (2) Synthesize a protective mucus layer and antimicrobial peptides[48,49], and (3) Secrete cytokines and chemokines allowing appropriate crosstalk with the underlying immune compartment. The intestinal immune system is involved in tolerogenic or inflammatory responses to the commensal microbiota or pathobionts/pathogens[50,51], and it represents the largest immune organ in the body. Intestinal epithelial cells, intraepithelial lymphocytes, and immune cells located in the lamina propria are involved in the modulation of immunity and inflammation by microbiota[52].

The impact of QS molecules on barrier function and the immune response has been mainly studied in the context of host-pathogen interactions, probably because most of the data rely on AHLs produced by *P. aeruginosa*. However, evidence of the presence of QS molecules in the healthy intestinal lumen has led to further study on their effects on the host compartment, including barrier function, inflammatory process, and carcinogenesis.

Effects of quorum-sensing molecules on intestinal epithelial barrier function

AHLs: 3-oxo-C12-HSL produced by *P. aeruginosa* is probably the QS molecule whose effects on the barrier function of epithelial cells have been the most studied during the last two decades[53]. *P. aeruginosa* synthesizes various virulence factors, which act synergistically with QS molecules to destabilize cell-cell junctions and promote bacterial transmigration across epithelial and endothelial barriers[54].

3-oxo-C12-HSL induces an increase in paracellular permeability to ions and macromolecules[55-60] (Table 2). This deleterious effect of 3-oxo-C12-HSL on barrier function is accompanied by an alteration of tight junctions (TJs) (Figure 3). In the Caco-2 intestinal epithelial cell line, 3-oxo-C12-HSL induced a decrease in the expression, as well as mislocalization, of the TJ proteins occludin, tricellulin, ZO- 1, ZO-3, JAM-A, and of the adherent junction proteins E-cadherin and β-catenin[55,58-61] (Table 2). Loss of occludin/ZO-1 and tricellulin/ZO-1 interaction at the plasma membrane suggested the dismantling of TJ protein complexes[61]. In addition, hyperphosphorylation of occludin, ZO-1, ZO-3, JAM-A, E-cadherin, and β-catenin on tyrosine residues (as well as serine and threonine for E-cadherin and ZO-1) was reported in the presence of 3-oxo-C12-HSL, whereas the serine and threonine residues of occludin, JAM-A and β-catenin were less phosphorylated[58,59].

Several signaling mechanisms, including p38 and p42/44 MAP kinases[59,60], Ca^{2+} release[57,58], matrix metalloproteinases MMP-2 and MMP-3 *via* protease-activated receptor (PAR) signaling[55] and oxidative stress[62], have been implicated in 3-oxo-C12-HSL effects on junctional proteins and on the concomitant increase in permeability (Table 2).

As discussed above, the most prominent AHL detected in the human intestinal ecosystem is unsaturated 3-oxo-C12:2-HSL[39]. Despite a high structural homology with the *P. aeruginosa* 3-oxo-C12-HSL, this intestinal AHL has recently been found to have opposite properties regarding the barrier function (Table 2). In contrast to 3-oxo-C12-HSL, 3-oxo-C12:2-HSL does not increase paracellular permeability in Caco-2/TC7 enterocytic cells[39,61]. Most importantly, 3-oxo-C12:2-HSL can limit TJ disruption induced by the proinflammatory cytokines interferon-gamma (IFN-γ) and tumor necrosis factor-α (TNF-α)[61] (Figure 3). In these conditions mimicking intestinal inflammation encountered, for example, in IBD, 3-oxo-C12:2-HSL maintains the interaction of the TJ transmembrane proteins occludin and tricellulin with their main cytoplasmic partner ZO-1. It limits cytokine-induced occludin and tricellulin ubiquitination and the interaction of these TJ proteins with the E3 ubiquitin ligase itch, suggesting stabilization of TJ complexes at the plasma membrane in inflammatory conditions. Altogether, these results show that "commensal" intestinal 3-oxo-C12:2-HSL mitigates the deleterious effects of the inflammatory environment on TJs, which are key actors in epithelial barrier function[61].

Epithelial barrier disruption may combine TJ alteration and an unrestricted passage, which occurs following epithelial damage, generated, for example, by cell apoptosis upon exposure to harmful molecules such as high doses of proinflammatory cytokines [63-65]. This TJ-independent breaking of the barrier allows translocation of large particles such as large proteins, entire bacteria, and viruses, which *a priori* cannot cross the epithelium through the paracellular route even in conditions where TJs are "open" [66]. The 3-oxo-C12-HSL produced by *P. aeruginosa* exerts cytotoxic effects, particularly through apoptosis induction, in numerous cell types, including the intestinal and

Table 2 Effects of quorum sensing molecules on different parameters of the intestinal epithelial barrier function

QS molecule	Effects	Ref.
Effects on the intestinal epithelial migration		
3-oxo-C12-HSL	Increased migration at low concentrations (1.5-12 μmol/L) *vs* inhibition at 200 μmol/L	Karlsson *et al*[72]
	Interaction with IQGAP1 and increase in Rac1/Cdc42 (1.5-200 μmol/L)	Karlsson *et al*[72]
Effects on the intestinal epithelial permeability and intercellular junctions		
3-oxo-C12-HSL	Increased permeability to ions and macromolecules (100-400 μmol/L)	Eum *et al*[55], Vikström *et al*[58-60], and Aguanno *et al*[61]
	Activation of p38 and p42/44 and calcium signaling (100-200 μmol/L)	Vikström *et al*[58-60]
	Decreased expression levels of tight junction genes (100-400 μmol/L); Disassembly of tight and adherens junctions (modification of their phosphorylation status and involvement of MMP-2 and -3)	Eum *et al*[55], Vikström *et al*[58-60], and Aguanno *et al*[61]
	Decreased levels of tight junction proteins occludin and tricellulin (100-400 μmol/L)	Eum *et al*[55]
	Decreased protein levels of extracellular matrix and tight junction proteins (400 μmol/L)	Tao *et al*[62]
3-oxo-C12:2-HSL	No deleterious effects on permeabilityProtection of tight junction integrity and maintenance of junctional complexes at the plasma membrane under pro-inflammatory conditions	Landman *et al*[39] and Aguanno *et al*[61]
3-oxo-C14-HSL	Decreased protein levels of extracellular matrix and tight junction proteins (400 μmol/L)	Tao *et al*[62]
Indole and indole derivatives	Decreased permeability to ions and increased expression of genes coding tight junction and cytoskeleton proteins	Bansal *et al*[76] and Shimada *et al*[77]
	Decreased permeability to macromolecules	Venkatesh *et al*[79]
	Increased transcripts levels of genes coding tight junction proteins	Shin *et al*[78]
Effects on the mucus layer components		
3-oxo-C12-HSL	Decreased MUC3 mRNA levels (30 μmol/L)	Taguchi *et al*[70]
	Decrease in Muc2 production in goblet cell-like cell line (100 μmol/L) *vs* increase in colonic cell line (400 μmol/L)	Tao *et al*[67]
Indole	Increased expression of genes involved in the production of mucins	Bansal *et al*[76]
Effects on intestinal epithelial cell viability		
3-oxo-C12-HSL	Mitochondrial dysfunction and induction of apoptosis in goblet cell-like cell line (100 μmol/L) and in colonic cell line (30-100 μmol/L)	Tao *et al*[67-69], and Taguchi *et al*[70]
	Induction of apoptosis, mitochondrial dysfunction, oxidative stress and blocking of cell cycle (400 μmol/L)	Tao *et al*[62]
3-oxo-C14-HSL	Induction of apoptosis, mitochondrial dysfunction, oxidative stress and blocking of cell cycle (400 μmol/L)	Eum *et al*[55], Vikström *et al*[58-60], Aguanno *et al*[61], and Tao *et al*[62]
CSF	Reduction of oxidative stress-induced cell death and loss of the epithelial barrier (involving HSP27 and p38/MAPK pathway)	Fujiya *et al*[74]

CSF: Competence and sporulation factor; HSL: Homoserine lactones; HSP27: Heat shock protein 27; IQGAP1: IQ motif containing GTPase activating protein 1; MAPK: Mitogen-activated protein kinase; MMP-2/-3: Matrix metalloproteinase-2/-3; MUC: Mucin; QS: Quorum sensing; Rac1/Cdc42: Ras-related C3 botulinum toxin substrate 1/cell division control protein 42 homolog.

colonic epithelial cell lines LS174T[67-69], Caco-2[70], and CT26[62] (Table 2 and Figure 3). Apoptosis triggered by 3-oxo-C12-HSL relies on oxidative stress and caspase-dependent processes[62,69], whereas short-chain C4-HSL does not exert any apoptotic effects[67]. Interestingly, the increase in paracellular permeability to macromolecules induced by 3-oxo-C12-HSL was dramatically exacerbated in Caco-2/TC7 cells cultured in the presence of IFN-γ and TNF-α or cocultured with THP-1 activated monocytic cells[61]. These synergistic effects on barrier disruption probably rely on epithelial cell apoptosis, as they are abolished by a caspase inhibitor (unpublished results). In contrast, intestinal AHL 3-oxo-C12:2-HSL neither exerts cytotoxic effects nor synergizes with proinflammatory cytokines to disrupt the epithelial barrier[61].

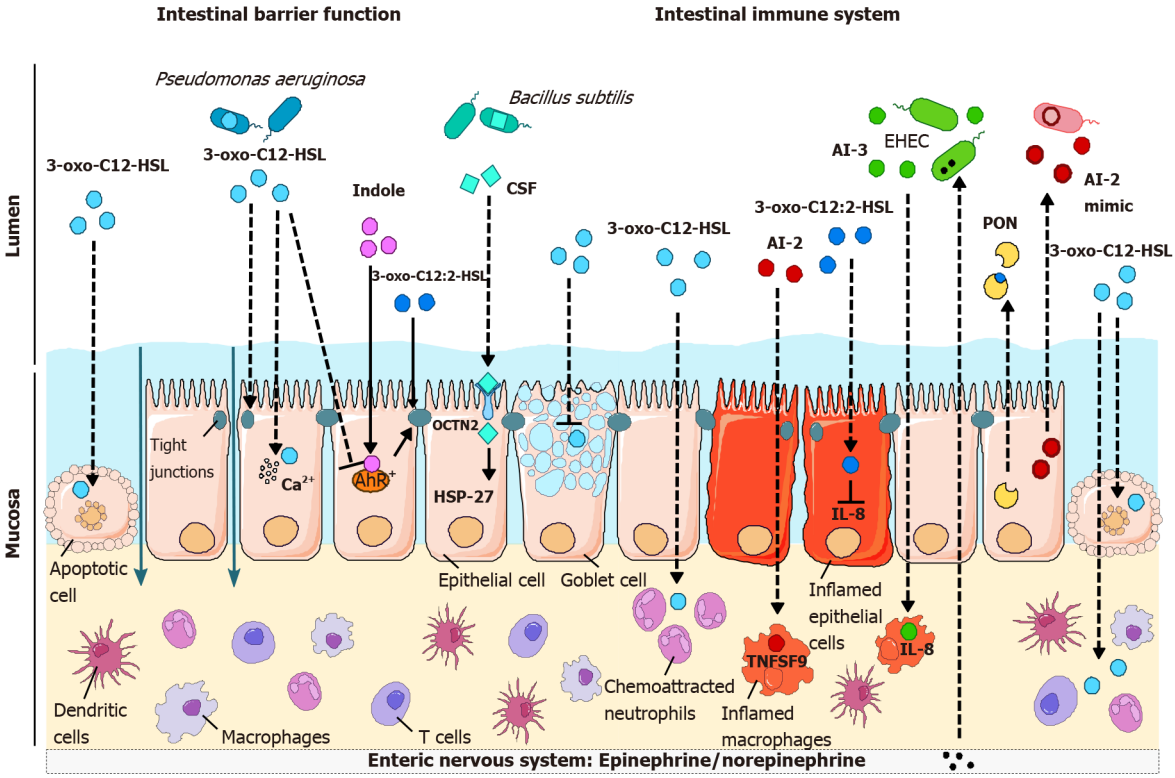


Figure 3 Effects of quorum sensing molecules on intestinal barrier function (see Table 2) and on the immune response (see Table 3). The *Pseudomonas aeruginosa* quorum sensing (QS) molecule 3-oxo-C12-HSL induces apoptosis in various cell types, including epithelial cells, promoting a breach in the intestinal barrier. In addition, 3-oxo-C12-HSL disrupts tight junctions, thus leading to increased paracellular permeability, and affects mucin production. Conversely, intestinal acyl-homoserine lactone 3-oxo-C12:2-HSL and the tryptophan metabolite indole protect tight junctions. *Bacillus subtilis* CSF, which binds to OCTN2, also promotes intestinal barrier integrity by reducing cell death through activation of HSP27 signaling. While 3-oxo-C12-HSL stimulates chemoattraction and phagocytosis in neutrophils and induces cell death, its pro- or anti-inflammatory effects on immune cells are more complex (see Table 3). Autoinducers (AI)-2 and AI-3 both exert proinflammatory effects on macrophages by inducing the expression of the immune mediators TNSF9 and interleukin (IL)-8, respectively, whereas 3-oxo-C12:2-HSL reduces IL-8 production by epithelial cells. It remains to be clarified how all these QS molecules could cross the intestinal barrier and/or reach immune cells *in vivo* in a physiological context, as illustrated by dotted lines. Last, just as QS molecules can impact eukaryotic cells, the host can interfere with QS: the hormones epinephrine/norepinephrine bind to the AI-3 receptor in EHEC; intestinal epithelial cells secrete an AI-2 mimic in addition to paraoxonase (PON) enzymes degrading homoserine lactones. CSF: Competence and sporulation factor; AI: Autoinducer; PON: Paraoxonase; AhR: Aryl hydrocarbon receptor; IL: Interleukin.

Epithelial injury accompanying acute inflammatory conditions is followed by a re-epithelialization phase, during which cell migration plays an important role[71]. Interestingly, 3-oxo-C12-HSL has been shown to dose-dependently modulate Caco-2 cell migration in a wound-healing assay and interact directly with the GTPase activating protein IQGAP1, stressing a potential role of AHL in cytoskeletal reorganization[72] (Table 2).

Another key actor of the intestinal physical barrier is the mucus layer, which is essential to maintain segregation between luminal microorganisms and the epithelium [49]. 3-oxo-C12-HSL induces reduced expression and production of Mucin2 in LS174T cells[67], as well as a decrease in the levels of MUC3 mRNA in the Caco-2 cell line cultivated in an undifferentiated state[70] (Table 2 and Figure 3). Interestingly, differentiated Caco-2 cells, which express higher levels of mucin 3, showed less sensitivity to 3-oxo-C12-HSL-induced apoptosis in the latter study, and the addition of mucin dose-dependently protected cells from apoptosis induced by this AHL[70].

It must be specified that all these studies on barrier function were carried out with high concentrations of AHLs (100-400 μmol/L) (Table 2), knowing that the concentration of 3-oxo-C12-HSL has been estimated to reach 600 μmol/L in biofilms of *P. aeruginosa*[73].

Gram-positive QS peptides: The effects of AIP (found in Gram-positive bacteria) on intestinal barrier function are much less documented than those of Gram-negative QS molecules. Whereas most of the studies on the effects of AIP on host inflammation describe the indirect effects of AI through the modulation of bacterial metabolism, one article reported the direct effects of AIP. Fujiya *et al*[74] reported that *Bacillus subtilis* AIP, named competence and sporulation factor (CSF), induces HSP27 expression and the p38/MAPK pathway and reduces cell death and the loss of the epithelial barrier

induced by oxidative stress (Table 2 and Figure 3). Inducible HSPs are needed under stress and help stabilize proteins to prevent denaturation[75]. *B. subtilis* is part of the normal microbiota; it is also used as a commercial probiotic and is beneficial to the host. Moreover, CSF seems to signal through a receptor named OCTN2 (organic cation/carnitine transporter 2), and polymorphisms of the gene encoding this receptor are part of the susceptibility locus of Crohn's disease[74].

Indole: Indole exerts a beneficial role on TJ protein expression in several intestinal epithelial cells[76-79] (Table 2). Oral administration of indole to germ-free mice, which display very low indole fecal levels, increased the expression of TJ and adherens junction-associated proteins in the colonic epithelium and improved their resistance to dextran sulfate sodium (DSS)-induced colitis[77]. Indole has been identified as an endogenous agonist of aryl hydrocarbon receptor (AhR), which can compete for receptor binding with well-known AhR ligands[80]; several studies have also stressed the key role of the AhR pathway in indole derivative protective effects[81-83]. Accordingly, several studies have shown that AhR activation strengthens the epithelial barrier by protecting TJs[82,84-87] (Figure 3) or by stimulating antimicrobial peptide production *via* interleukin (IL)-22[88,89].

Effects of QS molecules on immune response

In addition to their effects on the intestinal barrier, QS molecules were analyzed on different actors of the immune compartment of the intestine, which is involved in a complex crosstalk with the epithelial compartment to maintain an appropriate immune response toward the content of the intestinal lumen.

AHLs: Our group described that 3-oxo-C12:2-HSL, an AHL recently discovered in the human gut[39], exerts anti-inflammatory effects on intestinal epithelial cells. During inflammation, intestinal epithelial cells can secrete some cytokines, among which the chemokine IL-8 promotes the recruitment of neutrophils in the mucosa and participates in the acute-phase response[90,91]. In a study comparing the effect of 3-oxo-C12:2-HSL to 3-oxo-C12-HSL produced by *P. aeruginosa*, our group demonstrated in the human enterocytic Caco-2/TC7 cell line that 3-oxo-C12:2-HSL, but not 3-oxo-C12-HSL, attenuated the induction of IL-8 secretion induced by the proinflammatory cytokine IL-1β[39,92] (Table 3 and Figure 3). This potential anti-inflammatory effect of 3-oxo-C12:2-HSL is consistent with the hypothesis of a beneficial role of this AHL in gut ecosystems[39], as are its protective effects on TJ integrity. The impact of intestinal 3-oxo-C12:2-HSL on immune cells remains largely unknown.

The effects of 3-oxo-C12-HSL depend on the concentration and cell type studied[6,93]. Telford *et al*[94] showed that 3-oxo-C12-HSL inhibits the production of TNF-α and IL-12 [a cytokine involved in the T helper cell-1 type response (Th1-type response)] by lipopolysaccharide-activated macrophages at high concentrations and stimulates the production of antibodies, particularly immunoglobulin G1, which is an indicator of a Th2-type response at lower concentrations (Table 3). Conversely, Smith *et al*[95] showed that 3-oxo-C12-HSL activates and promotes the differentiation of naive T lymphocytes toward a Th1-like phenotype, while Ritchie *et al*[96] observed that 3-oxo-C12-HSL inhibits the differentiation of both Th1 and Th2 T lymphocytes (Table 3). Altogether, these results demonstrated that 3-oxo-C12-HSL is an immunomodulator of the Th1/Th2 response. 3-oxo-C12-HSL and two other QS molecules from *P. aeruginosa*, PQS (*Pseudomonas* quinolone signal), and HHQ (4-hydroxy-2-heptylquinoline), suppress both innate and adaptive immune responses acting on lymphoid cells, dendritic cells, and neutrophil monocytes/macrophages[22,97,98]. 3-oxo-C12-HSL and PQS decreased the production of the cytokines IL-12 and IFN-γ by activated dendritic cells, which in turn decreased T-cell proliferation and activity[98-100] while promoting the induction of regulatory T-cells[99] (Table 3). 3-oxo-C12-HSL provoked apoptosis of macrophages, neutrophils, and T lymphocytes through activation of caspases and the mitochondrial apoptosis pathway[101,102]. Several reports described inhibition of the nuclear factor-kappa B (NF-κB) pathway by QS molecules from *P. aeruginosa*[93,103-106] and/or the activation of signaling pathways such as p38 MAPK[105,107] (Table 3). It has been recently demonstrated that only long-chain AHLs such as 3-oxo-C12-HSL modulate the phenotype of dendritic cells and the type 2 immune response through mechanisms involving retinoic acid signaling and the protein kinase AKT [106].

The molecular mechanisms involved in the effects of QS molecules from *P. aeruginosa* on immune cells are independent of the Toll-like receptor pathway[105], which is a classical cell process involved in recognizing pathogen fragments. Some reports have indicated that the perception of AHL by mammalian cells involves the

Table 3 Effects of quorum sensing molecules on inflammation in different cell types

Cell type	QS molecule	Effects	Ref.
Effects on innate immune cells			
Macrophages	3-oxo-C12-HSL	Anti-inflammatory effects on IL-12 and TNF-α (0.1-100 μmol/L)	Telford *et al*[94]
		Increased TLR2 and TLR4 expression and decreased TNF-α production (1-100 μmol/L)	Bao *et al*[180]
		Pro-apoptotic effects (12-50 μmol/L)	Tateda *et al*[102]
		Increased phagocytosis (100 μmol/L)	Vikström *et al*[107]
		NF-κB inhibition (4.7 μmol/L)	Kravchenko *et al*[104]
		Dose-dependent anti-inflammatory effects (1-50 μmol/L)	Kravchenko *et al*[105]
		Involvement in p38/MAPK signaling (1-100 μmol/L)	Kravchenko *et al*[105], Vikström *et al*[107], Glucksam-Galnoy *et al*[181]
		Activation of the Unfolded Protein Response (6.25-100 μmol/L)	Zhang *et al*[182]
		Change in cell volume and shape (10-50 μmol/L)	Holm *et al*[183]
	Indole derivatives	Prevents the induction of pro-inflammatory cytokines	Krishnan *et al*[184]
	AI-2	Induction of the expression of cytokines, chemokines and TNFSF9	Li *et al*[41]
Monocytes	AI-3 and analogues	Increase in IL-8 secretion	Kim *et al*[22]
Dendritic cells	3-oxo-C12-HSL	Pro-apoptotic effects (100 μmol/L)	Boontham *et al*[185]
		No effect on IL-10 secretion (5-30 μmol/L)	Skindersoe *et al*[100]
		Increased IL-10 production (5-100 μmol/L)	Li *et al*[99]
		Decreased IL-12 secretion (5-100 μmol/L)	Li *et al*[99] and Skindersoe *et al*[100]
		Increased induction of Treg (5-100 μmol/L)	Li *et al*[99]
Neutrophils	3-oxo-C12-HSL	Chemoattraction (0.01-100 μmol/L)	Karlsson *et al*[186] and Zimmermann *et al*[187]
		Activation of MAPK signaling (12-50 μmol/L)	Tateda *et al*[102] and Singh *et al*[188]
		Increased phagocytosis (10 μmol/L)	Wagner *et al*[189]
		Pro-apoptotic effects (12-50 μmol/L)	Tateda *et al*[102]
Effects on adaptive immune cells			
T cells	3-oxo-C12-HSL	Inhibition of proliferation and activation (0.1-100 μmol/L)	Telford *et al*[94], Boontham *et al*[185], Gupta *et al*[190], and Hooi *et al*[191]
		Activation of naïve T cells towards Th1 phenotype (5 μmol/L)	Smith *et al*[95]
		Decreased secretion of IL-4 and IFN-γ (5 μmol/L)	Ritchie *et al*[96]
		Induction of apoptosis *via* the mitochondria pathway (100 μmol/L)	Jacobi *et al*[101]
		Induction of Treg (1-50 μmol/L)	Li *et al*[99]
	Indole derivatives	Re-programming into tolerogenic T cells	Cervantes-Barragan *et al*[192]
		Promotion of differentiation towards a regulatory type 1 phenotype	Aoki *et al*[193]
B cells	3-oxo-C12-HSL	Modulation of immunoglobulin production (0.1-100 μmol/L)	Telford *et al*[94] and Ritchie *et al*[194]
ILC	Indole derivatives	Promotion of IL-22 production	Zelante *et al*[83]
Effects on epithelial cells			
Pulmonary tract epithelial	3-oxo-C12-HSL	Induction of IL-8 production and NF-B activation	Smith *et al*[195]

cells		(100 μmol/L)	
		Increased expression levels of pro-inflammatory cytokines	Jahoor *et al*[115]
Intestinal epithelial cells	3-oxo-C12-HSL	Mitigation (1-10 μmol/L) or aggravation (> 50 μmol/L) of IL-8 expression induction	Peyrottes *et al*[92]
	3-oxo-C12:2-HSL	Attenuation of the induction of IL-8 expression (5-50 μmol/L)	Landman *et al*[39]

AI: Autoinducer; B cells: Lymphocytes B; HSL: Homoserine lactones; IFN-γ: Interferon-γ; IL: Interleukin; ILC: Innate lymphoid cells; MAPK: Mitogen-activated protein kinase; NF-κB: Nuclear factor-kappa B; QS: Quorum sensing; T cells: Lymphocytes T; Th: T helper; TLR: Toll like receptors; TNF-α: Tumor necrosis factor-α; Treg: Regulatory T cells.

bitter taste receptor T2R38[108-110], which is widely expressed in the human digestive tract from the tongue to the colon[111]. Polymorphisms in the *TAS2R38* gene may increase susceptibility to infections and colorectal cancer (CRC)[112]. It has been shown that these receptors use inflammatory pathways, which differ according to the cell type and their localization[113]. 3-oxo-C12-HSL binds to the transcription factor peroxisome proliferator-activated receptor γ[114], which has been proposed as a potential receptor for AHL and seems to be involved in AHL proinflammatory effects [115]. Recently, Moura-Alves *et al*[116] showed that QS molecules produced by *P. aeruginosa* modulated the activity of the transcription factor AhR, which plays an important role in regulating innate and adaptive immunity[117,118].

Overall, it has been demonstrated that QS molecules from *P. aeruginosa* have an immunosuppressive effect, allowing the pathogen to evade the immune system during infection. It remains to be determined whether endogenous intestinal 3-oxo-C12:2-HSL participates in controlling intestinal immunity in health and diseases and to decipher the underlying mechanisms.

AI-2 and AI-3: AI-2 is produced by both Gram-negative and Gram-positive bacteria and is mainly studied for its role in bacteria-bacteria communication and the virulence of pathogenic strains[8]. However, little is known about the effect of AI-2 on immune cells. In mice, AI-2 administration has no effect by itself on cytokine expression but aggravates lung inflammation during *P. aeruginosa* infection by interfering with QS molecules produced by this pathogen[119]. In cultured macrophages, AI-2 induces the expression of several cytokines and chemokines as well as the expression of TNF superfamily member 9 (TNFSF9), a protein involved in the immune response[41] (Figure 3).

The AI-3 system is mainly described in enterohemorrhagic *E. coli* and is therefore linked to the development of intestinal epithelial lesions, suggesting its proinflammatory activity[120] (Table 3 and Figure 3). Indeed, AI-3 and its analogs increase IL-8 secretion by THP-1 monocytes[22]. Given that the AI-3 structure has only been uncovered recently[22], the direct effect of this molecule on the host is poorly known so far. In addition, since the AI-3 bacterial receptor can recognize host-synthesized epinephrine/norepinephrine (Figure 3), one could suggest that adrenergic receptors could recognize AI-3[19]. However, it has been shown that AI-3 and its analogs do not activate or modulate adrenergic signaling[22].

Gram-positive AIP: The effects of Gram-positive AIP bacteria on inflammation are far less documented than those of Gram-negative QS. Moreover, most of the studies on the effects of AIP on host inflammation describe the indirect effects of AIs through the modulation of bacterial metabolism.

A study described that AIP could selectively cross intestinal epithelial cell Caco-2 cell monolayers[121]. Additionally, it has been reported that AIP can cross the highly selective blood-brain barrier *in vivo*[122]. These processes seem to be peptide-specific: *Clostridium acetobutylicum* AIP easily penetrates the blood-brain barrier, while *Streptococcus pneumonia*'s AIP crosses it poorly[122]. This shows that small molecules such as AIs affect the host's physiology beyond the gastrointestinal tract. For instance, it has been described that AIP has various effects on muscle inflammation as part of the gut-muscle axis. De Spiegeleer *et al*[121] performed an extensive screening of 75 QS molecules on muscle cells. They demonstrated both pro- and anti-inflammatory effects of four peptides from the genera *Staphylococcus*, *Streptococcus*, *Lactobacillus*, and *Bacillus*, and some of those peptides have been described in the gut.

Indole: Several studies have reported that indole exerts anti-inflammatory effects in the intestine and protects against pathogenic infection[123] (Table 3). AhR, an important contributor to the maintenance of innate and adaptive immunity, drives most of these effects, particularly in the intestinal mucosa[117,118,124].

Several reports have shown altered Trp metabolism in gut inflammation in humans and mice [81,125-127]. A decrease in endogenous indole was observed in human feces from subjects with celiac disease or IBD[127]. This was associated with a decrease in AhR activity in the intestinal mucosa. In parallel, an increase in Trp levels in the same samples suggested that the gut microbiota-dependent metabolism of Trp was altered [127]. In mouse models of celiac disease and IBD, the implantation of indole-producing bacteria increases AhR activity and protects them from gut inflammation[81]. Interestingly, Moura-Alves *et al*[116] showed that 3-oxo-C12-HSL and HHQ had an inhibitory effect on AhR activity and could compete with well-known activators of AhR. This observation raises the question of potential competition between several QS molecules for AhR-dependent modulation of innate and adaptive immunity.

Effects of QS molecules on carcinogenesis

There is growing evidence that gut microbiota dysbiosis plays a major role in CRC development[128]. Indeed, modifications of commensal gut microbiota in favor of opportunist bacteria promote intestinal inflammation, which is well known as a driver event in CRC onset[129,130]. Thus, the concept of the "bacterial driver-passenger model" highlights the crosstalk between host immunity and colonic microbiota[131]. For example, some driver pathogens, such as *Bacteroides fragilis,* have been proposed to promote a strong Th17 inflammatory response[132]. This proinflammatory microenvironment might favor colonization by opportunist pathogens such as *Fusobacterium spp.* Accordingly, Fusobacteria-dominant biofilms were associated with human CRC [133-135]. Altogether, these findings support that polymicrobial interactions and intercellular communications might play an important role in CRC development[136]. Nevertheless, how bacteria communicate with themselves and with the host during CRC remains poorly understood.

Recent findings provide new insights into the role of the QS molecule AI-2 in intercellular communication during CRC. First, the AI-2 concentration is increased in tumors compared to the surrounding normal tissue in human CRC[41]. These levels also correlate with the progression of the disease according to the CRC TNM (tumor node and metastasis) score[41]. Regarding the tumor immune microenvironment, the AI-2 concentration positively correlates with TNFSF9 expression, which is mainly expressed by tumor-associated macrophages, and negatively correlates with the CD4/CD8 ratio, suggesting that AI-2 associates with the antitumor response[41]. At the molecular level, it was demonstrated that AI-2 induces *in vitro* M1 polarization of U987-derived macrophages through the TNFSF9 signaling pathway[137]. These findings reveal that AI-2 could be an important factor linked to the immune tumor microenvironment and shed light on the role of the quorum-sensing system during CRC development and progression. Interestingly, mammalian epithelial cells are able to produce AI-2 analog molecules that mimic AI-2 effects (Figure 3), illustrating the complexity of bacteria-host crosstalk[45]. Thus, a better characterization of QS molecules involved in tumorigenesis might be an opportunity to improve our knowledge of the mechanisms underlying CRC development.

The host strikes back to QS

Interkingdom signaling works in two ways, as host cells are able to counterattack the QS system using several strategies.

As described above, as part of AI-3 signaling, host hormones such as epinephrine and norepinephrine can be recognized by EHEC and lead to the expression of virulence genes[19]. This AI-3/epinephrine/norepinephrine signaling is not restricted to EHEC, and the receptor QseC is also expressed, for example, by the intestinal pathogenic *Salmonella enterica* serovar Typhimurium[138] (Table 1). Recently, *in silico* analysis suggested that another catecholamine neurotransmitter, dopamine, can bind to QseC. However, no effect *in vitro* was measured[139]. This study of interkingdom signaling through hormones has been named "microbial endocrinology"[140].

Interestingly, there is evidence that human epithelial cells can produce AI-2 mimicking molecules. The study was conducted on Caco-2 intestinal epithelial cells, and the authors showed that an AI-2 mimic is produced not only when cells are in contact with bacteria but also after TJ disruption by calcium deprivation or DSS treatment[45]. This emphasizes how much AI-2 is a universal language between Gram-positive and Gram-negative bacteria and the host (Figure 3).

Hosts have also developed defense tools against QS, leading to a mechanism named quorum quenching. Mammals can synthesize enzymes named paraoxonases (PONs) that hydrolyze the lactone ring of long-chain AHLs[141] (Figure 3). There are three types of PONs (PON 1, PON2, PON3) that are highly conserved across species, and PON2 has greater activity on AHLs[142]. It has been demonstrated that PON2 is more highly expressed in the human jejunum than in other parts of the intestine[143]. Interestingly, PON1 and PON3 are expressed at lower levels in patients with Crohn's disease and ulcerative colitis than in healthy subjects[144]. A case-control study has also shown that carriage of the PON1 R192 allele in Ashkenazi Jewish may confer protection against the development of IBD. This allele was significantly less common among IBD Ashkenazi patients, with a significant odds ratio of 0.61[145].

QS IN THE GUT: FUTURE DIRECTIONS FOR THIS NEW PLAYER

Using QS to modulate gut microbiota: Application in gut ecosystem disorders

Gut dysbiosis is an imbalance in the composition of microorganisms inside the digestive tract, especially described with bacteria. This dysregulation has been shown to be a preponderant risk factor in several digestive and extra digestive diseases[146-149]. For example, in IBD and recurrent *C. difficile* infections, it is well known that the over- and underrepresentation of certain phyla can lead to a pathologic state[150,151]. Modulating the gut microbiota may be the key to treating or even preventing such diseases by restoring normobiosis. Fecal microbiota transplantation is now commonly used in the setting of *C. difficile* infections[152,153]. However, the lack of standardization and the safety and quality issues of this procedure call for the development of new strategies.

Theoretically, AHLs remain good candidates in this approach using natural molecules from QS to modulate microbiota composition and gut inflammation. As seen above, AHL signaling may involve different pathways that contribute to controlling intestinal inflammation, such as inhibition of NF-κB, modulation, inhibition of MAPK activation, increase in regulatory T cell induction, decrease in proinflammatory cytokines, and modulation of junctional complexes in the epithelial barrier. Indeed, using QS molecules could play a role in both components (gut microbiota and host responses) of gut ecosystem disorders observed in metabolic and inflammatory diseases. AHL-based QS devices already exist as therapeutic applications for the dynamic control of Gram-negative bacterial populations, especially in infectious diseases. Other QS molecules could be extended as potential clinical therapies for diseases related to the gut microbiota that involve biofilm formation and antibiotic resistance[154]. Research efforts must investigate the potential of this new trial.

In addition to therapeutic applications, one could consider QS molecules as reliable biomarkers for dysbiosis-related chronic diseases such as IBD or CRC. Indeed, it has been shown that the presence of some AI-1 QS molecules in the gut ecosystem directly correlates with bacterial group size[39]. AHLs could represent a biomarker of the bacterial level population acting as a magnifying glass for dysbiosis. In addition, AI-2 concentration increased during adenomas to colorectal transition and CRC progression [41]. This opens the perspective for using the QS system as a biomarker for the prevention and follow-up of chronic diseases.

For the future

Knowing which commensal bacteria carry QS systems, their site of production, their ability to be mobilized during dysbiosis, and their effect on the luminal or mucosal microenvironment are as many unresolved questions. The scientific community, together with gastroenterologists, needs to tackle these issues to pave the way for translation into clinical use. Future directions also involve designing dedicated QS derivatives targeting either the host cells or the bacterial compartment. Such QS derivatives have already been reported to control the epithelial cell inflammation pathway with a wider effect than natural 3-oxoC12:2 without bacterial-activating properties[92]. Using QS molecules as an approach to tackle the gut microbiota compartment has already been proven to be a successful strategy[42], thus leading to interesting perspectives. Considering the QS system as a new player in the gut ecosystem, it represents a control platform to shape the host's gut microbiota population and/or major physiological pathways.

CONCLUSION

In conclusion, the intestinal microbiota interacts mutually with epithelial and immune cells of the coevolved host in a beneficial, reciprocal relationship. The QS signaling of bacteria probably contributes substantially to establishing symbiotic interactions in certain dynamics of interaction between the different kingdoms. A better understanding of QS systems by researchers and gastroenterologists involved in describing and managing ecological disorders of the intestinal ecosystem is a new approach that opens up fascinating therapeutic opportunities.

ACKNOWLEDGEMENTS

We thank Rainteau D for his support and knowledge on quorum sensing. We acknowledge the Association François Aupetit for its unwavering support.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 14; 27(42): 7271-7284

DOI: 10.3748/wjg.v27.i42.7271

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

REVIEW

Hepatitis B virus/hepatitis D virus epidemiology: Changes over time and possible future influence of the SARS-CoV-2 pandemic

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Conflict-of-interest statement: All the authors of the manuscript declare they have no conflict of interest in connection with this paper.

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Abstract

Hepatitis D virus (HDV) is a defective liver-tropic virus that needs the helper function of hepatitis B virus (HBV) to infect humans and replicate. HDV is transmitted sexually or by a parenteral route, in co-infection with HBV or by super-infection in HBV chronic carriers. HDV infection causes acute hepatitis that may progress to a fulminant form (7%-14% by super-infection and 2%-3% by HBV/HDV co-infection) or to chronic hepatitis (90% by HDV super-infection and 2%-5% by HBV/HDV co-infection), frequently and rapidly progressing to cirrhosis or hepatocellular carcinoma (HCC). Peg-interferon alfa the only recommended therapy, clears HDV in only 10%-20% of cases and, consequently, new treatment strategies are being explored. HDV endemicity progressively decreased over the 50 years from the identification of the virus, due to improved population lifestyles and economic levels, to the use of HBV nuclei(t)side analogues to suppress HBV replication and to the application of universal HBV vaccination programs. Further changes are expected during the severe acute respiratory syndrome coronavirus-2 pandemic, unfortunately towards increased endemicity due to the focus of healthcare towards coronavirus disease 2019 and the consequently lower possibility of screening and access to treatments, lower care for patients with severe liver diseases and a reduced impulse to the HBV vaccination policy.

Key Words: Hepatitis B virus/hepatitis delta virus; SARS-CoV-2; COVID-19; Hepatitis delta virus infection; Hepatitis D; Hepatitis delta virus epidemiology

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Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Italy

Peer-review report's scientific quality classification

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

Received: May 2, 2021
Peer-review started: May 2, 2021
First decision: June 12, 2021
Revised: June 20, 2021
Accepted: October 25, 2021
Article in press: October 25, 2021
Published online: November 14, 2021

P-Reviewer: Azarbakhsh H, Cai J, Papazafiropoulou A, Samadder S, Sun C
S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Fan JR

Core Tip: There has been a tendency to a reduction in hepatitis D virus (HDV) endemicity in most countries in recent decades, mostly due to an improvement in population lifestyles and economic levels, to an extensive use of hepatitis B virus (HBV) nuclei(t)side analogues to suppress HBV replication and to the application of universal HBV vaccination programs. However, an increase in HDV endemicity is expected during the severe acute respiratory syndrome coronavirus-2 pandemic since healthcare is mostly diverted towards coronavirus disease 2019, with a reduced attention to liver disease, screening, access to treatment, care for patients with severe liver disease and the HBV vaccination policy.

Citation: Sagnelli C, Pisaturo M, Curatolo C, Codella AV, Coppola N, Sagnelli E. Hepatitis B virus/hepatitis D virus epidemiology: Changes over time and possible future influence of the SARS-CoV-2 pandemic. *World J Gastroenterol* 2021; 27(42): 7271-7284
URL: https://www.wjgnet.com/1007-9327/full/v27/i42/7271.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i42.7271

INTRODUCTION

A new antigenic reactivity was identified in hepatocytic nuclei by immunofluorescence technique on paraffinized liver sections of hepatitis B surface antigen (HBsAg) positive patients by Rizzetto *et al*[1] in the early 1970s in Turin, Italy, and called Delta. The Authors understood that this new reactivity was different from that expressed by the hepatitis B virus (HBV) core antigen (HBcAg) and hypothesized that it could be part of a new virus; with a careful interlacing of antisera on cryostat-cut biopsy sections, Delta reactivity was identified as a new antigen localized in the nucleus of the hepatocytes and yielding a fluorescent staining similar but not equal to HBcAg[1]. In Bethesda, United States, Rizzetto *et al*[2] developed a radioimmunoassay for the delta antigen-antibody system in serum and performed transmission studies in chimpanzees demonstrating the existence of a new a liver tropic virus [hepatitis D virus (HDV)][2]. Further studies mentioned below defined HDV as a defective virus that needs a helper function of HBV to infect humans and replicate.

This virus consists of the HDV antigen (HDAg, small and large antigen) and a circular single-strand RNA (HDV-RNA). It uses a human RNA polymerase II and a rolling-cycle mechanism like that of plant viroid to replicate. Being a defective virus, HDV needs the S-HBsAg, M-HBsAg and L-HBsAg proteins of HBV to produce its envelope and propagate. Coated with the HBV envelope, HDV uses the pre-S1 domain of L-HBsAg to bind NCTP (sodium taurocholate co-transporting polypeptide) and enter differentiated hepatocytes[3].

Eight genotypes of HDV have been identified at present, distributed differently in the world: Type 1 is present worldwide and prevalent in western Europe and North America, type 2 mainly in the Far East, Egypt and Iran, type 3 in the Amazon Basin, type 4 in Japan and Taiwan, types 5 to 8 in Africa but now spreading also in Western regions due to immigration flows[4]. In Western countries, HDV infection at present occurs mostly in elderly adults with chronic HBV infection or in young immigrants from endemic areas[5].

HDV is parenterally and sexually transmitted in co-infection with HBV or by super-infection in HBV chronic carriers. Both types of transmission cause acute hepatitis, self-limited with a clearance of both viruses in 95% of subjects simultaneously infected, while in the case of HDV super-infection in HBV chronic carriers the disease progresses to chronicity in nearly 90% of cases[6].

Acute hepatitis delta is clinically indistinguishable from other types of acute viral hepatitis and is usually mistaken for HBV acute hepatitis if the specific diagnostic tests [search for HDV RNA and immunoglobulin M (IgM) to HDV-Ag] are not performed. HDV super-infection in HBsAg chronic carriers causes massive acute exacerbation with a fulminant clinical course in 7%-14% of patients, two thirds of whom die of liver failure. The outcome is less severe in subjects acquiring HBV and HDV in co-infection. They may develop fulminant hepatitis in 2%-3% of cases and progression to chronicity in 1%-5%.

Chronic HDV infection is the most severe and rapidly progressive form of chronic hepatitis, leading to cirrhosis in 70% of patients within 5-10 years[7]. Furthermore, as compared with HBV mono-infection, HBV/HDV co-infection increases the risk up to three times of cirrhotic patients developing hepatocellular carcinoma[4,8,9].

Despite the importance of HDV infection, no treatment has been so far approved by the Food and Drug Administration. However, treatment with pegylated interferon alfa (Peg-IFN) is recommended by the major scientific societies for the study of the liver because it may clear HDV infection in 10%-20% of patients and may reduce liver fibrosis and the risk of hepatic decompensation and mortality. Because of its limited efficacy, its significant side effects, and the prolonged time of administration (1-2 years) Peg-IFN was used also in combination with ribavirin and/or HBV nucleoside analogues, but no improvement was obtained. Therefore, new treatment strategies are now under study with the use of peginterferon lambda, bulevirtide (entry inhibitor that targets NCTP), nucleic acid polymers (HBsAg secretion inhibitors), and lonafarnib (virus assembly inhibitor)[10].

In end-stage liver disease, HCC and fulminant hepatitis, liver transplant is the only therapeutic option. Comparing HBV mono-infected patients with the HBV/HDV co-infected, the latter show a better outcome if reinfection is prevented by the continuous administration nucleos(t)ide analogues and of hepatitis B immune globulins[11,12].

The epidemiology of HDV infection has changed a lot in nearly every country since its first identification 55 years ago[13-20]. This is related to changes in population lifestyle habits, variations in economic levels, extension of the use of HBV nucleos(t)ide analogues to treat HBV chronic carriers and, most importantly, to the effectiveness of universal vaccination campaigns against HBV infection in most countries. Further changes are expected in this era of the coronavirus disease 2019 (COVID-19) pandemic, which might involve, among others, a lower possibility of screening and access to treatment and a reduced effectiveness of the vaccination policy against HBV infection and of the management of patients with severe chronic hepatitis.

These epidemiological changes have great impact on the health quality of life of human beings and are the subject of this review.

HBV/HDV EPIDEMIOLOGY BEFORE SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS-2 PANDEMIC

The epidemiology of HDV infection has been mainly studied by assessing the prevalence of subjects with HDV infection among HBV chronic carriers because acute HDV hepatitis, either acquired in co-infection with HBV or by HDV super-infection in HBV chronic carriers, easily identifiable during outbreaks, often remains undiagnosed when it occurs in single subjects. In fact, the clinical presentation of HBV and HDV acute hepatitis is similar, and only specific tests (anti-HDV IgM and quantitative HDV RNA), infrequently performed in general practice, allow the differentiation between these etiologies. These tests, however, do not provide accurate results in epidemiological studies on chronic HDV infection, because their titers, elevated in acute phase of the infection, decrease markedly in the chronic phase. In addition, HDV RNA is typically determined home made only in some specialized laboratories and its quantification is not standardized. For these reasons, commercial standardized kits to detect serum anti-HD IgG have been used in almost all epidemiological studies on HDV chronic hepatitis.

HDV has spread all over the world, with a global disease burden around 62-72 million HBV/HDV co-infected subjects[13] and with an HDV seroprevalence among HBV carriers widely varying in different geographic areas[14,15].

EPIDEMIOLOGICAL INFORMATION OF HDV/HBV ACUTE HEPATITIS

Outbreaks of fulminant hepatitis have occurred in the Amazon Basin. in French Guyana, in Yucpa Indians of Venezuela from 1934 to 1955, and in the Central African Republic in 1997 as documented by the presence of Delta antigen in hepatocytic nuclei of autoptic liver specimens; small but similar outbreaks have occurred from 1955 to 1985 in northern Colombia and designated as hepatitis of Santa Marta (de Sierra Nevada)[16-24].

In patients with fulminant hepatitis, morphologic studies showed marked liver steatosis with characteristic "morular hepatocytes", cells with small droplets of fat and

central pycnotic nuclei. Worthy of note, some similarities in the clinical presentation of fulminant hepatitis Delta with that of yellow fever has led to the hypothesis that some of the outbreaks occurring in the past in the Amazon basin and attributed to yellow fever, may have been due to HBV/HDV co-infection[25].

In the mid-1980s, several cases of severe acute liver failure were described in Bangui, in the Central African Republic, associated with an HDV outbreak. At least 124 cases of fulminant hepatitis were observed among 154 jaundiced patients, 88% of whom died[23]. The disease was associated with specific pathological lesions (spongiocyte-associated hepatitis) involving lympho-plasmocytoid infiltrates, eosinophilic necrosis and massive macro- or micro-vacuolar steatosis[23] resembling the "morula-like" cells previously described in South American HDV outbreaks[24-26]. In Italy, two collections of sera from patients with acute hepatitis B collected in Naples in 1972 and 1974 were retrospectively evaluated and for the first time the IgM antibody to HDV (anti-HDV IgM) was detected in over 90% of cases. Most anti-HDV IgM-positive patients were also anti-HBc IgM-positive, suggesting an outbreak of HBV/HDV coinfection in Naples in those years[27].

THE INCIDENCE OF ACUTE HDV HEPATITIS IN HOSPITALIZED PATIENTS

Italy was estimated by a system of hospital surveillance in the period 1987-2010[28]. The incidence of primary HDV infection in the general population progressively decreased from 3.2/100000 inhabitants in 1987 to 0.2/100000 in 2010. The decrease in the incidence of HDV acute hepatitis and the parallel similar trend observed for HBV acute hepatitis in the same study were most likely an effect of the mass vaccination, which in 2010 had covered all Italian citizens aged from 0 to 31 years. In this period, however, three peaks of HDV epidemic were registered, in 1990 and 1993 in the age groups 14-25 and 25 or more and in 1997 only in the 15-24 age group, all due to the spread of HDV infection among intravenous drug users[29]. Apart from intravenous drug use (IVDU), other putative risk factors associated with the development of HDV acute hepatitis were family contacts with a chronic HBsAg carrier, promiscuous sexual activity, and cosmetic treatments with percutaneous exposure in 1992-1994, whereas those identified from 2008 to 2010 were dental therapy, cosmetic treatment with percutaneous exposure and promiscuous sexual activity.

Between December 2004 and January 2005, out of 110 patients with acute hepatitis in 8 city hospitals of Mongolia, 1.8% had HDV etiology by co-infection with HBV and 27.3% by HDV super-infection[30]. Again, in Mongolia from January 2012 to December 2014, 546 consecutive patients with acute hepatitis were observed, of whom 6% had HDV etiology by co-infection with HBV and 10.8% by HDV super-infection [31].

In Spain, 398 patients with HDV infection were observed from 1983 to 2008, 182 with acute hepatitis, of whom 84% by co-infection and 16% by super-infection, and 216 had chronic HDV infection[28]. The number of patients with acute hepatitis Delta strongly decreased across this study, while the number of those with chronic Delta infection showed a small decline. In addition, patients observed in the 1983–1995 period compared to those examined from 1996-2008 were significantly younger, born in Spain, mainly intravenous drug users, and more frequently had acute hepatitis. In this study, the percentage of immigrants (prevalently from Africa and Eastern Europe) increased from 1% of the first period to 28% in the second.

EPIDEMIOLOGICAL INFORMATION OF HDV/HBV CHRONIC INFECTION

In 2018, the World Health Organization considered these geographic areas HBV/HDV endemic regions: Central and West Africa, Central and Northern Asia, Vietnam, Mongolia, Pakistan, Japan, Chinese Taipei, the Pacific Islands (Kiribati and Nauru), the Middle East and Eastern Europe, Eastern Mediterranean regions, Turkey, the Amazonian Basin, and Greenland[32].

To offer the readers an overview of the worldwide variability of the prevalence of anti-HDV among HBV chronic carriers, we report data recorded at different times in some countries: 10.6%–37.5% in India (1985-2005)[33,34], 3.5%–13.1% in China (1987-1993)[35,36], 17.7% in Tunisia (1987-1994)[37], 8.3%–23.0% in Italy (1987 and 1997)[13,38], 21.8%–65.5% in Thailand (1988)[39], 48%-5.7% in Iran (1991-2005)[40], 8%–10.9% in Germany (1992-2006)[41], 16.6%–58.6% in Pakistan (1994 to 2001)[42], 8.6% in Saudi Arabia (1996-1997)[43], 4.4%–15.3% in Taiwan (1997-2012)[13,44], 56.5% in Mongolia

(2004)[13,44], 41.9% in Brazil (2005-2006)[45], 7%–45.5% in Turkey (2006-2009)[46], and 9.9% in Egypt (2013)[13,44] (Table 1).

A lower prevalence was reported for non-endemic areas, ranging from 6% in Japan (1991)[47], 2% in Jordan (1978-1985)[48], 4.1%-4.8% in Australia (1997-2016)[49,50], 4.2% in Greece (1997)[51], and 2.6%–8.5% in England (2000-2006)[13,44]. Notably, in the United States, the prevalence of HDV among HBV chronic carriers has been reported to range from 2% in native citizens to 50% in some foreign populations[52-54] (Table 1).

There was a gradual reduction in the percentage of HBsAg-positive subjects and a parallel decrease in the prevalence of cases with HBV/HDV chronic infection in several countries in the last 3 decades, primarily due to the application of universal vaccination programs. As an example, the prevalence of HDV infection in HBsAg-positive chronic carriers was very high in some Mediterranean countries (Greece, southern Spain, southern Italy) 5 decades ago[55], a prevalence that gradually declined, like in Italy, where the prevalence of HDV infection in HBV chronic carriers decreased from 24% in 1990 to 8.5% in 2006[38].

About 35 million people are infected with human immunodeficiency virus (HIV) in the world and among these the percentage of those with HBV/HDV co-infection varies from 1.4% to 19.7% in relation to the epidemiological diversity of these infections in individual nations[56-60]. Considering the high number of subjects with triple HBV, HDV and HIV infections, it is highly likely that some of them have contracted severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection, asymptomatic or symptomatic, although there are no data in the literature to the best of our knowledge.

REASONS FOR REDUCED PREVALENCE RATE OF HDV/HBV CHRONIC INFECTION

The impact of risk factors for acquiring HBV infection has changed over the past five decades around the world. Vertical transmission is no longer a risk factor for the transmission of HBV infection, due to the introduction of mass passive/active immunization of newborn babies. Furthermore, the impact of risk factors for HBV transmission through household contacts with HBsAg infected subjects has dropped in Western countries with the application of universal HBV vaccination and the reduction in family size. The epidemiological impact of other risk factors, such as the use of improperly sterilized medical and surgical instruments, transfusion of contaminated blood and blood products, the use of glass syringes and for men shaving at a barber's shop have dramatically decreased worldwide. IVDU and sexual transmission of HBV infection plays a major role in HBV transmission nowadays due to the exchange between drug addicts of syringes or other objects used for the preparation of drugs and to the infrequent or incorrect use of condoms in unsafe sexual activity. A marginal role is still played by cosmetic treatment with percutaneous exposure (piercing, tattooing, manicure, pedicure, and acupuncture) and dental treatment[61].

The current residual reservoir of HDV in Western countries comprises aging patients with advanced liver disease, young immigrants with chronic hepatitis Delta from areas where HDV infection remains endemic and drug users. The increase in the spread of HDV has recently been observed in some countries of Western Europe mostly regarding IV drug users and immigrants from Eastern Europe and Sub-Saharan Africa[44,62]. The recently increased HDV prevalence observed in the United Kingdom, France, and Germany reflects the increased prevalence in young immigrants from regions at high HBV/HDV prevalence. A high prevalence has been reported for immigrants from Equatorial Guinea living in Spain (20.9% among 1220 HBV chronic carriers).

Drug use remains a major risk factor, as seen by a study performed in Baltimore (United States) showing 50% HBV/HDV co-infection in IV drug users[54].

In some other geographic areas, however, the prevalence of HDV infection in HBV chronic carriers has remained high in the last two decades, such as in some eastern countries of the Mediterranean basin, the Middle East, central and northern Asia, western and central Africa, the Amazonian basin (Brazil, Peru, Venezuela, and Colombia), some Pacific islands[63] and Vietnam[64]. In a recent study in the western Brazilian Amazon region, 41.9% of HBV chronic carriers were found to be coinfected with HDV, with a prevalence of over 60% among individuals aged 20–39 years[45], suggesting the likelihood of sexual transmission. In a 2013 study from West Africa (Mauritania), 30% of HBV chronic carriers had the anti-HDV antibody[65] and among

Table 1 Worldwide variability of anti-hepatitis D virus in hepatitis B surface antigen chronic carriers

Country	Anti-HDV in HBsAg chronic carriers, % (years of study)	Ref.
India	10.6 (1985); 37.5 (2005)	[33,34]
China	3.5 (1987); 13.1 (1993)	[35,36]
Tunisia	17.7 (1987-1994)	[37]
Italy	23.0 (1987); 8.3 (2006)	[13,38]
Thailand[1]	8.3; 11.1; 65.5 (1988)	[39]
Iran	48.0 (1991); 5.7 (2005)	[40]
Germany	8.0 (1992); 10.9 (2006)	[41]
Pakistan	16.6 (1994); 58.6(2001)	[42]
Saudi Arabia	8.6 (1996-1997)	[43]
Taiwan	4.4 (1997); 15.3 (2012)	[13,44]
Mongolia	56.5 (2004)	[13,44]
Brazil	41.9 (2005-2006)	[45]
Turkey	7 (2006); 45.5 (2009)	[46]
Egypt	9.9 (2013)	[13,44]
Japan	6.0 (1991)	[47]
Jordan	2.0 (1978-1985)	[48]
Australia	4.1 (1997); 4.8 (2016)	[49,50]
Greece	4.2 (1997)	[51]
England	2.6 (2000); 8.5(2006)	[13,44]
United States	2 .0 (1950); 50.0 (2016)	[52-54]

[1]65.5% in intravenous drug abusers, 11.1% in chronic active hepatitis, and 8.3% in cirrhosis patients.
HDV: Hepatitis D virus; HBsAg: Hepatitis B surface antigen.

these, 62.2% were HDV-RNA-positive[66-68]. A recent study from Iran showed that in Zahedan, 17% of HBV chronic carriers were anti-HDV-positive[69]. A meta-analysis showed that 14.8% of asymptomatic HBsAg-positive patients in the eastern Mediterranean region were coinfected with HDV and that cirrhosis was common in these patients[70]. A study from Vietnam showed an anti-HDV prevalence of 10.7% in HBV chronic carriers[64], a prevalence much higher than expected based on previous reports[71]. These studies confirm the continuing high prevalence of HDV infection in the Amazonian basin, the Middle East, the eastern Mediterranean region, and western Africa, and show an unexpectedly increased prevalence in Vietnam. Instead, a low anti-HDV prevalence in HBV chronic carriers was reported from Egypt (4.7%)[72] and India (4.8%)[73,74].

The reports on the epidemiology of HDV infection are numerous, but at the same time are inadequate to have a complete overview of the spread of the Delta infection around the world[75,76].

First, HDV acute infection is infrequently recognized outside the outbreaks and the rate of chronic forms are likely to be underestimated for the unsatisfactory use of laboratory tests to identify HDV infection in HBsAg chronic carriers. In a nationwide study in the United States only 8.5% of the 25603 HBV patients investigated were ever tested for HDV. Manesis *et al*[51] reported that only one third of Greek patients with HBV chronic hepatitis have been tested for HDV infection[51]. Similarly, El Bouzidi *et al*[77] reported that only 40% of British patients with HBV chronic infection have been tested for anti-HDV[77]. In addition, most epidemiologic studies used anti-HDV to identify the seroprevalence of HDV infection, whereas HDV RNA was performed in some other studies to confirm the etiology and was used for epidemiological investigations.

Second, studies from the same country have reported a discrepant prevalence, probably due to significant geographic variations even within the same country. For

example, a meta-analysis from Turkey reported an anti-HDV seroprevalence of 4.8% in west Turkey, and 46.3% in south-east Turkey[78].

Finally, the time when the epidemiological survey was carried out is of considerable importance, due to the frequent variations over time in the lifestyle habits of the population of a geographic area and, even more, in the progress of anti-hepatitis B vaccination in the same area. As an example, HDV seroprevalence in chronic HBV carriers has largely declined in Italy over the last 50 years (from 23% in 1987, to 14% in 1992 and to 8.3% in 1997), indicating a substantial, progressive reduction in the endemicity level, due to improved socioeconomic conditions and the associated increase in the standards of hygiene, to a substantial reduction in family size, and, most importantly, to the mass vaccination campaign against HBV started in 1991[79-82]. More recent data, however, suggest that the prevalence of HDV in Italy has remained stable at nearly 8%[83].

Overall, HDV infection is still a major burden for public healthcare management in many countries. In recent times we have accumulated evidence that the prevalence of HDV has remained stable or has increased in many endemic and non-endemic geographic areas, due to an increase in migratory flows and to the persistent impact of other relevant risk factors like IVDU, intra-family spread and unsafe sexual activity, homosexual, and heterosexual[13,38,44,84,85]. Furthermore, outbreaks of HDV acute hepatitis are still occurring[80]. In this regard, it is useful to recall the important role of sex workers in the spread of HBV and HDV infections, as well as HIV and other sexually transmitted diseases. The prevalence of HBsAg among migrant sex workers in Chiangmai, Thailand, was 11.4%, but none of them showed anti-HDV antibodies [86].

Of note, most of these sex workers were migrants from Myanmar, born before the introduction of the universal HBV vaccination program in their native country. A meta-analysis on five studies, 4 from China and one from Vietnam, showed that Commercial sex workers have a greater risk to be anti-HDV positive than the external comparator population from the same geographic areas, with a pooled OR of 18.7 (95%CI: 6.70-51.17). In the same meta-analysis, a pooled OD ratio of 16.00 (95%CI: 3.94-64.92) indicates that MSM from Italy and Indonesia are at a greater risk to be anti-HDV positive than the comparator population from the same geographic areas, asymptomatic HBsAg carriers for Italy and normal subjects for Indonesia. No positive case was observed in MSM from a Burkina Faso study analyzed in the same contest [44].

SARS-COV-2 PANDEMICS: REDUCED OPPORTUNITY FOR SCREENING, MANAGEMENT, AND TREATMENT

Much of the assistance not linked to the SARS-CoV-2 pandemic has been severely compromised and/or downsized during this pandemic, a drastic reduction also affecting liver diseases. In Italy, 26% of hepatology wards have been converted to SARS-CoV-2 wards and another 33% have suffered a substantial reduction in the availability of beds. Screening and day hospital activities for liver diseases have been significantly reduced and the start of treatments for "new" HBV chronic hepatitis have been postponed in 23% of the open liver units[87]. Overall, hepatology activities, including the management of patients with compensated or decompensated liver disease, liver transplantation or HCC have been significantly reduced and/or suspended[34].

In April 2020, health centers in Burkina Faso, Gambia, and Tanzania, respectively, recorded a 71%, 83% and 95% decline in the numbers of "new" patients attending liver clinics, compared to the numbers recorded at the beginning of the same year, mainly due to the patient's fear of accessing health services[88].

A limitation or closure of health centers offering harm reduction services (such as needle exchange and opioid replacement therapy) due to SARS-CoV-2 pandemic have been reported from South Africa, with an increased risk in overdose and transmission of blood-borne infections[89].

In other countries in Sub-Saharan Africa, essential diagnostic tests to manage chronic hepatitis, rapid tests for HBsAg, alanine aminotransferase and hematological tests, have always been available but there has been a serious shortage of nucleic acid tests to detect HBV and HDV replications; therefore, people newly identified as HBsAg carriers may have missed the opportunity to be evaluated for viral load, connected to treatments, and finally treated[89]. For Sub-Saharan African countries, there has also been a strong reduction in tenofovir supply[89], with an increased risk

of an exacerbation of liver cell necrosis, the onset of drug resistance and transmission of HBV infection.

In Japan, Singapore and the United States, the number of physical examinations for HBV chronic hepatitis decreased significantly in 2020, compared to 2019, especially for more elderly subjects[90].

Numbers from England give clear information on the influence of the SARS-CoV-2 pandemic on screening for liver diseases, 2478 tests in 2019 and 1224 in 2020[91].

COVID-19 has also led to an increase in economic and public health disparities. The pandemic has caused a global recession, driving millions of people below the poverty line, not only in countries with low economic levels[92]. In the United States, 6.2 million people have lost their health insurance since the start of the pandemic, due to job losses, leading to a greater risk of acquiring viral hepatitis as well as other infectious diseases[93].

The World Hepatitis Alliance conducted a worldwide survey to assess the impact of the SARS-CoV-2 pandemic on patients with viral hepatitis: Job loss during this pandemic resulted in a strong impact on household monetary income in low- and middle-income countries, with a frequent lack of access to drugs. Indigenous communities in some developing countries were the most affected both by traffic restrictions with the inability to reach healthcare centers and to the difficulties in earning a living and paying for a physical examination[93,94].

In high-income countries, remote assistance through telemedicine and other virtual platforms has been strongly encouraged to maintain continuity of care[95]. However, in sub-Saharan Africa, many patients and healthcare providers do not have access to telemedicine solutions[88].

There is also an increased risk of HBV vertical transmission during the SARS-CoV-2 pandemic, due to a higher frequency of home births without prophylactic measures [92]. Childhood prophylaxis against HBV infection performed at birth is estimated to have prevented 310 million new HBV infections between 1990 and 2020[96], including 14 million HBV/HDV infections[44]. Preliminary data from the Institute for Health Metrics and Evaluation indicate that global levels of vaccination coverage progressively increased from 1990 to the beginning of 2020, but during 2020 it fell to the levels of the 1990s[97]. The reduction in vaccination coverage may lead to a high increase in the incidence of HBV infection in childhood, and to an increased risk of acquiring HDV infection in subsequent years.

CONCEIVABLE DEVELOPMENTS IN HBV/HDV EPIDEMIOLOGY DUE TO THE SARS COV-2 PANDEMIC

The SARS-CoV-2 pandemic has had a negative impact on the global health system and on the development of assistance programs for other diseases. Considering that 290 million people live with chronic HBV, it is easy to calculate that the unidirectionality of healthcare towards SARS-CoV-2 has strongly damaged the programs for screening, management, and treatment of HBV and HDV associated diseases worldwide.

No epidemiological data on HBV/HDV coinfection during the SARS-CoV-2 pandemic is available at present and few data on HBV infection are reported in the literature, although HBsAg positive subjects are at high risk of acquiring SARS-CoV-2 infection. Guan *et al*[98] reported a 2.1% HBsAg positivity in 1099 SARS-CoV-2 Chinese patients[98] while Chen *et al*[99] found a 4% HBsAg prevalence in 274 SARS-CoV-2 Chinese patients[99]. Instead, the HBV prevalence was 0.1% in 5700 (8/5700) SARS-CoV-2 patients in the United States[100].

Even in the absence of data on the epidemiology of HBV/HDV co-infection during the SARS-CoV-2 pandemic, mostly due to the short period elapsed, it seems useful to make some predictions based on the sudden great variation in lifestyle worldwide, imposed by the spread of this life-threatening pandemic.

The multiplicity of factors, favoring or hindering the spread of HBV and HDV infections, mostly created by the measures put in place by the Political or Healthcare Authorities of different countries to hinder the spread of SARS-CoV-2, makes it difficult to predict the epidemiological variations of these two infections in the coming years. In fact, the restrictions in movement imposed in many countries may have decreased HBV transmission due to the reduction, outside the family, of physical contact between infected subjects and those exposed. These restrictions, however, have induced a depression in large segments of the population, which has led to an increase in alcohol consumption, drug use and unprotected sex. Another adverse effect of the restrictions is the increased risk of child deliveries at home, which may involve the

lack of screening for HBV infection for mothers and an inadequacy in the prophylaxis of the newborn.

In addition, a significant part of the resources usually reserved for the different fields of medicine have been devolved to the screening of SARS-CoV-2 infection and to the assistance of COVID-19 patients. As a result, there has been a downsizing of care for liver diseases: A lower number of hepatic wards and day hospitals, a lower number of beds in hepatic wards still open, difficulty in managing patients with serious liver diseases and the recipients of liver graft. In addition, screening for HBV and HDV infections have been significantly reduced, and the start of treatment for "new" patients frequently postponed. Another important role in the reduction of healthcare facilities for liver patients was played by their fear of contracting SARS-CoV-2 infection in healthcare structures and in centers giving assistance to drug addicts. Moreover, many "new" cases of HDV infection may have gone undiagnosed, since the determination of HBV DNA, HDV DNA and IgM to HD-Ag has undergone a significant reduction in several countries for the considerable commitment of many laboratories in the diagnosis of SARS-CoV-2 infection and for the fear of patients attending them. Therefore, several HBsAg carriers and HBV/HDV patients may not have been identified, and if identified may not have received appropriate treatment.

The sum of these contrasting effects exerted by the measures restricting mobility on the spread of HBV and HDV infections makes it difficult to predict the extent and modalities of their spread in coming years. However, we have no doubt in advancing pessimistic forecasts, because in the continuing course of the SARS-CoV-2 pandemic, foreseeable for about another 2 years, all the negative elements reported above will remain, while the positive effects of movement restriction will gradually decrease, because such restrictions, already not respected in many countries, will be even less so in the future. We only trust in a more rapid and intense production of vaccines against SARS-CoV-2 infection and in universal vaccination campaigns for a future return to normality[101].

CONCLUSION

The impact of the SARS-CoV-2 pandemic on essential health services is of great concern. In fact, it is feared that most improvements in public health achieved in recent years could be wiped out, with negative repercussions on the provision of health services to the public, with a consequent reduction in the prevention of infectious diseases, diagnostic efficacy, and the application of therapeutic and rehabilitative treatments, and overall, of healthcare promotion. The reduction of essential healthcare services, due to the focus towards COVID-19, risks having serious negative effects on the health of the most vulnerable populations, such as children, the elderly, patients with chronic diseases, viral (HBV, HDV and hepatitis C virus hepatitis, HIV infection), inflammatory, degenerative, oncological, cardiac, autoimmune, *etc.*

The COVID-19 pandemic has required the use of enormous healthcare resources and, consequently, reduces the care of patients with other diseases, particularly those who need continuous clinical monitoring to avoid clinical deterioration such as patients with chronic HBV/HDV infection. For these patients, there has already been, and will occur even more in the next few years, a lack of diagnosis for some, delayed access to treatment or lack of continuity of antiviral therapy for others, and finally a lack in the surveillance for the possible emergence of liver cirrhosis and HCC and the need for liver transplantation. When the COVID-19 emergency draws to a close, there will be a resumption of health activities for all diseases neglected during the pandemic, including screening for HBV and HDV infection, and a false increase in the prevalence of these infections will occur because of the effect of the recovery of cases undiagnosed in 2020, 2021 and, for some countries with reduced vaccination activity against COVID-19 even in some subsequent years.

We remind those in charge of treating patients with chronic hepatitis B and those who have the related administrative responsibility that the interruption of drugs suppressing HBV replication in patients with HBV chronic hepatitis frequently leads to the exacerbation of liver cell necrosis, with a risk of death from acute liver failure. Furthermore, patients with chronic HBV/HDV infection are at risk of developing cirrhosis, HCC, and serious decompensation of the disease, and therefore require close clinical, biochemical and ultrasound monitoring, now made difficult by the COVID-19 emergency.

There is also the need for strategic adjustments to ensure specialist healthcare services to identify, monitor and treat patients with chronic HBV infection and those

with HBV/HDV coinfection.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 14; 27(42): 7285-7298

DOI: 10.3748/wjg.v27.i42.7285

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

MINIREVIEWS

Hemostasis testing in patients with liver dysfunction: Advantages and caveats

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Author contributions: Nguyen G and Frere C performed the literature search, analyzed the data and wrote the paper; all authors contributed to editing, reviewing and final approval of manuscript.

Conflict-of-interest statement: Authors declare that they have no competing interests.

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Abstract

Due to concomitant changes in pro- and anti-coagulant mechanisms, patients with liver dysfunction have a "rebalanced hemostasis", which can easily be tipped toward either a hypo- or a hypercoagulable phenotype. Clinicians are often faced with the question whether patients with chronic liver disease undergoing invasive procedures or surgery and those having active bleeding require correction of the hemostasis abnormalities. Conventional coagulation screening tests, such as the prothrombin time/international normalized ratio and the activated partial thromboplastin time have been demonstrated to have numerous limitations in these patients and do not predict the risk of bleeding prior to high-risk procedures. The introduction of global coagulation assays, such as viscoelastic testing (VET), has been an important step forward in the assessment of the overall hemostasis profile. A growing body of evidence now suggests that the use of VET might be of significant clinical utility to prevent unnecessary infusion of blood products and to improve outcomes in numerous settings. The present review discusses the advantages and caveats of both conventional and global coagulation assays to assess the risk of bleeding in patients with chronic liver disease as well as the current role of transfusion and hemostatic agents to prevent or manage bleeding.

Key Words: Hemostasis; Bleeding risk; Conventional tests; Thrombin generation; Viscoelastic tests; Hemostatic agents

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 17, 2021
Peer-review started: March 17, 2021
First decision: May 1, 2021
Revised: May 8, 2021
Accepted: October 20, 2021
Article in press: October 20, 2021
Published online: November 14, 2021

P-Reviewer: Chawla S, Skok P
S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H



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Core Tip: Patients with liver dysfunction have a "rebalanced hemostasis" which can easily be tipped toward either a hypo- or a hypercoagulable phenotype. Clinicians are often faced with the question whether patients with liver dysfunction undergoing invasive procedures or surgery and those having active bleeding require correction of hemostasis abnormalities. While conventional coagulation screening tests have numerous limitations and do not predict the risk of bleeding prior to high-risk procedures or during surgery, a growing body of evidence suggests that viscoelastic testing might be of significant clinical utility in this setting. The present review discusses the advantages and caveats of both conventional and global coagulation assays in patients with chronic liver disease and the current role of hemostatic agents to prevent and manage bleeding.

Citation: Nguyen G, Lejeune M, Crichi B, Frere C. Hemostasis testing in patients with liver dysfunction: Advantages and caveats. *World J Gastroenterol* 2021; 27(42): 7285-7298
URL: https://www.wjgnet.com/1007-9327/full/v27/i42/7285.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i42.7285

INTRODUCTION

The liver plays a crucial role in synthesizing numerous plasma proteins, including most of the clotting factors except factor VIII and von Willebrand factor (VWF)[1-3]. Liver cells also produce thrombopoietin (TPO), which is the primary regulator of megakaryopoiesis, accounting for approximately 90% of the overall platelet production[4]. Therefore, liver dysfunction results in decreased levels of circulating pro- and anticoagulant factors, decreased levels of circulating pro- and antifibrinolytic factors, and thrombocytopenia, which all worsen with disease severity. Alterations in platelet number and function are, however, partially compensated by elevated levels of VWF and decreased levels of ADAMTS13.

For many years, liver dysfunction has been considered as an acquired bleeding disorder, and patients with acute or chronic liver disease thought to be naturally "autoanticoagulated". Nevertheless, recent advances in the understanding of changes occurring in the hemostasis balance during liver dysfunction (summarized in Figure 1) support a paradigm shift from "liver disease-associated coagulopathy" to "rebalanced hemostasis"[1-3].

This overall "rebalanced hemostasis" is nevertheless fragile and can easily be tipped toward either a hypo- or a hypercoagulable phenotype, leading to either bleeding or thrombotic complications[5].

Clinicians are often faced with the question whether patients with liver dysfunction undergoing invasive procedures or surgery and those having active bleeding require correction of hemostasis abnormalities. While conventional coagulation tests do not accurately predict the risk of bleeding, a growing body of evidence now supports a role for viscoelastic testing (VET) to monitor hemostasis in this setting.

The present review will discuss: (1) the advantages and caveats of both conventional and global coagulation assays to assess the risk of bleeding in patients with chronic liver disease; and (2) the current role of transfusion and hemostatic agents to prevent and manage bleeding in patients with liver dysfunction.

EVALUATION OF THE HEMOSTASIS PROFILE OF PATIENTS WITH LIVER DYSFUNCTION

Platelet count

Thrombocytopenia, as defined by a platelet count < 150 × 10^9/L, is common in patients with chronic liver disease (CLD), particularly in those with portal hypertension. Its prevalence varies widely according to the underlying disease and its severity[6-8]. However, data regarding the association between thrombocytopenia and the risk of

Hemostasis changes that may favor thrombosis or bleeding in patients with liver dysfunction

	Favors thrombosis	Favors bleeding
Primary hemostasis	Increased levels of VWF Decreased levels of ADAMTS-13	Thrombocytopenia Platelet dysfunction Increased levels of NO and prostacyclin
Coagulation	Increased levels of factor VIII. Decreased levels of antithrombin, protein C and protein S	Dysfibrinogenemia Decreased levels of fibrinogen. Decreased levels of factor II, V, VII, IX, X, XI XII, XIII
Fibrinolysis	Decreased levels of plasminogen Increased levels of PAI-1	Decreased levels of α2-antiplasmin & TAFI Increased levels of t-PA

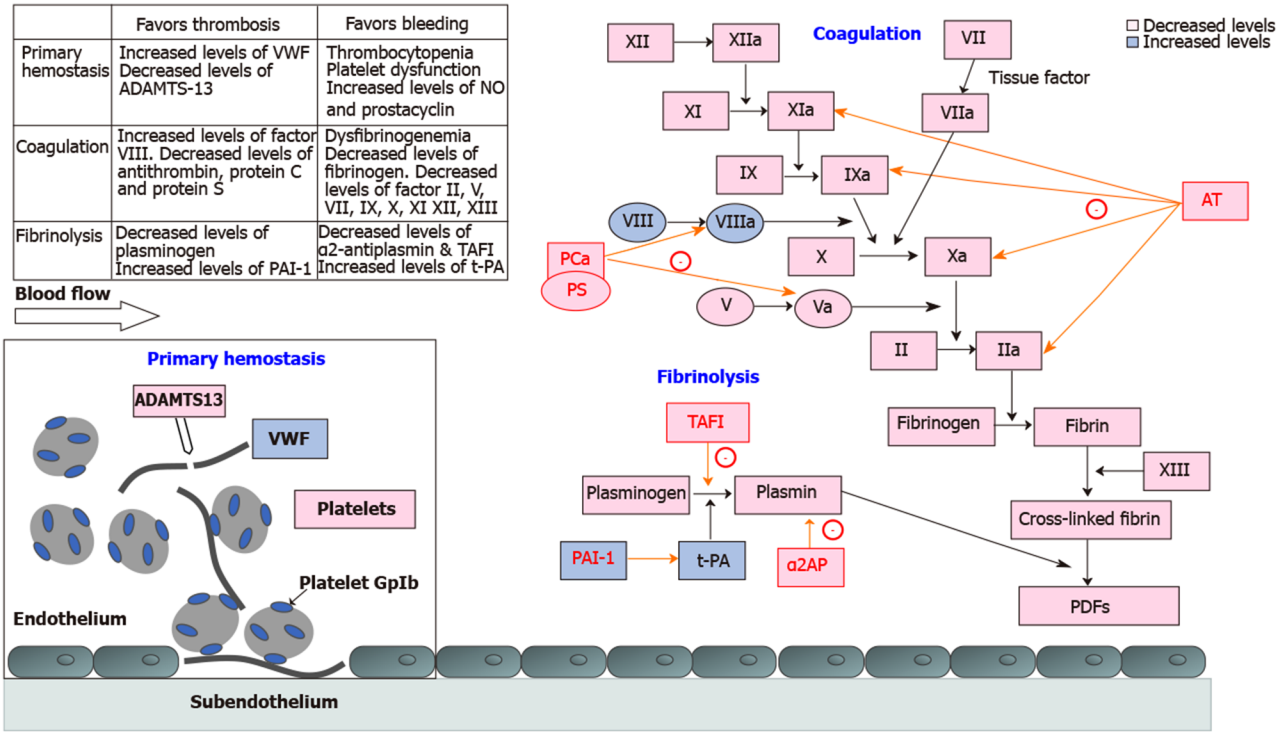


Figure 1 "Rebalanced" Hemostasis in patients with liver dysfunction. AT: Antithrombin; ADAMTS13: a disintegrin and metalloprotease with thrombospondin type I repeats-13; α2AP: α2-antiplasmin; PAI-1: Plasminogen activator inhibitor-1; PDFs: Fibrin degradation products; PC: Protein C; PS: Protein S; TAFI: Thrombin-activatable fibrinolysis inhibitor; t-PA: Tissue plasminogen activator; VWF: Von Willebrand factor.

bleeding in patients with liver dysfunction are conflicting.

In the PRO-LIVER study, 280 patients with cirrhosis were followed up for a median duration of 3 years. One hundred eighty-one (66%) patients had thrombocytopenia at inclusion while 23 (8%) of them had severe thrombocytopenia (defined as a platelet count < 50 × 10^9/L). During the follow-up, bleeding occurred in 52 (18.6%) patients. However, platelet count did not predict the onset of unprovoked bleeding[9]. Similarly, platelet count did not predict the risk of bleeding in a case series of cirrhotic patients undergoing invasive procedure[10]. Conversely, severe thrombocytopenia was associated with a high risk of periprocedural bleeding in patients with advanced liver disease undergoing invasive procedure[11]. Severe thrombocytopenia also independently predicted the onset of major bleeding [odds ratio (OR) 6.476, 95%CI: 1.386-30.255, P < 0.05] in a prospective study of 1493 critically ill patients with cirrhosis admitted to intensive care unit[12].

According to the 7th International Coagulation in Liver Disease Conference[13], platelet count alone is not sufficient to assess the risk of bleeding in patients with CLD. However, a platelet count < 50 × 10^9/L may be associated with a high risk of bleeding.

Global platelet functional tests

Global platelet functional tests are of low clinical utility in patients with liver dysfunction. Their use is limited by several factors including pre-analytical and analytical constraints, lack of standardization and their time-consuming nature. Moreover, none of them has been demonstrated to be clinically useful for stratifying the risk of bleeding in patients with liver dysfunction. The platelet function analyzer (PFA)-100® or PFA-200® closure time (CT) is the most widely used test to assess primary hemostasis. It measures the CT of an aperture within a membrane coated with either collagen/adenosine diphosphate or collagen/epinephrine under flow conditions. Importantly, this test is highly influenced by platelet count, VWF level and hematocrit, making the results challenging to interpret in many cases. Patients with stable cirrhosis or those with end-stage liver disease have been reported to have prolonged CT[14-16]. However, the prognostic value of CT in predicting bleeding complications in patients with liver dysfunction has yet never been investigated. Other functional platelet tests, such as light transmission aggregometry or whole blood aggregometry, have been extensively reviewed elsewhere[17-19].

The use of global platelet functional tests to assess the risk of bleeding in patients with CLD is not recommended by current guidelines.

Conventional coagulation tests

Conventional coagulation tests, such as the prothrombin time (PT)/international normalized ratio (INR) and the activated partial thromboplastin time (aPTT), are commonly used for screening of inherited bleeding disorders and for monitoring of anticoagulant therapy. However, they are of limited value in patients with liver dysfunction since they predict neither the risk of bleeding complications nor the efficacy of blood cell product transfusion or hemostatic agent infusion[1-3].

PT and aPTT are both performed on platelet-poor plasma by measuring the time for fibrin clot formation after addition of phospholipids, calcium, and a trigger agent (tissue factor for the extrinsic pathway and kaolin, celite or ellagic acid for the intrinsic pathway). These tests only evaluate the time to the start of fibrin polymerization, knowing that 90% to 95% of thrombin generation occurs after this step. Importantly, they are insensitive to coagulation inhibitors (*e.g.*, antithrombin, protein C and protein S), which are also decreased in patients with liver dysfunction who have a "rebalanced" hemostasis. Furthermore, they contain low amount of thrombomodulin which is required to activate protein C. Finally, they do not provide any insight on clot lysis.

PT and aPTT are influenced by the level of procoagulant factors [*e.g.* factors I (fibrinogen), II, V, VII and X for the PT and factors I (fibrinogen), II, V, VIII, IX, X, XI and XII for the aPTT]. With the exception of factor VIII, procoagulant factors are heterogeneously decreased in patients with liver dysfunction. Therefore, PT and aPTT are frequently prolonged in patients with acute or CLD. Nevertheless, thrombin generation has been demonstrated to be preserved in patients with liver dysfunction and prolonged PT/aPTT[20], due to a concomitant decrease in anticoagulant levels.

Although the INR is currently used as a prognostic factor when calculating the model for end stage liver disease (MELD) score (which includes serum bilirubin, INR, serum creatinine, dialysis, and serum sodium[21]), it is important to emphasize that it was exclusively designed to monitor vitamin K antagonist therapy. Several studies have shown that its use is not appropriate for patients with liver dysfunction since it may vary for a single sample according to the reagent used[22,23]. A prospective study of 29 consecutive patients listed for liver transplantation (LT) reported that the between-laboratory variability in INR determination had a significant impact on the calculated MELD score[24], further suggesting the need to establish a "modified INR" specific for liver diseases[25].

According to the 7th International Coagulation in Liver Disease Conference[13], the American Association for the study of liver diseases (AASLD)[26], and the American Gastroenterology Association (AGA)[27], the PT/INR and the aPTT should not be used for assessing the risk of bleeding or for guiding blood products transfusion in patients with CLD.

Fibrinogen levels (Clauss assay) are determined by measuring the time for fibrin clot formation in the presence of excess thrombin. The clotting time expressed in seconds is converted to mg/dL by using a calibration curve prepared by serial dilution of a reference plasma of known fibrinogen concentration. Fibrinogen levels may either be normal, increased or decreased in patients with liver dysfunction[3]. Dysfibrinogenemia has been observed in up to 76% of patients with cirrhosis, 78% of those with chronic active liver disease and 86% of those with acute liver failure[28]. In a prospective cohort study of 165 patients with cirrhosis, low fibrinogen levels were associated with decreased survival in univariate analysis[29]. Fibrinogen levels < 60 mg/dL were a strong independent predictor for major bleeding (OR 11.129, 95%CI: 1.189-104.173, $P < 0.05$) in critically ill patients admitted to intensive care unit[12]. In a series of 109 cirrhotic patients undergoing endoscopic variceal band ligation, patients who bled had significant lower median fibrinogen levels compared to those who did not bleed (146 mg *vs* 230 mg/dL, $P = 0.009$)[30]. A fibrinogen level cut-off of 179 mg/dL predicted bleeding with a sensitivity of 83.3% and a specificity of 73.0%[30]. Finally, in a retrospective study of 322 patients undergoing orthotopic LT, baseline fibrinogen levels were found to predict excessive transfusion[31]. Additional studies are warranted to determine the best fibrinogen level cut-off for predicting the risk of bleeding.

Global coagulation assay

Compared to conventional coagulation tests, global coagulation assays such as thrombin generation assays (TGA) or VET, which take into account the interactions between procoagulants, anticoagulants, platelet function and the fibrinolytic system,

are expected to better reflect the overall hemostasis profile, especially in patients with liver dysfunction who have a "rebalanced" hemostasis.

Thrombin generation assays

TGAs have been developed in the 1950s. The most widely used method is the calibrated automated thrombogram[32], which dynamically quantifies the total amount of thrombin generated in platelet-poor plasma or in platelet-rich plasma after initiating the coagulation by the addition of tissue factor, phospholipids and calcium. Tripodi *et al*[20] reported that the profile of thrombin generation measured without thrombomodulin was decreased in cirrhosis patients compared to healthy controls, while it was normal in the presence of thrombomodulin (which is required for protein C activation), demonstrating for the first time that patients with liver dysfunction have a "rebalanced hemostasis". Recent TGA studies (reviewed elsewhere by Lebreton *et al* [33]) further reported that patients with liver dysfunction may have an hypercoagulable phenotype which seems to correlate with the disease severity[33]. Studies assessing the ability of TGA in predicting bleeding or thrombotic complications in patients with liver dysfunction are yet lacking. For now, there is still a need for standardization and automation of TGA methods.

According to the 7th International Coagulation in Liver Disease Conference[13], the use of TGA in patients with CLD should be restricted to clinical research studies.

Viscoelastic tests

VETs, which are performed in whole blood, evaluate the *in vivo* dynamics of clot formation, clot stabilization and fibrinolysis. They are expected to provide a more accurate assessment of the overall hemostasis profile, and therefore to better predict the risk of bleeding and thrombosis. Nowadays, VETs are used as point-of-care testing and provide faster results than standard coagulation tests, which constitute an additional advantage.

Three methods are currently available, *i.e.*, thromboelastography (TEG, Hemonetics Corporation, Braintree, MA), thromboelastometry (ROTEM, TEM International GmbH, Munich, Germany) and sonorheometry (Quantra QPlus System, HemoSonics, LLC, Charlottesville, VA).

In patients with CLD, VET parameters correlate well with platelet count and fibrinogen levels (except in patients with fibrinogen levels < 100 mg/dL) but not with PT/INR and aPTT [34-42].

VETs have been mainly studied in patients with end-stage liver disease undergoing LT, but numerous studies have also been conducted in patients with less severe liver disease, including those well compensated.

Viscoelastic tests in liver transplantation

Historically, the first orthotopic LT was performed by Starzl *et al*[43] in 1963. The recipient was a 3-year-old boy with congenital biliary atresia. Interestingly, coagulation was monitored by performing serial thromboelastograms[43]. Twenty years later, Kang *et al*[44] first suggested that VET-guided transfusion could substantially reduce blood products administration during LT. In their series of 66 patients undergoing LT, VET-monitoring was associated with a significant reduction in the administration of red blood cell units (17 ± 12.9 units *vs* 26.7 ± 23.8 units in the historical cohort, $P < 0.05$), fresh frozen plasma (FFP) units (18.3 ± 12.5 units *vs* 26.7 ± 24.1 units, $P < 0.05$) and total volume infused (20.2 ± 11.2 L *vs* 31.4 ± 19.2 L, $P < 0.05$). However, VET-guided transfusion led to a significant increase in the administration of platelets (20.8 ± 12.8 units *vs* 14.1 ± 13.7 units, $P < 0.05$) and cryoprecipitate units (17.2 ± 8.5 units *vs* 10.2 ± 4.5 units; $P < 0.05$). Numerous non-randomized studies further confirmed these findings[45-47].

Only two small randomized controlled trials (RCTs) compared VET-guided transfusion to the standard of care (SOC) during LT (Table 1)[48,49]. Importantly, they used different VET methodologies and various algorithms for VET-guided transfusion. Wang *et al*[48] randomized 28 adult patients with cirrhosis undergoing LT in either a TEG-guided transfusion arm or a SOC arm. Intraoperative administration of FFP was significantly reduced in the TEG-guided transfusion arm (12.8 ± 7.0 units *vs* 21.5 ± 12.7 units in the SOC arm, $P < 0.05$), without significant difference in the administration of other blood products between the two arms. In the TEG-guided transfusion arm, there was a trend towards reduction in blood loss (4775 ± 4264 mL *vs* 6348 ± 3704 mL in the SOC arm, P = ns). More recently, Bonnet *et al*[49] randomized 82 adult patients with cirrhosis undergoing orthotopic LT in either a ROTEM-guided transfusion arm or a SOC arm. Median [interquartile range] intraoperative administration of blood

Table 1 Randomized controlled trials assessing viscoelastic (tests) in patients with cirrhosis undergoing liver transplantation or invasive procedures and for the management of active bleeding

Ref.	VET	Population	*n*	Exclusion criteria	Intervention in the VET arm	Intervention in the SOC arm	Blood product	Bleeding
Liver transplantation								
Wang *et al*[48], 2010	TEG	Adult patients with cirrhosis undergoing liver transplantation	28 patients (14 in each arm)	Unspecified	FFP titrated to maintain R time < 10 min; 6-8 pooled platelet units if MA < 55 mm; 5 pooled units of cryoprecipitate if alpha angle < 45 degrees	FFP titrated to maintain PT and APTT at less than one and a half times control; Platelets to maintain a platelet count ≥ 50 × 10^9 /L; Cryoprecipitate to maintain fibrinogen > 1 g/L	FFP use: 12.8 units in the TEG arm *vs* 21.5 units in the SOC arm ($P < 0.05$); RBC: no difference; Platelets use: no difference; Cryoprecipitate use: no difference	Trend towards reduction in blood loss in the TEG arm (not statistically significant)
Bonnet *et al*[49], 2019	ROTEM	Adult patients with cirrhosis undergoing orthotopic liver transplantation	82 patients (41 in each arm)	Pregnancy; congenital coagulopathy; patients participating in another study	2 FFP units if EXTEM CT < 110 s; 1 platelet unit if EXTEM MCF < 40 mm or A10 < 35 mm and FIBTEM A10 or MCF > 8 mm; Fibrinogen 3 g if FIBTEM A10 < 8 mm	2 FFP units if PT < 40% at baseline or an hepatic phase or hemorrhage; PT < 30% at declamping or end of surgery and no hemorrhage; 1 platelet unit if platelet count < 50 × 10^9/L at baseline or an hepatic phase or hemorrhageor if platelet count < 30 × 10^9/L at declamping or end of surgery and no hemorrhage; Fibrinogen 3 g if fibrinogen ≤ 1 g/L	FFP use: 6 patients in the TEG arm *vs* 19 patients in the SOC arm ($P = 0.002$); RBC use: no difference; Platelets use: no difference; Cryoprecipitate use: 29 patients in the TEG arm *vs* 12 patients in the SOC arm ($P < 0.001$)	No difference in revision surgery or postoperative hemorrhage at 24 and 48 h
Invasive procedure								
De Pietri *et al*[51], 2016	TEG	Adult patients with cirrhosis undergoing invasive procedures with an INR > 1.8 and/or platelet count < 50 × 10^9 /L	60 patients (30 in each arm)	Ongoing bleeding; current thrombotic events; antiplatelets or anticoagulants use; infection or sepsis; hemodialysis	FFP 10 mL/kg if R > 40 min; Platelets if MA < 30 mm	FFP10 mL/kg if INR > 1.8; Platelets if platelet count < 50 × 10^9/L	16.7% in the TEG arm *vs* 100% in the SOC arm ($P < 0.0001$)	1 post procedure bleeding after large volume paracentesis in the SOC arm
Vuyyuru *et al*[52], 2019	TEG	Adult patients with cirrhosis undergoing invasive liver-related procedures with INR > 1.8 and/or < 50 × 10^9/L	58 patients(29 in each arm)	Cancer; hemophilia; DIC; antiplatelets use; pregnancy; renal failure; blood products in the previous 7 d	FFP if R > 14 min; 6-8 pooled platelet units; if MA < 30 mm	FFP if INR > 1.8; 6-8 pooled platelet units; if platelet count < 50 × 10^9/L	31% in the TEG arm *vs* 100% in the SOC arm ($P < 0.001$)	No bleeding in any group
Rocha *et al*[53], 2020	ROTEM	Adult critically ill patients with cirrhosis undergoing CVC insertion	57 patients (19 per arm)	Acute liver failure; vonWillebrand's disease; anticoagulants use; patients participating in another study	FFP10 mL/kg if CT EXTEM > 80 s; 1 apheresis platelets unit if A10 EXTEM < 40 mm and A10 FIBTEM ≥ 10 mm; 1 unit/kg of cryoprecipitate if A10 ESTEM < 40 mm and A10 EXTEM < 10 mm	SOC arm: FFP 10 mL/kg if INR > 1.5 or aPTT > 50 s 1 unit/kg of platelets if platelet count < 50 × 10^9 /L; 1 unit/kg of cryoprecipitate if fibrinogen < 150 mg/dL; Restrictive arm: FFP 10 mL/kg if INR > 5; 1 unit/kg of platelets if	Significantly lower in the restrictive arm (15.8% *vs* 68.4% in the ROTEM arm; $P < 0.006$ and *vs* 73.7%; $P < 0.002$) in the SOC arm. No difference between ROTEM and SOC arms	No major bleeding in any group

					platelet count < 25 × 10^9/L		
Active bleeding							
Kumar *et al*[55], 2020	TEG	Adult patients with advanced liver cirrhosis presenting with nonvariceal upper gastrointestinal bleeding with INR > 1.8 and/or platelet count < 50 × 10^9/L	96 patients (49 in the TEG arm, 47 in the SOC arm)	Variceal bleed; postvariceal ligation; ulcer bleed, previous or current thrombotic events; anticoagulant therapy at the time of enrollment or that had been discontinued less than 7 d before evaluation for the study; hemodialysis in the previous 7 d; pregnancy; significant cardiopulmonary disease	FFP 10 mL/kg if R > 10 min; 6-8 pooled platelet units if MA < 55 mm; 5 pooled units of cryoprecipitate if α-angle < 45 degrees	FFP 10 mL/kg if INR > 1.8; 6-8 pooled platelet units if plateletcount < 50 × 10^9/L 5 pooled units of cryoprecipitate if fibrinogen < 80 mg/dL	Patients transfused with all three blood components: 26.5% in TEG *vs* 87.2% SOC (P < 0.001)	No difference in failure to control bleeding or rebleeding on day 5. No difference in mortality on day 5 and on day 42
Rout *et al* [56],2020	TEG	Adult patients with cirrhosis presenting with acute variceal bleeding with INR > 1.8 and/or plateletcount < 50 × 10^9/L	60 patients (30 in each arm)	Malignancy; hemophilia; DIC; antiplatelets use; pregnancy; blood products in the previous 7 d; shock; sepsis; acute-on-chronic liver failure, renal failure, encephalopathy	FFP 5mL/kg if R > 15 min; 3 pooled units of platelets if MA < 30 mm	FFP if INR > 1.8; Platelets if platelet count < 50 × 10^9/L	13.3% TEG *vs* 100% SOC (P < 0.001)	No difference in control of bleeding or rebleeding on day 5 between the two groups.Rebleeding on day 42 less in TEG (10%) than SOC (36.7% ; P = 0.012)

A10: Amplitude at 10 min; ACLF: Acute-on-chronic liver failure; aPTT: activated partial thromboplastin time; CCT: Conventional coagulation test; CT: Clotting time; CVC: Central venous catheter; DIC: Disseminated intravascular coagulation; INR: International normalized ratio; FFP: Fresh frozen plasma; MA: Maximum amplitude; R: Reaction time; ROTEM: Rotational thromboelastometry; SOC: Standard of care; TEG: Thromboelastography; VET: Viscoelastic test.

products was significantly decreased in the ROTEM-guided transfusion arm [3 (2–4) *vs* 7 (4–10) units in the SOC arm, P = 0.005]. FFP was administered less frequently in the ROTEM-guided transfusion arm (15% *vs* 46.3% in the SOC arm, P = 0.002). However, fibrinogen concentrates were administered more frequently (72.5% *vs* 29.3% in the SOC arm, P < 0.001). There was no difference in revision surgery or postoperative bleeding at 24 and 48 h.

Overall, the body of evidence supporting the use of VET to guide transfusion during LT remains poor. Nevertheless, VETs have been widely adopted in most large-volume academic transplant centers, as reported in a recent survey[50].

Viscoelastic tests during invasive procedures

Three small RCTs assessed the benefit of VET-guided prophylactic transfusion during invasive procedures (Table 1)[51-53]. De Pietri *et al*[51] randomized 60 adult cirrhosis patients with an INR > 1.8 and/or a platelet count < 50 × 10^9/L undergoing invasive procedures in either a TEG-guided prophylaxis transfusion arm or a SOC arm. Nearly half of patients (47%) underwent low-risk procedures such as paracentesis or thoracentesis. FFP alone (0% *vs* 53.3%, P < 0.001), platelets alone (6.7% *vs* 33.3%, P = 0.021) and overall blood products (16.7% *vs* 100%, P < 0.0001) were administered less frequently in the TEG-guided arm compared to the SOC arm. Post-procedure bleeding occurred in only 1 patient in the SOC arm after large-volume paracentesis.

A second RCT[52] randomized 58 adult cirrhosis patients with an INR > 1.8 and/or a platelet count < 50 × 10^9/L undergoing high-risk invasive procedures (83% of liver biopsy) in either a TEG-guided prophylaxis transfusion arm or a SOC arm. Transfusions of any blood products (31% *vs* 100%, P < 0.0001), and platelet transfusions (6.9% *vs* 42.4%, P < 0.001) were administered less frequently in the TEG-guided arm. There was no difference in the rate of patients who received FFP. No procedure-related bleeding was observed.

Finally, Rocha *et al*[53] randomized 57 adult critically ill patients with cirrhosis undergoing central venous catheterization in three arms, *i.e.*, a restrictive strategy arm,

a SOC arm, or a ROTEM-guided arm. The restrictive strategy decreased the rates of any blood component administration compared to the SOC (OR 0.07, 95%CI: 0.01-0.45, P = 0.002) and to the ROTEM-guided strategy (OR 0.09, 95%CI: 0.01-0.56, P = 0.006). There was no difference in bleeding, length of stay, mortality, and transfusion□related adverse events between the three arms.

Overall, the current evidence suggests that the use of VET-guided transfusion during invasive procedures might significantly decrease the prophylactic infusion of blood products. However, the benefit of VTE monitoring during low bleeding risk procedures remains debated.

Viscoelastic tests in patients with liver dysfunction and active bleeding

In a prospective study of 20 cirrhotic patients with active variceal bleeding, serial TEGs were performed daily for 7 d to assess the association between changes occurring in TEG profile and early rebleeding[54]. Patients who presented early rebleeding had longer median R (42 mm *vs* 24 mm, P < 0.001) and median K (48 mm *vs* 13 mm, P < 0.001) and smaller median alpha (12 *vs* 38, P < 0.001) on the day of rebleeding compared with the mean of all daily results in patients who did not rebleed[54].

Two small RCTs evaluated the benefit of using VETs to monitor transfusion in cirrhotic patients with active upper gastrointestinal bleeding (Table 1)[55,56]. The first one randomized 96 adult patients with advanced liver cirrhosis, coagulopathy (as defined by an INR > 1.8 and/or platelet count < 50 × 10^9/L) and active non-variceal upper gastrointestinal bleeding in either a TEG-guided transfusion arm or a SOC arm. The volume of FFP infused per patient was significantly lower in the TEG-guided arm (440 mL *vs* 800 mL in the SOC arm, P < 0.001). Moreover, the rate of patients who received blood products was significantly lower in the TEG-guided arm (26.5% *vs* 87.2% in the SOC arm, P < 0.001). The rates of control of bleeding, rebleeding, and mortality did not differ between the two arms[55]. The second RCT[56] randomized 60 adult patients with cirrhosis, coagulopathy (as defined by an INR > 1.8 and/or platelet count < 50 × 10^9/L) and active variceal upper gastrointestinal bleeding in either a TEG-guided transfusion arm or a SOC arm. A significant reduction in the rate of blood product infusion was observed in the TEG-guided arm (13.3% *vs* 100% in the SOC arm, P < 0.001). The rate of initial control of bleeding at the primary endoscopy and the rate of rebleeding at 5 d did not differ between the two arms.

Overall, a growing body of evidence indicates that VETs may be useful to guide transfusion, particularly in patients with active bleeding. Further studies to establish standardized algorithms and cut-off values to guide transfusion are warranted.

Nevertheless, some limitations of VTE leading to underestimate the true hemostatic potential should be acknowledged[57]. First, some VET parameters such as the maximum amplitude are frequently hypocoagulable in patients with liver dysfunction. This is mainly due to thrombocytopenia. However, it is important to notice that VETs are insensitive to VWF levels which are increased in patients with liver dysfunction, promoting platelet adhesion and partly compensating thrombocytopenia. Second, VETs are insensitive to the protein C system which requires the transmembrane protein thrombomodulin expressed on the luminal surface of endothelial cells to be activated. Third, VETs do not capture clot quality. Finally, most VETs parameters are obtained after the addition of an activator of the intrinsic pathway, which might not be relevant to study the physiological initiation of coagulation.

According to the 7th International Coagulation in Liver Disease Conference[13], the use of VETs in patients with CLD should be restricted to clinical research studies.

CURRENT ROLE OF HEMOSTATIC AGENTS IN PATIENTS WITH LIVER DYSFUNCTION

Platelet transfusion and thrombopoietin agonists

Platelet transfusion is the standard of care to transiently increase platelet count in patients undergoing invasive procedures or in those with active bleeding. A conventional threshold of 50 × 10^9/L, which is exclusively based on expert opinion, is recommended by the AASLD[26]and the AGA[27]. However, *in vitro* studies have suggested that platelet-dependent thrombin generation should be preserved in patients with cirrhosis having a platelet count above 56 × 10^9/L[58].

Due to platelet transfusion detrimental side effects, treatments stimulating endogenous production of functional platelets are increasingly used in CLD patients undergoing invasive procedures. Two second generation TPO receptor agonists

(avatrombopag and lusutrombopag) were recently approved by the Food and Drug Administration for the treatment of severe thrombocytopenia in this setting.

The ADAPT-1 and ADAPT-2 trials randomized a total of 435 CLD patients with severe thrombocytopenia (platelet count < 50 × 10^9/L) undergoing a diagnostic or therapeutic invasive procedure to receive either avatrombopag (n = 277) or placebo (n = 158) daily for 5 d[59]. The primary endpoint was the rate of patients not requiring platelet transfusions or rescue therapy for bleeding within 7 d following procedure. In ADAPT-1, the rate of patients who met the primary endpoint was significantly higher in patients receiving avatrombopag (66% in patients receiving 60 mg avatrombopag *vs* 23% in the placebo arm, and 88% in patients receiving 40 mg avatrombopag *vs* 38% in the placebo arm). Similarly, in ADAPT-2, the rate of patients who met the primary endpoint was significantly higher in patients receiving avatrombopag (69% in patients receiving 60 mg avatrombopag *vs* 35% in the placebo arm, and 88% in patients receiving 40 mg avatrombopag *vs* 33% in the placebo arm). There was no difference in serious adverse events between the two arms.

The L-PLUS 1 trial randomized 96 CLD patients with severe thrombocytopenia (platelet count < 50 × 10^9/L) undergoing an invasive procedure to receive either lusutrombopag (n = 48) or placebo (n = 48) daily for up to 7 d[60]. The primary efficacy endpoint was the rate of patients not requiring platelet transfusion before the invasive procedure. Seventy-nine percent of patients met the primary endpoint in the lusutrombopag arm *vs* 12% in the placebo arm (P < 0.001). Lusutrombopag was well tolerated. The L-PLUS 2 trial randomized 215 CLD patients with severe thrombocytopenia (platelet count < 50 × 10^9/L) undergoing an invasive procedure to receive either lusutrombopag (n = 108) or placebo (n = 107) daily for up to 7 d[61]. The procedure was scheduled 2 to 7 d after the last dose of lusutrombopag or placebo. The primary efficacy endpoint was the rate of patients not requiring platelet transfusion before the invasive procedure or rescue therapy for bleeding. Sixty-five percent of patients met the primary endpoint in the lusutrombopag arm *vs* 29 % in the placebo arm (P < 0.001). There was no difference in serious adverse events between the two arms.

Importantly, TPO receptor agonists have a slow onset of action (5 d or more) and are indicated only in stable CLD patients having severe thrombocytopenia t without active bleeding who undergo planned elective procedures. Performing platelet count on the day of the procedure is warranted.

FFP

FFP is commonly used to correct coagulation factor deficiencies in patients with active bleeding when coagulation tests are abnormal. However, there is currently insufficient evidence to support the prophylactic use of FFP in patients with CLD.

In an *in vitro* study of 58 patients with advanced cirrhosis (Child–Pugh B and C), addition of normal pooled plasma to plasmas from cirrhotic patients shortened both PT and aPTT (P < 0.0001), without significant change in endogenous thrombin potential (ETP) in presence of thrombomodulin (P = ns)[62]. In line with these results, a small sample study (n = 53) found that infusion of FFP increased the ETP by only 5.7%[63]. Two out of 53 (3.8%) patients had baseline ETP below normal values and infusion of FFP corrected thrombin generation in only one out of two patients.

The only large multicenter, RCT aiming to assess the efficacy and safety of prophylactic FFP infusion in CLD patients undergoing invasive procedures[64] was unfortunately interrupted due to inadequate enrollment.

Potential detrimental side effects of FFP, such as transfusion-related acute lung injury or volume expansion leading to portal hypertension exacerbation, should be considered.

Based on available evidence, the AASLD[26] and the AGA[27] do not recommend using prophylactic infusion of FFP in CLD patients undergoing invasive procedures or surgery.

Fibrinogen

Cryoprecipitates or fibrinogen concentrates are generally preferred over FFP to correct hypofibrinogenemia due to lower volume to infuse and best standardized fibrinogen content. Cryoprecipitates contain fibrinogen, von Willebrand factor and factor VIII. One unit of cryoprecipitate per 10 kg of body weight increases fibrinogen levels by approximately 50 mg/dL. In a randomized, multicenter, double-blind, controlled trial comparing preemptive fibrinogen administration to placebo during LT, preemptive fibrinogen infusion did not reduce blood products transfusion (RR 0.80, 95%CI: 0.57-1.13)[65]. In cirrhosis patients with active bleeding, maintaining fibrinogen levels above 120 mg/dL is generally required.

Recombinant activated factor VII

The use of activated recombinant factor VII (rFVIIa) is restricted to patients presenting massive bleeding.

Two RCTs aiming to assess the efficacy and safety of rFVIIa on upper gastrointestinal or variceal bleeding in cirrhosis patients reported no difference between the rFVIIa and the placebo arms in the composite primary endpoint of failure to control acute bleeding, rebleeding within the first 5 d, and death within the first 5 d [66,67]. However, patients with variceal bleeding receiving rFVIIa had a significantly lower rate of 42-d mortality compared to those receiving placebo (15% *vs* 29%, OR 0.31, 95%CI: 0.13-0.74)[67].

Consensus guidelines on the use of rFVIIa as an adjuvant treatment for massive bleeding do not recommend using rFVIIa in patients with Child-Pugh A cirrhosis (grade B evidence)[68]. Furthermore, they consider its benefit as uncertain in patients with more advanced liver disease (grade C evidence)[68].

Prothrombin complex concentrates

Data on the benefit of prothrombin complex concentrates (PCC) in patients with cirrhosis are limited to retrospective studies and case reports[69,70]. PCC infusion was reported to significantly reduce INR in critically ill cirrhosis patients, with lower amount of blood transfusion requirements compared to FFP. The ongoing double-blind, multicenter, placebo-controlled randomized PROTON trial comparing infusion of PCC *vs* placebo prior to surgery to reduce transfusion requirements in cirrhotic patients undergoing LT is expected to provide more data on the benefit of PCC in the near future[71].

Desmopressin

The 1-deamino-8-D-arginine vasopressin (DDAVP) is commonly used to prevent blood loss in a variety of bleeding disorders. It has also been demonstrated to improve platelet function in patients with severe renal failure. An early study reported that intranasal DDAVP was effective and safe in cirrhotic patients undergoing dental extraction and having thrombocytopenia < 50 × 10^9/L. However, later RCTs found no benefit of DDAVP administration in controlling acute variceal bleeding[72] or in preventing blood loss in CLD patients undergoing LT[73]. In line with these results, DDAVP administration did not improve VWF-dependent platelet adhesion in patients with cirrhosis in a flow-based model[74]. According to the AGA clinical practice guidelines, the use of DDVP in CLD patients should be restricted to those with concomitant end-stage renal disease[27].

Antifibrinolytic agents

Data regarding the benefits and risks of antifibrinolytic agents to prevent or manage bleeding in patients with CLD are conflicting. A metanalysis pooled the results of several RCTs assessing their efficacy and safety to prevent blood loss in patients undergoing LT[75]. In the pooled analysis, aprotinin or tranexamic acid (TXA) reduced red blood cell and FFP transfusion requirements compared with placebo, without increasing the rates of hepatic artery and venous thromboembolism[75]. The recent international, randomized, double-blind, placebo-controlled hemorrhage alleviation with tranexamic acid-intestinal system (HALT-IT) trial randomized 12009 patients with gastrointestinal bleeding to receive either TXA or matching placebo[76]. TXA did not decrease death from gastrointestinal bleeding [risk ratio (RR) 0.99, 95%CI: 0.82–1.18] but it increased the risk of venous thromboembolism (RR 1.85; 95%CI: 1.15-2.98)[76]. Conversely a recent pooled analysis of 11 RCT including the HALT-IT trial found that TXA significantly decreased the risk of mortality compared to control (RR 0.75, 95%CI: 0.57-0.96)[77].

Based on available evidence, the AGA suggests using antifibrinolytic agents for a short duration to control periprocedural bleeding in case of hyperfibrinolysis[27].

CONCLUSION

In patients with liver dysfunction, hemostasis is rebalanced due to concomitant changes in pro- and anti-coagulant mechanisms. In these patients, current conventional coagulation screening tests, such as the PT/INR and the aPTT, have numerous limitations and should not be used to predict the risk of bleeding prior to high-risk procedures. The introduction of global coagulation tests has been an important step

forward in the assessment of the overall hemostasis profile. There is nowadays a growing body of evidence suggesting that they might be of significant clinical utility to prevent unnecessary infusion of blood products and to improve outcomes in numerous settings. Further studies are, however, required to develop standardized algorithms and establish clinical practice guidelines for VET-guided transfusion in patients with liver dysfunction.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 14; 27(42): 7299-7310

DOI: 10.3748/wjg.v27.i42.7299

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

MINIREVIEWS

Colonoscopy-related colonic ischemia

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Author contributions: Fusaroli P designed the research study; Sadalla L and Lisotti A performed the research and wrote the article; Fuccio L analyzed the data for relevant intellectual content; All authors have read and approved the final manuscript.

Conflict-of-interest statement: All authors declare that there are no conflicts of interest.

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Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Italy

Peer-review report's scientific

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Abstract

Colonoscopy is a risk factor for colon ischemia. The colon is susceptible to ischemia due to its minor blood flow compared to other abdominal organs; the etiology of colon ischemia after colonoscopy is multifactorial. The causative mechanisms include splanchnic circulation impairment, bowel preparation, drugs used for sedation, bowel wall ischemia due to insufflation/barotrauma, and introduction of the endoscope. Gastroenterologists must be aware of this condition and its risk factors for risk minimization, early diagnosis, and proper treatment.

Key Words: Endoscopy; Colon ischemia; Colonoscopy; Bowel preparation; Mesenteric circulation; Ischemic colitis

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Core Tip: Colonic ischemia is the main form of vascular injury to the gastrointestinal tract and is characterized by sudden onset of nausea and abdominal pain, followed by bloody diarrhea. Among the different etiologies, colonoscopy has been proposed as a risk factor for colon ischemia.

Citation: Sadalla S, Lisotti A, Fuccio L, Fusaroli P. Colonoscopy-related colonic ischemia. *World J Gastroenterol* 2021; 27(42): 7299-7310

URL: https://www.wjgnet.com/1007-9327/full/v27/i42/7299.htm

DOI: https://dx.doi.org/10.3748/wjg.v27.i42.7299

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

Received: March 30, 2021
Peer-review started: March 30, 2021
First decision: June 23, 2021
Revised: July 6, 2021
Accepted: October 20, 2021
Article in press: October 20, 2021
Published online: November 14, 2021

P-Reviewer: Gkolfakis P, Imaeda H
S-Editor: Ma YJ
L-Editor: Filipodia
P-Editor: Ma YJ

INTRODUCTION

At the end of 2018, an elderly retired physician came to our attention because of worsening post-prandial epigastric pain, hyporexia and weight loss. Complete blood cell count showed moderate anemia (hemoglobin 10.2 g/dL). The patient had sought medical attention in the emergency room of our hospital 1 mo prior for the same symptoms. At that time, doctors mainly focused on premature atrial complexes and hypokalemia, as the patient was suffering from hypertensive cardiopathy and had undergone percutaneous transluminal coronary angioplasty. Comorbidities were mild chronic renal failure, type-2 diabetes mellitus, and stable metastatic hormone-sensitive prostate cancer.

Upon presentation to our unit, no remarkable physical findings were noted. Medications included low-dose aspirin, beta-blockers, anti-hypertensive agents (candesartan and amlodipine), simvastatin, glibenclamide, bicalutamide, and omeprazole. We performed percutaneous abdominal ultrasound and esophago-gastroduodenoscopy, which were unremarkable. As the patient was strongly determined not to neglect any diagnostic possibility, consent to undergo colonoscopy was given too. Being a fragile individual, we elected to administer the bowel preparation in our in-hospital facility with a 2-liter same-day ascorbate-based polyethylene glycol regimen. After the first laxative dose, the patient had syncope and was brought to the emergency room. No notable findings emerged; serum sodium was 147 mmol/L (136-145) while potassium concentration was normal. The patient was able to complete the preparation and was still determined to undergo a colonoscopy. The exam went smoothly and failed to show any relevant findings. The patient was discharged home in good condition and relieved as no malignancy was found.

However, 48 hours after the exam, disaster struck. The patient came back to the hospital suffering from severe abdominal pain of sudden onset. Urgent computed tomography showed complete thrombosis of the superior mesenteric artery, ileocolic arteries, and common hepatic artery (Figure 1). Large bowel loops were over-distended with a lack of parietal perfusion as per severe ischemic changes. Due to the rapidly deteriorating conditions, the patient died 24 h later despite intensive care.

Colonic ischemia, also described as ischemic colitis if an inflammatory component is present[1], is usually a benign condition that occurs when there is sudden and temporary hypoperfusion of the large bowel wall[2]. Colonic ischemia can usually be managed conservatively and must be differentiated from other causes of acute and chronic ischemic injury, such as emboli and thrombosis[3], which often require urgent intervention[4].

Ischemia occurs when blood supply is inadequate to fulfill the metabolic requirements of tissues and can occur in every district of the body[5]. When there is a decrease in blood flow, the affected tissues initially react with vasodilation. However, if hypoperfusion persists, vasoconstriction may ensue[6].

The colon is susceptible to ischemia due to its minor blood flow compared to other abdominal organs and to its decrease in perfusion when functionally inactive[7]. In the colon, ischemic injury displays a wide spectrum of manifestations. Reversible ischemia determines the rupture of the sub-epithelial microvasculature, which evolves in a colitis phase when the mucosa ulcerates and subepithelial hemorrhage is reabsorbed [8]. On the other hand, irreversible damage leads to necrosis of the bowel wall, gangrene, strictures, and ultimately to fulminant colitis[9].

Colonic ischemia represents the predominant form of vascular injury to the gastrointestinal tract with an estimated incidence ranging from 7.2 to 15.6 per 100000 population[10]. It is also one of the main causes of lower gastrointestinal bleeding[8]. Women seem affected more than men, especially among patients < 40 years of age [10]. However, the incidence of colonic ischemia is probably underestimated because many patients never seek medical attention due to its frequent presentation with mild symptoms[2].

Etiology

Colonic ischemia is multifactorial. Previous studies identified risk factors for colonic ischemia such as cardiovascular, pulmonary, and renal comorbidities, history of irritable bowel syndrome, and surgical interventions[10,11]. As far as drugs are concerned, immunomodulators and constipation-inducing drugs have been associated more frequently with colonic ischemia[12]. The use of vasopressors, alfa-adrenergic agents, diuretics as well as cocaine has also been described as contributing factors[8]. Additionally, in most cases, no specific cause of colonic ischemia can be determined [13].



Figure 1 Abdominal computed tomography with intravenous contrast, sagittal scan showing thrombosis of the superior mesenteric artery and the common hepatic artery (arrows).

Colonoscopy has been proposed as a risk factor for colonic ischemia although only few reports are available in this respect[14]. To our knowledge, no current epidemiologic data exist about colonic ischemia after colonoscopy.

Splanchnic vascular anatomy

Visceral blood supply is strictly connected to the function of the gastrointestinal organs. The celiac trunk, the superior mesenteric artery (SMA), and the inferior mesenteric artery (IMA), all of which originate from the abdominal aorta (Figure 2) [15], represent the main abdominal vessels.

The SMA and IMA sustain colon perfusion. The SMA is the largest abdominal artery supplying the inferior part of the duodenum, the entire jejunum and ileum, the proximal colon, and a portion of the pancreas[16]. The IMA feeds the distal colon through three different branches and the rectum with its terminal branch, the superior rectal artery[17].

These vessels are deeply interconnected through collateral branches to ensure visceral perfusion also in case of deficiency of a single vessel[18]. Despite this network, some segments of the colon can be more frequently affected by ischemia due to their location between two different vascular supplies. These are known as "water-shed areas", the most important being the splenic flexure (Griffith's point) and the sigmoid colon (Sudeck's point)[2].

COLONIC ISCHEMIA AFTER COLONOSCOPY

Colonoscopy is the gold standard for colorectal screening programs and the number of colonoscopies is progressively increasing worldwide[19]. This exam allows direct visualization of the colonic mucosa and allows resecting of pre-neoplastic lesions. Colonoscopy is considered relatively safe being associated with a perforation rate of about 4 in 10000 procedures, a major bleeding rate of 8 in 10000 procedures, and an estimated overall mortality of 0.007-0.07%[20,21].

An extensive search of the literature showed 25 reports of colonoscopy-related colonic ischemia[22]. The first report in 1990 described a young woman with a history of systemic lupus erythematosus who developed abdominal pain and bloody diarrhea after a diagnostic colonoscopy. Arteriography showed no abnormalities of mesenteric vessels while sigmoidoscopy showed severe inflammation with biopsies suggestive for necrotic areas, compatible with ischemic colitis. The authors suggested that the impairment in small circulation due to the connective tissue disease combined with the mechanical stress of the colonoscopy probably led to colonic ischemia[14]. Subsequently, more cases of ischemic colitis were published[23]. More recent literature

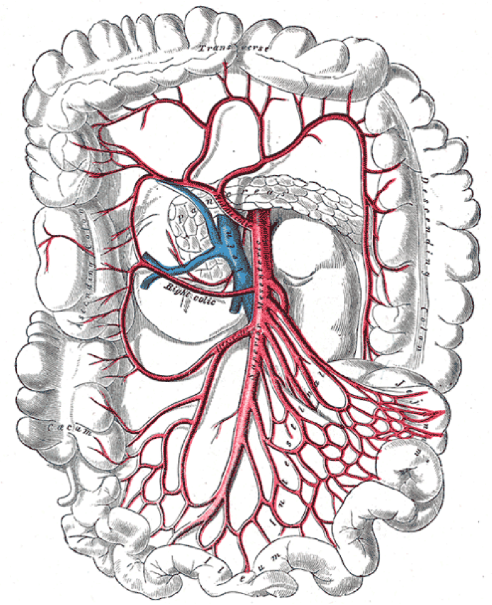


Figure 2 Splanchnic vascular anatomy, detail of colonic arteries (Case courtesy of Assoc Prof Craig Hacking, Radiopaedia.org, rID: 54523.

reported one case of SMA thrombosis and one case of SMA embolism[24]. Other case reports of colonic ischemia after colonoscopy described patients with cardiovascular comorbidities[25] or without any known risk factor[26].

Based on the previous considerations, we propose five different mechanisms by which colonoscopy-related colonic ischemia might arise.

Splanchnic circulation impairment

Colonic ischemia can develop in patients with bowel circulation impairment. Chronic mesenteric ischemia is predominantly related to atherosclerotic disease, patients with this condition usually being elderly and with a history of smoking[27]. A study reported an 18% prevalence of asymptomatic stenoses of abdominal vessels in individuals over 65 years[28]. Studies with magnetic resonance showed a significant postprandial reduction of mesenteric flow in patients with vascular stenoses compared to healthy subjects[29,30].

Moreover, conditions such as antiphospholipid syndrome, systemic lupus erythematosus and diabetes can determine an endothelial dysfunction of the colonic vasculature[8]. In these conditions, the involvement of the splanchnic vessels can range from mesenteric thrombosis in the largest veins to mild ischemic injury in the mesenteric microcirculation[23,31]. In all these cases, the colon suffers a condition of chronic ischemia. Therefore, when subjected to gas insufflation and mechanical compression during colonoscopy, the previously impaired blood flow can easily drop to a critical level leading to ischemic injury[14].

Bowel preparation

Adequate bowel cleansing is fundamental to perform a high-quality colonoscopy. Various combinations of volume, timing, and adjuvants have been validated for bowel preparations[32].

The isotonic polyethylene glycol (PEG) bowel preparations are generally considered safe as they minimize electrolyte and water loss[33]. Hypertonic bowel preparations include PEG solutions combined with osmotically active molecules to reduce the volume of total fluid, oral sulfate solutions, and magnesium picosulfate solutions. It is generally assumed that hypertonic preparations have potential risks of inducing serum electrolyte imbalance. Furthermore, as they bind with water molecules, PEG monomers cause fluid retention that can increase intraluminal pressure[34]. This effect, combined with an inadequate assumption of fluids during the preparation, can thus lead to dehydration[35] and potentially precipitate an ischemic injury. We found on the Federal Drug Administration Adverse Events Report System a study from Bielefeldt *et al*[36] describing bowel preparations as the suspected causative agents of 60 cases of colonic ischemia. Adjunctive drugs such as bisacodyl and magnesium

citrate are often administered with low volume preparations to improve colon cleansing. Of note, case reports of ischemic colitis have been reported following the intake of bisacodyl[32].

Drugs used for endoscopy

Sedation during colonoscopy aims to reduce discomfort and improve outcomes. Narcotics such as meperidine and fentanyl, benzodiazepines (midazolam) and propofol represent the most common drugs. Although considered safe, sedation has been associated with cardiovascular and pulmonary adverse events such as hypotension and hypoxemia with an overall frequency of 1.1 per 100 procedures[37].

Midazolam, alone or in combination with opioids, is frequently used in colonoscopy as it has a rapid onset and a short duration of action. However, it can induce cardiovascular effects such as vasodilation, depression of myocardial contractility, and hypotension. In a recent study, Kim *et al*[38] demonstrated a reduction of heart rate, systolic and diastolic blood pressure in patients undergoing colonoscopy with midazolam compared to no sedation. Given that hypoperfusion plays a key role in the multifactorial pathogenesis of colonic ischemia, the risk of anesthesia-related cardiovascular depression deserves consideration. In a recent prospective study, Wernli *et al*[39] found that the overall risk of complications associated with colonoscopy increases in patients receiving anesthesia services. Moreover, propofol is often used in combination with benzodiazepines and/or opioids with the combination effects on cardiovascular and respiratory depression still being under investigation.

Bowel wall ischemia due to insufflation/barotrauma

Gas insufflation fills the colon up to the cecum, which is the highest point when the patient is in the left lateral decubitus[40,41]. It is necessary to correctly inspect the mucosa but it leads to an increase in luminal pressure and consequently in vascular resistance. As a result, barotrauma should be considered the main cause of reduction in parietal blood flow during colonoscopy.

When the pressure on the vessels reaches 30-60 mmHg, it causes a reduction in parietal blood flow that can cause mucosal damage in about 20 min[26,42]. Light source air pumps generate a maximal pressure of 375 mmHg, which is reduced by 30%-40% when measured at the tip of the endoscope because of air leakage[43]. In a seminal study, Kozarek *et al*[44] showed that in human cadaver colon air pressures leading to serosal tears, pneumatosis and transmural rupture ranged between 52 and 226 mmHg. Caecum rupture was observed with 81 mmHg of air pressure while tearing of the sigma required pressure of 169 mmHg. In the same study, the authors measured the intraluminal pressure during routine colonoscopies. Intraluminal pressure ranged from 9 to 57 mmHg when the tip of the endoscope was free, while it reached a maximum of 138 mmHg when the tip was impacted against the bowel wall.

Advancement of the scope

Scope manipulation can traumatize the vascular pedicles and lead to mesocolon injury [45]. Anatomical conditions of the colon such as length, mobility, and redundancy can affect procedures such as colonoscopy[46]. The ascending and the descending colon are fixed and retroperitoneal, while the sigmoid and transverse colon are suspended in a double layer of peritoneum, the mesentery or mesocolon[47]. Excessive traction and torsion on the mesocolon can occur in the context of abdominal adhesions or during pull-back maneuvers, slide-by advancement, alpha maneuver, and straightening of the sigmoid loop[48]. The stress on the mesocolon can reduce blood flow, impair microcirculation and activate the inflammatory cascade, eventually leading to vascular thrombosis. Cases of splenic trauma due to colonoscopy are also described in the literature[49,50], suggesting caution with straightening maneuvers and manual compression of the abdominal wall.

CLINICAL PRESENTATION

The symptoms of acute colonic ischemia after colonoscopy do not differ from those of colonic ischemia per se. These include the sudden onset of abdominal pain associated with nausea, vomiting, bloating, and passage of bright bloody diarrhea within 24 h, which often evolve in melena[8]. Zizzo *et al*[22] found that abdominal pain and bloody diarrhea were present in 95% of patients reported to have colonic ischemia after colonoscopy. Moreover, it is assumed that a non-negligible number of cases may be paucisymptomatic and thereby go undetected.

Diagnosis

Given the scant literature focusing on the association between colonoscopy and colonic ischemia, we rely on the general evidence of ischemic colitis per se. In a patient with progressive abdominal pain and/or bloody diarrhea after colonoscopy, colonic ischemia related to the procedure must be suspected along with other possible adverse events (*e.g.*, perforation, post-polypectomy syndrome)[51]. Laboratory tests such as complete blood count, albumin, and acid-base status should be requested to help predict the severity of ischemia.

Computed tomography with intravenous and oral contrast can show signs of ischemia such as mesenteric fat stranding, bowel wall thickening, and abnormal wall enhancement, regardless of the disease severity[52].

Colonoscopy is also the gold standard for the optical diagnosis of colonic ischemia along with biopsies for histological confirmation. When signs of colonic ischemia are found, the endoscopic procedure should be halted at the most distal part of the affected segment[8]. In the existing reports of post-colonoscopy colonic ischemia, the diagnosis was confirmed with a second colonoscopy with biopsies in 85% of the cases [22]. Nevertheless, we believe that the indication to repeat colonoscopy must be weighed against clinical and radiological presentation in order not to worsen existing conditions.

Typical endoscopic signs of ischemia include patchy petechial hemorrhages, erosions, and edematous mucosal inflammation (Figure 3)[26]. Zuckerman *et al*[53] described a typical single linear ulcer running longitudinally along the anti-mesenteric left colonic wall[54]. Notably, Cheema *et al*[55] described a dynamic in-vivo appearance of bowel ischemia, where an intermittent blanching of the colonic mucosa was observed.

Treatment

Treatment of colonic ischemia after colonoscopy should be based on general knowledge of a disease that may vary from a mild to a severe and life-threatening condition. In the available literature, the majority of patients suffered from transient reversible ischemic colitis, which required just a conservative therapy[2]. In such cases, therapeutic options included general supportive measures, intravenous hydration, bowel rest, and correction of possible precipitating conditions including withdrawal of putative causative medications[36]. On the other hand, emergent surgery was needed in two cases of irreversible ischemia[22].

PREVENTION

Double-check colonoscopy indications and risk factors

Physicians must be aware of risk factors for colonic ischemia such as advanced age, recent vascular surgery, atherosclerotic and vascular disease such as a peripheral artery, cerebrovascular, coronary artery, and renovascular disease[8]. Additionally, a careful drug history should be obtained with particular attention to constipation-inducing agents[12]. A history of either coronary heart disease, peripheral obliterative arteriopathy, or ischemic stroke could be suggestive for the presence of mesenteric thrombosis while abdominal angina, as well as gastroparesis and gastric ulcers, could be indirect signs of chronic ischemia[4]. Doppler ultrasound and/or computed tomography angiography of the major abdominal arteries can be helpful when suspicion arises. Antiplatelet and anticoagulant agents should be managed according to the available clinical guidelines. As a result, whether to perform a colonoscopy in high-risk cases must always be weighed in every case by accurately balancing colonoscopy indications with the individual risk factors (Table 1).

Optimize bowel preparation

Bowel laxatives determine a large loss of fluids that can lead to volume depletion. Therefore, physicians should assess the presence of cardiovascular and renal comorbidities before recommending a specific bowel preparation, above all in elderly and fragile patients.

Phosphate-based preparations should be avoided due to their adverse effects on kidney function[32]. On the contrary, high volume PEG-based regimens account for a higher safety profile due to their osmotically balanced formulation and reduced sodium load[56]. However, the high volume accounts for poor tolerability in elderly people. Alternatively, low-volume PEG-based regimens should be considered[56]. In

Table 1 Summary of proposed pathogenetic factors for colonoscopy-related mesenteric ischemia and suggested interventions to reduce the risk

Pathogenetic factor	Mechanism of action	Type of ischemia	Prevention
Splanchnic circulation impairment	Chronic mesenteric ischemia (atherosclerosis, smoking habits), parietal vessels inflammation (connective tissue diseases, LES, antiphospholipid syndrome)	Vascular thrombosis; microcirculatory mild ischemic injury	Careful evaluation of medical history. Specific and indirect symptoms assessment. Antiplatelet agents according to guidelines. Consider pre-colonoscopy assessments (serum electrolyte, color-Doppler ultrasound)
Bowel preparation (hypertonic, isotonic, laxative)	Serum electrolyte imbalance, dehydration. Potential additional risk if laxative were used (*i.e.*, bisacodyl)	Multifactorial	Give specific information. Consider high-volume isotonic formulations; split-dose regimens; avoid bisacodyl-containing preparations
Sedation (midazolam, opioids, propofol)	Vasodilation, depression of myocardial contractility and hypotension	Multifactorial	Minimal sedation protocol (response to verbal stimulation, patent airways, spontaneous ventilation, and normal cardiovascular function) in high-risk patients. Consider prophylactic fluid infusion
Air insufflation/barotrauma	Increased luminal pressure and consequent vascular resistance	Non-occlusive mesenteric ischemia	Use carbon dioxide (CO_2) insufflation. Consider water-exchange colonoscopy technique
Scope manipulation	Mechanical stress on mesocolon, blood flow reduction, microcirculatory damage, and inflammatory cascade activation	Vascular thrombosis	Procedure interruption in case of intense discomfort or endoscopic findings of ischemia. Consider pediatric or "ultra-slim" colonoscopes. Reschedule or reconsider indication in case of complex exams

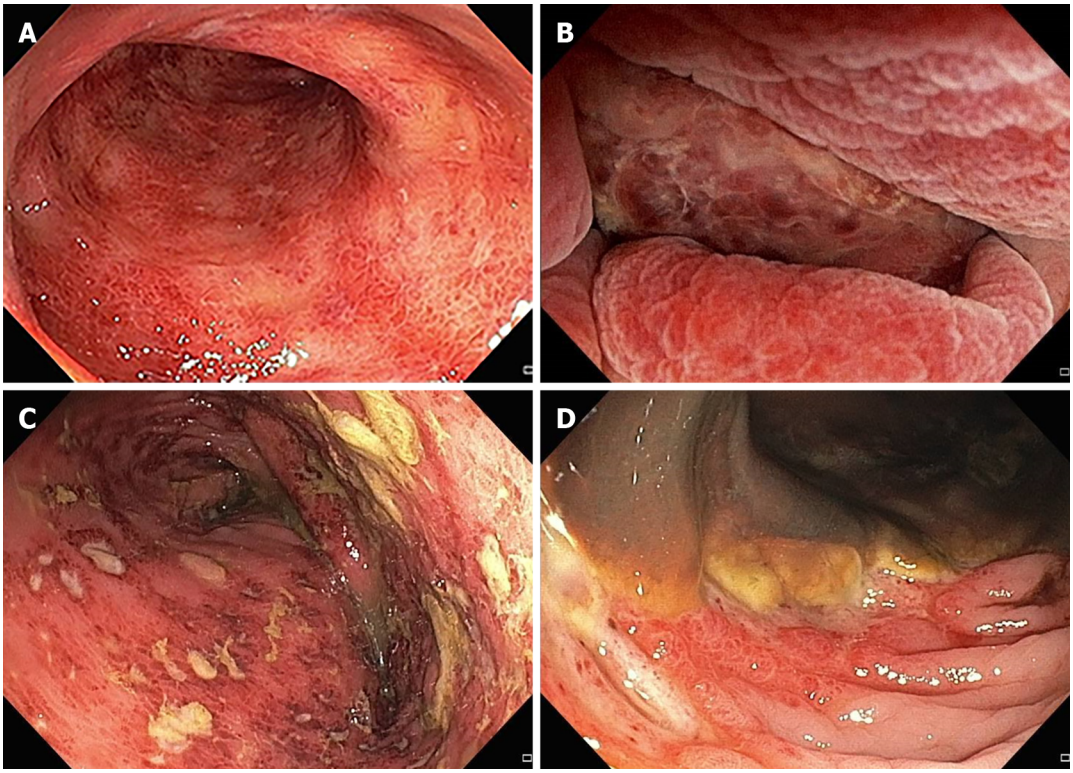


Figure 3 Endoscopic signs of ischemia, showing moderate diffuse erythema (A), severe erythema with mucosal edema and erosions (B), multiple ulcerations and inflammatory exudate (C), necrosis (D).

both cases, split regimens should always be advised with written and oral information to achieve the best patients' adherence and optimize the results[32].

As all bowel preparations can determine electrolyte imbalance, baseline serum testing should be obtained in patients with risk factors. Finally, to prevent dehydration induced by osmotic diarrhea, patients should be instructed to drink enough replacement water prior, during, and after colonoscopy[57]. Because of reports about bisacodyl-associated colonic ischemia, this drug should be avoided in patients with multiple risk factors[58].

Check sedation protocol

Sedation in colonoscopy is generally safe. However, sedation-related adverse events such as hypotension and hypoxemia could act as precipitating factors in individuals at risk for colonic ischemia. Therefore, physicians should understand the pharmacokinetics, pharmacodynamics, and the potential adverse effects of each sedative agent [59]. Patients should be evaluated before colonoscopy to assess their cardiopulmonary risk to better gauge the sedation protocol.

Minimal sedation, defined as a normal response to verbal stimulation and ability to maintain patent airways, spontaneous ventilation, and cardiovascular function[59] should be preferred in patients at risk for colonic ischemia. Unsedated endoscopy could also be considered in selected patients.

Monitoring during colonoscopy includes repeated assessment of blood pressure, heart rate, and pulse oximetry. Oxygen should be routinely administered to reduce the risk of hypoxemia.

Hypotension is considered a key factor for the development of colon hypoperfusion. Tang *et al*[60] found that, in patients undergoing sedated colonoscopy with fentanyl and midazolam, the presence of pre-procedural low blood pressure was a primary risk factor for the development of hypotension during the procedure. Moreover, there was no evidence to suspend antihypertensive drugs in the morning before colonoscopy to prevent procedural hypotension. Even if prophylactic intravenous fluid infusion did not prevent procedural hypotension in a study by Leslie *et al*[61], it could be considered in patients with pre-existent low blood pressure.

Minimize air insufflation

Although air insufflation through endoscopic light source air pumps has been the most common technique for luminal distension, it is now recognized that this method can potentially damage the colon[43]. On the other hand, carbon dioxide (CO_2) is rapidly absorbed from the bowel and eliminated through the lungs. In an animal study, Brandt *et al*[62] demonstrated that CO_2 insufflation minimized the reduction in blood flow and intraluminal pressure compared to air insufflation during colonoscopy. Moreover, it has been demonstrated that CO_2 insufflation is more advantageous than air in terms of reduced procedural and post-procedural pain[63,64]. Other studies showed less residual gas with CO_2 insufflation at 1 h after colonoscopy compared to air insufflation[65], and even suggested that CO_2 has a potential dilating effect on colonic microvasculature when insufflated at low pressure[66].

Water-assisted colonoscopy may represent another way to minimize air insufflation [40,67]. Moreover, as procedural pain is also reduced with this technique, the amount of sedation can be minimized[68]. Therefore, CO_2 insufflation and water-assisted colonoscopy should be adopted in patients with multiple risk factors for colonic ischemia.

Minimize mechanical trauma

The sudden observation of a dry and pale mucosa during a colonoscopy could represent a real-time sign of ischemia[55,69]. In these cases, interruption of the procedure should be always considered. Special attention should be taken in patients undergoing combined procedures such as same-session gastroscopy and colonoscopy.

Experienced endoscopists often rely on torqueing maneuvers to pass the sigmoid tract, thus stretching the mesentery. Using left/right controls can reduce the mechanical stress on the mesentery, as well as patient discomfort. Caution with straightening maneuvers and manual compression of the abdominal wall is also suggested[49]. Change of decubitus to right or supine position can be useful in difficult segments[19].

Consider switching to pediatric or ultraslim endoscopes in difficult cases as thinner instruments may allow negotiation of fixed angulations. Finally, if the patient still manifests discomfort, consider rescheduling the procedure with a more experienced operator and/or anesthesia care.

CONCLUSION

In our patient, the abdominal computed tomography performed after colonoscopy showed thrombosis of the main abdominal arteries. We suppose that in this fragile person, bowel preparation led to general dehydration and hypoperfusion. Subsequently, colonoscopy-related procedural factors (sedation, advancement of the scope, gas insufflation) could have precipitated a pre-existing state of chronic

mesenteric ischemia, leading to thrombosis of the colonic vascular supply, and to the fatal acute colonic injury.

In conclusion, colonoscopy-related colonic ischemia is a rare complication that needs to be considered in the differential diagnosis of abdominal pain after colonoscopy, particularly in elderly, fragile, and comorbid patients. Given the increasing number of colonoscopies performed every year in an aging population with multiple comorbidities, endoscopists must be aware of this dreaded adverse event and should adopt all the possible preventive measures.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 14; 27(42): 7311-7323

DOI: 10.3748/wjg.v27.i42.7311

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

ORIGINAL ARTICLE

Basic Study

Fusobacterium nucleatum colonization is associated with decreased survival of *helicobacter pylori*-positive gastric cancer patients

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Author contributions: Hsieh YY and Tung SY contributed equally to this work; Hsieh YY, Tung SY, Chang TS, Wei KL, Chen WM, and Wu CS recruited patients for the study and collected biopsies; Deng YF performed *in vitro* and sequencing experiments; Lai YH and Pan HY performed data and statistical analyses; Wu CS and Li C designed and supervised the study; Li C wrote the manuscript; All authors reviewed the manuscript.

Supported by Chiayi Chang Gung Memorial Hospital grants, including No. CORPG6G0021, No. CORPG6G0022 and No. CORPG6G0023 to Wu CS; No. CORPG6G0061, No. CORPG6G0062 and No. CORPG6G0063 to Tung SY; No. CORPG6G0071, No.

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Abstract

BACKGROUND

An increased amount of *Fusobacterium nucleatum* (*F. nucleatum*) is frequently detected in the gastric cancer-associated microbiota of the Taiwanese population. *F. nucleatum* is known to exert cytotoxic effects and play a role in the progression of colorectal cancer, though the impact of *F. nucleatum* colonization on gastric cancer cells and patient prognosis has not yet been examined.

AIM

To identify *F. nucleatum*-dependent molecular pathways in gastric cancer cells and to determine the impact of *F. nucleatum* on survival in gastric cancer.

METHODS

Coculture of *F. nucleatum* with a gastric cancer cell line was performed, and changes in gene expression were investigated. Genes with significant changes in expression were identified by RNA sequencing. Pathway analysis was carried out to determine deregulated cellular functions. A cohort of gastric cancer patients undergoing gastrectomy was recruited, and nested polymerase chain reaction was performed to detect the presence of *F. nucleatum* in resected cancer tissues. Statistical analysis was performed to determine whether *F. nucleatum* colonization

CORPG6G0072 and No. CORPG6G0073 to Hsieh YY.

Institutional review board statement: All data acquisition and use of clinical specimens in this study were performed in accordance with the Declaration of Helsinki. Resected gastric cancer specimens were obtained from Tissue Bank, Department of Medical Research, Chang Gung Memorial Hospital at Chiayi. All the patients participating in this study were informed about the study and signed a written informed consent.

Conflict-of-interest statement: The authors declare that they have no competing interests.

Data sharing statement: Participants gave informed consent for data sharing.

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Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Taiwan

Peer-review report's scientific quality classification

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

Received: April 11, 2021
Peer-review started: April 11, 2021
First decision: May 24, 2021
Revised: May 31, 2021
Accepted: September 14, 2021

affects patient survival.

RESULTS

RNA sequencing and subsequent pathway analysis revealed a drastic interferon response induced by a high colonization load. This response peaked within 24 h and subsided after 72 h of incubation. In contrast, deregulation of actin and its regulators was observed during prolonged incubation under a low colonization load, likely altering the mobility of gastric cancer cells. According to the clinical specimen analysis, approximately one-third of the gastric cancer patients were positive for *F. nucleatum*, and statistical analysis indicated that the risk for colonization increases in late-stage cancer patients. Survival analysis demonstrated that *F. nucleatum* colonization was associated with poorer outcomes among patients also positive for *Helicobacter pylori* (*H. pylori*).

CONCLUSION

F. nucleatum colonization leads to deregulation of actin dynamics and likely changes cancer cell mobility. Cohort analysis demonstrated that *F. nucleatum* colonization leads to poorer prognosis in *H. pylori*-positive patients with late-stage gastric cancer. Hence, combined colonization of *F. nucleatum* and *H. pylori* is a predictive biomarker for poorer survival in late-stage gastric cancer patients treated with gastrectomy.

Key Words: *Fusobacterium nucleatum*; *Helicobacter pylori*; Gastric cancer; Survival; Interferon; Mobility

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Core Tip: *Fusobacterium nucleatum* (*F. nucleatum*) is frequently enriched in the gastric cancer-associated microbiota. Here, we showed that *F. nucleatum* solicits a rapid but transient interferon response from gastric cancer cells. In addition, *F. nucleatum* leads to deregulation of the genes functioning in regulation of actin filament dynamics, likely changing cell mobility. In a patient cohort receiving gastrectomy, combined infection of *F. nucleatum* and *Helicobacter pylori* infection incurs poorer survival, indicating these two pathogens act synergistically to promote invasiveness of gastric cancer. Our finding suggests that *F. nucleatum* is a biomarker as treatment outcome predicator.

Citation: Hsieh YY, Tung SY, Pan HY, Chang TS, Wei KL, Chen WM, Deng YF, Lu CK, Lai YH, Wu CS, Li C. *Fusobacterium nucleatum* colonization is associated with decreased survival of *helicobacter pylori*-positive gastric cancer patients. *World J Gastroenterol* 2021; 27(42): 7311-7323
URL: https://www.wjgnet.com/1007-9327/full/v27/i42/7311.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i42.7311

INTRODUCTION

Disruption of the normal gastrointestinal flora is often associated with pathogenic conditions. In general, the extreme acidity and thick protective mucosa of the gastric environment limit the complexity and abundance of the microbiota and prevent direct gastric epithelium colonization by pathogenic microbes[1]. However, *Helicobacter pylori* (*H. pylori*), which is well recognized as a risk factor for gastric cancer, is able to penetrate the mucosa layer and establish long-term colonization[2]. Indeed, virulence factors produced by this bacterium facilitate the transformation of gastric mucosal cells and lead to a drastic increase in the risk of gastric cancer[2,3].

Although it is well established that *H. pylori* is more frequently found in gastric cancer patients than in noncancer controls[4], recent microbiota profiling studies have revealed that the abundance of *H. pylori* in the gastric microbiota is frequently decreased in gastric cancer patients compared with that in noncancer patients[5,6]. Our hypothesis to account for the decrease in *H. pylori* abundance in gastric cancer is microbial succession: Once colonization occurs, *H. pylori* creates a niche microenvir-

Article in press: September 14, 2021
Published online: November 14, 2021

P-Reviewer: Krzyżek P
S-Editor: Wu YXJ
L-Editor: Filipodia
P-Editor: Liu JH

onment on the gastric epithelium that facilitates the colonization of secondary settler bacteria. Accordingly, the predominance of *H. pylori* in the microenvironment can be replaced by other bacteria after a prolonged colonization period. It is possible that the secondary gastric microbes also participate in promoting the development of gastric cancer[7].

Advanced sequencing technology has enabled profiling of the microbiota without the need to isolate pure cultures, and in a previous study employing this experimental approach, we identified *Fusobacterium nucleatum* (*F. nucleatum*) as being enriched in the gastric cancer-associated microbiota. An increased presence of *F. nucleatum* in the colorectal cancer-associated microbiota has also been reported[8,9]. *F. nucleatum* colonization correlates with high microsatellite instability, disruption of the mismatch repair mechanism, and poor prognosis[10]. The genomic instability that is observed is likely mediated by the metabolites produced by *F. nucleatum*. One such metabolite is hydrogen sulfide, which has been shown to generate reactive oxygen species, induce DNA damage, and cause single-nucleotide mutations[11]. Hence, it is possible that *F. nucleatum* promotes oncogenesis by acting as a DNA-damaging agent. In fact, *F. nucleatum* has been shown to promote the growth and metastasis of colorectal cancer [12,13], and the level of *F. nucleatum* in the colorectal cancer-associated microbiota correlates with poor patient prognosis[14,15]. Therefore, *F. nucleatum* may be used as a prognostic biomarker for colorectal cancer.

Detection of *Fusobacterium* DNA using polymerase chain reaction (PCR) facilitates screening of colorectal cancer by increasing the sensitivity of the standard fecal immunochemical test[15]. Although the role of *F. nucleatum* in colorectal cancer has been intensively studied, it remains unclear whether this bacterium exerts a similar oncogenic effect on the gastric epithelium. In this study, a nested PCR-based method was developed to detect the presence of *F. nucleatum* in resected gastric cancer tissue specimens. Based on statistical analysis, we found that the risk of *F. nucleatum* colonization is greatly increased in patients with late-stage gastric cancer. Moreover, *F. nucleatum* colonization was associated with a poor prognosis in *H. pylori*-positive patients. Our findings suggest that invasion of *F. nucleatum* into the gastric cancer-associated microenvironment promotes gastric cancer aggressiveness and subsequently leads to poorer prognosis.

MATERIALS AND METHODS

Coculturing of F. nucleatum with gastric cancer cells

F. nucleatum strain ATCC25586[16] was obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan). The bacteria were cultured on EG culture medium containing 2.5 g Lab-Lemco powder, 10 g proticasepeptone, 5 g yeast extract, 4 g glucose, 0.5 g starch, 0.2 g L-cystine, 0.5 g L-cysteine HCl, 4 g Na_2HPO_4, 15 g Bacto-Agar, and 50 mL defibrinated horse blood per liter under anaerobic conditions using a BD GasPak system (Thermo Fisher Scientific, Waltham, MA, United States). The bacteria were scrapped from a plate and resuspended in Dulbecco's Modified Eagle's Medium. The number of cells per millimeter in the resuspended medium was determined using light microscopy. The correlation between the observed cell number and colony forming units was determined by reculturing the bacteria after serial dilutions. The gastric cancer cell line 008L-C2 used in this study was originally isolated from resected gastric cancer tissue. The cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. *F. nucleatum* strain ATCC25586 was cultured with 008L-C2 gastric cancer cells under anaerobic conditions at 37 °C and 5% CO_2 and harvested when the coculture experiment was performed. In coculture experiments, the initial multiplicity of infection (MOI) was 10 and 100. After 0, 24, and 72 hours of coculture with *F. nucleatum*, the cells were collected and washed twice with phosphate-buffered saline. Total RNA was extracted from the washed cells using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's protocol, and RNA expression analysis was conducted using next-generation sequencing.

RNA sequencing

The integrity and concentration of purified RNA samples were determined using capillary electrophoresis with a TapeStation 2200 instrument (Agilent, Santa Clara, CA, United States) and fluorometric analysis (Qubit fluorometer; Invitrogen, Waltham, MA, United States). Libraries for RNA sequencing analysis were prepared using a SureSelect strand-specific mRNA library preparation kit (Agilent); the manufacturer's

protocol was closely followed. The libraries were pooled and sequenced using a NextSeq 550 sequencer. Quality filtering, mapping, annotation, and calculation of gene expression levels were performed using CLC Genomic Workbench v.12.0.3 (Qiagen, Redwood City, CA, United States). The RNA level is expressed as transcripts per million. The sequencing data were deposited in the Sequence Read Archive, National Center for Biotechnology Information, United States. The BioProject ID is PRJNA-630089. The BioSample accession numbers are SAMN14823957, SAMN14823958, SAMN14823959, SAMN14823960, and SAMN14823961.

Gastric cancer specimens and DNA extraction

Resected cancer tissues from patients undergoing gastrectomy were obtained from Chiayi Chang Gung Memorial Hospital Tissue Bank. The acquisition and use of clinical specimens in this study were carried out in accordance with the Declaration of Helsinki. This study was approved by the Institutional Review Board of Chiayi Chang Gung Memorial Hospital (Institutional Review Board approval No. 201700169A3, No. 201700172A3, and No. 201700173A3). Frozen biopsies were briefly rinsed in phosphate-buffered saline to remove extra mucus. The rinsed specimens were immersed overnight in RNAlater reagent (Thermo Fisher Scientific) and stored at -80 °C. The biopsies were pulverized in TRI reagent (Thermo Fisher Scientific) and centrifuged to remove undissolved debris. Total DNA, including both cellular and microbial DNA, was extracted according to the manufacturer's protocol, and the concentration of DNA was determined by fluorometric quantification.

Detection of H. pylori and F. nucleatum

The tissue specimens were examined for the presence of *H. pylori* using a standard rapid urease test. The presence of *F. nucleatum* in the specimens was determined by nested PCR detection of the *NusG* gene. The forward and reverse primer sequences used for the first-stage PCR were 5′-TGTTAGAGGAAAGCCCAAGAAG and 5′-CTTCTTCCATAGGAATAGGGTCAG, respectively. The initial amplification cycle (denaturation) was as follows: 94 °C for 5 min; followed by 36 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension step at 72 °C for 5 min. The product of the first-stage amplification was directly used in the second-stage PCR. The forward and reverse primer sequences of the second-stage PCR were 5′-GCTTGAAATGGAAGCTACAAGAG and 5′-GGTCAGAACCAACTCCTACAAA, respectively. The second-stage PCR amplification cycle parameter was identical to that of the first-stage PCR. The sequence of the PCR product was determined by dideoxynucleotide sequencing to confirm that the amplified product is the *F. nucleatum NusG* target sequence. The PCR product was examined by nondenaturing 6% polyacrylamide gel electrophoresis and documented. Statistical analysis was carried out using SAS/STAT statistical analysis software v. 9.4 (College Station, TX, United States). Survival probability was calculated with Kaplan-Meier analysis.

RESULTS

In our previous metagenomic analysis, we discovered that *F. nucleatum* colonizes and becomes enriched in the gastric cancer-associated microbiota[17]. Among the 11 cancer biopsies collected in that study, four showed *F. nucleatum* colonization, suggesting that *F. nucleatum* is a frequent cocolonizer among gastric cancer patients in the southwestern region of Taiwan. Moreover, clinical data confirmed that *F. nucleatum* colonization is frequently observed in gastric cancer patients. Nevertheless, the pathogenic effects of *F. nucleatum* have not been investigated in gastric cancer cells. Thus, to explore the role of *F. nucleatum* in gastric cancer, we examined gastric cancer cell growth using an *in vitro* coculture system using the gastric cancer cell line 008L-C2, derived from resected gastric cancer tissue. During the incubation period, an increase in *F. nucleatum* abundance was observed under a microscope, though we did not determine the precise doubling time of *F. nucleatum*.

RNA sequencing analysis revealed that the presence of *F. nucleatum* is associated with dose- and time-dependent changes in the gene expression profile of cancer cells. After 24 h of coculture with *F. nucleatum* at a low MOI, only a limited number of marginally expressed genes (transcripts per million < 1) exhibited more than four-fold changes (Figure 1). In contrast, the expression level of a specific set of genes was strongly upregulated at a high MOI (100) (Figure 1). After 72 h of coculture, low-MOI treatment led to a significantly higher number of genes with more than four-fold increases in expression (Figure 1). However, the number of strongly upregulated genes

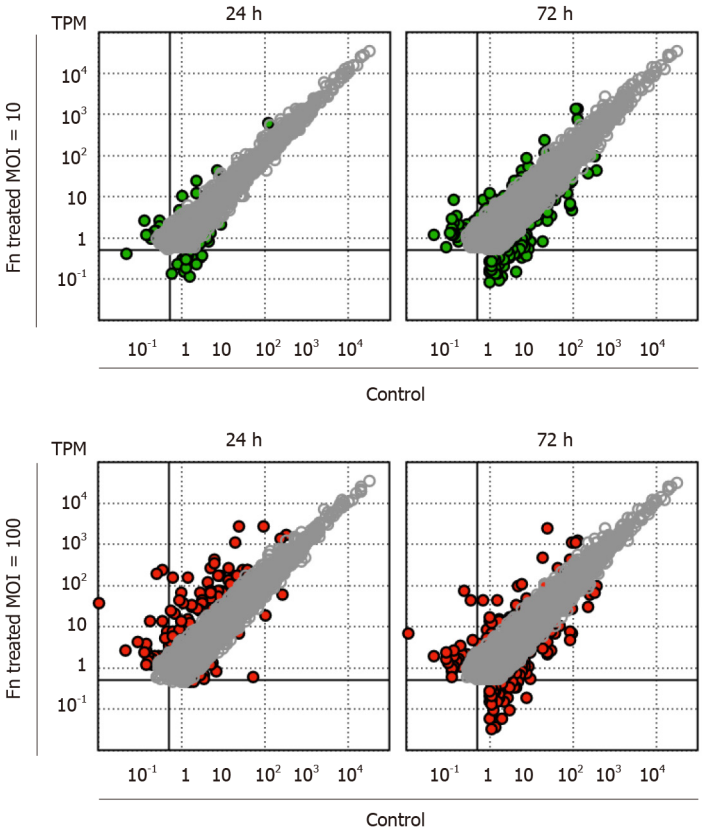


Figure 1 Changes in the gene expression profile of 008L-C2 cancer cells cocultured with *Fusobacterium nucleatum*. *Fusobacterium nucleatum* was added to the cell culture at multiplicity of infection (MOI) 10 and 100. After 24 h and 72 h of incubation, the cells were rinsed extensively to remove unattached bacteria. RNA was collected and analyzed using RNA sequencing. The cells collected at time 0 were used as control. Sequencing reads were trimmed and mapped to hg19 using CLC Genomic Workbench v.12.0.3. The colored dots (green for MOI = 10 and red for MOI = 100) are genes showing more than four-fold change at the respective incubation time. Genes with transcripts per million < 1 in all datasets and less than a four-fold change are not shown.

in high MOI-treated cells decreased after longer incubation, and there was an increased number of genes with more than four-fold decreased expression (Figure 1). Our results indicate a rapid and strong cellular response to a large amount of *F. nucleatum*. Additionally, *F. nucleatum* caused a prolonged change in gastric cancer cells.

Ontological analysis indicated that interferon and its response genes as well as inflammatory cytokines are immediately activated by high MOI treatment with *F. nucleatum*. The activated genes, including MX1, MX2, interferon induced protein (IFI)35, IFIT1, IFIT2, IFIT3, IFI44, IFI44L, IFITM1, IFITM3, IFIH1, and IFI6[18], are well-known interferon response genes. Furthermore, tripartite motif-containing 14[19,20], interferon -stimulated gene 15[21], ubiquitin specific peptidase 18[22], 2'-5'-oligoadenylate synthetase (OAS)1, OAS2, and OAS3[23,24] were found to be involved in interferon-dependent antiviral activity (Figure 2A and B). In addition to interferon response genes, simultaneous increases in interleukin (IL)6, IL8, IL32, C-X-C motif ligand (CXCL)1, CXCL2, and CXCL6 expression were observed in the high-MOI-treated cells after 24 h of exposure (Figure 2C). In contrast to the short-term activation profile observed for interleukins and chemokines, C-C ligand 2 remained highly expressed even after 72 h of exposure (Figure 2A). Not only genes involved in the regulation of immunological functions but also those related to cell mobility and adhesion were dysregulated by *F. nucleatum* colonization. For instance, epithelial-stromal interaction 1[25,26] and intercellular adhesion molecule 1[27], both of which are involved in cell-matrix interaction, were upregulated at 24 h and returned to basal levels after 72 h of incubation (Figure 2B). Other cytoskeleton and cell adhesion genes were activated in a distinct pattern, and actin alpha 2; actin, alpha cardiac muscle precursor 1; actin gamma 2, smooth muscle; calponin 1, endothelin 1, and cell migration inducing hyaluronan binding protein were continuously stimulated by *F. nucleatum* (Figure 2C). Increased expression of these genes was time dependent but not dose (MOI) dependent, indicating that chronic colonization with a small number of *F. nucleatum* bacteria may lead to long-term strong effects on cytoskeletal dynamics and cell mobility.

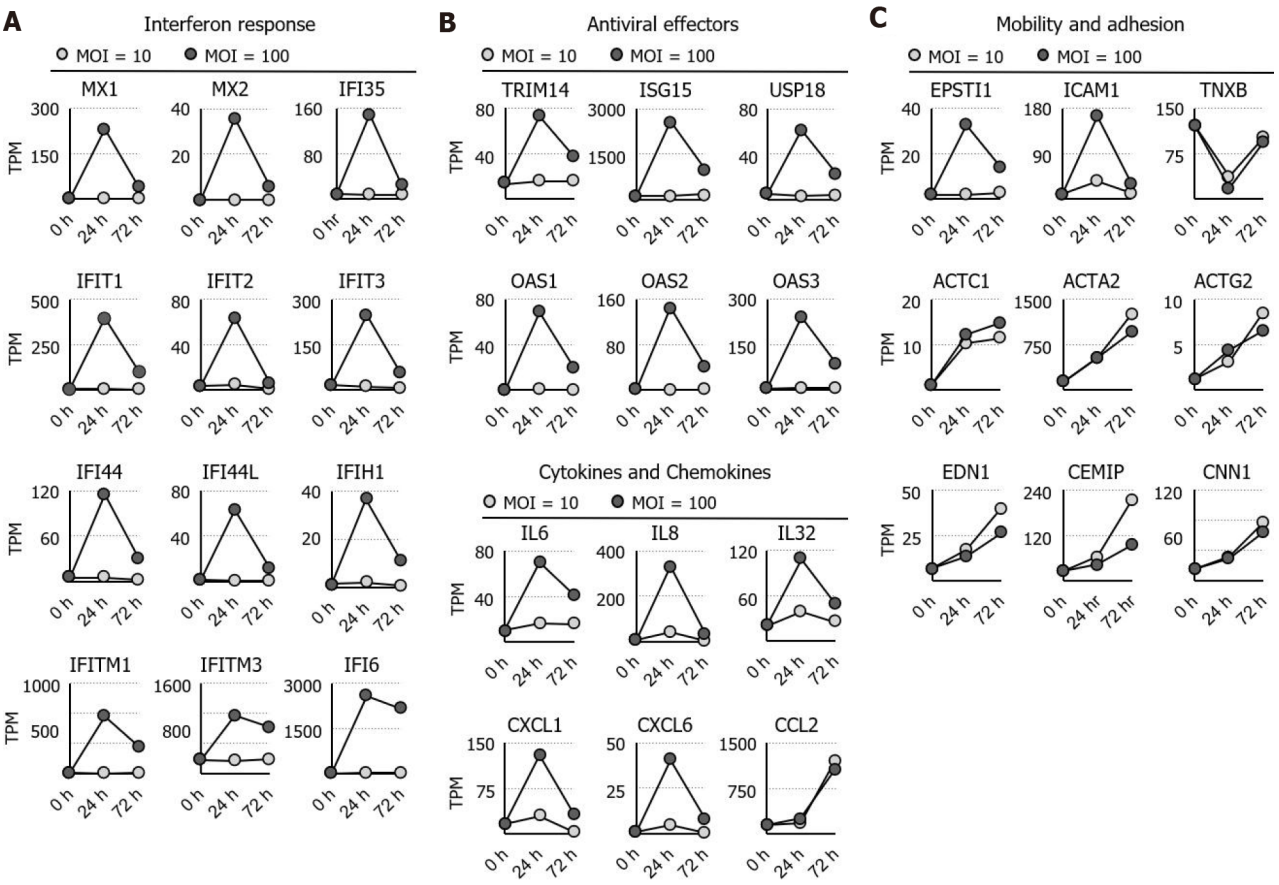


Figure 2 Identification and ontological analysis of the RNA sequencing dataset. A: The expression dynamics of the interferon response, cytokines, and chemokines genes are shown; B: The expression dynamic of the antiviral genes is shown; C: The expression dynamics of actin, its regulators, and genes involved in cell adhesion are shown. MOI: Multiplicity of infection; TPM: Transcripts per million.

Overall, our data show that coculturing gastric cancer cells with *F. nucleatum* results in a short-term immune response and continuous dysregulation of actin-related signaling *in vitro*. The change in actin cytoskeleton dynamics was associated with an increase in cell mobility, thereby promoting the invasiveness and metastasis of gastric cancer. Therefore, we investigated whether *F. nucleatum* colonization influences the survival of gastric cancer patients after gastrectomy. Gastric cancer progression is marked by tumor spread and distant metastasis, which are associated with increased cancer cell mobility. To test our hypothesis, we examined resected gastric cancer tissues from patients who underwent gastrectomy. The survival rate of these patients was 67%, which is higher than the 37% survival rate reported by Cancer Registry Annual Report, 2018, Taiwan. The clinical and pathological characteristics of the patient cohort are presented in Table 1.

To determine the presence of *F. nucleatum* in the specimens, we employed a nested PCR method with increased detection sensitivity and specificity. In a previous study utilizing conventional PCR, *F. nucleatum* was found at relatively low frequencies in gastric cancer specimens[28]. Our results obtained by metagenomic profiling indicated that the *F. nucleatum* frequency is much higher. The discrepancy could account for the higher frequency of *F. nucleatum* in the tested cohort, suggesting that the observed phenomenon is specific for this region of Taiwan. Nonetheless, the previously observed low frequency might be associated with methodological limitations resulting in the detection of lower amounts of *F. nucleatum*. This is a likely scenario, considering that resected cancer tissues may contain less surface mucosa. Nested PCR allowed for higher *F. nucleatum* detection sensitivity.

The detection target in this study was the highly conserved *NusG* gene of *F. nucleatum*[28,29]. The sizes of the first- and second-stage PCR products were expected to be 175 bp and 124 bp, respectively. The PCR product was resolved by acrylamide electrophoresis, and identity was confirmed by dideoxynucleotide sequencing. To validate the nested PCR protocol, we tested DNA specimens from our previous study. The method allowed us to identify the majority of *F. nucleatum*-positive specimens, except for those with an exceptionally low bacterial load. The positive identification

Table 1 The clinicopathalogical characteristics of the study cohort

Sex	Age	Stage	TNM classification	Tumor size	*H. pylori*	*F. nucleatum*
M	70	IA	pT1aN0	1.2 cm × 0.5 cm	Positive	Positive
F	78	IA	pT1bN0	7 cm × 5 cm	Positive	Negative
F	84	IA	pT1aN0Mx	2 cm × 2 cm	Positive	Negative
M	73	IA	pT1aN0Mx	0.5 cm × 0.3 cm	Positive	Negative
M	73	IA	pT1aN0	1.5 cm × 0.3 cm	Positive	Negative
M	73	IA	pT1aN0MX	6 cm × 6 cm	Negative	Negative
M	64	IA	pT1bN0MX	9.5 cm × 1.5 cm	Negative	Negative
M	67	IA	pT1bN0	2.5 cm × 2 cm	Positive	Negative
M	66	IA	pT1bN0	0.8 cm × 0.5 cm	Positive	Negative
M	65	IA	pT1bN0	3 cm × 2.7 cm	Positive	Negative
F	68	IB	pT2N0Mx	6 cm × 4 cm	Negative	Positive
M	73	IB	pT2N0Mx	1.5 cm × 1.5 cm	Positive	Positive
M	60	IB	pT2N0	4.5 cm × 4 cm	Positive	Positive
F	68	IB	pT2N0	3 cm × 1.8 cm	Positive	Negative
F	67	IB	pT2N0	1.5 cm × 1.0 cm	Negative	Negative
M	71	IB	pT1bN1MX	2 cm × 1.8 cm	Positive	Negative
M	69	IB	pT2N0	2.2 cm × 2.1 cm	Positive	Negative
F	86	IIA	pT3N0Mx	5 cm × 4.5 cm	Positive	Positive
F	87	IIA	pT3N0Mx	7.5 cm × 7.0 cm	Negative	Positive
M	68	IIA	pT3N0	4 cm × 3.5 cm	Positive	Positive
M	58	IIA	pT2N1	7 cm × 6 cm	Positive	Negative
M	50	IIA	pT2N1MX	3.5 cm × 3.5 cm	Negative	Negative
M	71	IIA	pT3N0	1 cm × 1 cm	Positive	Negative
F	69	IIB	pT3N1	1.2 cm × 1.8 cm	Positive	Positive
M	61	IIB	pT4aN0	2 cm × 1.8 cm	Positive	Positive
M	74	IIB	pT2N2	4.5 cm × 2.0 cm	Positive	Negative
M	64	IIB	pT4aN0	2.6 cm × 2.0 cm	Positive	Negative
M	75	IIB	pT2N2	4.2 cm × 4.0 cm	Negative	Negative
M	76	IIIA	pT3N2MX	5 cm × 4.5 cm	Positive	Positive
M	79	IIIA	pT3N2Mx	3.5 cm × 3.5 cm	Negative	Negative
M	51	IIIA	pT4N1	8 cm × 6 cm	Positive	Negative
M	63	IIIA	pT3N2	3.2 cm × 2.2 cm	Negative	Negative
M	87	IIIB	pT3N3b	6.5 cm × 5 cm	Positive	Positive
F	64	IIIB	pT3N3	7 cm × 5 cm	Positive	Negative
F	65	IIIB	pT4aN2	12 cm × 10 cm	Negative	Negative
F	78	IIIB	pT3N3b	3 cm × 2 cm	Positive	Negative
M	59	IIIB	pT4bN1Mx	8.5 cm × 7.5 cm	Positive	Negative
M	58	IIIB	pT3N3aMX	3 cm × 3 cm	Negative	Negative
M	87	IIIB	pT4aN2	3 cm × 2 cm	Positive	Negative
M	83	IIIB	pT4aN2	6 cm × 5 cm	Negative	Negative
M	69	IIIB	pT4aN2	6 cm × 5 cm	Positive	Negative

M	65	IIIB	pT3N3b	5.5 cm × 4 cm	Positive	Negative
F	73	IIIC	pT4N3a	4.5 cm × 4 cm	Positive	Positive
F	74	IIIC	pT4a N3b	2.0 cm × 2.0 cm	Positive	Positive
M	57	IIIC	pT4bN2Mx	4 cm × 4 cm	Positive	Positive
M	67	IIIC	pT4aN3b	8 cm × 7.5 cm	Positive	Positive
F	53	IIIC	pT4bN3a	6 cm × 5 cm	Negative	Negative
F	72	IIIC	pT4aN3a	7 cm × 2 cm	Negative	Negative
F	79	IIIC	pT4aN3a	4.5 cm × 2.5 cm	Negative	Negative
M	52	IIIC	pT4bN3aMX	8 cm × 6 cm	Positive	Negative
M	76	IIIC	pT4aN3a	2.0 cm × 1.8 cm	Positive	Negative
M	71	IIIC	pT4aN3a	4.8 cm × 4.5 cm	Positive	Negative
F	47	IV	pT4aN3aM1	4.5 cm × 4.5 cm	Positive	Positive
M	47	IV	pT4bN3aM1	6 cm × 3 cm	Negative	Positive
M	69	IV	pT4N3M1	5 cm × 4.5 cm	Positive	Positive
M	73	IV	pT3N3bM1	12 cm × 10.5 cm	Positive	Positive
M	75	IV	pT1bN1M1	2.2 cm × 2.0 cm	Positive	Negative
M	66	IV	pT4aN3bM1b	7 cm × 4 cm	Positive	Negative
M	75	IV	pT4bN3bM1	3 cm × 3 cm	Positive	Negative
M	70	IV	pT3N3aM1	9 cm × 8 cm × 2.5 cm	Negative	Negative

M: Male; F: Female; TNM: Tumor, Node, Metastasis; *H. pylori*: *Helicobacter pylori*; *F. nucleatum*: *Fusobacterium nucleatum*.

rate was 90%, with no false-positives. Thus, we used nested PCR to assess the presence of *F. nucleatum* in the resected gastric cancer tissues used in this study (Figure 3A). The results showed 19 of the 60 examined specimens to be positive for *F. nucleatum*. The other 41 specimens were negative or showed only marginal PCR product levels, all of which were defined as *F. nucleatum* negative. Accordingly, the proportion of *F. nucleatum*-positive patients was approximately one-third of the cohort. The results are consistent with our previous study examining upper endoscopic-collected gastric biopsies.

The risk of *F. nucleatum* colonization was analyzed against clinical characteristics and cancer stages (Figure 3B), indicating that older age (≥ 75) is not associated with the risk of *F. nucleatum* colonization (P value = 0.636). Although *H. pylori* colonization appears to be associated with a higher risk of *F. nucleatum* colonization, no statistical significance was obtained (P value = 0.208). The risk of *F. nucleatum* colonization was increased in late-stage gastric cancer patients, suggesting that the microenvironment during cancer progression is more suitable for colonization and growth of *F. nucleatum* (P value = 0.012). Interestingly, we found that the risk of *F. nucleatum* colonization was significantly decreased in male patients (P value = 0.02), which may be associated with the lifestyle or hormonal status of the patients and requires confirmation in larger and/or independent gastric cancer patient cohorts.

We then analyzed the impact of *F. nucleatum* on the survival of gastric cancer patients. Although results showed that *F. nucleatum*-positive patients had a lower survival rate, this was not significant (Figure 4A). Stage I cancer patients with a nearly 100% 5-year survival rate were then excluded from the survival analysis, but significance was still not reached while poorer survival outcomes were maintained (Figure 4A). As noted above, patients who undergo gastrectomy have good treatment outcomes, and it is likely that oncogenic factors, including pathogenic gastric microbiota, are reduced after surgery. Despite the lack of statistical significance, our analysis shows that *F. nucleatum* colonization may adversely impact treatment outcome.

Approximately one-third of the patients in our cohort tested positive for *H. pylori* colonization, and our previous study demonstrated that *F. nucleatum* is likely a secondary settler of the gastric microbiota after *H. pylori* colonizes the gastric epithelium. Consecutive or simultaneous colonization of *H. pylori* and *F. nucleatum*

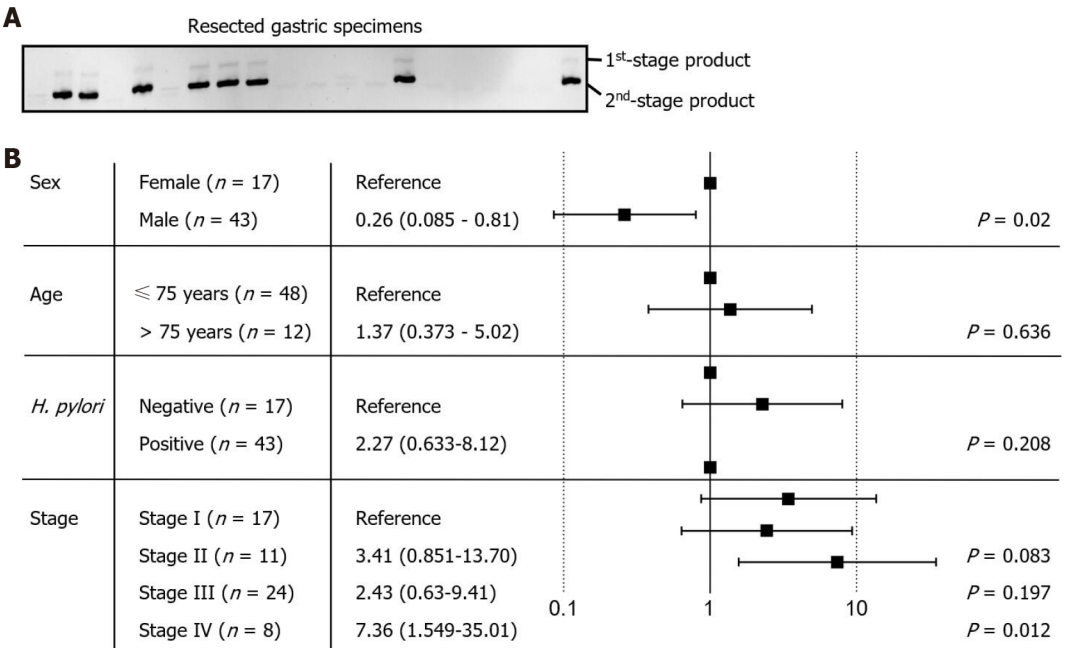


Figure 3 Identification of *Fusobacterium nucleatum* in the resected gastric cancer tissues. A: *Fusobacterium nucleatum* (*F. nucleatum*) was detected by amplification of the conserved *NusG* gene sequence using nested polymerase chain reaction method. The end point product was analyzed using 6% nondenaturing polyacrylamide gel electrophoresis; B: The relative risk of *F. nucleatum* colonization was analyzed against the sex, age, status of *Helicobacter pylori* colonization, and cancer stage. Data analysis is shown as Forest plot.

might have synergistic effects and promote cancer progression. To test this hypothesis, we analyzed the impact of *F. nucleatum* on the survival of *H. pylori*-positive patients. Our data demonstrate that patients positive for both *H. pylori* and *F. nucleatum* had a poorer survival outcome than those who were positive for *H. pylori* alone (Figure 4B). Similar results were observed when stage I cancer patients were excluded from the analysis (Figure 4B). Therefore, the presence of *F. nucleatum* colonization negatively impacts gastric cancer prognosis. Our analysis confirms that *F. nucleatum* colonization is frequent in patients with advanced-stage gastric cancer, with an unfavorable effect on patient survival.

DISCUSSION

F. nucleatum is an opportunistic pathogen mainly residing in the oral cavity[30]. Although it is likely that oral-resident microbes are regularly passed to the stomach through dietary intake, they are unable to colonize the rest of the gastric microenvironment under normal physiological conditions. However, *H. pylori* colonization and the growth of carcinoma cells create a suitable microenvironment[31,32] and likely allow *F. nucleatum* invasion. Our previous study, which utilized 16S metagenomic analysis, showed that *F. nucleatum* can be present in the gastric cancer-associated microbiota[17]. In the present study, we employed nested PCR to determine the existence of *F. nucleatum* in resected gastric cancer tissues. The results confirmed our previous finding, showing *F. nucleatum* colonization in approximately one-third of gastric cancer patients in southwestern Taiwan. Whether *F. nucleatum* colonization is common remains to be investigated.

Analysis of our *in vitro* coculture experiment indicated that *F. nucleatum* evokes two distinct cellular responses. Based on dosage dependence and expression pattern, these two responses are likely activated through independent signaling pathways. One is an immediate response that peaked at 24 h to 48 h of incubation and declined to a near-unstimulated level after 72 h. This immediate response was induced only by a high amount of *F. nucleatum* and marked by activation of interferon response genes, antiviral genes, cytokines, and chemokines. Other inflammation-inducing pathogens, such as *H. pylori*, may collaborate with *F. nucleatum* to sustain expression of these genes. The second response involved activation of actin and genes that regulate cell mobility. High expression of these gene products has multiple effects in promoting cancer progression, especially in increasing cell mobility and promoting distant

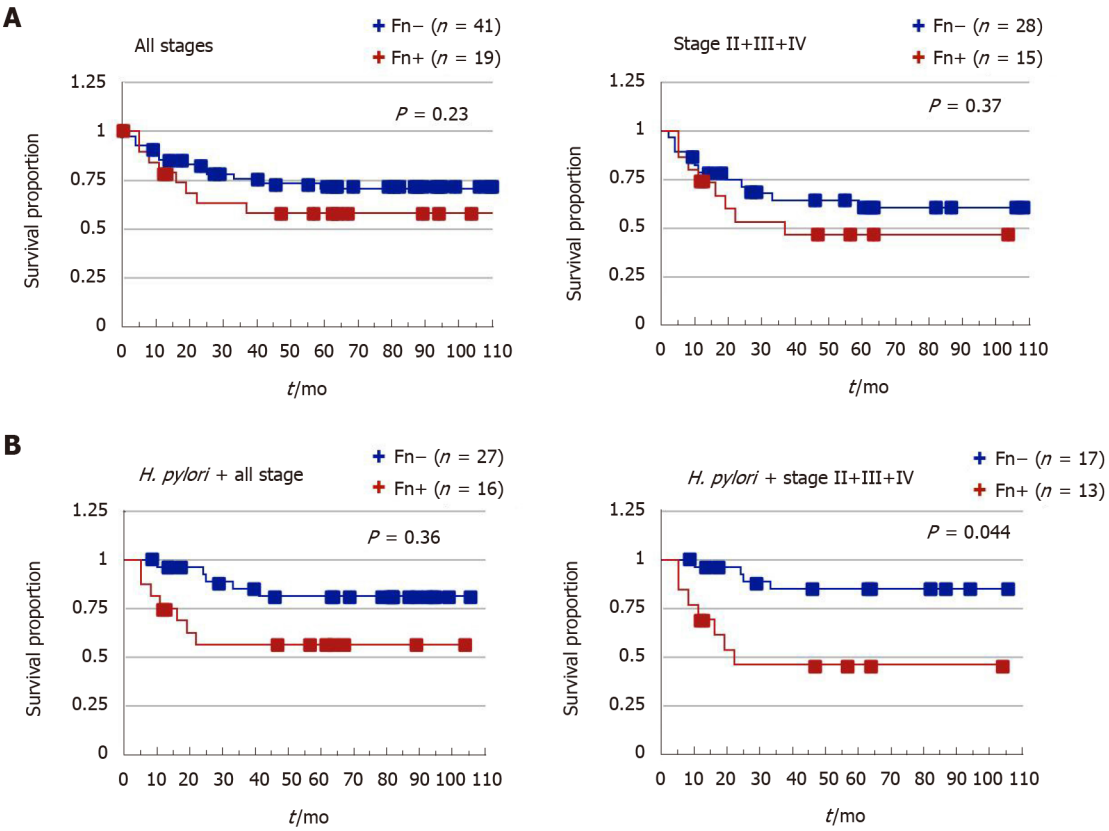


Figure 4 Analysis of *Fusobacterium nucleatum* colonization in gastric cancer patients. A: The study cohort was divided into *Helicobacter pylori* (*H. pylori*)-positive and negative groups according to the result of nested-polymerase chain reaction analysis. Survival probability was calculated using Kaplan-Meier analysis in all or stage II, III, and IV cancer patients with at least 1-year follow-up; B: Survival probability was calculated in stages II, III, and IV cancer patients.

metastasis[33-34]. Overall, our experiment demonstrates that colonization by *F. nucleatum* activates specific signaling pathways and promotes aggressiveness in gastric cancer cells.

After determining the prevalence of *F. nucleatum* colonization in gastric cancer patients, we evaluated its clinical impact. All enrolled patients underwent gastrectomy, in which the main cancerous lesions were completely removed. Gastrectomy also results in extraction of cancer-associated microbiota, minimizing the negative impact of pathogenic microbes. The average 5-year survival in our study cohort was approximately 60%, indicating the effectiveness of treatment. Notably, survival analysis showed that *F. nucleatum* colonization negatively impacts the survival outcome of *H. pylori*-positive patients. It is possible that *F. nucleatum* sequentially and/or synergistically cooperates with *H. pylori* to promote gastric cancer progression. Our analysis indicates a complex interaction between multiple pathogens and gastric cancer cells. Moreover, our data suggest that *F. nucleatum* colonization may serve as a prognostic biomarker for *H. pylori*-positive patients.

Our experimental findings show that *F. nucleatum* colonization in the gastric microbiota is a common event in gastric cancer patients. The risk of colonization appears to increase toward the later stages of gastric cancer progression. Cocolonization of *F. nucleatum* with *H. pylori* results in poorer survival than that observed for patients with *H. pylori* colonization alone. As the majority of gastric microbiota components are presumably removed during gastrectomy, preliminary exposure to *F. nucleatum* may exert a long-term impact on the aggressiveness of cancer cells. Our findings indicate that *F. nucleatum* may precondition and promote gastric cancer progression.

CONCLUSION

In this study, we found that *F. nucleatum* is able to alter actin filament dynamics to promote cell mobility. Additionally, the prevalence of *F. nucleatum* colonization in gastric cancer tissues is much higher than previously thought. Importantly, cocolon-

ization of *F. nucleatum* with *H. pylori* was found to lead to reduced survival in gastric cancer patients. Our study suggests that *F. nucleatum* increases the aggressiveness of gastric cancer and negatively impacts the prognosis of gastric cancer patients.

ARTICLE HIGHLIGHTS

Research background

Our previous research identified *Fusobacterium nucleatum* (*F. nucleatum*) as an opportunistic pathogen frequently found in the gastric cancer-associated microbiota. *F. nucleatum* has been demonstrated to promote carcinogenesis and metastasis of colorectal cancer. However, the role of *F. nucleatum* in gastric cancer remains unclear.

Research motivation

It is our goal to determine the impact of *F. nucleatum* colonization to progression of gastric cancer.

Research objectives

The objective of current study is to identify the impact of *F. nucleatum* to the cellular function of gastric cancers and to the prognosis of gastric cancer patients.

Research methods

F. nucleatum-induced expression change of a patient-derived gastric cancer cell line was profiled by RNA sequencing and ontological analysis. The presence of *F. nucleatum* in patients' tumor tissue was determined by nested polymerase chain reaction. Statistical analysis of *F. nucleatum* colonization status was performed to determine the correlation with clinical characterization and patients' survival.

Research results

F. nucleatum induces a drastic but temporary interferon response and prolonged deregulation of actin and its regulators from gastric cancer cells. A survey of clinical specimens showed that approximately one-third of gastric cancer patients are positive for *F. nucleatum*. Survival analysis showed that the combined colonization of *Helicobacter pylori* (*H. pylori*) and *F. nucleatum* leads to poorer survival of late-stage patients.

Research conclusions

The actin filament dynamic change caused by *F. nucleatum* colonization likely promotes cell mobility and cancer metastasis. This observation is correlated with the finding that *F. nucleatum* colonization leads to poor survival of *H. pylori*-positive late-stage patients.

Research perspectives

F. nucleatum colonization leads to poorer survival of gastrectomy-received patients. Our findings indicate the importance of tumor-associated microbiota to the progression of gastric cancer.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 14; 27(42): 7324-7339

DOI: 10.3748/wjg.v27.i42.7324

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

ORIGINAL ARTICLE

Basic Study

Detailing the ultrastructure's increase of prion protein in pancreatic adenocarcinoma

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Author contributions: Bianchini M and Giambelluca MA contributed equally to this work; Morelli L and Fornai F contributed equally to this work; Bianchini M, Giambelluca MA, Scavuzzo MC, Morelli L and Fornai F studied the conception and design; Bianchini M, Giambelluca MA, Scavuzzo MC, Di Franco G, Guadagni S, Palmeri M, Furbetta N, Gianardi D, Funel N, Ricci C, Gaeta R, Pollina LE,

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Abstract

BACKGROUND

Recent evidences have shown a relationship between prion protein (PrPc) expression and pancreatic ductal adenocarcinoma (PDAC). Indeed, PrPc could be one of the markers explaining the aggressiveness of this tumor. However, studies investigating the specific compartmentalization of increased PrPc expression within PDAC cells are lacking, as well as a correlation between ultrastructural evidence, ultrastructural morphometry of PrPc protein and clinical data. These

Falcone A, Vivaldi C and Di Candio G contributed to data acquisition; Morelli L and Fornai F are responsible for data analysis and interpretation, and revised the manuscript critically; Bianchini M, Giambelluca MA, Scavuzzo MC, Di Franco G, Guadagni S, Palmeri M, Furbetta N, Gianardi D, Funel N, Pollina LE, Falcone A, Vivaldi C, Di Candio C, Biagioni F and Busceti CL drafted the manuscript; all authors approved the final manuscript.

Supported by Tizzi Foundation and Arpa Foundation (www.fondazionearpa.it).

Institutional review board statement: The study was approved by Ethics committee of "Area Vasta Nord Ovest (CEAVNO)".

Informed consent statement: All patients signed an informed consent to authorize the scientific use of the collected data.

Conflict-of-interest statement: The authors declare that they do not have any conflict of interest.

Data sharing statement: No additional data available.

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Specialty type: Oncology

Country/Territory of origin: Italy

Peer-review report's scientific quality classification

Grade A (Excellent): 0

Grade B (Very good): B, B

data, as well as the quantitative stoichiometry of this protein detected by immuno-gold, provide a significant advancement in understanding the biology of disease and the outcome of surgical resection.

AIM

To analyze quantitative stoichiometry and compartmentalization of PrPc in PDAC cells and to correlate its presence with prognostic data

METHODS

Between June 2018 and December 2020, samples from pancreatic tissues of 45 patients treated with pancreatic resection for a preoperative suspicion of PDAC at our Institution were collected. When the frozen section excluded a PDAC diagnosis, or the nodules were too small for adequate sampling, patients were ruled out from the present study. Western blotting was used to detect, quantify and compare the expression of PrPc in PDAC and control tissues, such as those of non-affected neighboring pancreatic tissue of the same patient. To quantify the increase of PrPc and to detect the subcellular compartmentalization of PrPc within PDAC cells, immuno-gold stoichiometry within specific cell compartments was analyzed with electron microscopy. Finally, an analysis of quantitative PrPc expression according to prognostic data, such as cancer stage, recurrence of the disease at 12 mo after surgery and recurrence during adjuvant chemotherapy was made.

RESULTS

The amount of PrPc within specimen from 38 out of 45 patients was determined by semi-quantitative analysis by using Western blotting, which indicates that PrPc increases almost three-fold in tumor pancreatic tissue compared with healthy pancreatic regions [242.41 ± 28.36 optical density (OD) *vs* 95 ± 17.40 OD, $P < 0.0001$]. Quantitative morphometry carried out by using immuno-gold detection at transmission electron microscopy confirms an increased PrPc expression in PDAC ductal cells of all patients and allows to detect a specific compartmentalization of PrPc within tumor cells. In particular, the number of immuno-gold particles of PrPc was significantly higher in PDAC cells respect to controls, when considering the whole cell (19.8 ± 0.79 particles *vs* 9.44 ± 0.45, $P < 0.0001$). Remarkably, considering PDAC cells, the increase of PrPc was higher in the nucleus than cytosol of tumor cells, which indicates a shift in PrPc compartmentalization within tumor cells. In fact, the increase of immuno-gold within nuclear compartment exceeds at large the augment of PrPc which was detected in the cytosol (nucleus: 12.88 ± 0.59 particles *vs* 5.12 ± 0.32, $P < 0.0001$; cytosol: 7.74. ± 0.44 particles *vs* 4.3 ± 0.24, $P < 0.0001$).

RESULTS

In order to analyze the prognostic impact of PrPc, we found a correlation between PrPc expression and cancer stage according to pathology results, with a significantly higher expression of PrPc for advanced stages. Moreover, 24 patients with a mean follow-up of 16.8 mo were considered. Immuno-blot analysis revealed a significantly higher expression of PrPc in patients with disease recurrence at 12 mo after radical surgery (360.71 ± 69.01 OD *vs* 170.23 ± 23.06 OD, $P = 0.023$), also in the subgroup of patients treated with adjuvant CT (368.36 ± 79.26 OD in the recurrence group *vs* 162.86 ± 24.16 OD, $P = 0.028$), which indicates a correlation with a higher chemo-resistance.

CONCLUSION

Expression of PrPc is significantly higher in PDAC cells compared with control, with the protein mainly placed in the nucleus. Preliminary clinical data confirm the correlation with a poorer prognosis.

Key Words: Pancreatic ductal adenocarcinoma; Prion protein; Western blotting; Electron microscopy; Cellular compartmentalization; Neuroinvasion

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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 14, 2021
Accepted: October 25, 2021
Article in press: October 25, 2021
Published online: November 14, 2021

P-Reviewer: Şurlin VM, Yamanaka K
S-Editor: Wu YXJ
L-Editor: A
P-Editor: Wu YXJ



Core Tip: Pancreatic ductal adenocarcinoma (PDAC) is an extremely lethal cancer and we are far away from understanding its biology. Recent *in vitro* evidence hypothesizes some role of cellular prion protein (PrPc) in PDAC carcinogenesis. We found that this protein is over-expressed *in vivo* within PDAC tissue of surgically resected patients, here we quantify the stoichiometric increase and provide evidence for a shift towards a nuclear compartmentalization. Such a protein amount is associated with poorer post-surgical prognosis and neuro-invasion. Thus, PrPc might be a key in the puzzled evidence of PDAC biology leading to better comprehend and cure such an aggressive disorder.

Citation: Bianchini M, Giambelluca MA, Scavuzzo MC, Di Franco G, Guadagni S, Palmeri M, Furbetta N, Gianardi D, Funel N, Ricci C, Gaeta R, Pollina LE, Falcone A, Vivaldi C, Di Candio G, Biagioni F, Busceti CL, Morelli L, Fornai F. Detailing the ultrastructure's increase of prion protein in pancreatic adenocarcinoma. *World J Gastroenterol* 2021; 27(42): 7324-7339
URL: https://www.wjgnet.com/1007-9327/full/v27/i42/7324.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i42.7324

INTRODUCTION

Pancreatic cancer is currently the fourth most frequent cause of death among cancers with a prevalence, which is increasing, mostly in western countries, where it is estimated to become the second prevalent cause of death from cancer by 2030[1]. The overall median survival of patients with pancreatic ductal adenocarcinoma (PDAC) is 6 mo and the 5-year survival rate is less than 10%[2,3].

Surgical resection is still the only approach with a curative intent, but it is feasible for less than 20% of patients at diagnosis, while 80% of cases are considered too advanced for surgery, and this is based on regional infiltration or distant metastasis [4]. Even in surgically eligible cases, the 5-years survival rate is extremely low, since patients experience early local or distant relapse. The aggressiveness of the disease is mainly related to the extensive local infiltration and to the early lymphatic and hematogenous spread, which rely on the high propensity of these cells to produce neuro-invasion.

In this scenario, an in-depth knowledge of the biology of the disease is fundamental. The comprehension of the mechanisms involved in tumorigenesis is needed, in order to discover early diagnostic tools, and novel therapeutic strategies to improve patients' prognosis. When comparing PDAC with other tumors, the biology of the disease is scarcely investigated, and it remains largely unknown.

Recent evidence demonstrates that cellular Prion Protein (PrPc) is overexpressed either *in vitro* and *in vivo* in PDAC cells and that it interacts with several pathways, enhancing cellular growth, tumoral proliferation and invasion[5-7].

Still, the occurrence of increased PrPc was described so far by using quite general approaches, in the absence of subcellular localization of the protein and without a quantitative, stoichiometric count of protein particles within cancer cells. Thus, we felt it mandatory to provide a quantitative measurement of the increase in PrPc, by using ultrastructural stoichiometry, which is more reliable in protein analytical detection compared with immunohistochemistry. Again, understanding cell compartmentalization of PrPc, and whether this is shifted within PDAC cells is key to establish its potential role in the biology of disease. This is expected to improve the knowledge about the intrinsic role of PrPc in PDAC pathogenesis. So far, studies focusing on these aspects based on an *in vivo* approach are lacking. Again, it is fundamental to strengthen whether a higher expression of PrPc in PDAC cells does associate with specific clinical phenotypes of PDAC. Therefore, the aim of this study is to quantify the increase in PrPc and analyze the subcellular localization of PrPc in PDAC cells from surgically resected patients. These findings are correlated with clinical data, in order to dissect a potential role of PrPc as a biological marker to predict disease severity.

MATERIALS AND METHODS

Patients and specimens

Samples from tumors of patients surgically treated with pancreatic resections at our Institution were collected between January 2018 and December 2020. Written informed consent was obtained from patients to use their surgical specimens and clinical pathological data for research purposes.

All patients had a preoperative suspicion of PDAC. Preoperative evaluation included medical history, physical, laboratory and radiological examinations, computed tomography (CT) and magnetic resonance imaging, often with magnetic resonance cholangiopancreatography. In addition, abdominal ultrasound with and without contrast, endoscopic ultrasonography (EUS), and fine-needle aspiration (FNA) during EUS were also performed in selected patients. Preoperative data included age and gender.

All the specimens were frozen intraoperatively and further sliced and scored for histology. Tissue from pancreatic nodules other than adenocarcinomas were ruled out from the present study.

Similarly, we could not proceed when tumor specimens were too small. When PDAC diagnosis was confirmed, the pathologist took specimens from the pancreatic tumor and from normal pancreatic tissue. From each specimen (either from controls or tumor tissues) a part was fixed and kept in glutaraldehyde and paraformaldehyde for electron microscopy analysis, while the other one was rapidly frozen and kept at -80°C for storage until for western blotting analyses (SDS-PAGE immunoblotting) to be carried out.

Histological data included: (1) Histological phenotype of the tumor; (2) Grade of differentiation; (3) Tumor size; (4) Number of harvested lymph nodes; (5) Number of metastatic lymph nodes; and (6) Occurrence of angio-invasion and peri-neural infiltration.

Patients were staged according to the T and N definitions proposed for the AJCC 8th edition[8]. Proposed T-stage definitions are the following: T1 ≤ 2 cm maximal diameter, T2 > 2 ≤ 4 cm maximal diameter, T3 > 4 cm maximal diameter, T4 = locally unresectable. Extra-pancreatic extension was not included in T-stage definitions. The N-staging included the following: N0 = node negative, N1 = 1-3 nodes positive for metastatic disease, N2 ≥ 4 nodes positive for metastatic disease.

Pathology, post-operative disease outcome and oncologic follow-up were prospectively retrieved and organized in a specific database. For our clinical analysis patients with at least 12 mo from surgical resection were considered. The degree of PrPc expression in PDAC was reported and compared also on the basis of cancer stage according to AJCC 8th edition.

SDS-PAGE immunoblotting

Pancreatic tissue to be immunoblotted was homogenized in ice-cold lysis buffer containing 50 mmol/L Tris-HCl (pH = 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% SDS, 0.1% IGEPAL (NP40) and Complete Protease Inhibitor Cocktail Tablet (leupeptin, pepstatin, aprotinin) (Santa Cruz Biotechnology, Dallas, TX, United States). Then tissue was sonicated, and homogenates were centrifuged at 5000 x g for 5 min. An aliquot of supernatant was used to determine the protein concentration by a protein assay kit (Sigma-Aldrich, St. Louis, MO).

Samples (25 μg) were separated on 4%-20% sodium dodecyl sulfate-polyacrylamide gel. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Biorad; Milano, Italy). The membrane was then immersed in blocking solution containing PBS with 0.05% Tween-20 (PBS-T) and 5% not fat dried milk (Sigma) for 1 h at room temperature. Then the membrane was incubated overnight at 4°C with primary antibody anti-protein PrPc (1:2000, Abcam, Cambridge, United Kingdom) diluted in PBS-T containing 2% BSA (Sigma). The blots were washed three times with PBS-T and incubated for 1 h with goat anti-rabbit horseradish peroxidase-labelled secondary antibody (1:2000; KPL, Maryland, United States) diluted in PBS-T containing 2% not fat dried milk (Sigma).

The bands were visualized with enhanced chemiluminescence reagents (Immuno-Star HRP Substrate; Bio-RadLaboratories) and image analysis was carried out by ChemiDoc System (Bio-RadLaboratories). β-Actin was used as an internal standard for semi-quantitative protein measurement, so-called "house-keeping protein". Densitometric analysis was performed with ImageJ software and the unit of measure was the optical density (OD). Western blotting of PDAC tissues were compared with control tissues.

Semi-thin sections

When preparing embedded pancreas tissue blocks for electron microscopy analysis, firstly we carried out semi-thin sections in order to better focus on those areas of the tissue where ductal and parenchymal area could be evidenced. Each semi-thin section owning a thickness range of 3-6 μm was cut at ultra-microtome. After cutting, slices were picked up with an iron loop 1 cm long and 2 mm thick. By using the loop, the slice was moved into a drop of distilled water lying on a glass slide and it was then placed on a hot plate at approximately 60°C to be dried. Then, 1 or 2 drops of a toluidine blue staining solution were added on the semi-thin slice. When the edge of the staining drop switched the color from blue to metallic gold, the slide was removed quickly from hot plate to be rinsed with distilled water. Finally, these slides were mounted by using the mounting medium DPX to be analyzed by a Nikon Eclipse 80i light microscope, which was connected to the NIS Elements software for image analysis (Nikon, Tokyo, Japan).

Electron microscopy

For electron microscopy small fragments of normal and tumoral pancreatic tissue were fixed in 0.1% glutaraldehyde and 2 % paraformaldehyde in phosphate buffer pH = 7.4 for 90 min, creating a fixing solution minimally covering antigen epitope, while fairly preserving tissue architecture.

After washing for 10 min in buffer, samples were post-fixed in 1% OsO4 buffered solution for 1 h at 4°C. Then samples were dehydrated in a series of increasing ethanol concentration (50%, 70%, 90% 95%, 100%) followed by propylene oxide for 20 min. Afterward, samples were embedded in a mixture of Epon-Araldite and propylene oxide (ratio of 1:1 overnight at room temperature) and finally they were embedded in pure Epon-Araldite resin for 72 h at 60°C. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with Jeol Jem 100SX transmission electron microscope (TEM) (Jeol, Tokyo, Japan) at an acceleration voltage of 80 kV.

Post–embedding immunocytochemistry

Post-embedding procedure was carried out on ultrathin sections collected on nickel grids. Grids were washed in PBS and incubated in a blocking solution containing 10% goat serum and 0.2% saponin for 20 min, at room temperature, then they were incubated with a primary antibody solution containing anti-rabbit Prion protein PrPc (Abcam, diluted 1:50), with 0.2% saponin and 1% goat serum in a humidified chamber, overnight, at 4°C. After washing in PBS, grids were incubated with secondary anti-rabbit antibodies conjugated with gold particles (20 nm mean diameter, BB International Crumlin, United Kingdom) which were diluted 1:40 in PBS containing 0.2 % saponin and 1% goat antiserum for 1 h, at room temperature. Slices used as methodological controls were incubated with secondary antibody only.

After washing in PBS, grids were incubated on droplet of 1% glutaraldehyde for 3 min; an additional extensive washing of grids with distilled water was carried out to remove an excess of salt traces. Sections were stained with uranyl acetate and lead citrate and examined at TEM.

From each experimental group (Controls and PDAC) 20 grids were observed each one containing a mean of 5 cells: In particular we selected the region in which the cellular ducts were present. In order to measure the distribution of the immuno-gold particles, first we counted the total number of gold particles within each cell, then their numbers in nucleus and in cytosol. TEM analysis was performed at a magnification of 6000-8000x which allowed the concomitant visualization of immuno-gold particles and all cell organelles, using higher magnification when it was necessary to better visualize the immuno-gold particles and lower magnification when an ensemble view of the whole ultrastructure was requested for our analysis. The expression of PrPc was revealed by counting the immuno-gold particles in whole cells, nucleus and cytosol both in control and PDAC groups.

Statistical analysis

Continuous variables with normal distribution are expressed as the mean ± SD of the mean and they were compared by using Student's *t* test. A *P* value equal or lower than 0.05 was arbitrarily considered as not due to biological variability (H_0 hypothesis rejected). The software used for such a statistical analysis was SPSS (Statistical Production and Service Solution for Windows, SPSS Inc., Chicago, IL, United States), version 23.

RESULTS

Patients

Surgical specimens of 45 patients were collected. 7 of them were ruled out because of the small dimension of the tumor that prevented an adequate sampling or because the diagnosis of PDAC was excluded at frozen section.

The samples from 38 PDAC patients which were included in the analysis, 19 (50%) were from male, while 19 (50%) from female. The mean age was 72.7 ± 7.9 years (range 52-87). Histological exam confirmed the presence of PDAC in all these 38 cases. The grading of the pancreatic tumor was "moderately differentiated" in 35/38 cases (92.1%), "poorly differentiated" in 3/38 (7.9%). The mean tumor size was 3.2 ± 1.1 cm (range 1.5-6.5 cm). The mean harvested lymph nodes were 34.2 ± 15 (range 14-79). Metastatic lymph-nodes occurred in 30/38 cases (78.9%) with a number of metastatic lymph-nodes of 4.5 ± 4.9 (range 1-23). The presence of angio-invasion was reported in 5/38 cases (13%), while the presence of peri-neural infiltration was reported in 33/38 cases (86.8%). Three cancer stage groups were identified according to pTNM, stage I (n = 8, 21.1%), stage II (n = 14, 36.8%) and stage III (n = 16, 42.1%). These data are shown in Table 1.

Expression of PrPc in pancreatic tissue at western blotting

PrPc was markedly expressed in tumor pancreatic tissues, while the expression in non-cancer tissues was scarce, with a significant difference (242.41 ± 28.36 OD *vs* 95 ± 17.40 OD, $P < 0.0001$) (Figure 1).

Semi-thin sections

When observing representative semi-thin sections at different magnification (Figure 2) the difference between control and PDAC pancreas was strikingly evident, mostly at the level of ductal tissue. In detail, the pancreas from control at low magnification possesses a quite homogeneous staining at toluidine blue, where ductal areas are simply evident as empty roundish areas surrounded by cell staining as much as those in the neighboring parenchyma (Figure 2A). This was further evidenced at higher magnification showing a pale toluidine staining of ductal cells (Figure 2B). Conversely, in the pancreas affected by PDAC, low magnification showed a highly non-homogeneous tissue where ductal regions were markedly stained. Also, neighboring areas possess a scattered toluidine staining (Figure 2C). At high magnification, the ductal cells from PDAC are overwhelmed and they tend to occlude the ductal lumen with multiple cell layers with an abnormal shape and abundant cell protrusions (Figure 2D). This ductal tissue was the topographical reference to proceed with electron microscopy analysis.

Electron microscopy

The ultrastructure of pancreatic healthy cells owns normal architecture with well-preserved cell compartments and well-defined membranes. The zymogen granules maintain their integrity, which suggests fair biochemical activity (Figure 3A). Conversely, PDAC cells (Figure 3B) possess severe cell pathology, which is concomitant with a marked derangement of vacuolar compartment and damaged organelles.

Expression of PrPc immuno-gold particles within pancreatic tissue

Immuno-cytochemistry shows increased PrPc expression in PDAC cells of all patients. In particular the number of immuno-gold particles was significantly higher in PDAC cells compared with controls (Figure 4) either considering the whole cell (19.8 ± 0.79 particles *vs* 9.44 ± 0.45, $P < 0.0001$) (Figure 5A), or the nuclear compartment (12.88 ± 0.59 particles *vs* 5.12 ± 0.32, $P < 0.0001$) (Figure 5C) or the cytosol (7.74. ± 0.44 particles and 4.3 ± 0.24, $P < 0.0001$, respectively) (Figure 5B).

Within PDAC cells, PrPc was more abundant in the nucleus compared with the cytosol (12.88 ± 0.59 particles *vs* 7.74. ± 0.44, $P < 0.0001$) (Figure 5D). Conversely, in normal cells no difference was noticeable for the placement of PrPc within nucleus and cytosol (5.12 ± 0.32 particles and 4.3 ± 0.24, $P > 0.05$, respectively).

Clinical data

When correlating within PDAC group the amount of PrPc expression with specific cancer stages, a significantly higher expression of PrPc for advanced stages was detected. In particular, PrPc expression at Western Blotting was 161.69 ± 63.92 OD in stage I, 173.25 ± 76.5 OD in stage II and 346.86 ± 55.26 OD in stage III ($P = 0.0042$).

Table 1 Patients and postoperative data

Number of patients, *n*	38
Pancreatic ductal adenocarcinoma, *n* (%)	38 (100)
Mean tumor dimension, cm	3.2 1.1 (1.5-6.5)
Mean harvest lymph nodes, *n*	34.2 15 (14-79)
Mean metastatic lymph nodes, *n*	4.5 4.9 (1-23)
Angioinvasion, *n* (%)	5 (13)
Perineural infiltration, *n* (%)	33 (86.8)
T status, *n* (%)	
T1	2 (8.7)
T2	14 (60.9)
T3	7 (30.4)
T status, *n* (%)	
T1	3 (7.9)
T2	24 (63.2)
T3	11 (28.9)
N status, *n* (%)	
N0	7 (18.4)
N1	15 (39.5)
N2	16 (42.1)
Stage, *n* (%)	
I	8 (21.1)
II	14 (36.8)
III	16 (42.1)
Patients with available follow-up, *n*	24
Patients with disease recurrence at 12 months, *n* (%)	13 (54.1)
Patients treated with adjuvant CT, *n* (%)	21 (87.5)
Recurrence during CT, *n* (%)	11 (52.4)

CT: Chemotherapy.

When considering clinical data retrieved from our follow-up, patients surgically treated in the last 12 mo were ruled out from correlation with disease prognosis. Overall, 27 patients were considered, of which 3 were ruled out for missing information.

For the remaining 24 patients, the mean follow-up was 16.8 mo (range 5.6-34.4 mo). Median overall survival and disease-free survival were 15.9 mo and 11.2 mo, respectively. The 12-mo recurrence rate was 54.1% (n = 13). Comparing patients with relapse at 1 year with those without evidence of the disease, the difference in PrPc expression at immuno-blotting was statistically significant between the two groups (360.71 ± 69.01 OD *vs* 170.23 ± 23.06 OD, P = 0.023) (Figure 6A).

Moreover, 21 patients out of 24 received adjuvant chemotherapy (CT). Of these, 10/21 (47.6%) were without evidence of disease relapse at 12 mo, while 11/21 (52.4%) experienced a relapse of the disease. Detailing our analysis of PrPc expression correlated with disease recurrence a significant higher PrPc expression was detected for patients who experienced a relapse, despite the administration of adjuvant CT compared with those receiving CT without evidence of disease at follow-up (368.36 ± 79.26 OD *vs* 162.86 ± 24.16 OD, respectively, P = 0.028) (Figure 6B).

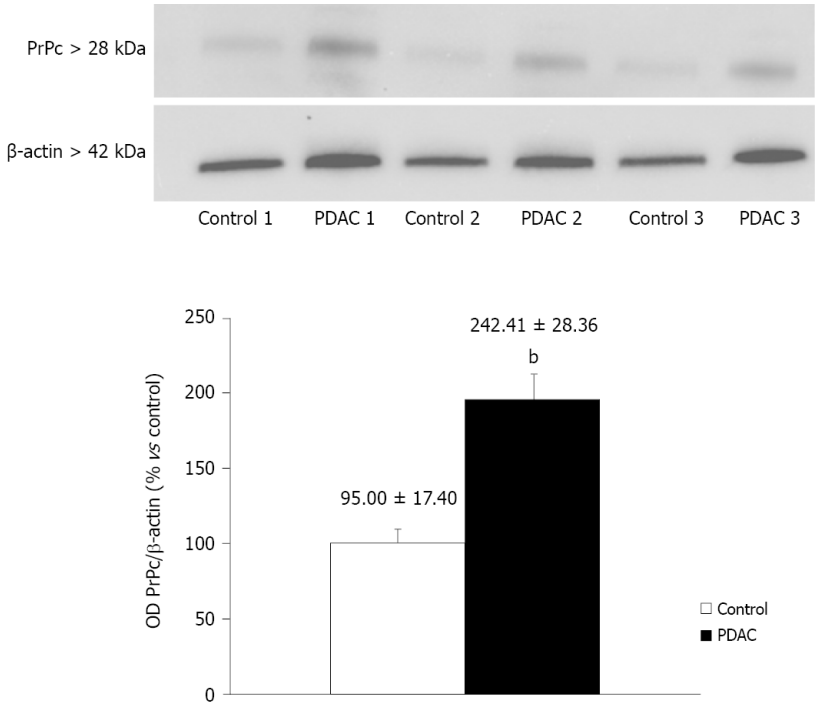


Figure 1 Immuno-blots for prion protein and the house keeping protein b-actin in control tissues and pancreatic ductal adenocarcinoma tissues. $^{b}P < 0.0001$. Comparisons between two groups were made by using Student *t*-test. Values are given as the mean ± SD.

DISCUSSION

Cellular prion protein is a cell surface glycoprotein, firstly discovered as the normal isoform of the scrapie prion protein (PrP^{Sc}), the infectious agent of Transmissible Spongiform Encephalopathies. Despite the normal isoform is a highly conserved glycoprotein present in all vertebrates, which indicates some intrinsic and fundamental roles in cell homeostasis, studies about the physiology of PrPc have long been overlooked.

Only recently some authors focused on the role of PrPc and many paths have been opened[11]. PrPc was proposed to protect neurons against cell death and oxidative stress[12], to control copper metabolism[13], to regulate cell cycle[14], synaptic transmission[15], cell adhesion[16], and activation of the immuno-system[17]. It is remarkable that PrPc plays a role in pluripotency and differentiation of embryonic stem cells[18], cell proliferation and differentiation[19-24] and muscle cell regeneration [25].

In detail, the discovery of a role of this protein in regulating the cell cycle led some authors to focus on studying a relationship between PrPc and carcinogenesis. It is now well established that PrPc may sustain cancer cell proliferation in various types of cancers[26]: Gastric[27], colon cancer[28-30], as well as glioblastoma[31-33]. Overall, the contribution of PrPc to cancer cell proliferation fully fits with a gain of its physiological function in normal cells, where it controls the activation of several effectors associated with cell growth[24,34]. A second field of investigation focuses on the correlation between PrPc and chemo-resistance. High PrPc expression levels are indeed associated with increased resistance to various types of agents in glioblastoma [35], gastric[27,36-38], breast[39-41], and colon cancer[29,42,43]. According to several studies this effect could be related to the activation of several pathways that lead to over expression of MDR1 (multidrug-resistance protein 1)[44]. For instance it was recently found that PrPc confers doxorubicin resistance to breast cancer cells[45].

PrPc also promotes cancer cells invasion/migration. In fact, elevated PrPc levels confer enhanced invasive properties to glioblastoma[46], gastric[47], breast[40,48], colon[49], lung[50] and melanoma[51] cell lines.

Finally, PrPc directly induces proliferation and confers resistance to apoptosis in cancer stem cells[18], by dysregulating their interactions with surrounding environment and thus causing cancer stem cells proliferation[52]. As with proliferation, the contribution of PrPc to cancer stem cells self-renewal may be envisioned as a diversion of its physiological role into normal stem cell maintenance[18].

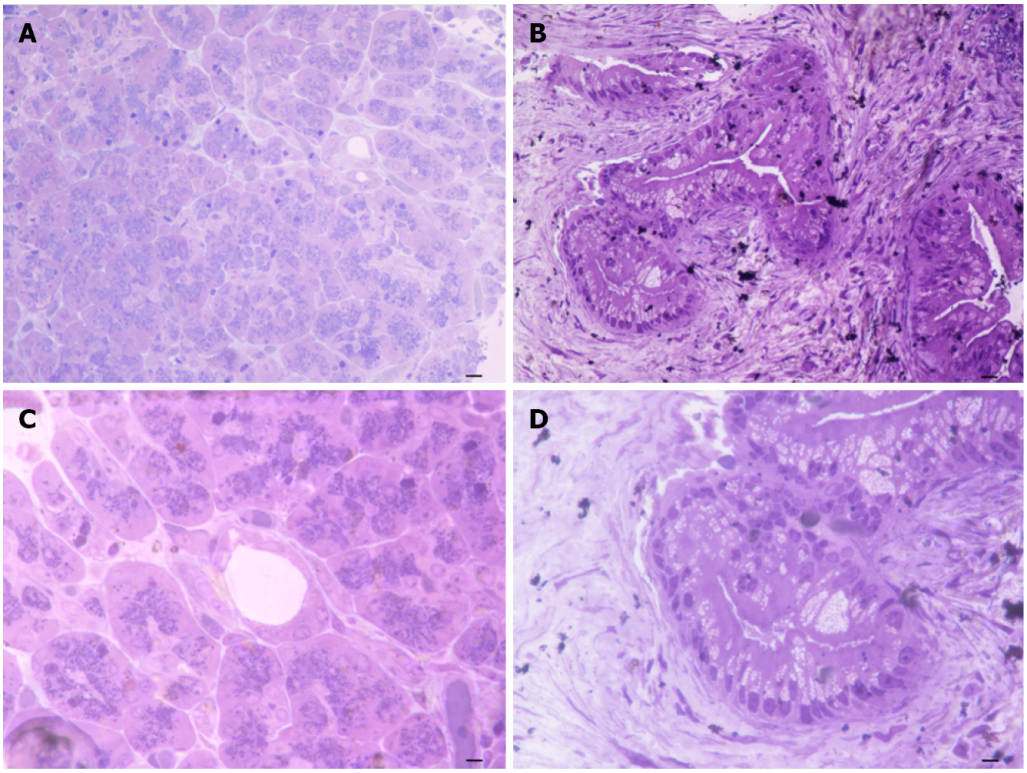


Figure 2 Semi-thin sections of control and pancreatic ductal adenocarcinoma pancreas. A: Pancreas from control tissue at low magnification (magnification: 20 ×, scale bar: 12.5 μm). The ductal areas are evident as empty roundish areas surrounded by cell staining as much as those in the neighboring parenchyma; B: The normal pancreatic tissue characteristics are more evident at higher magnification, where there is a pale toluidine staining of ductal cells (magnification: 40 ×, scale bar: 6.25 μm); C: In pancreatic ductal adenocarcinoma (PDAC) tissue a highly non-homogeneous tissue is present and ductal regions are markedly stained (magnification: 20 ×, scale bar: 12.5 μm); D: PDAC tissue at higher magnification (magnification: 40 ×, scale bar: 6.25 μm). The ductal cells are overwhelmed and they tend to occlude the ductal lumen.

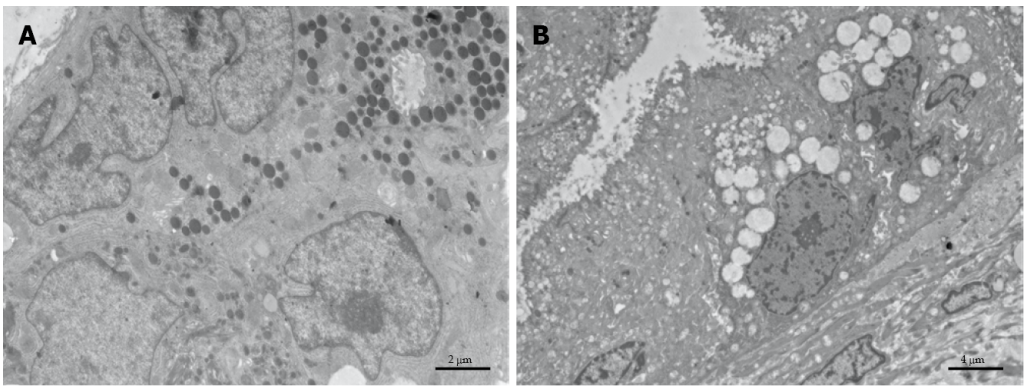


Figure 3 Ultrastructural organization. A: Pancreatic normal tissue. In pancreatic healthy tissue the cells surrounding ductal systems are well preserved. The cellular organelles, nuclei and secretory zymogen granules have normal architecture (magnification: 2000 ×, scale bar: 2 μm); B: Pancreatic normal tissue. The cells surrounding duct have increasing loss of cellular architecture (magnification: 1000 ×, scale bar: 4 μm).

Collectively, the involvement of PrPc in various aspects of cancer progression may be viewed as directly related to its physiological role in normal cells. Prion-mediated changes may represent initiating events that promote the emergence of the hallmarks of cancer, including self-sufficiency in growth signals, insensitivity to antigrowth signals, tissue invasion and metastasis, limitless replicative potential and inhibition of apoptosis[53]. Moreover, over-expression of PrPc has been found to be related to a higher chemoresistance[54]. This is likely to have clinical implications: from a therapeutic perspective, reducing PrPc expression may be beneficial, as it was documented for glioblastoma[55] or colon cancer[56]. Besides, alternative opportunities may ensue from a better knowledge of the signals upregulating PrPc expression in cancer cells.

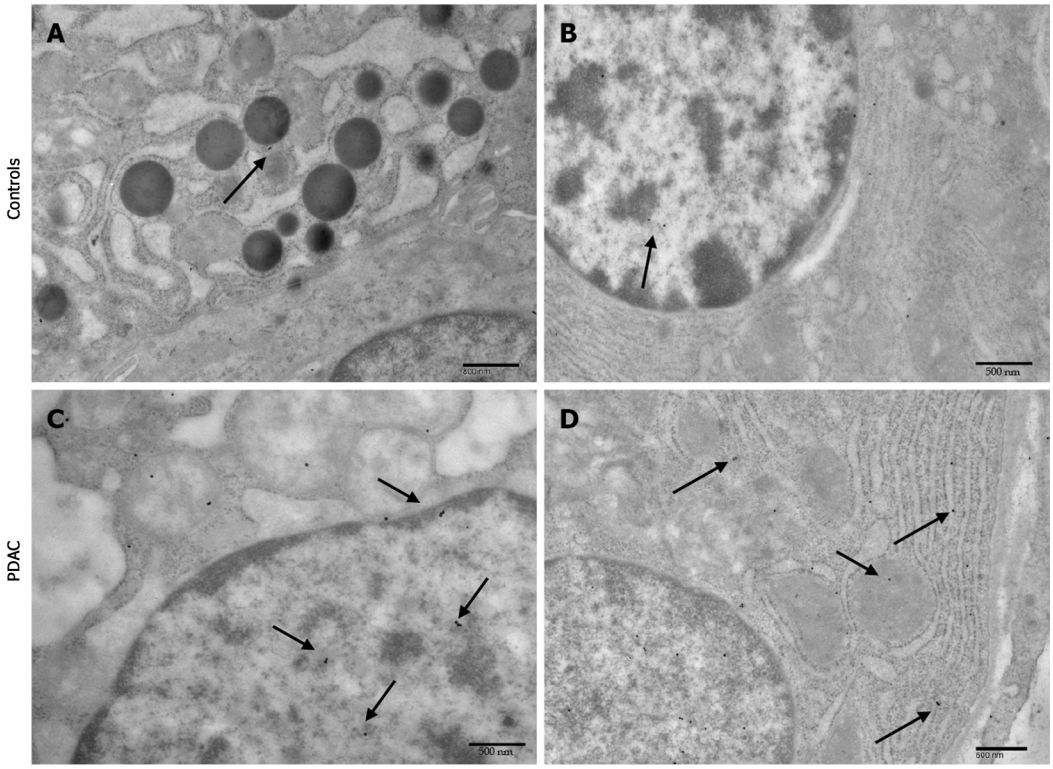


Figure 4 Prion protein immunocytochemistry in controls and pancreatic ductal adenocarcinoma. A: Prion protein immunocytochemistry in controls. Few particles of gold prion protein (PrPc) are evident (arrows) in the cytosol (magnification: 6000 ×, scale bar: 800 nm); B: Few particles of gold PrPc are evident (arrows) in the nucleus (magnification: 8000 ×, scale bar: 500 nm); C: Prion protein immunocytochemistry in pancreatic ductal adenocarcinoma. The arrows highlight some of the PrPc immuno-gold particles in the nucleus (magnification: 8000 ×, scale bar: 500 nm); D: The arrows highlight some of the PrPc immuno-gold particles in the cytosol (magnification: 7000 ×, scale bar: 600 nm).

In particular, looking to PDAC, a deeper comprehension of its role may add a significant piece of evidence in the puzzle of this highly aggressive pathology, with a potentially relevant clinical implication. The present study indicates a higher expression of PrPc in PDAC tissues *in vivo*, and provides the first evidence of the cellular localization of this protein. A higher amount of PrPc specifically within PDAC cells is now confirmed in a wider pool of patients compared with our recent *in vivo* study[7].

Moreover, the present study provides a stoichiometric measurement of the protein *is situ* within PDAC cells and indicates a shift in its sub-cellular placement in PDAC. Such a latter novelty was provided by ultrastructural morphometry, and immuno-gold staining under transmission electron microscopy. For instance, such an approach allowed to detect a misplacement of PrPc towards the nuclear compartment, which is in line with its strong effects on cancer-related gene expression. In particular, in PDAC cells the number of PrPc immuno-gold particles was two-fold higher compared with controls, although such an increase is more relevant in the cell nucleus, where it rises up to three-fold of the amount measured in controls. When comparing PrPc compartmentalization in PDAC cells and normal ductal pancreatic cells, the nuclear concentration of PrPc is 1.65-fold higher compared with cytosol, while in the normal cells there is no significant difference between nucleus and cytosol.

From one hand, this may be due to a marked ongoing over-expression of PrPc gene (PRNP) in PDAC cells. In fact, the first hint of a link between PrPc and pancreatic cancer dates back to the early 2000s, when PRNP was identified as one of the 30 genes mostly expressed in pancreatic cancer cell lines when compared with normal cells[57]. On the other hand, such a preferential nuclear compartmentalization may disclose a specific role of PrPc in the biology of PDAC. So far, the evidences from PDAC cell cultures show that PrPc act as a cell surface glycoprotein to activate specific intracellular pathways and signaling that bring to a proliferative effect[5]. However, the detection of a peculiar and prominent concentration in the nucleus suggests an involvement of PrPc in regulating directly gene expression, acting as a nucleo-junctional interplay in order to modulate the transcriptional activity of different pathways involved in carcinogenesis. In fact, PrPc could have a role in signaling complexes that contribute to a regulation of proliferation and cell-to-cell adhesion.

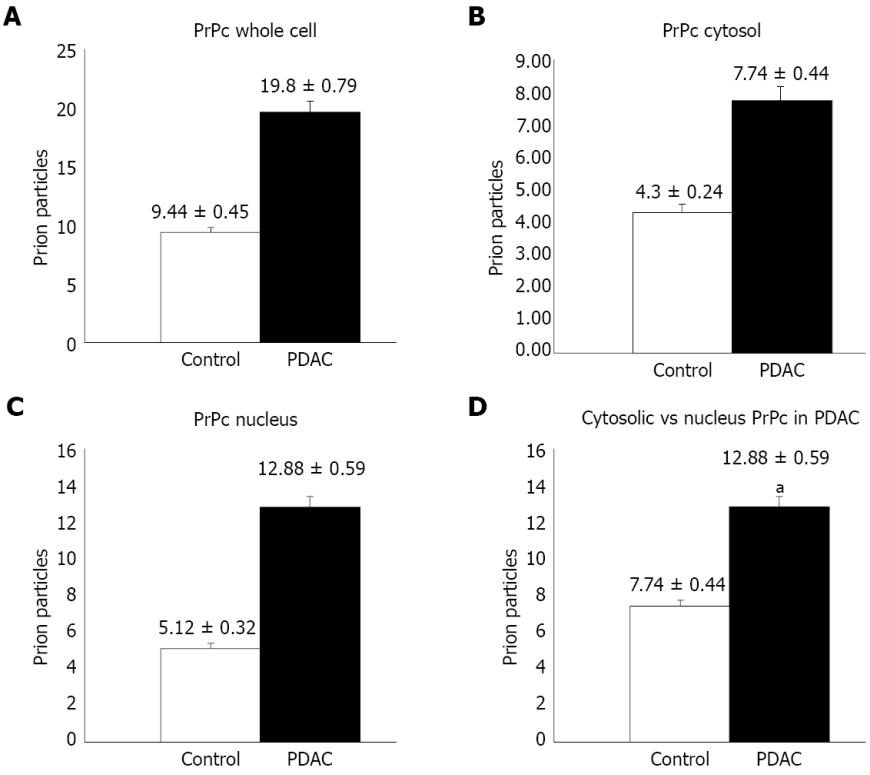


Figure 5 Prion protein immuno-gold particles count. A: Prion protein immuno-gold particles count in whole cells (controls *vs* pancreatic ductal adenocarcinoma); B: Prion protein immuno-gold particles count in cytosol (controls *vs* pancreatic ductal adenocarcinoma); C and D: Prion protein immuno-gold particles count in nuclear compartment. A significative difference in prion protein (PrPc) immunogold particles is observed between pancreatic ductal adenocarcinoma (PDAC) and control tissues (C); Even when analyzing the location only in PDAC cells, the nuclear compartment has a significantly higher PrPc concentration (D). [a]*P* < 0.0001. Comparisons between two groups were made by using Student *t*-test. Values are given as the mean ± SD.

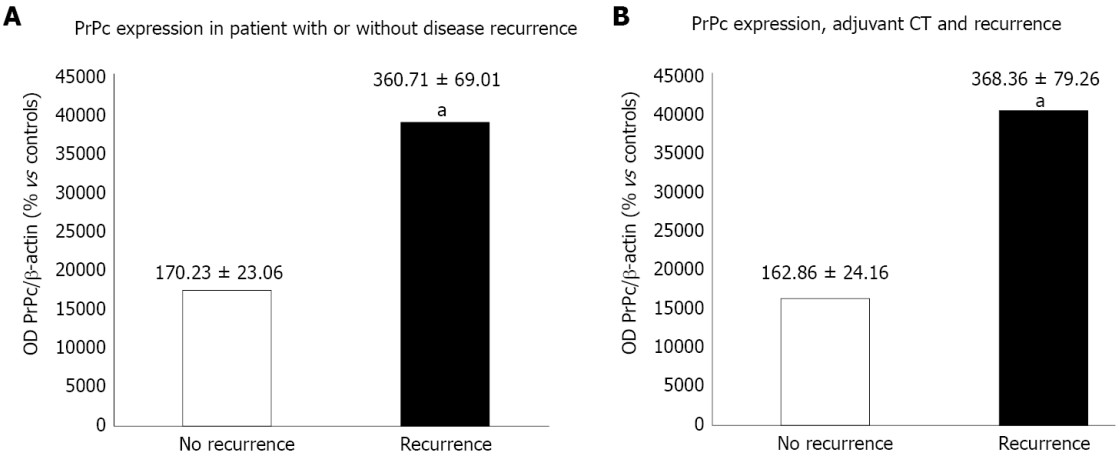


Figure 6 Prion protein expression. Comparisons between two groups were made by using Student *t*-test. Values are given as the mean ± SD. A: Prion protein expression according to the presence of disease recurrence after surgery. The histogram compares the expression of prion protein with western blot in specimen from patients surgically resected for pancreatic ductal adenocarcinoma with no evidence of disease at 12-mo follow-up (*n* = 11) with those from patients with disease recurrence (*n* = 13). [a]*P* = 0.023. B: Prion protein expression according to the presence of disease recurrence after surgery in patients treated with adjuvant chemotherapy. The histogram compares the expression of prion protein with western blot in specimen from patients surgically resected for pancreatic ductal adenocarcinoma with no evidence of disease at 12 mo and treated with adjuvant chemotherapy (CT) (*n* = 10) with those from patients treated with adjuvant CT with disease recurrence (*n* = 11). [a]*P* = 0.028.

Recently an association has been found in enterocytes between nuclear PrPc and Wnt and Hippo pathways, which are modulated by cell contacts and are de-regulated at high frequency in many human cancers. In this way, PrPc should be considered as an actor in oncogenic processes through its role in the dynamics of cell-to-cell junctions because its nuclear localization could lead to modulate transcriptional activity of Wnt and Hippo effectors, some of the pathways clearly involved in carcinogenesis[58].

The relevance of these findings in clinical setting is supported by the evidence of a more aggressive behavior of PDAC depending on the amount of PrPc expression. As already demonstrated in our previous study[7], the expression of PrPc correlates with predicted patients' prognosis based on cancer stage according to pathology results. These data are encouraging, since they are confirmed also in a wider group of patients. Moreover, by further collecting early clinical data, we were able to validate such a hypothesis. In fact, in the subgroup of patients with a relapse of the disease after a surgical R0 resection, the expression of PrPc was significantly higher compared with those patients without evidence of relapse.

Moreover, these data indicate a relationship between PrPc and chemo-resistance, since the relapse during adjuvant CT was significantly higher in patients according to their expression of PrPc at western blot.

This is the first project that investigates the sub-cellular nuclear compartmentalization of increased PrPc expression with electron microscopy *in vivo* in patients with PDAC treated with surgery. Our findings could open new research avenues: in fact, being PrPc markedly expressed in the nuclear compartment, studies should investigate whether this protein is involved in specific activation of some unknown nuclear pathways that can lead to a higher cancer aggressiveness and de-differentiation. Similarly, the correlation of PrPc expression with a chemo-resistant phenotype could be used not only for prognostic purposes. For instance, patients may be stratified for prognosis according to PrPc expression in the resected specimen. This may involve also the development of novel therapeutic outcomes. In fact, specific therapeutic agents designed to target PrPc metabolism should be able to reduce a pathological cell growth and to revert chemo-resistance.

CONCLUSION

The study confirms our previous data, highlighting *in vivo* in patients PDAC tissue the role of PrPc in PDAC aggressiveness. The evidence of a peculiar nuclear compartmentalization of this protein in the cellular nuclei of PDAC cells is in line with *in vitro* data from literature showing over-expression of PRNP gene in PDAC cells, and suggests the presence of some, still unknown, molecular pathways triggered by PrPc in the nuclear compartment. This extends the influence of PrPc beyond its role as cell-membrane glycoprotein. Finally, PrPc expression seems to be associated with a greater risk of relapse after radical surgery and in shifting the cancer towards a phenotype with a higher chemo-resistance. These data provide a step forward in the comprehension of PDAC biology, confirming that PrPc is likely to represent a marker of disease severity and a determinant of PDAC biology. Further studies are needed to validate these results and to investigate the molecular mechanism of PrPc in PDAC pathogenesis and its potential clinical application.

ARTICLE HIGHLIGHTS

Research background

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal human cancers, but its molecular basis is still poorly understood. Among the several new targets for the comprehension of its biology, cellular Prion protein (PrPc) deserves particular mention, since it seems to be involved also in tumorigenesis.

Research motivation

Recent evidences have shown a relationship between PrPc expression and PDAC, with a possible role of this protein in the molecular basis of PDAC aggressiveness itself.

Research objectives

The present study aimed to further analyze the occurrence of PrPc within PDAC tissues, by investigating the specific compartmentalization of PrPc within PDAC cells, which is a fundamental aspect in order to provide a significant advancement in understanding the biology of disease. Moreover, we aimed to correlate the presence of PrPc with clinical data in order to find an association with patients' prognosis.

Research methods

Samples from pancreatic tissues of 45 patients treated with pancreatic resection PDAC at a single institution were collected. Immune-gold stoichiometry within specific cell compartments was analyzed with electron microscopy in order to elucidate the subcellular compartmentalization of PrPc within PDAC cells. Western blotting was used to detect, quantify and compare the expression of PrPc in PDAC and control tissues, such as those of non-affected neighboring pancreatic tissue of the same patient. Data from western blot analysis where also used to perform a correlation with prognostic data from patients' follow-up.

Research results

Immune-electron microscopy highlighted an increased PrPc expression in PDAC ductal cells of all patients and allowed to detect a peculiar compartmentalization of PrPc within tumor cells, with a specific increase of PrPc in the nucleus. Furthermore, semi-quantitative analysis by using Western blotting, showed that PrPc increased almost three-fold in tumor pancreatic tissue compared with healthy pancreatic regions, with a significantly higher expression of PrPc in patients with disease recurrence at 12 mo after radical surgery, also in the subgroup of patients treated with adjuvant CT, thus revealing a possible higher chemoresistance.

Research conclusions

Our study provides evidence for a correlation between PrPc expression in PDAC and a worse biological behavior, with a higher recurrence rate and chemo-resistance. Moreover, it provides for the first-time the evidence of a peculiar subcellular compartmentalization of PrPc itself within PDAC cells.

Research perspectives

PrPc could be a molecular marker associated to PDAC aggressiveness and ominous prognosis. Its nuclear compartmentalization suggests the activation of specific, but still unknown, molecular pathways involved in the biology of the disease and further studies in this sense are necessary, since specific therapeutic agents designed to target PrPc metabolism should be able to reduce a pathological cell growth and to revert chemo-resistance.

ACKNOWLEDGEMENTS

The authors thank King SB for the Language Editing.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 14; 27(42): 7340-7349

DOI: 10.3748/wjg.v27.i42.7340

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

ORIGINAL ARTICLE

Basic Study

Gut microbiome composition can predict the response to nivolumab in advanced hepatocellular carcinoma patients

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Author contributions: Won EJ conceived of the study, participated in the study design and data analysis, and was responsible for writing and submission of the final manuscript; Kim MJ and Lee YJ carried out the experimental studies; Chung MW, Cho SB, Joo Y, Yun YW and Kim MJ analyzed and interpreted the data; Yun YW interpreted the statistical data; Hwang JE, Bae WK, Chung IJ, Shin MG and Shin JH were responsible for patient care and sample acquisition; All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

Supported by the National Research Foundation of Korea

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Abstract

BACKGROUND

Immunotherapy has revolutionized the clinical outcomes of intractable cancer patients. Little is known about the intestinal nonpathogenic bacterial composition of hepatocellular carcinoma (HCC) patients treated by immunotherapy.

AIM

To determine whether there is a correlation between gut bacterial composition and prognosis in HCC patients.

METHODS

From September 2019 to March 2020, we prospectively collected fecal samples and examined the gut microbiome of 8 advanced HCC patients treated with nivolumab as a second- or third-line systemic treatment. Fecal samples were collected before the start of immunotherapy. Fecal samples of patients with progression during treatment were collected at the time of progression, and fecal

(NRF) Grant funded by the Ministry of Education, Science, and Technology, No. NRF-2019R1C1C1004605, No. NRF-2019M3E5D1A02067953, and No. NRF-2021M3E5D1A0201518021.

Institutional review board statement: This study was carried out in accordance with all relevant institutional guidelines. The Ethics Committee of Chonnam National University Hwasun Hospital approved this study (CNUHH-2019-134) and written informed consent was obtained from all subjects.

Conflict-of-interest statement: All authors declare having no conflicts of interest.

Data sharing statement: No additional data are available.

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Specialty type: Gastroenterology and hepatology

Country/Territory of origin: South Korea

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: May 20, 2021
Peer-review started: May 20, 2021
First decision: June 13, 2021
Revised: June 18, 2021
Accepted: November 3, 2021

samples of patients who showed good response to nivolumab were collected after 5-7 mo as follow-up. Metagenomic data from 16S ribosomal RNA sequencing were analyzed using CLC Genomics Workbench. Microbiome data were analyzed according to therapeutic response.

RESULTS

All 8 patients were male, of which 6 had underlying chronic hepatitis B. A higher Shannon index was found in the responders than in the non-responders after nivolumab therapy (P = 0.036). The unweighted beta diversity analysis also showed that the overall bacterial community structure and phylogenetic diversity were clearly distinguished according to therapeutic response. There was no significant difference in the diversity or composition of the patient gut microbiome according to the immunotherapy used. Several taxa specific to therapeutic response were designated as follows: *Dialister pneumosintes, Escherichia coli, Lactobacillus reteri, Streptococcus mutans, Enterococcus faecium, Streptococcus gordonii, Veillonella atypica, Granulicatella* sp., and *Trchuris trichiura* for the non-responders; *Citrobacter freundii, Azospirillum* sp. and *Enterococcus durans* for the responders. Of note, a skewed *Firmicutes/Bacteroidetes* ratio and a low *Prevotella/Bacteroides* ratio can serve as predictive markers of non-response, whereas the presence of *Akkermansia* species predicts a good response.

CONCLUSION

The current presumptive study suggests a potential role for the gut microbiome as a prognostic marker for the response to nivolumab in treatment of HCC patients.

Key Words: Microbiome; Nivolumab; *Firmicutes/Bacteroidetes* ratio; *Prevotella/Bacteroides* ratio; Hepatocellular carcinoma; Prognosis

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Core Tip: Immune check point inhibitors are known to be an effective treatment option for advanced hepatocellular carcinoma (HCC), not only for second-line, but also as a first-line treatment. However, there are few predictive or prognostic markers for which patient group will have a good treatment response to immunotherapy or systemic therapy for HCC until now. Our study shows that non-responders to nivolumab in HCC patients have dysbiotic fecal composition, whereas a high *Prevotella/Bacteroides* ratio can predict a better response to nivolumab, highlighting a potential role for the gut microbiome as a prognostic marker for the response to nivolumab therapy.

Citation: Chung MW, Kim MJ, Won EJ, Lee YJ, Yun YW, Cho SB, Joo YE, Hwang JE, Bae WK, Chung IJ, Shin MG, Shin JH. Gut microbiome composition can predict the response to nivolumab in advanced hepatocellular carcinoma patients. *World J Gastroenterol* 2021; 27(42): 7340-7349
URL: https://www.wjgnet.com/1007-9327/full/v27/i42/7340.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i42.7340

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver, and it is now the second leading cause of cancer death worldwide[1]. Curative HCC treatment is only feasible in the early stages and involves local ablative procedures, surgical resection, or liver transplantation. For patients not amenable to curative therapy and in those with metastatic disease, other systemic treatments, such as sorafenib, are available. Beyond the limits of standard therapies, immunotherapy has been introduced for the oncological treatment of various solid malignancies. Nivolumab, as the first programmed cell death protein-1 (PD-1) agent, has shown great promise in treatment of various cancers, including melanoma and squamous cell carcinoma of the head and neck[2,3]. Recently, it was approved as the second-line agent for advanced HCC patients who have experienced sorafenib failure[4,5]. In line

Article in press: November 3, 2021
Published online: November 14, 2021

P-Reviewer: Tun HM
S-Editor: Gong ZM
L-Editor: A
P-Editor: Gong ZM

with this growing clinical relevance, stratification of patients receiving nivolumab therapy is now required to predict the prognosis and tumor aggressiveness.

There is mounting evidence that the gut microbiota can influence and modulate host immune responses; thus, microbiome profiling has been revealed as a predictor for response to immunotherapy among different groups and in different countries[6-8]. Moreover, some immunostimulatory bacterial species, including *Akkermansia muciniphila*[6], *Bifidobacterium longum*[7] and *Bacteroidetes fragilis*[8], were reported to elicit systemic immune responses and to reprogram the tumor microenvironment in mouse tumors treated with anti–cytotoxic T-lymphocyte-associated-4 and/or anti–PD-1 antibodies. The human gut microbiota has been shown to be associated with clinical responses to anti-PD-1/programmed death ligand 1 (PD-L1) immunotherapy in melanoma, non-small cell lung cancer, and renal cell carcinoma. However, the association between gut microbiota and response to nivolumab therapy is still not clear.

This study aimed to elucidate the impact of the gut microbiota on the prediction of prognosis in advanced HCC patients receiving nivolumab immunotherapy.

MATERIALS AND METHODS

Study design and patients

A total of 8 patients who received nivolumab (from September 2019 to August 2020) as second- or third-line treatment after sorafenib failure were included in the study. Tumor response was assessed using the Response Evaluation Criteria in Solid Tumors version 1.1[9]. The study was approved by the Chonnam National University Hwasun Hospital Institutional Review Board (CNUHH-2019-134). All patients signed a written informed consent form based on the principles of the Declaration of Helsinki. Patients were classified based on disease status (absence or progression at 12 mo after initiation of nivolumab therapy) and overall response (complete response, partial response, or stable disease for > 6 mo). Non-responders were those showing disease progression or stable disease for < 6 mo as well as those who died.

Collection of fecal samples and microbiome analysis

Fecal samples were collected prospectively, according to International Human Microbiome Standards guidelines (SOP_03_V1) at two time points — before the first nivolumab injection (< 1 mo, T0) and at the 3-mo follow-up (T1). The samples were immediately transferred on ice to our clinic and immediately stored at -80 °C until sample processing. Fecal genomic DNA was extracted as previously described[6-8]. DNA was extracted using the Cica Geneus® DNA Prep Kit (Kanto Chemical, Tokyo, Japan) following the manufacturer's instructions. The fecal microbiome was assessed by sequencing various regions (V3-V4) of the 16S ribosomal RNA bacterial gene at months 0 and 2. Briefly, polymerase chain reaction amplification was performed using 16S universal primers targeting the V3-V4 region of the bacterial 16S ribosomal gene. The joint pair length was set to encompass 467 base pair amplicons using the 2 × 300 paired-end MiSeq kit (Illumina, San Diego, CA, United States). For each sample, a sequencing library was generated by adding sequencing adapters. Detection of the sequencing fragments was performed using MiSeq technology by Macrogen (Seoul, South Korea).

Metagenomic and network analysis

The targeted metagenomic sequences from the microbiota were analyzed using the bioinformatics pipeline established by Vaiomer (Labège, France) using the FROGS guidelines. Low-depth samples (less than 9000 sequences per sample) were removed from the analysis. Sequences were trimmed and merged and then clustered into operational taxonomic units (OTUs) using CLC Genomics Workbench v. 10.1.1 and CLC Microbial Genomics Module v. 2.5 (Qiagen, Hilden, Germany). Taxonomic assignment of these sequences was carried based on the National Center for Biotechnology Information taxonomy database, with an OTU cutoff of 3%. The most abundant sequences were considered representative of each cluster and assigned to a taxonomy level based CLC Microbial Genomics default values.

Alpha diversity metrics (richness and Shannon's index) were calculated using the phyloseq R package based on rarefied OTU counts[10]. The beta diversity index was defined as the difference between the total number of species across the two groups and the number of species common to both[11]. Exploratory analysis of beta-diversity (between-sample diversity) was performed based on the Bray-Curtis measure of

dissimilarity as a principal coordinate analysis.

For the hierarchical cluster analysis, Bray-Curtis metrics and complete linkage clustering were implemented. At deeper taxonomic levels (from the phylum to genus level), we performed linear discriminant analysis (LDA) effect size analysis based on the non-parametric factorial Kruskal-Wallis sum rank test, to detect bacterial taxa with significantly different abundance between responders and non-responders. Then, LDA effect size was used to estimate the size effect of each differentially abundant taxon based on the criteria of LDA ≥ 3.0 and $P < 0.05$. Volcano plots showed the estimated log 2-fold difference in OTU abundance between responders and non-responders.

Statistical analysis

Alpha diversity metrics were compared by Mann-Whitney tests; for comparisons involving more than two groups, Bonferroni's correction was applied. The LDA was performed, and the volcano plots generated, at http://huttenhower.sph.harvard.edu/galaxy/. Two-tailed student's *t* test and the χ^2 test were used to test for differences in the phenotypical characteristics of the microbiome using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, United States).

RESULTS

Patients and sample

A single-site correlative study design was used to investigate the effects of gut microbiota on the efficacy of nivolumab in 8 adult HCC patients (Supplementary Table 1). All 8 patients received nivolumab as second- or third-line treatment after sorafenib failure. The median age was 62.5 (interquartile range: 58.0-66.25) years overall, and was not significantly different between the groups. All patients were male. Six patients (75%) were chronically infected with hepatitis B virus (HBV), and four patients (50%) had alcohol-related liver disease. All patients were Barcelona Clinic Liver Cancer stage C and stage IV by the modified Union for International Cancer Control stage system. While all 3 patients of the non-responder group had vascular invasion, only 1 patient (20%) had vascular invasion in the responder group. Presence of biliary invasion was 66.7% (2/3) in the non-responder group and 20% (1/5) in the responder group. The percentage of patients with an alpha-fetoprotein score ≥ 400 ng/mL at baseline was 66.7% (2/3) and 40% (2/5), each. Most patients (87.5%) were Child-Pugh class A, and only 1 patient in the good response group was Child-Pugh class B. All patients received three or more prior therapies before nivolumab.

Metagenome analysis

A total of 2027154 good-quality reads with a mean length of 301 base pairs were generated. A higher Shannon index was found in the responders when compared to the non-responders after nivolumab therapy ($P = 0.036$), reflecting a significantly higher species richness in the former group (Figure 1A and B). In contrast, there was no significant difference in alpha diversity within the same patients according to the nivolumab therapy (Figure 1C). The unweighted beta diversity analysis also showed that the overall bacterial community structure and phylogenetic diversity were similar between T0 and T1, but the responders and non-responders were clearly distinguished (Figure 2).

To identify the bacterial taxa associated with a good prognosis, different taxonomical levels were compared using LDA, and 36 and 4 species were found to be differentially abundant in the responders and non-responders, respectively (Figure 3). Of the bacterial species, *Ruminococcus gnavus* was abundant in the non-responders, while several bacterial taxa, including *Clostridia, Prevotella 9*, *Rikenellaceae, Alistipes,* the *Christensenellaceae R-7* group, *Dialister, Muribaculaceae, Desulfovibrionales, Deltaproteobacteria,* the *Eubacterium coprostanoligenes* group, *Acidaminococcaceae,* the *Lachnospiraceae NK4A136* group, *Roseburia, Mitsuokella, Ruminiclostridium 9, Marinifilaceae, Lachnospiraceae,* and *Ruminococcaceae* groups other than *Ruminococcus gnavus* were highly enriched in the responders. A volcano plot also showed several bacterial species specifically associated with the prognosis of nivolumab therapy in the HCC patients. *Dialister pneumosintes, Escherichia coli, Lactobacillus reteri, Streptococcus mutans, Enterococcus faecium, Streptococcus gordonii, Veillonella atypica, Granulicatella* sp., and *Trchuris trichiura* were specific to the non-responders, while *Citrobacter freundii, Azospirillum* sp., and *Enterococcus durans* were specific to the responders.

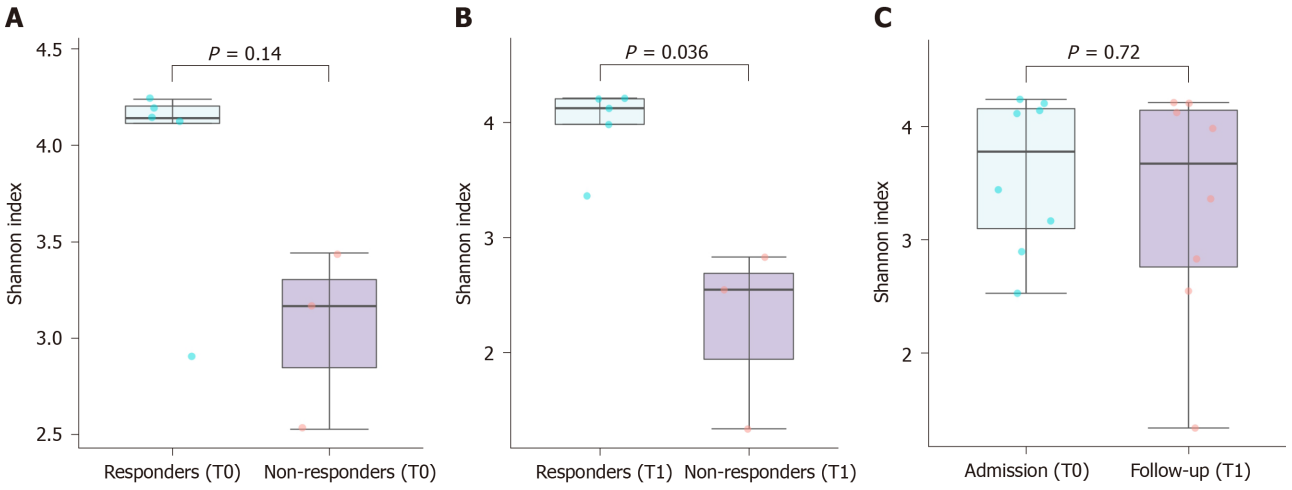


Figure 1 Alpha diversity indices (Shannon index) of hepatocellular carcinoma patients based on treatment and prognosis. A: The Shannon index of the non-responders on admission (T0) was not significantly lower than that of the responders (P = 0.14); B: The Shannon index of the non-responders was significantly lower than that of the responders after nivolumab therapy (T1); C: Nivolumab therapy did not alter the Shannon index. HCC: Hepatocellular carcinoma.

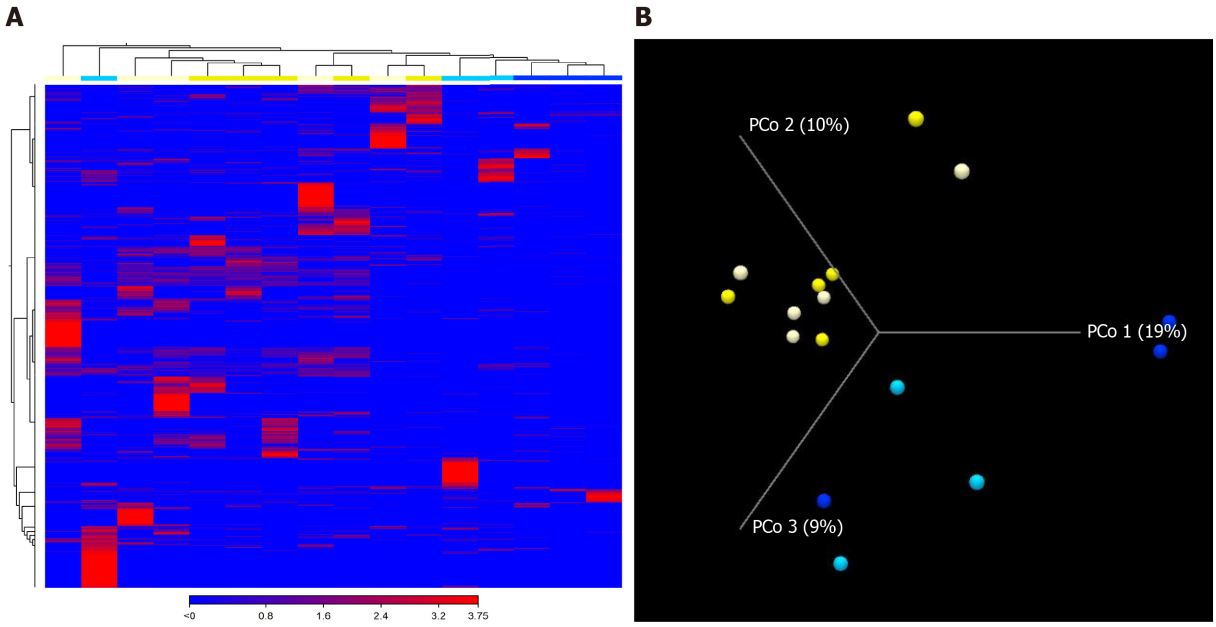


Figure 2 Composition of the gut microbiome in hepatocellular carcinoma patients is associated with the response to nivolumab. A: Heatmap showing the abundance of operational taxonomic units in responders (yellow) and non-responders (blue). The original comprehensive figure, including the names of bacterial taxa, is presented as Supplemental Figure 1; B: Unweighted beta diversity analysis showed that the overall bacterial community structure and phylogenetic diversity at T0 (light yellow and light blue) and T1 (yellow and blue) were similar; distinct clusters were not observed for responders (yellow) and non-responders (blue). Statistical values obtained by the PERMANOVA test are presented in Supplemental Table 2.

The gut microbiota composition in HCC patients was also described at the phylum and genus levels (Figure 4). At the phylum level, a skewed *Firmicutes/Bacteroidetes* ratio (< 0.5 or > 1.5) was more frequently found in the non-responders than in the responders (66.7% *vs* 10%, $P < 0.05$). The mean ratio of *Prevotella* species to *Bacteroides* species (P/B ratio) was significantly higher in the responders than in the non-responders (22.99 *vs* 2.312, $P = 0.024$). In addition, the presence of *Akkermansia* species was observed in two responders only, indicating that this could be a useful prognostic marker of the response to nivolumab therapy in advanced HCC patients.

DISCUSSION

A number of clinical trials investigating the therapeutic potential of manipulation of

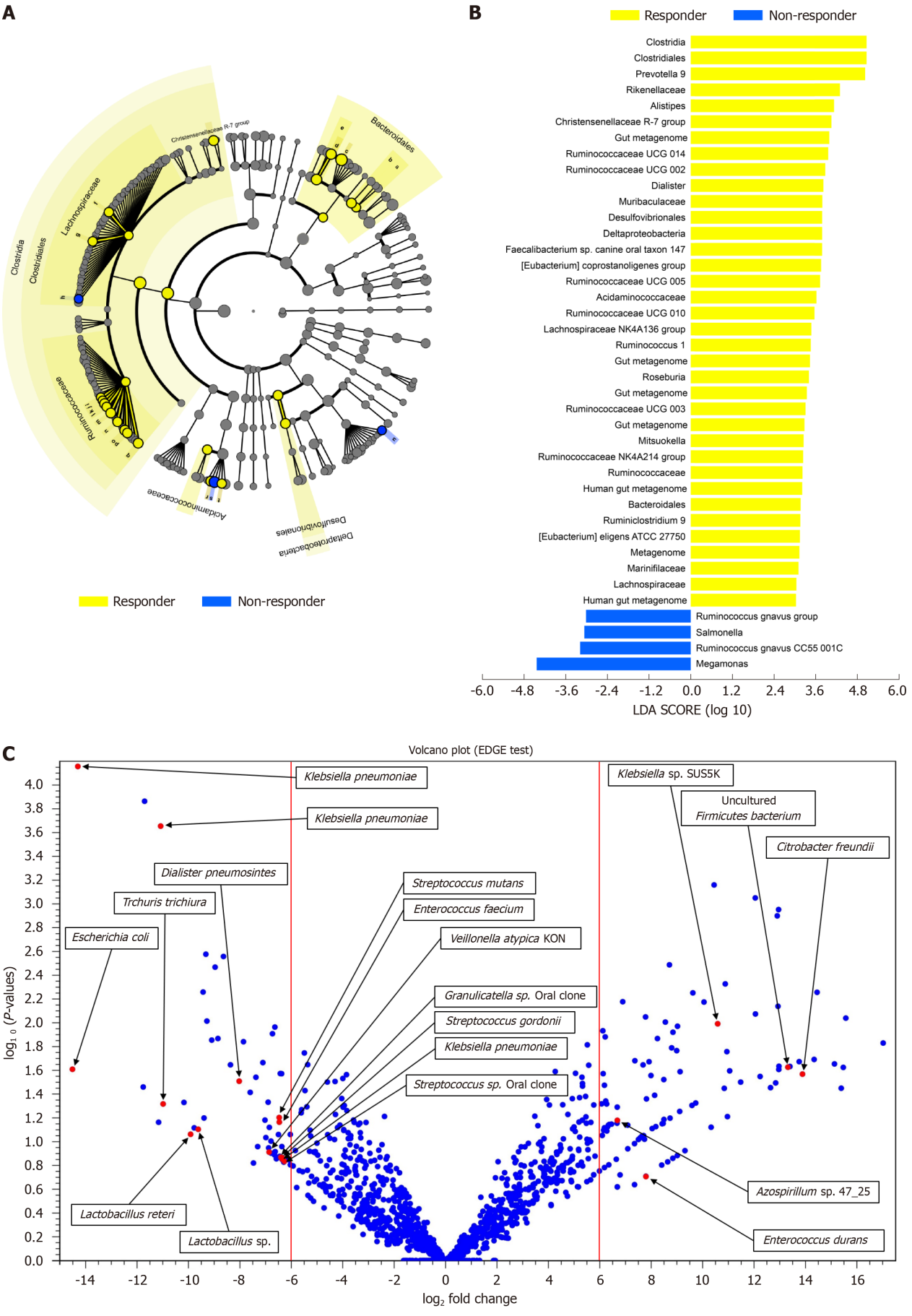


Figure 3 Specific bacterial taxa are associated with the prognosis of nivolumab therapy in hepatocellular carcinoma patients according to linear discriminant analysis effect size analysis. A: Taxonomic cladogram based on linear discriminant analysis (LDA) using effect size (LEfSe) showing

differences in fecal taxa. Dot size is proportional to the abundance of the taxon. Letters correspond to the following taxa: (a) *Marinifilaceae*, (b) *Muribaculaceae*, (c) *Prevotella 9*, (d) *Alistipes*, (e) *Rikenellaceae*, (f) the *Lachnospiraceae NK4A136* group, (g) *Roseburia*, (h) the *[Ruminococcus]gnavus* group, (i) *Ruminiclostridium 9*, (j) the *Ruminococcaceae NK4A214* group, (k) *Ruminococcaceae UCG-002*, (l) *Ruminococcaceae UCG-003*, (m) *Ruminococcaceae UCG-005*, (n) *Ruminococcaceae UCG-010*, (o) *Ruminococcaceae UCG-014*, (p) *Ruminococcus 1*, the (q) *[Eubacterium]coprostanoligenes* group, (r) *Dialister*, (s) *Megamonas*, (t) *Mitsuokella*, and (u) *Salmonella*; B: LDA scores for the differentially abundant taxa in the fecal microbiome of responders (yellow) and non-responders (blue). The length denotes the effect size for a taxon. $P = 0.05$ for the Kruskal-Wallis test; LDA score > 3; C: Volcano plot showing several bacterial taxa specifically related to the prognosis of nivolumab therapy in hepatocellular carcinoma patients. LDA: Linear discriminant analysis.

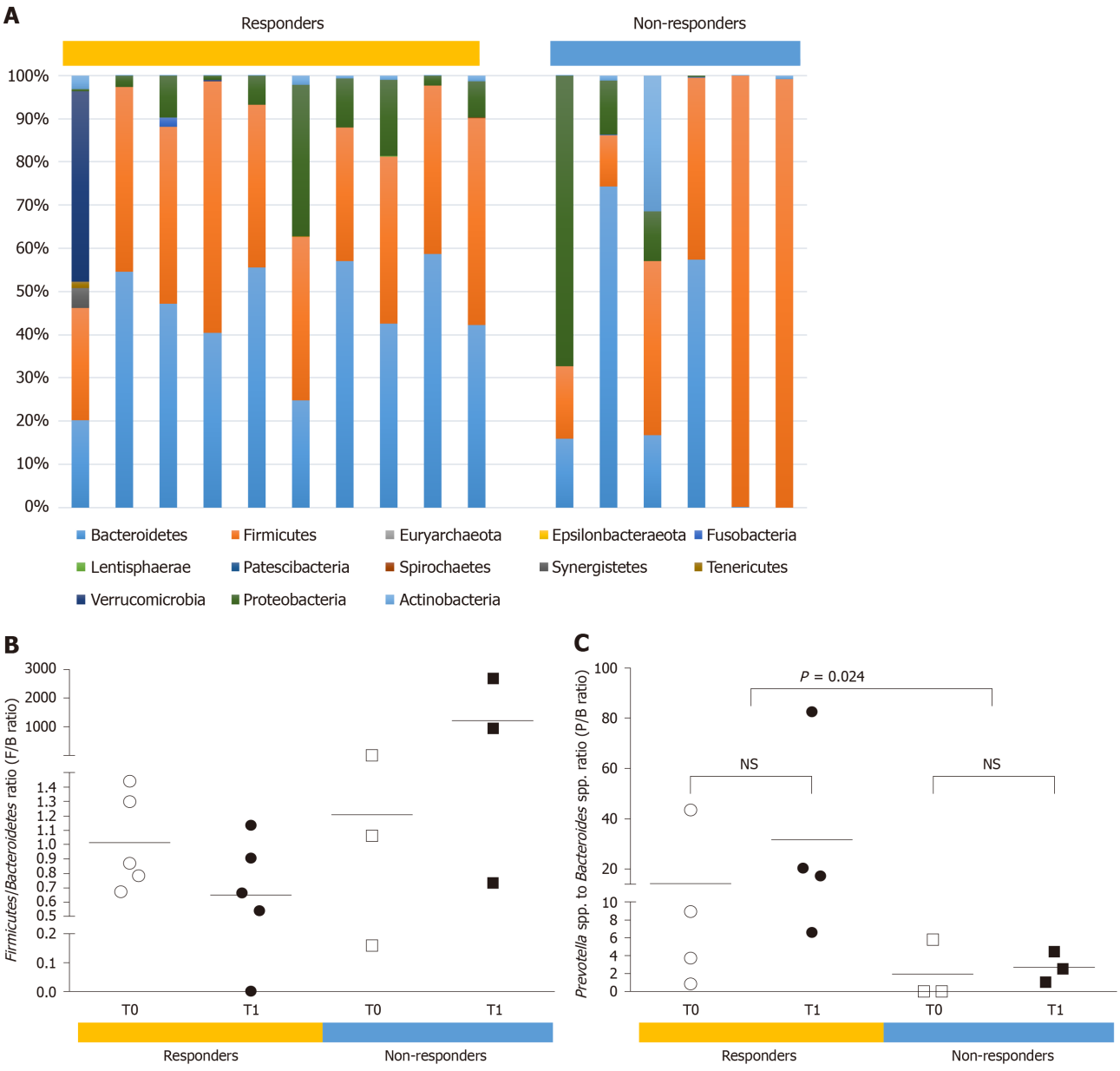


Figure 4 Potential prognostic markers of the response to nivolumab therapy in hepatocellular carcinoma patients. A and B: At the phylum level, a skewed *Firmicutes/Bacteroidetes* ratio (< 0.5 or > 1.5) was more frequent in the non-responders than the responders (66.7% *vs* 10.0%, $P = 0.018$); C: At the genus level, the ratio of *Prevotella* species to *Bacteroides* species (P/B ratio) was a prognostic marker of the response to nivolumab in hepatocellular carcinoma patients. The responders showed a significantly higher mean P/B ratio than the non-responders (22.99 *vs* 2.312, $P = 0.024$). NS: Statistically non-specific.

the gut microbiota in non-HCC cancer patients has already begun[6-8,12]. Regarding HCC, however, only two Chinese studies have been reported to date[13,14]. Reduced alpha diversity was found in the non-responders in those studies, in line with our data. In addition, we found that several bacterial taxa, such as *Dialister pneumosintes, Escherichia coli, Lactobacillus reteri, Streptococcus mutans, Enterococcus faecium, Streptococcus gordonii, Veillonella atypica, Granulicatella* sp., and *Trchuris trichiura*, were specific to non-responders, while *Citrobacter freundii, Azospirillum* sp., and *Enterococcus durans*

were specific to responders. The gut bacteria associated with the therapeutic response did not overlap with those in previous studies[13,14]. This might be partly due to differences in the techniques used to analyze samples, the reference databases, and geographical or racial/ethnic differences. While this study has not proven the clinical efficacy and benefit of our approach, it is feasible that it could reduce the therapeutic failure rate of nivolumab. Furthermore, it is not clear whether the aforementioned findings regarding the role of the gut microbiome in antitumor immune responses in animal models and patients with other tumor types also apply to patients with HCC.

Beyond dysbiosis, we suggested additional potential prognostic markers of the response to nivolumab therapy in HCC patients. We found that in all the responders, the *Firmicutes/Bacteroidetes* ratio ranged from 0.54 to 1.44 (mean of 0.88), whereas 66.7% of non-responders exhibited a highly skewed ratio (< 0.5 or > 1.5). Previous studies have shown elevated *Firmicutes/Bacteroidetes* ratio in the gut microbiota to be associated with obesity and older age[15], suggesting that it could be the result of dysbiosis arising from adaptation of individual microbial communities to long-term metabolic dysfunction[16]. Until recently, few studies have discussed the association between the *Firmicutes/Bacteroidetes* ratio and gastrointestinal cancer. Yu *et al*[17] reported that, in a rat model, precancerous lesions of gastric cancer had the highest *Firmicutes/Bacteroidetes* ratio. A reduced *Firmicutes/Bacteroidetes* ratio was also found to be related to the progression of liver diseases (liver cirrhosis and primary liver cancer) [18]. Notably, our data strongly support the notion that a highly skewed *Firmicutes/Bacteroidetes* ratio can be used to predict a lack of response to nivolumab therapy. This could facilitate the selection of appropriate immunotherapies for advanced HCC patients.

Furthermore, we found that a high P/B ratio was a better prognostic marker of the response to nivolumab therapy outcome in HCC patients. Overall, we found that responders had a significantly higher P/B ratio than non-responders. This observation is partly supported by a recent study reporting an elevated P/B ratio in fecal samples obtained from advanced-stage gastrointestinal cancer patients receiving anti-PD-1/PD-L1 and an improved response to anti-PD-1/PD-L1 treatment[19]. In line with previous studies, we also found that the presence of *Akkermansia* species can serve as a good prognostic marker of the response to nivolumab therapy in advanced HCC patients[5,13]. Although we did not analyze the gut microbiome of non-HCC patients, but previous studies have shown that patients with HCC and underlying cirrhosis typically present with profound dysbiosis. Thus, it is tempting to speculate that underlying dysbiosis could contribute to immunotherapy failure in some patients and that gut microbiome modulation may have even more profound effects in HCC than other tumors.

In this study, all 5 responders and 1 of 3 non-responders were infected by HBV. However, this may reflect the relatively higher proportion of HBV-related HCC in the Korean population in general, rather than the positive impact of HBV infection on nivolumab outcome. The association of HBV in nivolumab treatment response is not clear yet. For further consideration, in the CheckMate 040 data, the efficacy and safety of nivolumab treatment in sorafenib-experienced patients with advanced HCC were comparable between the HBV-infected group and the non-infected group[5].

A major limitation of this study is the small size of its cohort, which did not provide sufficient statistical power. This preliminary data should be interpreted with caution and further studies enrolling larger numbers of subjects may, thus, reveal additional microbial patterns. In addition, translation of a prognosis-associated microbial signature may not be straightforward, and thus several inherent variabilities between individuals within each cohort should be also considered as potential confounding factors. Nevertheless, our data highlights the promising possibility that a feasible approach may be to combine several microbial features for prediction of nivolumab treatment.

CONCLUSION

The current study suggests that non-responders to nivolumab have dysbiotic fecal composition. Moreover, a high P/B ratio predicted a better response to nivolumab in HCC patients, albeit that cohort was relatively small. Further studies should help define the role of these bacteria and their potential as novel biomarkers. New insights into the pathophysiological relevance of intestinal dysbiosis in the prognosis of HCC may lead to innovative therapeutic solutions, such as supplementation with probiotics, to prevent primary resistance to therapy.

ARTICLE HIGHLIGHTS

Research background

Systemic chemotherapy for hepatocellular carcinoma (HCC) has an important role, and immunotherapy in HCC is challenging.

Research motivation

Predictive or prognostic markers for systemic treatment for HCC have not been elucidated.

Research objectives

To investigate the correlation between gut microbiome and treatment response in advanced HCC.

Research methods

Patients who were treated with nivolumab for HCC were identified from one tertiary hospital in South Korea from September 2019 to August 2020. Metagenomic data from 16S ribosomal RNA sequencing were analyzed according to therapeutic response.

Research results

A higher Shannon index was found in the responders than in the non-responders after nivolumab therapy (P = 0.036). The unweighted beta diversity analysis also showed that the overall bacterial community structure and phylogenetic diversity were clearly distinguished according to therapeutic response. A skewed *Firmicutes/Bacteroidetes* ratio and a low *Prevotella/Bacteroides* ratio can serve as predictive markers of non-response, whereas the presence of *Akkermansia* species predicts a good response.

Research conclusions

Gut microbiome has a potential role as a prognostic marker for the response to nivolumab in the treatment of HCC patients.

Research perspectives

Microbiome study before and/or follow up of treatment with immunotherapy for HCC patients could be a prognostic marker, and it can be a criterion for selecting which systemic treatment should be used for each patient as part of precision medicine.

ACKNOWLEDGEMENTS

We would like to thank all participants in this study.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 14; 27(42): 7350-7361

DOI: 10.3748/wjg.v27.i42.7350

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

ORIGINAL ARTICLE

Retrospective Cohort Study

Presentation, patterns and predictive value of baseline liver tests on outcomes in COVID-19 patients without chronic liver disease

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Supported by National Institute on Aging of the National Institute of Health, No. R24AG06419; National Library of Medicine of the National Institutes of Health, No. R01LM012836.

Institutional review board statement: This study protocol was approved by the Northwell Health Institutional Review Board.

Informed consent statement: Informed consent statement was waived.

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Abstract

BACKGROUND

Coronavirus disease 2019 (COVID-19) infection is known to cause abnormal hepatic enzymes. The long term consequences of such elevations are uncertain.

AIM

To assessed the prevalence and prognostic value of initial liver enzymes in a large cohort of COVID-19 patients.

METHODS

We reviewed electronic medical records of 10614 COVID-19 patients without known chronic liver disease who were admitted to our health system from March 1, 2020, to April 30, 2020. We analyzed baseline demographics and liver chemistries. The primary outcome was in-hospital mortality, and the secondary outcome was a composite of in-hospital mortality or need for mechanical ventilation.

RESULTS

Subjects with abnormal liver tests had increased risks of mortality and composite outcome when compared to patients with normal measurements on unadjusted analysis and after adjustment for demographic factors.

Conflict-of-interest statement: The authors declare no conflicts of interest related to the content of the manuscript.

Data sharing statement: The data that support the findings of this study are available on request from COVID19@northwell.edu. The data are not publicly available due to restrictions as it could compromise the privacy of research participants.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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Specialty type: Gastroenterology and hepatology

Country/Territory of origin: United States

Peer-review report's scientific quality classification

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

Received: January 30, 2021
Peer-review started: January 30, 2021
First decision: June 4, 2021
Revised: July 12, 2021
Accepted: October 24, 2021
Article in press: October 24, 2021
Published online: November 14, 2021

CONCLUSION

In our diverse patient population, liver enzyme abnormalities are associated with increased mortality and the need for mechanical ventilation in subjects without chronic liver disease. Cholestasis patients are at the greatest risk for poor outcomes.

Key Words: COVID-19; Liver enzymes; Outcomes; Predictors

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Core Tip: We believe that our paper is an important contribution to the literature for the following reasons: (1) The cohort size is the largest to date; (2) We show the importance of initial liver tests in predicting outcomes; (3) In this large cohort, the finding of initial cholestatic pattern of injury being most predictive of poor outcome has not yet been described; and (4) This is a cohort from a large urban health system in the United States (New York) whose subject demographics reflect more the population seen in the United States. The other publications listed below are more uniform populations not representative of what our practitioners see in daily practice

Citation: Bernstein D, Roth N, Kim A, Epstein M, Hirschwerk D, Kvasnovsky CL, Satapathy SK. Presentation, patterns and predictive value of baseline liver tests on outcomes in COVID-19 patients without chronic liver disease. *World J Gastroenterol* 2021; 27(42): 7350-7361
URL: https://www.wjgnet.com/1007-9327/full/v27/i42/7350.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i42.7350

INTRODUCTION

The outbreak of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), first reported in Wuhan, China[1], was declared a global health emergency in January 2020 by the World Health Organization (WHO)[2].

Liver enzymes have been reported to be elevated in 15%-76% of patients with COVID-19 infection[3-14]. While most reported liver enzyme abnormalities have been mild, severe acute hepatitis and severe cholestasis has been reported secondary to COVID-19 infection[15,16]. The degree of enzyme elevation and patterns of liver enzymes can indicate patients' outcomes such as the need for mechanical ventilation and in-hospital mortality[3,12-17] but are not well described in a large, multi-ethnic cohort. Our study reports the results of our analysis of these factors in the largest cohort to date of hospitalized adults with COVID-19 infection without chronic liver disease.

MATERIALS AND METHODS

Study population and data collection

We obtained the medical records and compiled data from our electronic medical record on all patients with documented COVID-19 infection who were admitted to 12 hospitals in New York City, Long Island, and Westchester County, New York, within the Northwell Health system from the period of March 1, 2020, to April 30, 2020. A confirmed case was defined as a positive reverse transcriptase polymerase chain reaction for SARS-CoV-2 on a specimen obtained through nasopharyngeal swabbing, including if an initial test result was negative but repeat testing was positive. We collected the following demographic information: Age, sex, race, ethnicity, presence of co-morbid conditions, and body mass index (BMI). Race and ethnicity data were collected by self-report in pre-specified fixed categories. Baseline laboratory testing was defined as the first measurement available within 24 h of presentation. For this study, we excluded children under 18 years of age, patients missing a baseline value for serum alanine aminotransferase (ALT), and patients with known chronic liver

P-Reviewer: Hasan M, Lukito AA
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

disease. Exclusion for chronic liver disease was based on initial identification of this group of patients using ICD-10 codes and followed by manual chart review to confirm a diagnosis of chronic liver disease.

This study was supported by the Northwell Health COVID-19 Research Consortium and was approved by the Institutional Review Board for the Feinstein Institutes of Medical Research at Northwell Health as minimal-risk research using data collected for routine clinical practice, with the requirement for informed consent waived.

Statistical analysis

Liver enzymes, namely, serum levels of aspartate aminotransferase (AST), ALT, alkaline phosphatase, and total bilirubin levels were stratified into 4 groups: (1) Within normal limits; (2) Greater than the upper limit of normal (ULN) to less than or equal to 4 times the ULN; (3) Greater than 4 times the ULN to less than or equal to 10 times the ULN; and (4) Greater than 10 times the ULN. As *per* the American Association for the Study of Liver Diseases expert consensus recommendations, we defined the ULN for ALT as 25 U/L for women and 35 U/L for men[18]. The ULNs of other liver chemistries were defined using standard definitions and based on our health system laboratory's ULNs, which were: 40 U/L for AST, 125 U/L for alkaline phosphatase, and 1.2 mg/dL for total bilirubin. We classified patients who had more severe liver enzyme abnormalities into three patterns of liver injury: Hepatocellular (defined as ALT > 3 × ULN and alkaline phosphatase ≤ 2 × ULN), cholestatic (defined as alkaline phosphatase > 2 × ULN and ALT ≤ 3 × ULN), or mixed (defined as ALT > 3 × ULN and alkaline phosphatase > 2 × ULN)[7].

The primary outcome of interest was in-hospital mortality. The secondary outcome was a composite outcome of the need for mechanical ventilation or in-hospital mortality.

We summarized each continuous variable using its median and interquartile range (IQR). Categorical variables were summarized using counts and percentages. Comparisons between groups were assessed using Wilcoxon rank sum tests, chi-squared tests, and Fisher exact tests as appropriate. For survival analyses, we censored patients as alive without the event of interest on their date of hospital discharge or at 28 d of follow-up, whichever was earlier. Due to the low numbers of patients with elevations in alkaline phosphatase or bilirubin, we analyzed survival based on normal *vs* elevated measurements rather than the previously defined four categories. We analyzed survival using Kaplan-Meier survival curves using log-rank tests and estimated hazard ratios (HRs) and 95% confidence intervals (CIs) using univariate and multivariate Cox proportional hazards models. Multivariate models were adjusted for age, sex, race, ethnicity, BMI (< 30 kg/m^2 *vs* ≥ 30 kg/m^2), and presence of co-morbid conditions (hypertension, diabetes mellitus, coronary artery disease, chronic obstructive pulmonary disease, heart failure, chronic kidney disease, end-stage renal disease, and malignancy). We checked the proportional hazards assumption for each variable included in our Cox regression models using graphical assessment of the Kaplan-Meier survival curves and log[-log(survival)] *vs* log(time) graphs to look for parallel curves and by ensuring that Schoenfeld residuals were independent of time. Because the variables of age, sex, and race violated the proportional hazards assumption, we included interaction terms with time for those variables in our multivariate models. We used two-sided tests with alpha = 0.05. Statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC).

RESULTS

There were 11265 patients with COVID-19 hospitalized from March 1, 2020, to April 30, 2020. After excluding 106 children who were under 18 years old, 300 patients without baseline measurements of AST and ALT, and 245 patients with chronic liver disease, our study population included 10614 patients. Median length of hospital stay was 6 d (range: 0-58 d, IQR: 3-11 d). Baseline characteristics of the study population are described in Table 1. The median age was 65 years (range: 18-107 years, IQR: 54-77 years). The majority of patients were male (59%), white (38%), and non-Hispanic (21%). The most common comorbidities were hypertension (58%), obesity (39%), and diabetes (36%).

More than half of patients had elevations in AST (59%) and ALT (54%) on presentation, whereas alkaline phosphatase and bilirubin levels were elevated for only 13% and 5% of patients, respectively (Table 1). Most transaminase elevations were in group 2. 0.8% had ALT > 10 × ULN. 76% had AST higher than ALT on presentation.

Table 1 Baseline characteristics of the study population (*n* = 10614)

Demographics	
Sex	
Male	6243 (58.8%)
Female	4371 (41.2%)
Race	
White	4037 (38.0%)
Black	2248 (21.2%)
Asian	914 (8.6%)
Other/multiracial	2942 (27.7%)
Unknown	473 (4.5%)
Ethnicity	
Non-Hispanic or Latino or other/unknown	8338 (78.6%)
Hispanic or Latino	2276 (21.4%)
Age (yr)	65 (54-77)
Body mass index (kg/m^2)[1]	28.3 (24.9-32.6)
< 30	5120 (61.4%)
≥ 30	3220 (38.6%)
Presence of co-morbid conditions	
Hypertension	6204 (58.5%)
Diabetes mellitus	3764 (35.5%)
Coronary artery disease	1328 (12.5%)
Heart failure	832 (7.8%)
Malignancy	791 (7.5%)
Chronic obstructive pulmonary disease	608 (5.7%)
Chronic kidney disease (stage I-IV)	391 (3.7%)
End-stage renal disease	434 (4.1%)
Liver chemistries (within 24 h)	
AST (IU/L)[1]	46 (31-72)
Normal	4377 (41.3%)
> 1 to ≤ 4 × ULN	5713 (53.8%)
> 4 to ≤ 10 × ULN	437 (4.1%)
> 10 × ULN	85 (0.8%)
ALT (IU/L)	33 (21-56)
Normal	4883 (46.0%)
> 1 to ≤ 4 × ULN	5159 (48.6%)
> 4 to ≤ 10 × ULN	484 (4.6%)
> 10 × ULN	88(0.8%)
Alkaline phosphatase (IU/L)[1]	75 (59-98)
Normal	9242 (87.1%)
> 1 to ≤ 4 × ULN	1333 (12.6%)
> 4 to ≤ 10 × ULN	35(0.3%)
> 10 × ULN	2 (0.02%)

Bilirubin (mg/dL)[1]	0.5 (0.4-0.7)
Normal	10119 (95.4%)
> 1 to ≤ 4 × ULN	486 (4.6%)
> 4 to ≤ 10 × ULN	5 (0.05%)
> 10 × ULN	3 (0.03%)

[1]Missing data for some patients: Body mass index (n = 2274), alkaline phosphatase (n = 2), aspartate aminotransferase (n = 2), and bilirubin (n = 1).
Data summarized as n (%) for categorical variables and median (interquartile range) for continuous variables. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ULN: Upper limit of normal.

1160 patients (10.95) had severe hepatic enzyme elevations. Of these, 936 (8.9%) had a hepatocellular pattern, 133 (1.3%) a cholestatic pattern, and 91 (0.9%) a mixed pattern. The prevalence of hepatic test elevations differed based on sex, ethnicity, race, and presence of comorbidities (Table 2) Males were more likely to have elevations in AST (64.8% *vs* 50.1%, $P < 0.001$) and bilirubin (5.9% *vs* 2.8%, $P < 0.001$) but less likely to have elevations in alkaline phosphatase (12.0% *vs* 14.2%, $P = 0.001$) than females. There was no difference in ALT levels between males and females ($P = 0.34$).

In Kaplan-Meier survival analyses, any elevated liver chemistry was associated with increased mortality (Figure 1) or need for mechanical ventilation (Figure 2).

Having an elevated AST was associated with a higher risk of in-hospital mortality (unadjusted HR 1.34, 95%CI: 1.22-1.47, $P < 0.001$; adjusted HR 1.67, 95%CI: 1.49-1.86, $P < 0.001$) and in-hospital mortality or need for mechanical ventilation (unadjusted HR 1.34, 95%CI: 1.18-1.52, $P < 0.001$; adjusted HR 1.77, 95%CI: 1.51-2.08, $P < 0.001$). Increasing severity of AST abnormalities was associated with incrementally poor survival and need for mechanical ventilation (Table 3), and the trend remained significant after adjustment for age, sex, race, ethnicity, and the presence of co-morbid conditions. Compared to patients with normal AST, patients with AST > 10 × ULN were three times as likely to have in-hospital mortality (adjusted HR 2.64, 95%CI: 1.73-4.04, $P < 0.001$) and three times as likely to have in-hospital mortality or need for mechanical ventilation (adjusted HR 3.38, 95%CI: 1.78-6.40, $P < 0.001$).

In unadjusted models, having an elevated ALT appeared to be associated with a lower risk of in-hospital mortality (unadjusted HR 0.85, 95%CI: 0.78-0.92, $P < 0.001$) and the composite outcome (unadjusted HR 0.73, 95%CI: 0.65-0.83, $P < 0.001$). However, after adjusting for the confounding factors of age, sex, race, ethnicity and the presence of co-morbid conditions, an elevated ALT was associated with a 1.2 times higher risk of in-hospital mortality (adjusted HR 1.21, 95%CI: 1.09-1.34, $P < 0.001$) and the composite outcome (adjusted HR 1.21, 95%CI: 1.04-1.41, $P = 0.02$). Elevation in alkaline phosphatase was associated with a similar modestly increased risk of in-hospital mortality (adjusted HR 1.29, 95%CI: 1.12-1.48, $P < 0.001$) and in the composite outcome (adjusted HR 1.39, 95%CI: 1.12-1.72, $P = 0.003$). The associations between elevated bilirubin levels and outcomes became attenuated and lost significance after adjustment for confounding factors (Table 3).

The pattern of liver enzyme abnormalities affected outcomes. Patients with initial AST higher than ALT had a higher risk of in-patient mortality (adjusted HR 1.72, 95%CI: 1.46-2.02, $P < 0.001$) and the composite outcome of in-patient mortality or need for mechanical ventilation (adjusted HR: 1.84, 95%CI: 1.41-2.40, $P < 0.001$), compared to patients with AST less than or equal to ALT. Compared to patients with normal or non-severe elevations in ALT and alkaline phosphatase (with ALT < 3 × ULN and alkaline phosphatase ≤ 2 × ULN), patients with a severe cholestatic liver injury had a higher risk of in-patient mortality (adjusted HR 1.57, 95%CI: 1.07-2.28, $P = 0.02$) and the composite outcome of in-patient mortality or need for mechanical ventilation (adjusted HR 2.05, 95%CI: 1.17-3.58, $P = 0.01$). A hepatocellular or mixed pattern of liver injury was not associated with outcomes in adjusted models.

DISCUSSION

Our study shows that initial, abnormal liver enzymes are associated with poor outcomes in patients with COVID-19 infection in a diverse, multi-ethnic patient population. More than half of our patients upon presentation to the hospital had abnormal levels of serum aminotransferases, and a small proportion had elevations of

Table 2 Correlations between baseline liver chemistry elevations and demographic characteristics of the study population

	Elevated ALP	*P* value	Elevated AST	*P* value	Elevated ALT	*P* value	Elevated Bilirubin	*P* value
Sex		0.001		< 0.001		0.34		< 0.001
Female	619 (14.2%)		2191 (50.1%)		2336 (53.4%)		124 (2.8%)	
Male	751 (12.0%)		4044 (64.8%)		3395 (54.4%)		370 (5.9%)	
Race		< 0.001		< 0.001		< 0.001		0.51
Asian	134 (14.7%)		613 (67.1%)		533 (58.3%)		43 (4.7%)	
Black	228 (10.1%)		1332 (59.3%)		1146 (51.0%)		117 (5.2%)	
Other/multiracial	468 (15.9%)		1845 (62.7%)		1836 (62.4%)		139 (4.7%)	
Unknown	74 (15.6%)		300 (63.4%)		304 (64.3%)		17 (3.6%)	
White	466 (11.6%)		2145 (53.2%)		1912 (47.4%)		178 (4.4%)	
Ethnicity		< 0.001		< 0.001		< 0.001		0.51
Hispanic or Latino	398 (17.5%)		1410 (62.0%)		1450 (63.7%)		97 (4.3%)	
Non-Hispanic or Latino	864 (11.3%)		4384 (57.3%)		3852 (50.4%)		361 (4.7%)	
Unknown or other	108 (15.7%)		441 (64.0%)		429 (62.3%)		36 (5.2%)	
BMI (kg/m2)		< 0.001		0.08		< 0.001		< 0.001
< 30	730 (14.3%)		2996 (58.5%)		2694 (52.6%)		269 (5.3%)	
≥ 30	336 (10.5%)		1946 (60.4%)		1885 (58.5%)		111 (3.5%)	
Diabetes mellitus		0.03		< 0.001		< 0.001		< 0.001
No	920 (13.4%)		1158 (60.7%)		3897 (56.9%)		356 (5.2%)	
Yes	450 (12.0%)		2077 (55.2%)		1834 (48.7%)		138 (3.7%)	
Hypertension		< 0.001		< 0.001		< 0.001		0.36
No	642 (14.6%)		2683 (60.9%)		2649 (60.1%)		215 (4.9%)	
Yes	728 (11.7%)		3552 (57.3%)		3082 (49.7%)		279 (4.5%)	
Coronary artery disease		0.69		0.006		< 0.001		0.09
No	1194 (12.9%)		5501 (59.3%)		5184 (55.8%)		420 (4.5%)	
Yes	176 (13.3%)		734 (55.3%)		547 (41.2%)		74 (5.6%)	
Congestive heart failure		< 0.001		< 0.001		< 0.001		< 0.001
No	1223 (12.5%)		5811 (59.4%)		5419 (55.4%)		432 (4.4%)	
Yes	147 (17.7%)		424 (51.0%)		312 (37.5%)		62 (7.5%)	
COPD		0.24		< 0.001		< 0.001		0.46
No	1301 (13.0%)		5939 (59.4%)		5504 (55.0%)		462 (4.6%)	
Yes	69 (11.4%)		296 (48.7%)		227 (37.3%)		32 (5.3%)	
CKD (stage I-IV)		0.82		0.001		< 0.001		0.59
No	1321 (12.9%)		6036 (59.1%)		5582 (54.6%)		478 (4.7%)	
Yes	49 (12.5%)		199 (50.9%)		149 (38.1%)		16 (4.1%)	
End-stage renal disease		< 0.001		< 0.001		< 0.001		0.09
No	1265 (12.4%)		6044 (59.4%)		5621 (55.2%)		481 (4.7%)	
Yes	105 (24.2%)		191 (44.0%)		110 (25.4%)		13 (3.0%)	
Malignancy		0.58		< 0.001		< 0.001		0.049
No	1273 (13.0%)		5827 (59.3%)		5380 (54.8%)		446 (4.5%)	
Yes	97 (12.3%)		408 (51.7%)		351 (44.4%)		48 (6.1%)	

ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; CKD: Chronic kidney disease; COPD: Chronic obstructive pulmonary disease.

Table 3 Associations between liver chemistries and outcomes

	In-hospital mortality				In-hospital mortality or need for mechanical ventilation			
	Unadjusted analysis-HR (95%CI)	*P* value	Adjusted analysis[1]-HR (95%CI)	*P* value	Unadjusted analysis-HR (95%CI)	*P* value	Adjusted analysis[1]-HR (95%CI)	*P* value
AST								
Normal	Ref.		Ref.		Ref.		Ref.	
> 1 to ≤ 4 × ULN	1.29 (1.17-1.42)	< 0.001	1.65 (1.47-1.85)	< 0.001	1.29 (1.13-1.46)	< 0.001	1.73 (1.47-2.04)	< 0.001
> 4 to ≤ 10 × ULN	1.66 (1.38-1.99)	< 0.001	1.69 (1.33-2.14)	< 0.001	1.67 (1.27-2.20)	< 0.001	2.09 (1.42-3.07)	< 0.001
> 10 × ULN	3.50 (2.56-4.78)	< 0.001	2.64 (1.73-4.04)	< 0.001	3.89 (2.48-6.10)	< 0.001	3.38 (1.78-6.40)	< 0.001
ALT								
Normal	Ref.		Ref.		Ref.		Ref.	
> 1 to ≤ 4 × ULN	0.84 (0.77-0.91)	< 0.001	1.21 (1.09-1.34)	< 0.001	0.72 (0.63-0.81)	< 0.001	1.20 (1.02-1.40)	0.02
> 4 to ≤ 10 × ULN	0.88 (0.71-1.09)	0.24	1.22 (0.93-1.61)	0.15	0.79 (0.57-1.09)	0.15	1.40 (0.87-2.26)	0.16
> 10 × ULN	1.71 (1.20-2.45)	0.003	1.34 (0.81-2.22)	0.26	1.42 (0.80-2.51)	0.23	1.36 (0.58-3.19)	0.48
Alkaline phosphatase								
Normal	Ref.		Ref.		Ref.		Ref.	
Elevated	1.27 (1.14-1.43)	< 0.001	1.29 (1.12-1.48)	< 0.001	1.19 (1.01-1.42)	0.04	1.39 (1.12-1.72)	0.003
Bilirubin								
Normal	Ref.		Ref.		Ref.		Ref.	
Elevated	1.55 (1.32-1.82)	< 0.001	1.12 (0.92-1.37)	0.27	1.69 (1.34-2.13)	< 0.001	0.96 (0.71-1.30)	0.79
AST:ALT ratio								
≤ 1	Ref.		Ref.		Ref.		Ref.	
> 1	2.08 (1.82-2.37)	< 0.001	1.72 (1.46-2.02)	< 0.001	2.61 (2.13-3.19)	< 0.001	1.84 (1.41-2.40)	< 0.001
Pattern of liver injury[2]								
Normal or non-severe	Ref.		Ref.		Ref.		Ref.	
Hepatocellular	0.93 (0.80-1.09)	0.39	0.90 (0.74-1.11)	0.33	0.84 (0.66-1.07)	0.15	1.18 (0.85-1.65)	0.33
Mixed	1.14 (0.74-1.75)	0.56	1.06 (0.65-1.73)	0.82	1.00 (0.52-1.93)	0.99	1.02 (0.44-2.37)	0.96
Cholestatic	1.17 (0.85-1.61)	0.33	1.57 (1.07-2.28)	0.02	1.25 (0.81-1.93)	0.31	2.05 (1.17-3.58)	0.01

[1]Adjusted for age (time-varying), sex (time-varying), race (time-varying), ethnicity, and comorbidities (obesity, hypertension, diabetes, coronary artery disease, chronic kidney disease, end-stage renal disease, chronic obstructive pulmonary disease, heart failure, and cancer).
[2]Defined as: Normal or non-severe [alanine aminotransferase (ALT) ≤ 3 × ULN and alkaline phosphatase ≤ 2 × upper limit of normal (ULN)], hepatocellular (ALT > 3 × ULN and alkaline phosphatase ≤ 2 × ULN), mixed (ALT > 3 × ULN and alkaline phosphatase > 2 × ULN), and cholestatic (ALT ≤ 3 × ULN and alkaline phosphatase > 2 × ULN).
AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; CI: Confidence interval; HR: Hazard ratio; ULN: Upper limit of normal.

bilirubin and alkaline phosphatase. The predominant pattern of liver injury was hepatocellular. Of those who presented with abnormal transaminases, the overwhelming majority presented with enzymes one-four times the ULN and had an AST greater than ALT. Elevations in either ALT, AST, alkaline phosphatase or bilirubin were associated with increased mortality or need for mechanical ventilation.

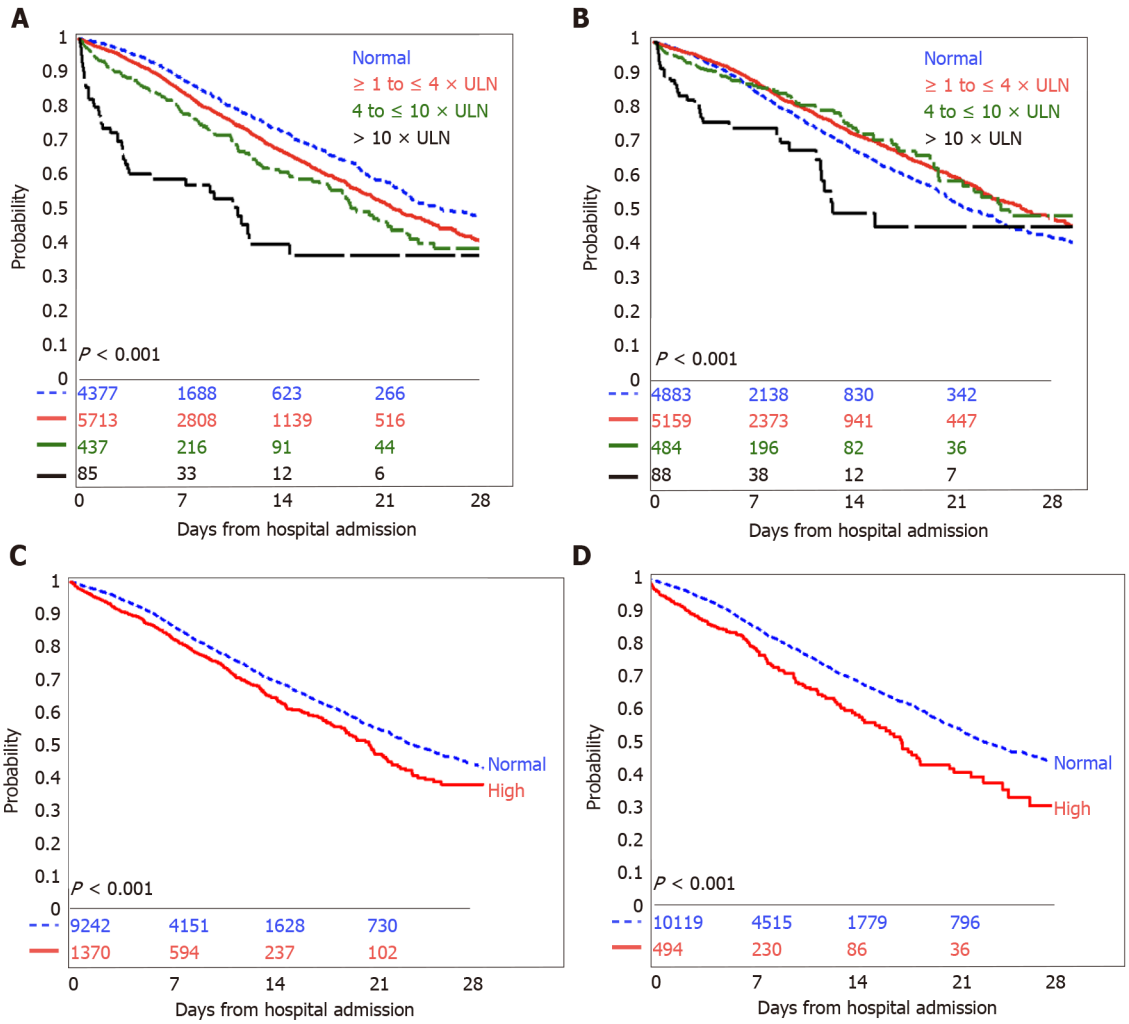


Figure 1 Kaplan-Meier curves for in-hospital mortality based on liver chemistries. A: Aspartate aminotransferase; B: Alanine aminotransferase; C: alkaline phosphatase; D: Bilirubin. ULN: Upper limit of normal.

Initial AST elevations appear to be the more significant predictor of mortality and the need for mechanical ventilation than elevations in ALT, alkaline phosphatase and total bilirubin with these risks increasing as AST elevations were more severe, even when adjusted for baseline demographics and the presence of co-morbidities. The mechanism behind this is unclear and still needs to be determined. One study noted AST-dominant aminotransferase elevation is common in COVID-19, can mirror disease severity, and appears to reflect true hepatic injury[12]. In this largest cohort of patients reported so far, we have demonstrated that AST predominant hepatic injury is common and associated with survival outcomes.

It has been postulated that abnormal liver enzymes in COVID-19 infection may be related to a direct cytotoxicity from active viral replication of SARS-CoV-2 in the liver, immune-mediated liver damage secondary to the systemic inflammatory response syndrome, hypoxic changes induced by respiratory failure, vascular changes due to COVID-19 induced thrombotic disease, endothelitis, right heart failure or drug induced liver injury[6,7,19-21]. SARS-CoV-2 virions have been detected in portal vein vessel lumens and endothelial cells by in situ hybridization[21]. SARS-CoV-2 enters host cells through the cell receptor, angiotensin converting enzyme 2 (ACE2). Cells with high ACE2 levels are associated with more severe COVID disease. ACE2 is highly expressed in the lung, cholangiocytes and hepatic vessel endothelial cells with some but less expression in hepatocytes. The high ACE2 expression in cholangiocytes may explain the severe cholestatic disease seen in the subset of patients presenting with this liver enzyme pattern[16,21]. Hypoxia is associated with increased expression of ACE2 receptors in hepatocytes and cholangiocytes[21]. While our study is unable to differentiate between a direct viral effect or an immune-induced response, we show that patients with COVID-19 infection have a high prevalence of abnormal liver chemistries prior to the administration of any COVID-19 specific medications.

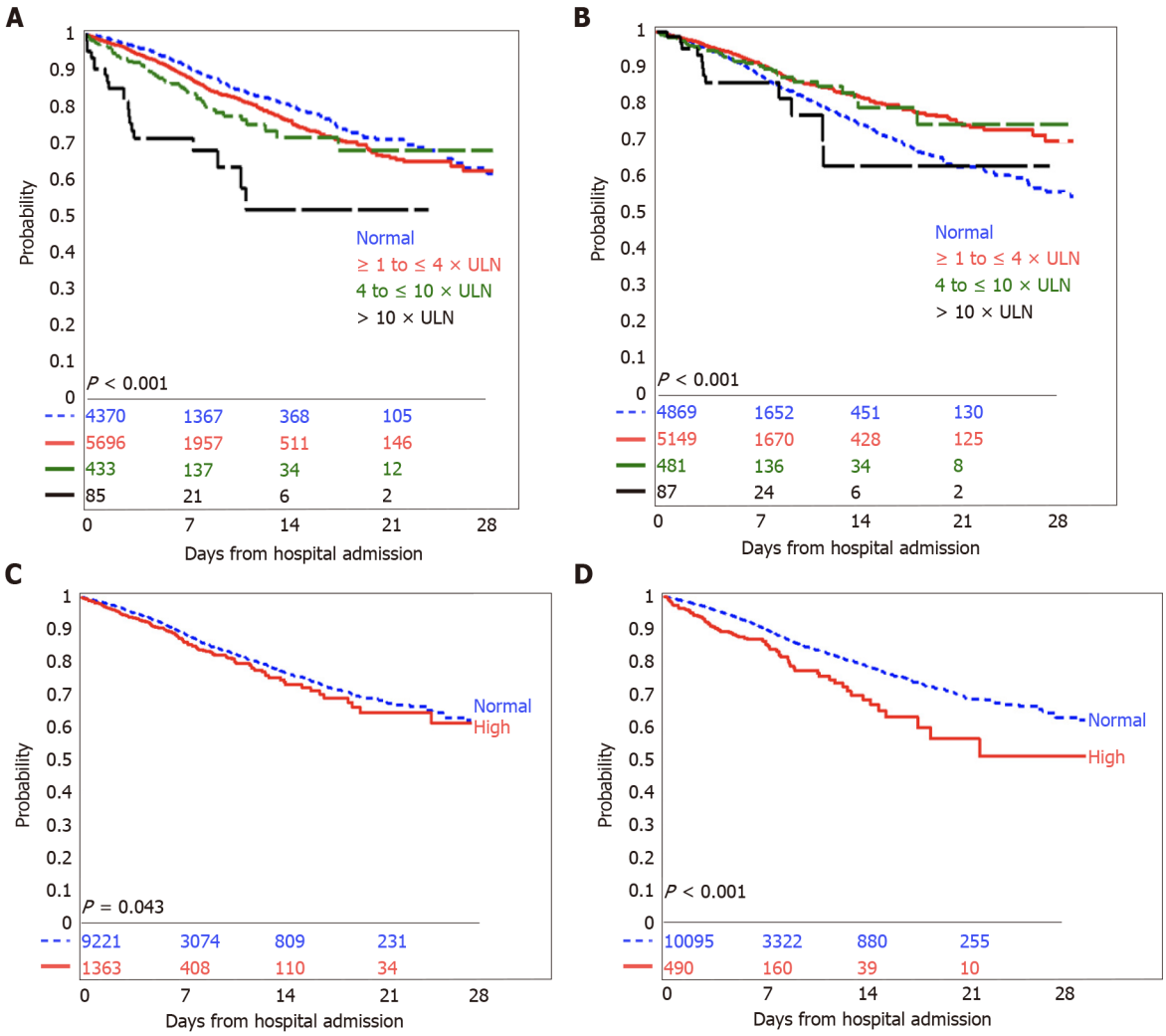


Figure 2 Kaplan-Meier curves for in-hospital mortality or need for mechanical ventilation based on liver chemistries. A: Aspartate aminotransferase; B: Alanine aminotransferase; C: Alkaline phosphatase; D: Bilirubin. ULN: Upper limit of normal.

Previous published smaller studies have reported that the pattern of liver injury, either hepatocellular or mixed hepatocellular-cholestatic, appears to be more common in people with severe COVID-19[6-8,14,22]. Based upon our strict definition of these patterns, we found that the hepatocellular pattern of injury on presentation was the most common type of injury noted and that this pattern was not associated with outcomes in an adjusted model. While an initial presentation of severe cholestasis was uncommon, it was associated with a higher risk of in-patient mortality or the need for mechanical ventilation. This is an important finding and should alert caregivers of the risk of decreased survival with this specific pattern of presentation.

Our study does have several limitations. All data was collected from the electronic health record database, as the large size of the cohort precluded a manual review of all cases. Baseline laboratory tests collected were defined as within 24 h of presentation to the emergency department, but this definition does not take into account the length of patient symptoms prior to presentation. Our study specifically evaluated initial laboratory data prior to the initiation of any multimodal treatments such as patient positioning, supplemental oxygen and medical therapies, both standard of care and experimental as our sites had access to several clinical trials medications. While initiation of therapies during hospital admission may have influenced overall outcomes, it is beyond the scope of this paper to assess the effects of individual therapies on outcomes. The absence of data on patients who remain hospitalized at the final study date may have biased the survival outcomes of the study.

Our study specifically excluded patients with known chronic liver disease. This was done through review of ICD-10 diagnostic codes followed by manual chart review as needed for confirmation. Despite this methodology, it remains a possibility that our cohort did include patients with undiagnosed non-alcoholic fatty liver disease, as the

prevalence of obesity and diabetes in our population was 39% and 36%, respectively, and both of these co-morbidities are associated with this condition. Diabetic patients in our study had significantly lower prevalence of AST and ALT elevations than non-diabetics. In contrast, compared to non-obese patients, obese patients had a higher prevalence of ALT elevations but not the other measured liver chemistries. These findings may make it unclear whether the presence of undiagnosed non-alcoholic fatty liver disease affects the risk of liver injury, survival or the need for mechanical ventilation in patients infected with COVID-19 and further study is warranted.

CONCLUSION

In this largest cohort of hospitalized COVID-19 patients reported so far, we have shown an increased mortality and the need for mechanical ventilation is associated with hepatic test elevations, and in particular those with cholestasis, these findings should be taken into consideration during the initial evaluation of COVID-19 patients, both in the in-patient and out-patient setting and patients with significantly elevated liver enzymes should be prioritized for future treatments as these treatments become available.

ARTICLE HIGHLIGHTS

Research background

Liver enzyme abnormalities are commonly seen in coronavirus disease 2019 (COVID-19) infection. We assessed the prevalence and prognostic value of the initial liver enzymes in patients admitted to hospital with COVID-10 infection.

Research motivation

At the time of the writing of this manuscript, our health system had data on 10614 individual patients admitted with COVID-10 infection. We wanted to assess the prevalence of liver enzyme abnormalities in these patients and determine if any particular enzyme pattern would predict prognosis.

Research objectives

Determine the prevalence of abnormal liver enzymes in patients admitted to the hospital with COVID-19 infection. Determine the prognostic value of initial liver enzymes on mortality and/or the need for mechanical ventilation. Determine if any particular abnormal liver enzyme pattern was most predictive of poor outcome in COVID-19 infection.

Research methods

Review of electronic medical records of 10614 patients admitted to the hospital with COVID-19 infection.

Research results

Elevated liver enzymes are common upon initial hospital presentation of COVID-19 infection.

Research conclusions

Increased mortality and the need for mechanical ventilation is associated with elevated hepatic enzymes in COVID-19 patients without chronic liver disease.

Research perspectives

This is an important study which highlights the importance of initial liver enzyme patterns in predicting outcomes. Health care workers should be aware of these findings to better triage COVID-19 patients.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 November 14; 27(42): 7362-7375

DOI: 10.3748/wjg.v27.i42.7362

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

ORIGINAL ARTICLE

Retrospective Cohort Study

Survival and outcomes for co-infection of chronic hepatitis C with and without cirrhosis and COVID-19: A multicenter retrospective study

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Author contributions: Afify S and Omran DA were the guarantors and designed the study; Afify S, Maher R, Eysa B, Abdel Ghaffar MM, Abdelhalim A and Edris MA participated in the acquisition of the data; Abo-Elazm OM participated in the analysis, and interpretation of the data; Shousha HI and Hamid FA drafted the initial manuscript; Afify S, Maher R, Omran DA, and Eysa B revised the article critically for important intellectual content.

Institutional review board statement: This study was approved by the research ethics committee of the General Organization for Teaching Hospitals and Institutes(number ITH00123) 22July 2020).

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Abstract

BACKGROUND

Chronic liver disease, particularly cirrhosis, is associated with worse outcomes in patients infected with coronavirus disease 2019 (COVID-19).

AIM

To assess outcomes of COVID-19 infection among patients with pre-existing hepatitis C with or without liver cirrhosis.

METHODS

This multicenter, retrospective cohort study included all cases of confirmed co-infection of severe acute respiratory syndrome coronavirus 2 and chronic hepatitis

Informed consent statement: All study subjects gave written informed consent before study inclusion.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest to report.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Egypt

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 21, 2021
Peer-review started: March 21, 2021
First decision: April 29, 2021
Revised: May 12, 2021
Accepted: October 24, 2021
Article in press: October 24, 2021
Published online: November 14, 2021

P-Reviewer: Beraldo RF, Gupta T
S-Editor: Ma YJ
L-Editor: A

C with or without liver cirrhosis who were admitted to six hospitals (Al-Sahel Hospital, Al-Matareya Hospital, Al-Ahrar Hospital, Ahmed Maher Teaching Hospital, Al-Gomhoreya Hospital, and the National Hepatology and Tropical Medicine Research Institute) affiliated with the General Organization for Teaching Hospitals and Institutes in Egypt. Patients were recruited from May 1, 2020, to July 31, 2020. Demographic, laboratory, imaging features, and outcomes were collected. Multivariate regression analysis was performed to detect factors affecting mortality.

RESULTS

This retrospective cohort study included 125 patients with chronic hepatitis C and COVID-19 co-infection, of which 64 (51.20%) had liver cirrhosis and 40 (32.00%) died. Fever, cough, dyspnea, and fatigue were the most frequent symptoms in patients with liver cirrhosis. Cough, sore throat, fatigue, myalgia, and diarrhea were significantly more common in patients with liver cirrhosis than in non-cirrhotic patients. There was no difference between patients with and without cirrhosis regarding comorbidities. Fifteen patients (23.40%) with liver cirrhosis presented with hepatic encephalopathy. Patients with liver cirrhosis were more likely than non-cirrhotic patients to have combined ground-glass opacities and consolidations in CT chest scans: 28 (43.75%) *vs* 4 (6.55%), respectively (P value < 0.001). These patients also were more likely to have severe COVID-19 infection, compared to patients without liver cirrhosis: 29 (45.31%) *vs* 11 (18.04%), respectively (P value < 0.003). Mortality was higher in patients with liver cirrhosis, compared to those with no cirrhosis: 33 (51.56%) *vs* 9 (14.75%), respectively (P value < 0.001). All patients in Child-Pugh class A recovered and were discharged. Cirrhotic mortality occurred among decompensated patients only. A multivariate regression analysis revealed the following independent factors affecting mortality: Male gender (OR 7.17, 95%CI: 2.19–23.51; P value = 0.001), diabetes mellitus (OR 4.03, 95%CI: 1.49–10.91; P value = 0.006), and liver cirrhosis (OR 1.103, 95%CI: 1.037–1.282; P value < 0.0001). We found no differences in liver function, COVID-19 disease severity, or outcomes between patients who previously received direct-acting antiviral therapy (and achieved sustained virological response) and patients who did not receive this therapy.

CONCLUSION

Patients with liver cirrhosis are susceptible to higher severity and mortality if infected with COVID-19. Male gender, diabetes mellitus, and liver cirrhosis are independent factors associated with increased mortality risk.

Key Words: COVID-19; Egypt; Outcome; Liver cirrhosis; Chronic hepatitis C

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Core Tip: Chronic liver disease, particularly cirrhosis, is associated with worse outcomes in patients infected with coronavirus disease 2019 (COVID-19). This study examined the impact of COVID-19 infection on patients with chronic hepatitis C during the first COVID-19 peak in Egypt. This retrospective cohort study was performed in six Egyptian hospitals. We found that cirrhotic patients had higher rates of pneumonia, severe COVID-19, and mortality. Cirrhotic mortality was observed among decompensated patients only. Male gender, diabetes mellitus, and liver cirrhosis were independent factors associated with increased mortality risk in Egyptian patients with COVID-19 and chronic hepatitis C.

Citation: Afify S, Eysa B, Hamid FA, Abo-Elazm OM, Edris MA, Maher R, Abdelhalim A, Abdel Ghaffar MM, Omran DA, Shousha HI. Survival and outcomes for co-infection of chronic hepatitis C with and without cirrhosis and COVID-19: A multicenter retrospective study. *World J Gastroenterol* 2021; 27(42): 7362-7375
URL: https://www.wjgnet.com/1007-9327/full/v27/i42/7362.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i42.7362

P-Editor: Ma YJ

INTRODUCTION

On March 11, 2020, WHO declared severe acute respiratory syndrome coronavirus 2 (SARS-COV-2), also known as coronavirus disease 2019 (COVID-19), as the sixth pandemic of the 21st century[1]. As of January 2021, the virus has caused more than 85 million confirmed infections and about 2 million deaths worldwide[2]. In Egypt, about 140000 confirmed infections and about 8000 deaths were recorded by the Egyptian Ministry of Health and Population as of January 2021[3]. Lung lesions can cause major damage in those infected with COVID-19, and liver injury also has been reported[4]. Studies conducted in Wuhan early in the epidemic outbreak found that up to 50% of infected patients had abnormal liver enzymes. Zhang *et al*[5] showed that SARS-CoV-2 infection occurs in 2%–11% of patients with pre-existing liver conditions.

COVID-19's adverse effects on the liver could be explained by both direct cytopathic injury and indirect effects of the virus[4]. It is well-established that SARS-CoV-2 uses angiotensin-converting enzyme receptors to gain entry into cells. These receptors are much more abundant in cholangiocytes (59.70%) than in hepatocytes (2.60%)[6]. The bile duct epithelium also plays an important role in regeneration after injury and immune response. Thus, the indirect effects may be due to exposure to multiple insults. For example, in severe cases admitted to intensive care, hemodynamic instability can lead to hypoperfusion and ischemic liver injury. Pneumonitis-associated hypoxia can lower mean arterial pressure, causing a synergistic effect contributing to this ischemic insult[4]. Another contributing factor could be toxic effects of medications (*e.g.*, steroids, non-steroid anti-inflammatory drugs, antibiotics, anticoagulants, antivirals), which are associated predominantly with hepatocellular rather than cholestatic liver injury[6,7]. Finally, immune dysregulation can lead to systemic inflammatory response syndrome, or cytokine storm, which is the release of inflammatory mediators (*e.g.*, interleukins IL-6 and IL-1, TNF- α, and interferon) that can cause and exacerbate liver injury[8].

Hepatitis C virus remains the most common etiology of overt and occult chronic liver diseases, liver cirrhosis, and risk of hepatocellular carcinoma in Egypt[9]. Chronic liver disease is associated with immune dysregulation and multiple system involvement (*e.g.*, cardiomyopathy, hepatopulmonary syndrome, and coagulopathy)[9]. Patients in Child-Pugh classes B and C and those with higher MELD scores have much higher mortality rates than patients in class A or with lower MELD scores[10]. Importantly, the cause of death in most of these patients is respiratory failure rather than acute on top of chronic liver failure[10].

Transaminitis, which is abnormal levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), is the most frequent and direct cause of disease severity in patients without cirrhosis[4]. Decompensation with worsening liver symptoms (*e.g.*, ascites, spontaneous bacterial peritonitis, hepatic encephalopathy, and variceal hemorrhage) has been reported in patients with chronic liver disease and is associated with a high risk of death. Interestingly, decompensation occurs without respiratory symptoms[11]. This study aimed to demonstrate the impact of COVID-19 infection on patients with pre-existing hepatitis C with or without liver cirrhosis during the first COVID-19 peak in Egypt.

MATERIALS AND METHODS

Study design and patient selection

This multicenter, retrospective cohort study included all cases of confirmed co-infection of SARS-CoV-2 and chronic hepatitis C with or without liver cirrhosis who were admitted to six hospitals (Al-Sahel Hospital, Al-Matareya Hospital, Al-Ahrar Hospital, Ahmed Maher Teaching Hospital, Al-Gomhoreya Hospital, and the National Hepatology and Tropical Medicine Research Institute) in the General Organization for Teaching Hospitals and Institutes in Egypt. Patients were recruited from May 1, 2020, to July 31, 2020. The diagnosis of COVID-19 was based on a positive RT-PCR from nasopharyngeal swabs. Patients with negative RT-PCR results or those who did not undergo the swab were excluded.

Ethics statement

The authors assert that all procedures contributing to this work complied with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. All procedures involving human subjects and patients were approved by the research

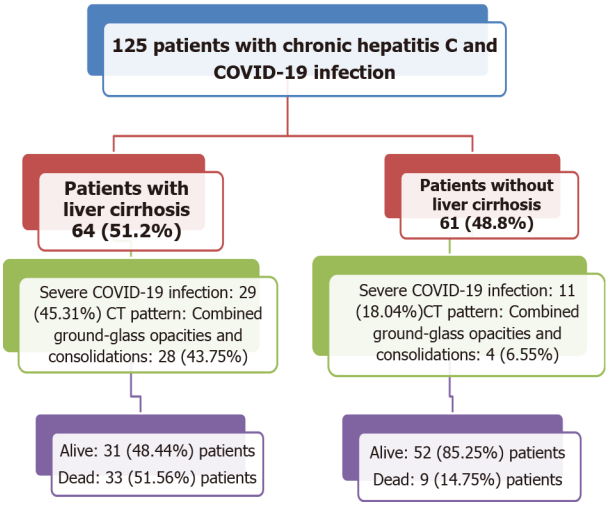


Figure 1 Flow chart of the study cohort.

ethics committee of the General Organization for Teaching Hospitals and Institutes. Written informed consent was obtained from all patients.

Methods

Baseline demographic data collected included age, gender, cigarette smoking status, and comorbidities. Other information recorded included general respiratory and gastrointestinal symptoms, chest CT scans, and laboratory results (complete blood count, liver and renal function, coagulation, D-dimer, ferritin, and C-reactive protein). We also included treatments administered to the patients and COVID-19 disease classification and outcome. COVID-19 severity was categorized as mild, moderate, or severe, according to the management protocol of the Egyptian Ministry of Health and Population[12]. Mild cases were symptomatic with lymphopenia or leucopenia and no radiological lung affection by pneumonia. Moderate cases were symptomatic with radiological features of pneumonia with or without leucopenia and lymphopenia. Severe and critical cases included any of the following: respiratory rate > 30 per minute; SaO_2 < 92 in room air; PaO_2/FiO_2 ratio < 300; chest radiology showing > 50% lung affection or progressive lung affection within 24 to 48 h; or critically ill at SaO_2 < 92, respiratory rate > 30 per minute, or PaO_2/FiO_2 ratio < 200 despite oxygen therapy. Severe and critical cases were indicated for intensive care unit (ICU) admission. Treatments were applied according to the protocol[12].

Statistical analysis

Data were analyzed using SPSS version 25 (SPSS Inc., Chicago, IL, United States). Numerical data are expressed as mean and standard deviation or median and range, as appropriate. Qualitative data are expressed as frequency and percentage. The Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, Student's *t*-test or the Mann-Whitney test (non-parametric *t*-test) was used to compare groups, as appropriate. A P value ≤ 0.05 was considered significant. To study possible associations between selected variables (gender, liver cirrhosis, and diabetes mellitus) and mortality, we fitted multiple logistic regression models. The results are expressed as the odds ratio (OR) with 95% confidence interval (CI).

RESULTS

This study included 125 patients infected with chronic hepatitis C virus and COVID-19 (Figure 1). Of those, 64 (51.2%) patients had liver cirrhosis: 25 (39.06%) were classified as Child-Pugh class A, 22 (34.38%) as class B, and 17 (26.56%) as class C. Patient residences included five Egyptian governorates: Cairo, Giza, Al-Behera, Al-Qalubya, and Al-Menofeya. Over half (61.2%) were older than 60 years, and most (68.8%) were men. Table 1 presents the baseline demographic features. Regarding COVID-19 symptoms, five (4.0%) patients were asymptomatic. The most common symptoms

Table 1 Baseline demographic features of patients (*n* = 125 patients)

Item	*n* (%)
Egyptian governorate	
Cairo	78 (62.4)
Al-Menofeya	17 (13.6)
Giza	14 (11.2)
Al-Qalubya	9 (7.2)
Al-Behera	7 (5.6)
Age (yr)	
20 to 30	1 (0.8)
30 to 40	6 (4.8)
40 to 50	10 (8.1)
50 to 60	31 (25.0)
60 to 70	35 (28.2)
70 to 80	35 (28.2)
80 to 90	6 (4.8)
Gender	
Male	86 (68.8)
Female	39 (31.2)
Cigarette smoking	10 (8.0)
History of contact with COVID-19 case	32 (25.6)
Diabetes mellitus	52 (41.6)
Hypertension	52 (41.6)
Direct-acting antiviral therapy treatment	
Not previously treated	108 (86.4)
Sustained virological response	17 (13.6)
COPD	1 (0.8)
Coronary artery disease	12 (9.6)
Acute kidney injury	1 (0.8)
Chronic renal insufficiency	8 (6.4)
Heart failure	2 (1.6)
Bronchial asthma	1 (0.8)
CT pattern	1 (0.8)
Consolidations and ground-glass opacities	31 (24.8)
Ground-glass opacities	94 (75.2)
Lesion distribution on CT	
Bilateral	122 (97.6)
Unilateral	3 (2.4)
COVID-19 case severity	
Moderate	86 (68.8)
Severe	39 (31.2)
Admission zone	
ICU	24 (19.2)

Intermediate care	6 (4.8)
Ward	95 (76.0)

COVID-19: Coronavirus disease 2019; COPD: Chronic obstructive pulmonary disease; CT: Computed tomography; ICU: Intensive care unit.

were dyspnea (80 patients, 64.0%), fever (78 patients, 62.4%), and cough (55 patients, 44.0%), and five (4.0%) had diarrhea. Table 2 summarizes the patient symptoms. Table 3 summarizes the baseline laboratory results and treatment protocols among the studied patients.

We compared the characteristics of patients with and without liver cirrhosis. Fever, cough, dyspnea, and fatigue were the most frequent symptoms in patients with liver cirrhosis. Cough, sore throat, fatigue, myalgia, and diarrhea were significantly more common in patients with liver cirrhosis than non-cirrhotic patients. There was no difference between patients with and without cirrhosis regarding comorbidities. Fifteen (23.4%) patients with liver cirrhosis presented with hepatic encephalopathy (Table 4).

Patients with liver cirrhosis were more likely to show combined ground-glass opacities and consolidations in their chest CT scans: 28 (43.75%) *vs* 4 (6.55%), respectively (P value < 0.001). These patients also were more likely to present with severe COVID-19 infection: 29 (45.31%) *vs* 11 (18.04%), respectively (P-value 0.003), compared to patients without liver cirrhosis. Mortality was higher in patients with liver cirrhosis: 33 (51.56%) *vs* 9 (14.75%), respectively (P value < 0.001) (Table 5). All patients classified as Child-Pugh class A recovered and were discharged, whereas 18 (45%) mortalities occurred among patients considered class B and 15 (37.5%) among those considered class C.

A multivariate logistic regression revealed that male patients were more likely than female patients to die, after adjusting for liver cirrhosis and diabetes mellitus status (OR 7.166, 95%CI: 2.185–23.506; P value 0.001). Mortality was four times more likely among patients with diabetes mellitus, after adjusting for other factors in the model (OR 4.029, 95%CI: 1.488–10.906; P value 0.006). Patients with liver cirrhosis also were more likely to die (OR 1.103, 95%CI: 1.037–1.282; P value 0.0001), compared to those without cirrhosis (Table 6).

Within our cohort, 17 (13.6%) patients received direct-acting antiviral therapy (DAA) and achieved sustained virological response before acquiring COVID-19 infection. Among them, 10 (15.6%) patients had liver cirrhosis and seven (11.5%) did not. Three (17.6%) DAA recipients had severe COVID-19 disease, and the rest had moderate COVID-19 disease on admission. Regarding liver function, COVID-19 disease severity, and outcome, we found no difference between patients who previously received DAA and those who did not (Table 7).

DISCUSSION

Little research has assessed outcomes of patients co-infected with chronic hepatitis C virus and COVID-19, and existing studies include either a small number of patients or report data from patients with chronic liver disease and multiple underlying etiologies (viral and non-viral). This is the first Egyptian study reporting the outcome of SARS-CoV-2 infection in patients with isolated chronic hepatitis C as the etiology of their underlying chronic liver disease. We found a substantially increased incidence (23.43%) of hepatic encephalopathy in our cirrhotic patients infected with COVID-19. The 1-year cumulative incidence of hepatic encephalopathy in liver cirrhosis ranges from 0% to 21%[13]. We also found that the severity of COVID-19 symptoms and mortality rates were significantly higher in patients with liver cirrhosis. All patients assigned to Child-Pugh class A recovered and were discharged, but mortalities occurred among patients assigned to Child-Pugh class B or class C on admission. Male gender, diabetes mellitus, and liver cirrhosis were independent factors affecting mortality in our cohort.

In our study, fever (67.2%), cough (57.8%), dyspnea (56.3%), and fatigue (32.8%) were the most frequent symptoms in patients with liver cirrhosis, followed by diarrhea (7.8%). Among patients with cirrhosis, 6.3% were asymptomatic. Iavarone *et al*[14] studied 50 patients with liver cirrhosis with hepatitis C, hepatitis B, non-viral (*e.g.*, alcoholic), or multiple etiologies and reported fever in 64%, fatigue in 60%, dyspnea in

Table 2 Symptoms of the studied patients

Symptom	*n* (%)
Dyspnea	80 (64.0)
Fever	78 (62.4)
Cough	55 (44.0)
Fatigue	26 (20.8)
Disturbed consciousness	22 (17.6)
Sore throat	15 (12.0)
Newly developed hepatic encephalopathy	15 (12.0)
Hepatocellular carcinoma	15 (12.0)
Myalgia	12 (9.6)
Current ascites	9 (7.2)
History of hematemesis from esophageal varices	9 (7.2)
Asymptomatic	5 (4.0)
Diarrhea	5 (4.0)
Arthralgia	5 (4.0)
Anorexia	4 (3.2)
Nausea	3 (2.4)
Vomiting	3 (2.4)
Loss of taste	2 (1.6)
Hemoptysis	1 (0.8)
Abdominal pain	1 (0.8)
Loss of smell	1 (0.8)
Jaundice	1 (0.8)
Rhinorrhea	1 (0.8)

42%, and cough in 36%, followed by diarrhea in 10% and no symptoms in 12% of patients. In our study, 15 (23.4%) patients with liver cirrhosis presented with hepatic encephalopathy, compared with 11 (22%) of patients in Iavarone *et al*[14]. For ALT levels in our cohort, 58 (46.4%) were normal, 40 (32%) were elevated 1-2 times above the upper limit of normal, 7 (5.6%) were elevated 2-3 times above the upper limit, 20 (16%) were three times above normal, and 1 patient was five times over the upper limit. Iavarone *et al*[14] define ALT elevation at five times over the upper limit of normal as hepatic flare.

Our results revealed higher mortality among male patients, compared to female patients. Nasiri *et al*[15] similarly reported COVID-19-related mortality to be higher among males, and cohorts from China, Italy, Denmark, and the United States confirmed these findings[16-20]. Underlying sex-related mechanisms could include chromosomal immunological response, lifestyle (alcohol, smoking, and obesity), and comorbidities[19]. We also found that mortality was significantly higher in patients with liver cirrhosis, particularly those with decompensated cirrhosis. Boettler *et al*[21] reported that chronic viral hepatitis did not seem to raise the risk of a severe COVID-19 in a study by Guan *et al*[22], which included patients in China with chronic hepatitis B only. Shalimar *et al*[23] found no difference in mortality among patients with and without cirrhosis, but their sample size was small. An Italian study by Mangia *et al*[24] found that cirrhosis of metabolic origin, older age, leucopenia, and lymphopenia were risk factors for mortality. They also suggested that the very low prevalence of SARS-CoV-2 infection in patients with chronic hepatitis C infection could play a protective role against SARS-CoV-2 infection.

A meta-analysis by Váncsa *et al*[25], which included mainly studies of chronic hepatitis B in China, reported that liver failure and platelet count could predict in-hospital mortality with high specificity and lactate dehydrogenase with moderate

Table 3 Baseline laboratory results and treatments of the studied 125 patients

	mean ± SD/*n* (%)
Pulse rate	92 ± 17
Temperature	37 ± 1
Respiratory rate	23 ± 5
Oxygen saturation	93 ± 5
Hemoglobin (gm/dL)	12 ± 2
Platelet count (× 1000/cmm)	183 ± 107
Total leucocyte count (× 1000/cmm)	10 ± 8
Neutrophil : Lymphocyte ratio	1 ± 0
INR	1.16 ± 0.29
PTT	33.9 ± 13.2
Serum creatinine (mg/dL)	1.39 ± 1.43
Serum sodium (mEq/L)	137 ± 9
Serum potassium (mEq/L)	4.18 ± 0.77
Total bilirubin (mg/dL)	3 ± 5
Direct bilirubin (mg/dL)	2.1 ± 3.8
Serum albumin (g/dL)	3.2 ± 0.7
Alanine Transaminase (U/L)	58 ± 53
Aspartate Transaminase (U/L)	92 ± 123
Alkaline phosphatase (U/L)	262 ± 226
Serum ferritin (ng/mL)	667 ± 483
D-dimer (mg/mL)	1560 ± 2503
Fibrinogen (mg/dL) × 100 if presented in g/L	77
Azithromycin	72 (57.6)
Paracetamol	45 (36.0)
Supplementary vitamin C	85 (68.0)
Zinc	69 (55.2)
Colchicine	2 (1.6)
Lactoferrin	18 (14.4)
Other antibiotics	71 (56.8)
Steroids	38 (30.4)
Hydroxyl-chloroquine	29 (23.2)
Low-molecular weight heparin	56 (44.8)
Warfarin	3 (2.4)

PTT: Partial thromboplastin time; INR: International normalized ratio.

specificity. Singh *et al*[26] found that patients with cirrhosis had a higher relative risk of mortality and a higher risk of hospitalization, compared to patients without liver disease. Another study by Galiero *et al*[27] described outcomes in 35 patients with liver cirrhosis, though they did not compare cirrhotic *vs* non-cirrhotic patients, they found that male sex, chronic liver disease, and malignancies were independent factors of poor prognosis in hospitalized patients with COVID-19. They also reported that patients with advanced chronic liver disease had worse clinical conditions compared to patients with no liver disease[27].

Table 4 Baseline features and symptoms of patients with and without liver cirrhosis

Variable		No liver cirrhosis61 (48.8%)	Liver cirrhosis 64 (51.2%)	*P* value
Gender	Male	42 (68.9%)	44 (68.8%)	0.57
	Female	19 (31.1%)	20 (31.3%)	
Age (yr)	20 to 30	0 (0.0%)	1 (1.6%)	0.004
	30 to 40	3 (4.9%)	3 (4.8%)	
	40 to 50	0 (0.0%)	10 (15.9%)	
	50 to 60	19 (31.1%)	12 (19.0%)	
	60 to 70	14 (23.0%)	21 (33.3%)	
	70 to 80	21 (34.4%)	14 (22.2%)	
	80 to 90	4 (6.6%)	2 (3.2%)	
Cigarette smoking		2 (3.2%)	8 (11.5%)	0.06
Diabetes mellitus		24 (39.3%)	28 (43.8%)	0.71
Hypertension		23 (37.7%)	29 (45.3%)	0.46
COPD		0 (0.0%)	1 (1.6%)	1
Coronary artery disease		5 (8.2%)	7(10.9%)	0.76
Chronic renal insufficiency		4 (6.6%)	4 (6.3%)	1
Heart failure		0	2 (3.1%)	0.49
Hepatocellular carcinoma		3 (4.9%)	12 (18.8%)	0.026
Esophageal varices		0 (0.0%)	9 (14.1%)	0.003
Asymptomatic		1 (1.6%)	4 (6.3%)	0.36
Fever		35 (57.4%)	43 (67.2%)	0.27
Cough		18 (29.5%	37 (57.8%)	0.002
Dyspnea		44 (72.1%)	36 (56.3%)	0.09
Sore throat		2 (3.3%)	13 (20.3%)	0.005
Hemoptysis		0 (0.0%)	1 (1.6%)	1
Fatigue		5 (8.2%)	21 (32.8%)	0.001
Anorexia		1 (1.6%)	3 (4.7%)	0.62
Diarrhea		0 (0.0%)	5 (7.8%)	0.05
Nausea		1 (1.6%)	2 (3.1%)	1
Vomiting		1 (1.6%)	2 (3.1%)	1
Abdominal pain		1 (1.6%)	0 (0.0%)	0.48
Arthralgia		1 (1.6%)	4 (6.3%)	0.36
Myalgia		1 (1.6%)	11 (17.2%)	0.004
Loss of taste		0 (0.0%)	2 (3.1%)	0.49
Loss of smell		0 (0.0%)	1 (1.6%)	1
Disturbed consciousness		3 (4.9%)	19 (29.7%)	0.001
Hepatic encephalopathy		0 (0.0%)	15 (23.4%)	0.033
Direct-acting antiviral therapy with sustained virological response before COVID-19 infection		7 (11.5%)	10 (15.6%)	0.61

COPD: Chronic obstructive pulmonary disease; COVID-19: Coronavirus disease 2019.

In a retrospective multicenter study from 16 hospitals in China that included 21

Table 5 Comparison between patients with and without liver cirrhosis

		No liver cirrhosis 61 (48.8%)	Liver cirrhosis 64 (51.2%)	*P* value
Hemoglobin (mg/dL)		12 ± 2	12 ± 2	0.85
Platelet count (× 1000/cmm)		193 ± 107	171 ± 107	0.36
TLC (× 1000/cmm)		12 ± 10	9 ± 5	0.31
Serum creatinine (mg/dL)		1.58 ± 1.89	1.21 ± 0.76	0.59
Total bilirubin (mg/dL)		3 ± 4	3 ± 5	0.79
Serum albumin (g/dL)		3.3 ± 0.7	3.1 ± 0.6	0.36
Alanine transaminase (U/L)		67 ± 67	50 ± 40	0.21
Aspartate transaminase (U/L)		60 ± 37	101 ± 137	0.89
CT pattern	Consolidations and ground-glass opacities	4 (6.55%)	28 (43.75%)	< 0.001
	Ground-glass opacities	57 (93.45%)	36 (56.25%)	
Lesion distribution on CT	Bilateral	58 (95.1%)	64 (100.0%)	0.11
	Unilateral	3 (4.9%)	0	
COVID-19 severity	Moderate	50 (81.96%)	35 (54.69%)	0.003
	Severe	11 (18.04%)	29 (45.31%)	
Azithromycin		33 (54.1%)	39 (60.9%)	0.47
Paracetamol		16 (26.2%)	29 (45.3%)	0.04
Supplementary vitamin C		37 (60.7%)	48 (75.0%)	0.12
Supplementary zinc		33 (54.1%)	36 (56.3%)	0.85
Lactoferrin		3 (4.9%)	15 (23.4%)	0.004
Other antibiotics		28 (45.9%)	43 (67.2%)	0.019
Anticoagulants	LMWH	28 (45.9%)	28 (43.8%)	1
	Warfarin	1 (1.6%)	2 (3.1%)	1
Steroids		16 (26.2%)	22 (34.4%)	0.339
Alive (discharged)		52 (85.25%)	31 (48.44%)	< 0.001
Died at the hospital		9 (14.75%)	33 (51.56%)	

TLC: Thin layer chromatography; CT: Computed tomography; COVID-19: Coronavirus disease 2019.

Table 6 Multivariate regression analysis of factors affecting mortality

	B	S.E.	*P* value	Odds ratio	95%CI	
					Lower	Upper
Male	1.969	0.606	0.001	7.166	2.185	23.506
Diabetes mellitus	1.393	0.508	0.006	4.029	1.488	10.906
Liver cirrhosis	2.274	0.515	0.0001	1.103	1.037	1.282
Constant	-1.879	0.604	0.002	0.153		

patients with COVID-19 and hepatitis B virus-related liver cirrhosis (Child-Pugh classes A, B, and C in 16, 3, and 2 cases, respectively), mortalities occurred in patients assigned to class A (3; 60.0%) and C (2; 40.0%)[10]. In another multinational cohort study of 745 patients from 29 countries, Marjot *et al*[28] found that liver cirrhosis was present in 386 patients: 171 (44%) in Child-Pugh class A, 124 (32%) in class B, and 91 (24%) in class C. Mortality rates significantly increased with worsening scores: 33 (19%) in class A, 44 (35%) in class B, and 46 (51%) in class C died. Age, Child-Pugh

Table 7 Comparison between patients with COVID-19 who received or did not receive previous direct-acting antiviral therapy

		Did not receive previous DAA (*n* = 108)	Received previous DAA (*n* = 17)	*P* value
COVID-19 severity	**Moderate**	72 (66.7%)	14 (82.4%)	0.26
	Severe	36 (33.3%)	3 (17.6%)	
Admission zone	**ICU**	22 (20.4%)	2 (11.8%)	0.52
	Intermediate care	6 (5.6%)	0 (0.0%)	
	Ward	80 (74.1%)	15 (88.2%)	
Vital status	**Alive**	73 (67.6%)	12 (70.6%)	1
	Dead	35 (32.4%)	5 (29.4%)	
Total bilirubin (mg/dL)		1.00 (1-20)	1.30 (1-6)	0.84
Direct bilirubin (mg/dL)		0.9 (0.2-15.6)	0.9 (0.1-2.7)	0.93
Serum albumin (g/dL)		3.1 (1.7-5)	3.15 (2.4-3.8)	0.45
Alanine transaminase (U/L)		41.5 (13-324)	49 (18-175)	0.54
Aspartate transaminase (U/L)		46.5 (10- 549)	53 (16-415)	0.42

DAA: Direct-acting antiviral; COVID-19: coronavirus disease 2019; ICU: Intensive care unit.

class, and alcoholic liver disease were the independent factors affecting mortality in their study, and hepatic encephalopathy occurred in 104 (27%) of their patients[28]. Another study from 13 Asian countries studied 43 patients with liver cirrhosis and reported worsening liver disease and increased hepatic complications in patients with COVID-19 infection (P value < 0.05); they found that a baseline Child-Pugh score ≥ 9 was associated with higher mortality (area under the ROC 0.94, hazard ratio 19.2, 95%CI: 2.3–163.3; P value < 0.001). The independent factors affecting mortality in their study were increased serum bilirubin and AST/ALT ratio[29].

The exact mechanism causing poor outcomes among patients with COVID-19 and preexisting liver disease remains unknown; however, interactions between local liver injury and systemic disturbances seem likely culprits, especially in patients with cirrhosis. Patients with liver cirrhosis are theoretically more susceptible to poor outcomes from COVID-19-related liver injury. Moreover, patients with advanced liver disease exhibit immune deficiency and systemic inflammation, as reflected by activated circulating immune cells and increased serum levels of pro-inflammatory cytokines. These factors can predispose this population to cytokine storms[26]. Furthermore, some cirrhotic patients may have an underlying hepato-pulmonary syndrome, portopulmonary hypertension, or hydrothorax, which can increase the risk of respiratory failure, as indicated in a study by Oyelade *et al*[30].

Sun *et al*[31] suggested SARS-CoV-2-related direct cytotoxicity (*i.e.*, severe inflammatory response leading to immune-mediated liver damage), hypoxic hepatitis due to anoxia (particularly in patients with severe COVID-19), drug□induced liver injury (especially related to the use of antiviral agents such as lopinavir, ritonavir, remdesivir, chloroquine, tocilizumab, umifenovir, and traditional Chinese preparations), and reactivation of pre-existing chronic liver disease as possible mechanisms of hepatic injury. They also found that liver biopsy specimens from deceased patients with severe COVID-19 showed moderate microvascular steatosis and mild lobular and portal activity, indicating that liver injury could have been caused by either SARS-CoV-2 infection or drug-induced liver injury[31].

A limitation to our study is its retrospective nature and the limited number of patients. It remains unknown whether liver injuries and mortalities among patients with chronic hepatitis C and COVID-19 co-infection are due to patients' pre-existing chronic conditions or the impact of SARS-CoV-2 infection. We found that patients with decompensated hepatitis C-related cirrhosis were at higher risk of COVID-19-related mortality. Hepatic encephalopathy could be the presentation of underlying SARS-CoV-2 infection in patients with liver cirrhosis. The underlying etiology of chronic liver disease also could impact COVID-19 disease course and outcome. Further studies comparing outcomes and prognostic factors in patients with isolated underlying etiologies of chronic liver disease are therefore encouraged, rather than combining them into one group, which could obscure relevant risk factors for disease severity and

outcomes.

CONCLUSION

In Egypt, among patients with chronic hepatitis C who also were infected with COVID-19, those with cirrhosis had higher rates of pneumonia, severe COVID-19, and mortality, though cirrhotic mortality occurred among decompensated patients only. Male gender, diabetes mellitus, and liver cirrhosis were the independent factors affecting mortality in these patients.

ARTICLE HIGHLIGHTS

Research background

Coronavirus disease 2019 (COVID-19) disease severity and outcomes are affected by pre-existing chronic liver disease, particularly cirrhosis. Patients with decompensated liver cirrhosis (Child-Pugh classes B and C) are severely affected, with higher mortality rates than patients with compensated disease.

Research motivation

Comprehensive research on the outcome of COVID-19 in patients with isolated etiology of pre-existing chronic liver disease is needed to understand the clinical presentations and outcomes.

Research objectives

This study aimed to demonstrate the impact of COVID-19 factors affecting mortality among patients with pre-existing hepatitis C with or without liver cirrhosis during the first peak of the pandemic in Egypt.

Research methods

This multicenter retrospective cohort study included 125 patients with COVID-19 at six quarantine hospitals in Egypt from May 1, 2020, to July 31, 2020. Clinical, laboratory features, COVID-19 severity, and outcomes were recorded. A regression analysis was performed to detect factors affecting mortality.

Research results

Fever, cough, dyspnea, and fatigue were the most frequent symptoms in patients with liver cirrhosis. Cough, sore throat, fatigue, myalgia, and diarrhea were significantly more common in patients with liver cirrhosis than in non-cirrhotic patients. Fifteen (23.4%) patients with liver cirrhosis presented with hepatic encephalopathy. Patients with liver cirrhosis were more likely to exhibit combined ground-glass opacities and consolidations in their chest CT scans and more likely to present with severe COVID-19 infection, compared to patients without liver cirrhosis. Mortality was higher among patients with liver cirrhosis: 33 (51.56%) *vs* 9 (14.75%), respectively (P value < 0.001. All patients in Child-Pugh class A recovered and were discharged, and mortalities occurred among patients in Child-Pugh classes B and C. A multivariate logistic regression revealed that male gender, diabetes mellitus, and liver cirrhosis were independent factors affecting mortality. Regarding liver function, COVID-19 disease severity, and outcomes, we found no difference between patients who previously received direct acting antiviral therapy (and achieved sustained virological response) and patients who did not receive such therapy.

Research conclusions

Patients with decompensated hepatitis C virus-related liver cirrhosis are at higher risk of severe COVID-19 disease and mortality. Male gender, diabetes mellitus, and liver cirrhosis are the independent factors affecting mortality.

Research perspectives

Male gender, diabetes mellitus, and liver cirrhosis significantly increased mortality in patients with COVID-19 and isolated hepatitis C virus-related chronic liver disease. Previous achievement of sustained virological response after direct acting antiviral therapy for chronic hepatitis C does not impact COVID-19 disease severity, outcome,

or the results of liver function tests.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 14; 27(42): 7376-7386

DOI: 10.3748/wjg.v27.i42.7376

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

ORIGINAL ARTICLE

Retrospective Study

Endoscopic ultrasound features of autoimmune pancreatitis: The typical findings and chronic pancreatitis changes

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Author contributions: Zhang SY and Feng YL contributed equally to this work and should be considered as co-first authors; Zhang SY, Wu X, Guo T, Tang SJ and Yang AM designed the study; Zhang SY and Feng YL collected and analyzed the data; Zou L and Lai YM collected the clinical information; Zou L collected the endoscopic images; Wu X, Guo T, Jiang QW and Wang Q collected the images; Zhang SY, Feng YL, Wang Q and Tang SJ analyzed the images; Zhang SY wrote the manuscript; Feng YL and Tang SJ revised the manuscript; Yang AM approved the final version of the manuscript.

Supported by Beijing Science and Technology Program, No. Z181100001618013; Peking Union Medical College Education Reform Program, No. 2019zlgc0116.

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Abstract

BACKGROUND

Few studies have fully described endoscopic ultrasound (EUS) features of newly diagnosed autoimmune pancreatitis (AIP) involving both typical findings and chronic pancreatitis (CP) features. The typical EUS findings are prevalent in the diffuse type AIP but may not be as common for the focal type, and the differences between the diffuse and focal AIP need to be specified.

AIM

To demonstrate the EUS features of newly diagnosed AIP and the difference between diffuse and focal AIP.

METHODS

This retrospective single center study included 285 patients of newly diagnosed type 1 AIP following the international consensus diagnostic criteria, with the EUS procedures accomplished before corticosteroid initiation. We explored the EUS features and compared the typical AIP and CP features between the diffuse and focal AIP cases. The Rosemont criteria were employed for CP features definition and CP change level comparison.

RESULTS

For the typical AIP features, there were significantly more patients in the diffuse group with bile duct wall thickening (158 of 214 cases, 73.4% *vs* 37 of 71 cases, 52.1%, $P = 0.001$) and peripancreatic hypoechoic margin (76 of 214 cases, 35.5% *vs*

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Peking Union Medical College Hospital (approval number S-K1613).

Conflict-of-interest statement: The authors declare no conflicts of interest for this article.

Data sharing statement: No additional data are available.

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Specialty type: Gastroenterology and hepatology

Country/Territory of origin: China

Peer-review report's scientific quality classification

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

Received: June 13, 2021
Peer-review started: June 13, 2021
First decision: July 14, 2021
Revised: July 27, 2021
Accepted: October 25, 2021
Article in press: October 25, 2021
Published online: November 14, 2021

P-Reviewer: Arcidiacono PG, Emmanuel J, Fujimori N, Nakai Y
S-Editor: Ma YJ
L-Editor: Filipodia
P-Editor: Ma YJ

5 of 71 cases, 7.0%, $P < 0.001$). For the CP features, there were significantly more patients in the focal group with main pancreatic duct dilation (30 of 214 cases, 14.0% *vs* 18 of 71 cases, 25.3%, $P = 0.03$). The cholangitis-like changes were more prevalent in the focal cases with pancreatic head involvement. The CP change level was relatively limited for newly diagnosed AIP cases in both groups.

CONCLUSION

This study demonstrated the difference in the typical AIP and CP features between diffuse and focal AIP and indicated the limited CP change level in newly diagnosed AIP.

Key Words: Endoscopic ultrasound; Autoimmune pancreatitis; Rosemont criteria; Chronic pancreatitis

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Core Tip: The endoscopic ultrasound (EUS) features of newly diagnosed autoimmune pancreatitis (AIP) involving both typical findings and chronic pancreatitis (CP) features have rarely been described. The EUS typical features of AIP can help to differentiate diffuse AIP from classic CP and differentiate focal AIP from pancreatic cancer. This study demonstrated the EUS features of newly diagnosed AIP and the difference in the typical AIP features and CP features between the diffuse and focal AIP cases on the basis of the largest number of cases and indicated the relatively limited CP change in newly diagnosed AIP cases.

Citation: Zhang SY, Feng YL, Zou L, Wu X, Guo T, Jiang QW, Wang Q, Lai YM, Tang SJ, Yang AM. Endoscopic ultrasound features of autoimmune pancreatitis: The typical findings and chronic pancreatitis changes. *World J Gastroenterol* 2021; 27(42): 7376-7386
URL: https://www.wjgnet.com/1007-9327/full/v27/i42/7376.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i42.7376

INTRODUCTION

In 1995, Yoshida *et al*[1] first proposed the concept of autoimmune pancreatitis (AIP) as a unique disease entity, which can be divided into two subtypes. Type 1 AIP, which is the most common type in Asia, is the pancreatic manifestation of immunoglobulin (Ig)G4-related disease and is often associated with extrapancreatic disorders, especially IgG4-associated cholangitis[2].

AIP can be divided into diffuse type and focal type based on the image findings[3]. With endoscopic ultrasound (EUS) as an effective tool to find parenchymal and main pancreatic ductal (MPD) changes, typical AIP features can be observed for diffuse type AIP, such as diffuse enlargement of the whole pancreas with diffuse hypoechoic parenchyma and hypoechoic peripancreatic margin[4-6]. But the typical EUS findings for AIP may not be as common for the focal type and differences between the diffuse and focal type need to be demonstrated.

As a special form of chronic pancreatitis (CP), almost all CP features could be recognized in AIP cases during EUS examination[4-6]. After recurrent attack or prolonged inflammatory injury from AIP, advanced CP features such as parenchymal calcification/atrophy and MPD calculi will emerge from the diffuse enlarged hypoechoic pancreas[7-9]. So, from newly diagnosed AIP to advanced CP, we need a measure to describe the level of CP change.

Therefore, we conducted this single center retrospective study for a detailed description of the EUS features of newly diagnosed AIP patients and demonstration of the difference between diffuse and focal AIP, and we tried to compare the CP change level in both groups *via* the Rosemont criteria based on all CP features.

MATERIALS AND METHODS

Patients

Patients diagnosed with AIP undergoing EUS before the initiation of corticosteroid treatment at the Peking Union Medical College Hospital from January 2012 to August 2018 were included in this retrospective study, and patients who had a history of alcoholism or recent acute pancreatitis onset (within 3 mo) were excluded. All diagnoses was made following the International Consensus Diagnostic Criteria (ICDC; see Supplement Figure)[10]. The study was approved by the Ethics Committee of Peking Union Medical College Hospital (approval number S-K1613).

EUS feature definitions

All EUS procedures were performed by experienced endoscopists (XW, DSW, TG, QWJ; all with over 5 years EUS experience) with radial or linear echoendoscopes (frequency 5-7.5 MHz; GF-UCT260 or GF-UE260, Olympus, Tokyo, Japan) and ultrasound workstations (EM-2, Olympus, Tokyo, Japan, and Aloka ProSound F75, Hitachi, Tokyo, Japan). EUS images and videos were stored as digital data, and the image analysis was completed by SYZ and YLF in a blinded manner independently, in which LZ selected all image videos for each case from the database, removed the patient's information and diagnosis and showed them to SYZ and YLF. If disagreement existed between the two investigators, the third one (QW) would decide the image interpreting results.

We defined the typical EUS features of AIP, including diffuse enlargement (the pancreas is divided into three parts: head, body, and tail; cases with more than one part of the pancreas enlarged were defined as "diffuse enlargement" and grouped as the "diffuse type"), focal enlargement (cases with less than one part of the pancreas enlarged were defined as "focal enlargement" and grouped as the "focal type"), diffuse hypoechoic area (DHA), focal hypoechoic areas, bile duct wall thickening, extrahepatic bile duct dilation, intrapancreatic bile stenosis, peripancreatic lymphadenopathy, peripancreatic hypoechoic margins, lobular outer margins and peripancreatic vessel involvement (Figure 1).

We used the Rosemont criteria to describe the CP features in AIP[11]. The parenchymal changes included hyperechoic foci (HF), hyperechoic strand (HS), lobularity with/without honeycombing, cystic lesions and calcifications (Figure 2). The MPD changes included MPD stones, duct irregularity, dilation and hyperechoic duct margins (Figure 2).

All definitions are interpreted in detail in Supplementary Table 1.

The Rosemont criteria application for CP change

We applied the Rosemont criteria to describe the level of CP change in newly diagnosed AIP patients of both diffuse and focal types.

Statistical analysis

Descriptive analysis was used to describe the clinical and EUS features of AIP patients. Categorical variables were presented as n (%), and continuous variables were presented as the mean ± SD or the median and the interquartile range, depending on the distribution. The distribution of the continuous variables was checked using a visual inspection of the histogram. Statistical analysis was accomplished with SPSS 23.0 (IBM, NY, United States). The differences in EUS features and clinical factors between the diffuse and focal AIP cases were compared using Student's t test/Mann-Whitney U test (for continuous variables, depending on distribution) or χ^2 test (for categorical variables). Multivariate analysis using the binary logistic regression including variables identified to be significant ($P \leq 0.10$) at univariate analysis. A two-tailed P value of less than 0.05 was considered to be statistically significant.

RESULTS

Demographics and clinical characteristics

A total of 285 patients were included in this study, with 230 male patients (80.7%) and a median age of 62 [interquartile range 54, 68] years. The mean follow-up duration was 26 [interquartile range 12, 51] mo. All patients were diagnosed with type 1 AIP according to the ICDC criteria. The clinical data (including symptoms and laboratory tests) are shown in Table 1. All items were comparable between the diffuse and focal

Table 1 Comparison of patients' demographics and clinical manifestations before corticosteroid treatment

	All (*n* = 285)	Diffuse type (*n* = 214)	Focal type (*n* = 71)	*P* value
Sex	230 (80.7)	179 (83.6)	51 (71.8)	0.03
Age	62 (54, 68)	62 (55, 68)	59 (53, 68)	0.12
Follow-up time (mo)	26 (12, 51)	28 (13, 51)	21 (9, 51)	0.13
Symptoms				
Abdominal pain	73 (26.0)	51 (23.8)	22 (31.0)	0.23
Jaundice	106 (37.2)	81 (37.8)	25 (35.2)	0.69
Number of involved organs[1]	1 (1, 2)	1 (1, 2)	1 (1, 2)	0.47
Laboratory tests				
ALT (U/L) (9-50)	47 (18, 133)	64 (18, 184)	30 (15, 83)	0.01
TBil (μmol/L) (5.1-22.2)	20.6 (12.2, 55.6)	21.4 (12.9, 68.7)	14.5 (11.6, 45.2)	0.08
IgG (mg/dL) (700-1700)	1590 (1140, 2070)	1630 (1130, 2140)	1510 (1130, 1760)	0.37
IgG4 (mg/dL) (8-140)	558.0 (280.5, 1270.0)	605.5 (253.5, 1457.5)	458.0 (301.0, 1050.0)	0.26
CA 19-9 (U/L) (0-34.0)	20.3 (7.9, 61.0)	23.4 (10.5, 74.1)	12.6 (6.7, 36.5)	0.01

[1]Involved organs included salivary gland, lacrimal gland, lung, kidney, liver, bile duct, retroperitoneal fibrosis and prostate gland.
Results presented as median (interquartile range) or *n* (%). ALT: Alanine transaminase; CA: Carbohydrate antigen; TBil: Total bilirubin; Ig: Immunoglobulin.

group, except for alanine transaminase and carbohydrate antigen 19-9, which were higher in the diffuse group.

EUS features and differences

The EUS features of all patients were shown, and the differences between the diffuse and focal types of AIP were compared (Table 2).

There were 214 cases of diffuse type and 71 cases of focal type AIP; among focal cases, more lesions were located in the pancreatic head (50 cases, 70.4%), while less in the body (8 cases, 11.3%) and tail (13 cases, 18.3%).

For the typical AIP features, there were significantly more patients with DHA in the diffuse group (197 of 214 cases, 92.1% *vs* 16 of 71 cases, 22.5%, $P < 0.001$), while there were significantly more patients with focal hypoechoic areas in the focal group (0 of 214 cases, 0% *vs* 59 of 71 cases, 83.1%, $P < 0.001$), which was consistent with the original definitions. For cholangitis-like changes, there were significantly more patients with bile duct wall thickening (158 of 214 cases, 73.4% *vs* 37 of 71 cases, 52.1%, $P = 0.001$) in the diffuse group. For peripancreatic changes, there were significantly more patients with peripancreatic hypoechoic margins (76 of 214 cases, 35.5% *vs* 5 of 71 cases, 7.0%, $P < 0.001$) in the diffuse group.

In the focal group, the cholangitis-like changes (bile duct wall thickening, intrapancreatic bile duct stenosis and extrahepatic bile duct dilation) were more prevalent in cases with pancreatic head involvement compared to those with body or tail involvement ($P < 0.001$, $P = 0.01$ and $P = 0.02$, respectively) (Supplementary Table 2).

For the CP features, parenchymal changes were all comparable in the diffuse and focal group. For MPD changes, there were significantly more patients with MPD dilation in the focal group than in the diffuse group (30 of 214 cases, 14.0% *vs* 18 of 71 cases, 25.3%, $P = 0.03$).

In the logistic regression analysis for the diffuse AIP with the EUS findings, the DHA [odds ratio (OR) = 11.23, 95% confidence index (CI): 3.07–41.03; $P < 0.001$], bile duct wall thickening (OR = 4.44, 95%CI 2.49–7.93; $P < 0.001$) and peripancreatic hypoechoic margin (OR = 4.34, 95%CI: 1.93–9.80; $P < 0.001$) were all predictors of the diffuse AIP (Table 3).

The Rosemont criteria application for CP change

The Rosemont criteria were applied to describe the CP change level. Only a small portion of patients was diagnosed as "suggestive of CP" (12.1% in the diffuse group *vs* 15.5% in the focal group), and patients with advanced CP change ("consistent with

Table 2 Comparison of endoscopic ultrasound features between the diffuse and focal types of autoimmune pancreatitis patients

EUS findings	All (*n* = 285)	Diffuse type (*n* = 214)	Focal type (*n* = 71)	*P* value
Typical findings				
DHA	213 (74.7)	197 (92.1)	16 (22.5)	< 0.001
FHA	59 (20.7)	0 (0)	59 (83.1)	< 0.001
Bile duct changes				
Bile duct wall thickening	195 (68.4)	158 (73.4)	37 (52.1)	0.001
Intrapancreatic bile duct stenosis	165 (57.9)	131 (61.2)	34 (47.9)	0.05
Extrahepatic bile duct dilation	122 (42.8)	97 (45.3)	25 (35.2)	0.14
Peripancreatic changes				
Peripancreatic lymphadenopathy	89 (31.2)	72 (33.6)	17 (23.9)	0.13
Peripancreatic hypoechoic margin	81 (28.4)	76 (35.5)	5 (7.0)	< 0.001
Lobular outer margin	40 (14.0)	34 (15.9)	6 (8.5)	0.12
Peripancreatic vessel involvement	21 (7.4)	16 (7.5)	5 (7.0)	0.90
Chronic pancreatitis changes				
Parenchymal changes				
HF	271 (95.1)	202 (94.4)	69 (97.2)	0.53[1]
HS	174 (61.1)	131 (61.2)	43(60.6)	0.92
Cystic lesion	18 (6.3)	14 (6.5)	4 (5.6)	1.00[1]
Parenchymal calcification	3 (1.1)	2 (0.9)	1 (1.4)	1.00[1]
Lobularity with honeycombing	26 (9.1)	19 (8.9)	7 (9.9)	0.80
Lobularity without honeycombing	48 (16.8)	36 (16.8)	12 (16.9)	0.99
Main pancreatic duct changes				
MPD calculi	1 (0.4)	0 (0)	1 (1.4)	0.56[1]
MPD dilation	48 (16.8)	30 (14.0)	18 (25.3)	0.03
Diffuse stenosis/irregularity	29 (10.2)	20 (9.3)	9 (12.7)	0.42
Focal stenosis	11 (3.9)	6 (2.8)	5 (7.0)	0.15[1]
Hyperechoic duct margin	119 (41.8)	91 (42.5)	28 (39.4)	0.65

[1]Fisher's exact test.

Results presented as *n* (%). DHA: Diffuse hypoechoic area; EUS: Endoscopic ultrasound; FHA: Focal hypoechoic area; HF: Hyperechoic foci; HS: Hyperechoic strand; MPD: Main pancreatic duct.

Table 3 The logistic regression for predictors of diffuse autoimmune pancreatitis

Effect	Odds ratio (95%CI)	*P* value
DHA	11.23 (3.07, 41.03)	< 0.001
Bile duct wall thickening	4.44 (2.49, 7.93)	< 0.001
Peripancreatic hypoechoic margin	4.34 (1.93, 9.80)	< 0.001

CI: Confidence index; DHA: Diffuse hypoechoic area.

CP") were even more rare (0.9% *vs* 1.4%) (Table 4). The CP change level was similar for newly diagnosed AIP cases in the diffuse and focal groups.

Table 4 The Rosemont Criteria for description of chronic pancreatitis change in newly diagnosed autoimmune pancreatitis patients

Rosemont criteria	Diffuse type (*n* = 14)	Focal type (*n* = 71)	*P* value
Consistent with CP	2 (0.9)	1 (1.4)	0.45
Suggestive of CP	26 (12.1)	11 (15.5)	
Indeterminate of CP	174 (81.3)	58 (81.7)	
Normal	12 (5.6)	1 (1.4)	

Results presented as *n* (%). CP: Chronic pancreatitis.

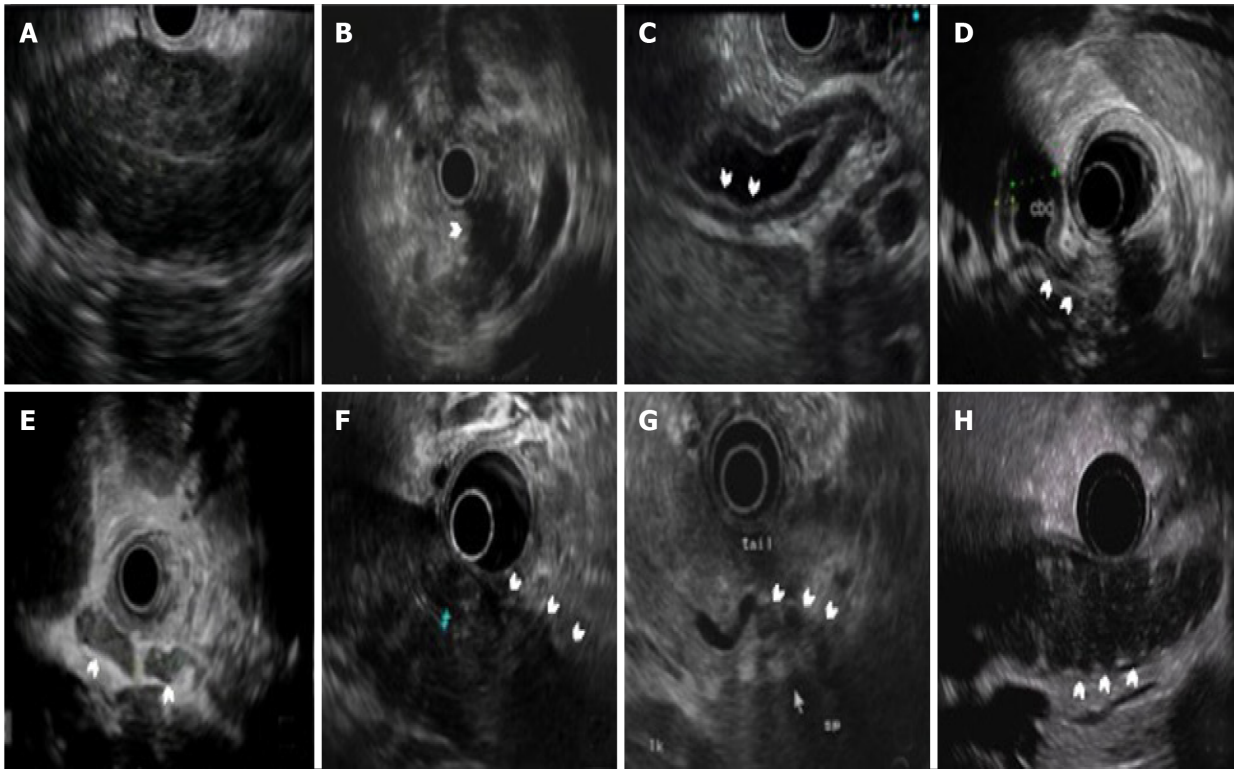


Figure 1 The typical endoscopic ultrasound findings in autoimmune pancreatitis. The white arrows show the following: A: Diffuse hypoechoic area of the pancreatic body; B: Focal hypoechoic area of the pancreatic body; C: Thickened wall of the common bile duct; D: Intrapancreatic bile duct stenosis; E: Peripancreatic lymphadenopathy; F: Peripancreatic hypoechoic margin; G: Peripancreatic vessel involvement with stenosis of the splenic vein; H: Lobular outer margin.

DISCUSSION

Type 1 AIP is a special form of CP that is pathologically characterized by an abundance of lymphoplasmacytic infiltrates, fibrosis and obliterative phlebitis[2]. EUS can detect abnormalities in the parenchyma and pancreatic duct that are possibly not visible by other modalities. To date, this is the largest single center retrospective study demonstrating EUS features in newly diagnosed type 1 AIP patients, which not only describes the typical findings and the CP features of AIP but also figures out the difference between the diffuse and focal type.

We demonstrated the typical EUS features in AIP, including diffuse or focal hypoechoic area, bile duct changes due to IgG4-associated cholangitis and peripancreatic changes. In fact, the typical EUS findings (such as diffuse enlargement of the pancreas, DHA, bile duct wall thickening or stenosis and peripancreatic hypoechoic margin) were still prominent manifestations for AIP patients, which was consistent with previous studies (Supplement Table 3). More patients in the diffuse group showed typical features, especially for bile duct wall thickening and peripancreatic hypoechoic margin due to the profound inflammatory change[6]. In the multivariate regression analysis for the diffuse AIP, we demonstrated the predictors of the diffuse AIP: the DHA, bile duct wall thickening and peripancreatic hypoechoic margin (all $P <$

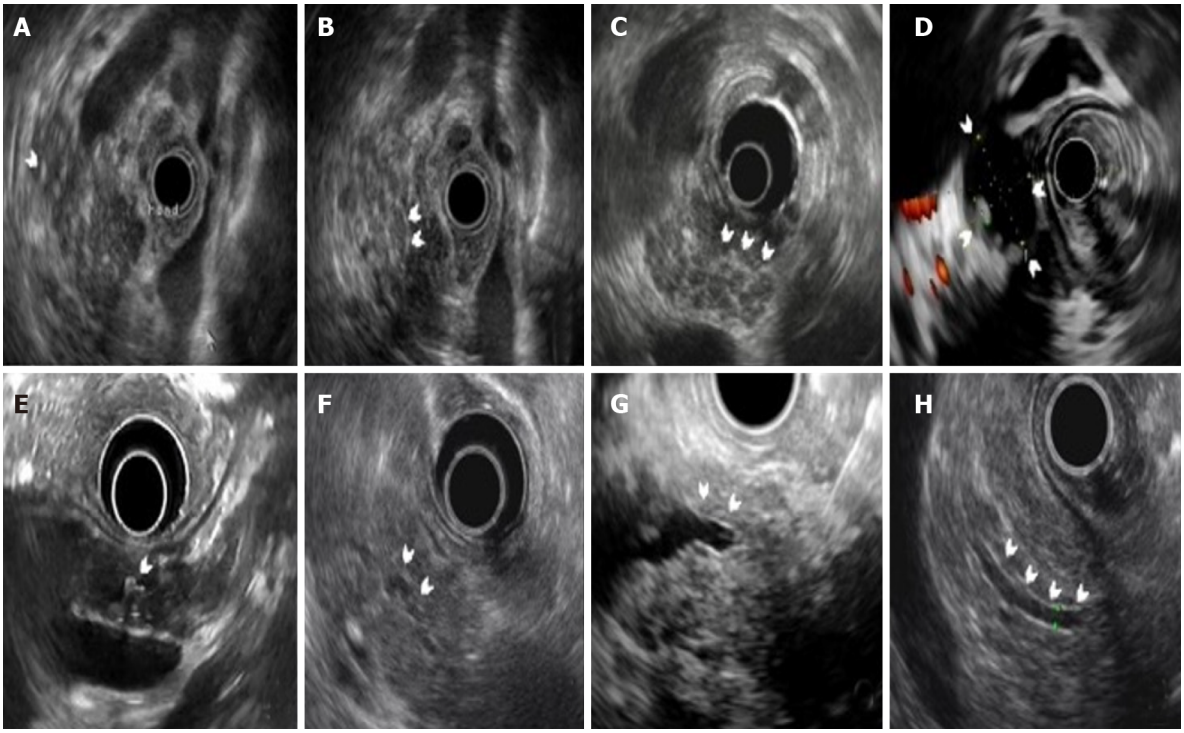


Figure 2 The pancreatic parenchymal and duct changes of chronic pancreatitis in autoimmune pancreatitis. The white arrows show the following: A: Multiple hyperechoic foci; B: Hyperechoic strands in pancreatic head; C: Multiple lobularities with honeycombing; D: Cystic lesion connecting to the main pancreatic duct (MPD); E: MPD stone with acoustic shadow; F: Diffuse irregularity change of the MPD; G: Focal stenosis and upstream dilation of the MPD; H: Hyperechoic duct margin.

0.001), which are all typical EUS findings of AIP rather than CP features.

It is also notable that despite hypoechoic parenchyma in the enlarged part of the focal type the rest of the parenchyma also showed hypoechogenicity and hyperechoic foci/strands under EUS, which implied the dynamic change of the parenchyma possibly caused by the spontaneous remission of AIP[12]. But we did not define these cases as "diffuse type" to avoid confusion with the ICDC, which emphasizes the diffuse or focal "enlargement" of the pancreas.

Bile duct changes often happen in AIP, as shown in this study. Bile duct wall thickening (73.4% in the diffuse group, 66.0% in the focal cases with pancreatic head involved and 19.0% in the focal cases without pancreatic head involved) resulting from IgG4-associated cholangitis and intrapancreatic bile duct stenosis (61.2% in the diffuse group, 58.0% in the focal cases with pancreatic head involved and 23.8% in the focal cases without pancreatic head involved) that possibly caused by both bile duct wall thickening and extrinsic pancreatic compression[13] are both more common in the diffuse group (almost all cases have head involvement) and focal AIP cases with pancreatic head involved.

The CP changes of AIP were also fully explored in this study. All parenchymal changes were comparable in the diffuse and focal groups. In previous studies, Farrell *et al*[4] showed that lobularity existed in 7.1% (1/14) of patients[7]. Hoki *et al*[5] demonstrated that the occurrence of HF, HS, lobularity, cystic lesions and calcifications were 32% (8/25), 56% (14/25), 8% (2/25), 16% (4/25) and 16% (4/25), respectively. Okabe *et al*[6] found that HF existed in all patients (32/32), with a lower incidence rate of HS and lobularity (81.3% and 53.1%, respectively). As for MPD changes, there was no difference between the two groups except for MPD dilation, which was more frequently seen in the focal group (14.0% *vs* 25.3%, $P = 0.03$) and seemed to be more prevalent in the focal cases with pancreatic head involved (32.0% in head involved cases *vs* 9.5% in non-head involved cases, $P = 0.05$). Previous studies reported that MPD dilation was present in 12%-37% of AIP patients, which was often located proximally to the AIP affected area where the MPD or surrounding parenchyma was involved, while hyperechoic duct margins and diffuse stenosis/irregularity were present in approximately 12% (3/25) and 40% (10/25) of patients, respectively[8,9] (Supplementary Table 3). The reason for the different incidence rate of parenchymal and MPD changes may be that AIP patients are possibly at different clinical stages (early or advanced) in these studies[14]. After the recurrent

attack or prolonged inflammatory damage, pancreatic duct stones or parenchymal calcifications may be formed, which will make the shape of AIP more similar to advanced CP[7-9].

The focal AIP accompanied by MPD dilation, sometimes also by peripancreatic lymphadenopathy and vessel involvement, is difficult to differentiate from pancreatic carcinoma. There are some EUS features, like bile duct wall thickening and peripancreatic hypoechoic margin that are relatively specific for focal AIP patients[5]. Several noninvasive EUS methods have been developed for differential diagnosis but without satisfactory sensitivity or specificity[15,16]. A prediction model with multiple EUS features could help to differentiate focal AIP from pancreatic carcinoma[17]. The EUS-guided fine-needle aspiration (EUS-FNA) procedure should be considered as the first choice to diagnosis focal AIP or rule out malignancy[18-21]. The diagnostic accuracy of EUS-FNA is between 45%–78%[22]. In this study, 92 initial "non-diagnostic" AIP cases received EUS-FNA procedures, among whom 36 cases (39.1%) got level 1 and 2 histological evidence (Supplementary Figure). All cases receiving EUS-FNA got the final diagnosis of "definite" AIP according to the ICDC.

To date, endoscopic retrograde pancreatography is included as part of the diagnosis of AIP in the Japanese guideline and the ICDC[10,23]. Exploring quantitative and qualitative parenchymal and ductal change, EUS is a reliable method to diagnose CP, which is statistically comparable to the endoscopic retrograde pancreatography and the Cambridge criteria (the gold standard in the past)[24-26]. So, we tried to describe the CP change level of newly diagnosed AIP cases with the CP features in the Rosemont criteria and found that only a small portion of patients was diagnosed as "suggestive of CP" (12.1% in the diffuse group *vs* 15.5% in the focal group), and patients with more advanced CP change ("consistent with CP") were even more rare (0.9% *vs* 1.4%). Therefore, the CP change is relatively limited for most newly diagnosed AIP cases that were probably in the early stage of disease, while advanced CP findings may happen in the long-term recurrent attacks[8,14]. EUS can detect the early parenchymal fibrosis of CP (like HF and HS) in AIP cases, which changes dynamically after corticosteroid therapy[6]. As the tool for accessing the fibrosis degree of the pancreas, the EUS findings of CP may be used for predicting the pancreatic atrophy and diabetes exacerbation, which needs further investigation[27].

This study had limitations. First, this was a single center retrospective study, and all AIP patients included in this study were diagnosed with type 1 AIP. Therefore, selection bias inevitably existed. Second, the EUS-FNA diagnosis accuracy in this study was somehow lower than previously reported (about 39.1% for level 1 and 2 histological evidence), which might be because the fine needle biopsy needles were used sparingly[22] (Supplementary Table 4). The long time period of the study (our center did not have fine needle biopsy needles until 2015) might explain the reason that we did use the fine needle biopsy needles (22G Procore and 20G Procore, COOK, United States) except in a low proportion (12.0% in diffuse AIP patients and 29.9% in focal AIP cases). However, the FNA needles still have the clinical significance of ruling out malignancy in AIP patients[21]. Third, MPD dilation was defined as "> 3 mm in the head, > 2 mm in the body, >1 mm in the tail" in the Rosemont criteria, but in the elderly population of AIP patients, the normal range of MPD diameter might be larger [28]. Therefore, we might have overestimated the incidence rate of MPD dilation. Lastly, the "dilated side branches" of the pancreatic duct in the Rosemont criteria were relatively difficult to evaluate without endoscopic retrograde pancreatography, so they were not included in this study.

CONCLUSION

In conclusion, this study demonstrated the EUS features of newly diagnosed AIP and the difference in the typical AIP features and CP features between diffuse and focal AIP on the basis of the largest number of cases and indicated the relatively limited CP change in newly diagnosed AIP cases *via* the Rosemont criteria.

ARTICLE HIGHLIGHTS

Research background

Few studies have fully described the endoscopic ultrasound (EUS) features of newly diagnosed autoimmune pancreatitis (AIP) involving both typical findings and chronic

pancreatitis (CP) features. The typical EUS findings are prevalent in diffuse AIP but may not be as common for the focal type, and the differences between diffuse and focal AIP need to be specified. The EUS typical features of AIP (especially the cholangiopathy-like features) can help to differentiate diffuse AIP from classic CP and differentiate focal AIP from pancreatic cancer.

Research motivation

This is the largest single center retrospective study demonstrating EUS features in newly diagnosed type 1 AIP patients that not only describes the typical findings and the CP features of AIP but also figures out the difference between the diffuse and focal types.

Research objectives

The authors conducted this single center retrospective study for a detailed description of the EUS features of newly diagnosed AIP patients and demonstration of the difference between diffuse and focal AIP, and we tried to compare the CP change level in both groups *via* the Rosemont criteria based on all CP features.

Research methods

This retrospective single center study included 285 patients of newly diagnosed type 1 AIP following the international consensus diagnostic criteria, with the EUS procedures accomplished before corticosteroid initiation. We explored the EUS features and compared the typical AIP and CP features between the diffuse and focal AIP cases. The Rosemont criteria were employed for CP features definition and CP change level comparison.

Research results

For the typical AIP features, there were significantly more patients in the diffuse group with bile duct wall thickening and peripancreatic hypoechoic margin. In the multivariate regression analysis for diffuse AIP, we demonstrated the predictors of diffuse AIP: the DHA, bile duct wall thickening and peripancreatic hypoechoic margin (all $P < 0.001$), which are all typical EUS findings of AIP rather than CP features. For the CP features, there were significantly more patients in the focal group with main pancreatic duct dilation. The cholangitis-like changes were more prevalent in the focal cases with pancreatic head involvement. The CP change level was relatively limited for newly diagnosed AIP cases in the diffuse and focal groups.

Research conclusions

This study demonstrated the EUS features of newly diagnosed AIP and the difference in the typical AIP features and CP features between the diffuse and focal AIP cases on the basis of the largest number of cases. It indicated the relatively limited CP change in newly diagnosed AIP cases *via* the Rosemont criteria.

Research perspectives

EUS can detect the early parenchymal fibrosis of CP in AIP cases, which changes dynamically after corticosteroid therapy. As the tool for accessing the fibrosis degree of the pancreas, the EUS findings of CP may be used for predicting pancreatic atrophy and diabetes exacerbation, which needs further investigation.

ACKNOWLEDGEMENTS

The authors would like to thank Yue-Lun Zhang, PhD (Central Research Laboratory, Peking Union Medical College Hospital) for his professional assistance in statistical analysis and all the nurses for their expert assistance during the EUS procedures.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 14; 27(42): 7387-7401

DOI: 10.3748/wjg.v27.i42.7387

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

ORIGINAL ARTICLE

Observational Study

Long-term clinical outcomes of lipiodol marking using standard gastroscopy for image-guided radiotherapy of upper gastrointestinal cancers

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Author contributions: Be KH, Zorron Cheng Tao Pu L, Khor R, Lim Joon D, Chandran S, Efthymiou M and Vaughan R conceptualized and designed the study; all authors were involved in data collection, analyses, or both; all authors were involved in the interpretation of the results; Be KH, Zorron Cheng Tao Pu L, Khor R and Chandran S drafted the manuscript; Lim Joon D, Ng SP, Efthymiou M and Vaughan R carried the critical revision of the article for important intellectual content; and all authors read and approved the final version of the manuscript.

Institutional review board statement: The study was reviewed and approved by the

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Abstract

BACKGROUND

Image-guided radiotherapy (IGRT) has significantly improved the precision in which radiotherapy is delivered in cancer treatment. Typically, IGRT uses bony landmarks and key anatomical structures to locate the tumor. Recent studies have demonstrated the feasibility of peri-tumor fiducials in enabling even more accurate delineation of target and normal tissue. The use of gold coils as fiducials in gastrointestinal tumors has been extensively studied. However, placement requires expertise and specialized endoscopic ultrasound equipment. This article reports the long-term outcomes of using a standard gastroscopy to inject liquid fiducials for the treatment of oesophageal and gastric tumors with IGRT.

AIM

To assess the long-term outcomes of liquid fiducial-guided IGRT in a cohort of oesophageal and gastric cancer patients.

Austin Health Institutional Review Board (Approval No. LNR/18/Austin/254).

Informed consent statement: Informed consent was waived.

Conflict-of-interest statement: Dr. BE on behalf of all authors has nothing to disclose.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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Specialty type: Gastroenterology and hepatology

Country/Territory of origin: Australia

Peer-review report's scientific quality classification

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

Received: July 5, 2021
Peer-review started: July 5, 2021
First decision: July 13, 2021
Revised: July 26, 2021
Accepted: October 27, 2021
Article in press: October 27, 2021
Published online: November 14, 2021

P-Reviewer: An T, Chien CR, Link

METHODS

A retrospective cohort study of consecutive adults with Oesophagogastric cancers referred for liquid fiducial placement before definitive/neo-adjuvant or palliative IGRT between 2013 and 2021 at a tertiary hospital in Melbourne, Australia was conducted. Up to four liquid fiducials were inserted per patient, each injection consisting of 0.2-0.5mL of a 1:1 mixture of iodized oil (Lipiodol; Aspen Pharmacare) and n-butyl 2-cyanoacrylate (Histoacryl®; B. Braun). A 23-gauge injector (Cook Medical) was used for the injection. All procedures were performed by or under the supervision of a gastroenterologist. Liquid fiducial-based IGRT (LF-IGRT) consisted of computer-assisted direct matching of the fiducial region on cone-beam computerised tomography at the time of radiotherapy. Patients received standard-IGRT (S-IGRT) if fiducial visibility was insufficient, consisting of bone match as a surrogate for tumor position. Radiotherapy was delivered to 54Gy in 30 fractions for curative patients and up to 45Gy in 15 fractions for palliative treatments.

RESULTS

52 patients were referred for liquid fiducial placement within the study period. A total of 51 patients underwent liquid fiducial implantation. Of these a total of 31 patients received radiotherapy. Among these, the median age was 77.4 years with a range between 57.5 and 88.8, and 64.5% were male. Twenty-seven out of the 31 patients were able to have LF-IGRT while four had S-IGRT. There were no complications after endoscopic implantation of liquid fiducials in our cohort. The cohort overall survival (OS) post-radiotherapy was 19 mo (range 0 to 87 mo). Whilst the progression-free survival (PFS) post-radiotherapy was 13 mo (range 0 to 74 mo). For those treated with curative intent, the median OS was 22.0 mo (range 0 to 87 mo) with a PFS median of 14.0 mo (range 0 to 74 mo). Grade 3 complication rate post-radiotherapy was 29%.

CONCLUSION

LF-IGRT is feasible in 87.1% of patients undergoing liquid fiducial placement through standard gastroscopy injection technique. Our cohort has an overall survival of 19 mo and PFS of 13 mo. Further studies are warranted to determine the long-term outcomes of liquid-fiducial based IGRT.

Key Words: Image-guided radiotherapy; Lipiodol; Gastroscopy; Gastric cancer; Oesophageal cancer; Fiducial

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Core Tip: Based on a cohort of 31 patients who had undergone lipiodol fiducial implantation through standard gastroscopy and received radiotherapy, fiducial-based image-guided radiotherapy (IGRT) was possible in 87.1%. Our cohort had an overall survival of 19 mo and progression-free survival of 13 mo. Further studies are warranted to determine the long-term outcomes of liquid-fiducial based IGRT.

Citation: Be KH, Khor R, Lim Joon D, Starvaggi B, Chao M, Ng SP, Ng M, Zorron Cheng Tao Pu L, Efthymiou M, Vaughan R, Chandran S. Long-term clinical outcomes of lipiodol marking using standard gastroscopy for image-guided radiotherapy of upper gastrointestinal cancers. *World J Gastroenterol* 2021; 27(42): 7387-7401
URL: https://www.wjgnet.com/1007-9327/full/v27/i42/7387.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i42.7387

INTRODUCTION

In 2020, an estimated 1.69 million cases of oesophageal and gastric cancers were diagnosed worldwide, equating to nearly one in every twelve new diagnoses of cancer. Together esophageal and gastric cancers were responsible for approximately

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1.31 million cases of cancer-related mortality[1]. In Australia, the projected age-standardised cancer-related death rates of oesophageal and gastric cancers were 7.8 in 2020, ranking in the top 5 cancer-related death[2]. As with all cancers, patients with earlier-stage tumors display a better prognosis. Unfortunately, most gastrooesophageal cancers are diagnosed at later stages, often warranting chemoradiotherapy with or without surgery[3].

Radiotherapy is one of the main treatment modality in the neoadjuvant and definitive treatment of oesophageal cancers[4-6]. Currently, its use in gastric cancer is largely limited to the post-operatively setting, however more evidence in the neoadjuvant setting is emerging with the near completion of a randomized control trial[7,8]. As such, radiotherapy either as the primary therapy or as an adjunct continues to have an important role in the treatment of patients with oesophageal and gastric cancer[9,10].

A known limitation to the delivery of radiotherapy to patients with upper gastrointestinal malignancies is that early tumors can be difficult to identify on computerized tomography (CT) scans, impairing the delineation of the tumor for radiotherapy treatment design. F-18 Fluorodeoxyglucose positron emission tomography (FDG-PET) can assist in defining the metabolically active tumor volume, but some studies indicate that up to 20% of oesophageal carcinomas are not FDG-PET avid[11]. Furthermore, the mobility of the oesophagus and stomach particularly with respiratory motion and its different physiological states such as fasting and fed states need to be considered during radiotherapy[12,13]. Fiducial placements of markers potentially allow a more accurate definition of the radiotherapy field. The use of fiducial markers to help guide radiotherapy has been demonstrated as feasible in many solid cancer treatments including the prostate, pancreas and hollow viscus of the gastrointestinal tract[12,14,15]. Fiducial-based image-guided radiotherapy (F-IGRT) in the treatment of prostate cancer has been demonstrated to have advantages of optimising radiation dosage to the tumor, whilst minimising radiation exposure to normal tissue[16,17]. Comparatively, the data for the gastrointestinal tract is less robust. A recent meta-analysis, although showing high efficacy and safety of endoscopic ultrasound (EUS)-guided fiducials for pancreatic cancer radiotherapy, highlights that more information is needed regarding the impact of fiducials on long-term clinical outcomes[18]. The insertion of fiducials within the gastrointestinal tract using EUS guidance, although attaining high technical success rates, has substantial limitations with its broad adoption in clinical practice. These include high costs, the necessity of a highly trained endoscopist (who can perform EUS) and a cumbersome solid fiducial loading method[19].

A new type of fiducial was introduced by our group to address these limitations of solid fiducials implantation. Iodized oil-based liquid fiducials are less expensive and can be inserted by an endoscopist who perform gastroscopies. This was described for the first time in our pilot study in 2016[20], in which we demonstrated that similar results could be achieved compared to solid fiducials. Although the data from this study has enabled conclusions on the safety and technical efficacy of the use of liquid fiducials, long-term efficacy data focused on the patients that received LF-IGRT was not available. Hence, this study presents data on long-term follow-up of patients with gastroesophageal cancers who received F-IGRT based on liquid fiducials placed with standard gastroscopy injection technique.

MATERIALS AND METHODS

Ethical approval

This is a retrospective cohort study of consecutive adults with oesophageal and gastric cancers referred to the endoscopy unit at Austin Health, Melbourne, Australia for liquid fiducial placement before IGRT between January 2013 and January 2021. A database of all patients referred to the endoscopy unit for liquid fiducial placement before IGRT was prospectively maintained. The study was approved after institutional board review (Austin Research Ethics Committee: H2013/04975). Informed consent was waived; patient confidentiality was maintained and protected.

Patient selection

Inclusion criteria were patients with: (1) A management plan discussed in a multi-disciplinary team meeting for radiotherapy for oesophageal (squamous carcinoma or adenocarcinoma) or gastric cancer; and (2) Referred to the endoscopy team for placement of fiducial markers.

Exclusion criteria were patients that did not have liquid fiducials inserted (*e.g.*, deemed as unfit for endoscopic procedure); patients that did not have radiotherapy after placement of fiducials (*e.g.*, declining clinical status) and patients that had surgery before radiotherapy after fiducial placement. In addition, patients who had incomplete treatment and outcome data (*e.g.*, due to loss of follow up) were excluded from our analysis.

Clinical data collection

Patient clinical data including diagnosis, functional performance status as defined by Eastern Cooperative Oncology Group (ECOG)[21], the American Joint Committee on Cancer (AJCC) 8th edition staging[22], and treatment outcomes were retrospectively collected from patient medical records, endoscopy, radiology, surgical and histopathology reports. The national health database (©Australian Digital Health Agency) was used to assess whether the patient was still alive.

Information for patients treated at other centers was requested from their treating radiation oncologists. When information on the measured primary and secondary outcomes was available, these patients were included in the analysis.

Progression-free survival (PFS) was assessed based on patient disposition at their latest oncological follow-up appointment.

The endoscopic procedure

The endoscopic procedure aims to insert a total of four fiducials per patient (2 proximal and 2 distal edges of the tumor), each injection consisting of 0.2-0.5mL of a 1:1 mixture of iodized oil (Lipiodol; Aspen Pharmacare) and n-butyl 2-cyanoacrylate (Histoacryl®; B. Braun). If a tumor was obstructing the passage of the gastroscope, only fiducials on the proximal edge were placed. In addition, if a tumor was extending to the level of the cricopharyngeal, it was not technically possible to insert fiducials at the proximal edge and only distal edges were placed. A 23-gauge injector (Cook Medical®) was used for these injections. All procedures were performed by or under the supervision of a gastroenterologists. All procedures were done under sedation, which was performed by an accredited anaesthetist. The endoscope used for all procedures was a standard gastroscope [(GIF-H1180 and H190; Olympus©), Melbourne, Victoria, Australia], and did not require the aid of fluoroscopy. Patients were routinely observed after the procedure for 1 h after which they were discharged if there were no significant adverse events.

The injection technique

Three 2-mL syringes with a Luer Lock™ that can securely be locked onto the end of the injector needle are required; two are filled with iodized oil and only one will contain the iodised oil/n-butyl 2-cyanoacrylate mixture (1 mL: 1 mL).

Step 1: The 23-gauge injector is primed with the iodised oil only outside the patient.

Step 2: When the endoscopist is ready to inject, the injector is passed down the accessory channel of the gastroscope and further primed with the iodized oil/n-butyl 2-cyanoacrylate mixture, ideally within the stomach.

Step 3: A total of four-point injections (0.2-0.5 mL each) are made into the edges of the tumor. Two injections are placed proximally and another two placed distally into the edges of the tumor, when possible.

Step 4: Once marking is completed, the injector should be flushed with the syringe containing iodised oil only to prevent accidental gluing of the accessory channel, again ideally within the stomach.

Step 5: The gastroscope is then withdrawn with the needle retracted but the injector tip itself is slightly out of the distal tip of the gastroscope.

Step 6: Once the gastroscope is removed from the patient, the injector is cut at the port end so that it can be pulled through from the distal tip.

Step 7: The gastroscope accessory channel is subsequently flushed with water. Images and a video of these steps can be found in a previous publication[20].

Fiducial IGRT

Following insertion of liquid fiducial markers, the patient underwent CT simulation a minimum of 24 h after insertion. The gross target volume (GTV) was defined using the fiducial markers, endoscopic report and correlative imaging (*e.g.*, diagnostic FDG-PET/CT and CT).

For patients who received definitive treatment, a high dose clinical target volume (CTV) included the GTV plus a 1 cm margin, clipped at anatomic boundaries. A low dose CTV included the GTV plus a 3 cm margin in the cranio-caudal (C-C) axis and 1

cm in other planes (clipped to anatomic boundaries), plus regional lymph nodes at the discretion of the radiation oncologist. A 1 cm planning target volume (PTV) margin was used. The high dose PTV was treated to 54Gy in 30 fractions, and the low dose PTV was assigned 46Gy in 30 fractions. Treatments were planned using the Monaco treatment planning system (Elekta, Stockholm).

For patients who had palliative treatments, the CTV was defined using a 1 cm margin, clipped at anatomical boundaries, with a 1 cm PTV margin. A range of prescription doses were used depending on patient disposition, ranging from 30Gy in 10 fractions to 45Gy in 15 fractions.

Liquid fiducial-based IGRT (LF-IGRT) was performed when the implanted liquid fiducials could be adequately visualized at the time of radiotherapy treatment on cone-beam CT (CBCT). LF-IGRT was performed each fraction using the Elekta XVI software (Elekta Synergy, XVI version 5, Elekta AB, Stockholm, Sweden) online image verification software and an Elekta linear accelerator. A grey value match was performed on an area including the fiducial markers. If liquid fiducials could not be located on the CBCT scan, then patients were treated with S-IGRT. S-IGRT utilized a grey value match on the vertebrae only.

PFS and complications post radiotherapy

Postprocedural follow-up was assessed through outpatient radiation oncology appointments. Data on patients seen at our center were retrieved from electronic medical records. Patients who had the fiducials implanted at our center but had their follow-up elsewhere had their treating radiation oncologist contacted for information. Data on PFS, late complications from fiducial placement and radiotherapy complications (as per the National Cancer Institute Common Terminology Criteria for Adverse Events v4.0.[23]) were assessed.

Outcome Measures

Primary Outcome: The primary outcome was the overall survival (OS) and PFS of patients who received IGRT after liquid fiducial placement for the treatment of a gastroesophageal tumor. The OS and PFS were referenced to the time of radiotherapy completion. Progression was defined as radiographic or histologic progression (*e.g.*, from recurrence detected on gastroscopy), coded as either local or distant in location.

Secondary outcomes: Secondary outcomes included the technical success of liquid fiducial guiding radiotherapy, adverse events rate and subgroup analyses based on radiotherapy treatment (LF-IGRT and S-IGRT), treatment intent (curative and palliative), tumor type (oesophageal and gastric) and oesophageal histology.

Key definitions: Technical success was defined as the successful delivery of LF-IGRT after the placement of liquid fiducial(s) through standard gastroscopy technique.

Gastroesophageal junction cancers classified as Siewert types I and II were analyzed as oesophageal cancers in accordance with the 8th edition of the AJCC staging guidelines[22]. Gastroesophageal junction cancers classified as Siewert type III were analyzed as gastric cancers.

Functional performance status was defined by the ECOG performance status[21].

Statistical analysis

Data are summarized as median and ranges for continuous data, and as frequency and percentages for categorical data. For continuous data, comparisons were done using the Mann-Whitney *U* test or Independent Samples Kruskal-Wallis test based on the normality assumption. For categorical data, Fisher's Exact test and Likelihood Ratio Chi-Squared test were used as per high prevalence of expected cells with a count less than 5. P value of < 0.05 was considered significant. Survival rates were estimated using the Kaplan-Meier method. Statistical analyzes were performed with SPSS statistical software (IBM Corp. 2020. IBM SPSS Statistics, Version 26.0. Armonk, NY) and JMP v16.0 (SAS Institute Inc).

RESULTS

Population

A total of 52 patients were referred to the endoscopy unit for liquid fiducial placement for IGRT over eight years between January 2013 and January 2021.

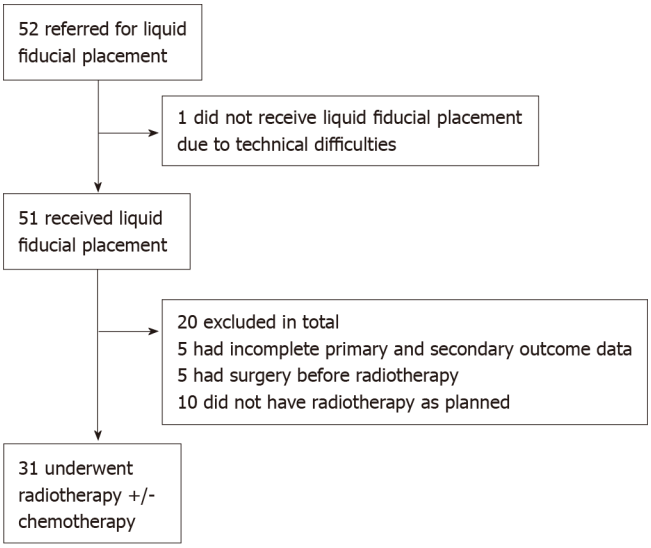


Figure 1 Study flowchart.

1 patient did not have liquid fiducial placement after endoscopic assessment as the tumor was obstructive and extended up to the level of the oropharynx and deemed unsafe to proceed. The majority of patients (98.0%) were able to have at least one edge marked with liquid fiducials, with a large proportion (77.4%) having both at distal and proximal edges marked.

51 patients had liquid fiducials inserted during the study period. Of these, 20 were excluded from our analysis. Five underwent radiotherapy at other centers and their clinical data were not available for analysis. Five had surgery prior to radiotherapy and ten did not have radiotherapy as anticipated. For instance, some patients were treated on the TOPGEAR trial and were randomized to no adjuvant therapy[8]. Therefore, data on the use of endoscopically-placed liquid fiducials during radiotherapy was available for 31 patients (Figure 1).

Our cohort of 31 patients had a median age of 77 years (range 57.5 to 88.8). The majority (71.0%) had oesophageal cancers, with a significant subset (72.7%) of these with squamous cell carcinoma (SCC). Only three patients in our cohort had gastroesophageal junction cancer of which two were classified as Siewert type III. Most patients (67.7%) had locally advanced disease without lymph node involvement or metastatic disease. Most of the cohort had an ECOG score of 0 or 1.

Three (9.6%) of patients received neoadjuvant chemoradiotherapy prior to oesophagectomy for oesophageal SCC. A large proportion (38.7%) of our cohort received definitive chemoradiotherapy, whilst a further 25.8% received definitive radiotherapy only. Detailed demographics and treatments are summarized in Table 1.

Technical success

Twenty-seven out of the 31 patients (87.1%) received LF-IGRT. Patients commenced radiotherapy after a median period of 18 d (range 9 to 44) of fiducial placement. The cohort median duration of radiotherapy was 30 d (range 14 to 47). Details on F-IGRT and S-IGRT subgroups are shown in Table 2.

OS and PFS

On the close-out date of 24/06/2021, 54.8% of patients were alive. The cohort OS post-radiotherapy was 19 mo (range 0 to 87 mo). Whilst the PFS post-radiotherapy was 13 mo (range 0 to 74 mo). For those treated with curative intent, the median OS was 22.0 mo (range 0 to 87 mo) with a PFS median of 14.0 mo (range 0 to 74). Nineteen patients were alive at 5 years. The 5-year survival rate for oesophageal cancer was 42.5% and for gastric cancer was 55.6% for this cohort. Kaplan-Meier curves for the overall cohort OS and PFS, treatment intent and type of cancer and histology are described in Figures 2, 3 and 4 respectively. Details on OS, PFS and 5-year survival for each subgroup analysis for the type of radiotherapy treatment (LF-IGRT and S-IGRT), treatment intent (curative and palliative), tumor type (oesophageal and gastric) and oesophageal histology are described in Table 3.

Table 1 Patient demographics and treatment

Variables		Median/*n*	Range/%
	Age	77.4	57.5-88.0
	Male	20	64.5
Site of cancer	Oesophageal	21	67.7
	GOJ (Siewert I/II)	1	3.2
	GOJ (Siewert III)	2	6.5
	Gastric	7	22.6
Type of cancer	Oesophageal SCC	16	51.6
	Oesophageal adenocarcinoma	6	19.4
	Gastric adenocarcinoma	9	29.0
Endoscopy data	Patients with both proximal and distal fiducial placed	24	77.4
	Patients with solely proximal or distal fiducials placed	7	22.6
ECOG	0	13	41.9
	1	11	35.5
	2	7	22.6
Stages[1]	LN negative without distant metastasis	21	67.7
	LN positive without distant metastasis	6	19.4
	Distant metastasis present	3	9.6
Radiotherapy	Fiducial seen on CBCT	29	93.5
	Fiducial-based IGRT	27	87.1
Treatment intent	Neoadjuvant chemotherapy with oesophagectomy	3	9.7
	Definitive chemoradiotherapy	12	38.7
	Palliative chemoradiotherapy	3	9.7
	Definitive radiotherapy	8	25.8
	Palliative radiotherapy	5	16.1

[1]Staging as per AJCC 8th edition. T-stage was not available for all patients as endoscopic ultrasound is not routinely performed at our center.
SCC: Squamous cell carcinoma; ECOG: Eastern Cooperative Oncology Group; GOJ: Gastro-oesophageal junction; LN: Lymph Node; CBCT: Cone-beam computerized tomography; IGRT: Image-guided radiotherapy.

Of note, 12 patients had disease progression during the study period. Seven of these patients had local progression whilst five had distal disease progression.

Adverse events

No early or late adverse events occurred following the insertion of the fiducials as assessed prior to discharge on the day of the procedure and on subsequent radiation oncology follow-ups, respectively. Nine patients experienced grade three adverse events which were odynographia, dysphagia, nausea, dehydration, febrile neutropenia and lung infection during their treatment. No patient experienced grade four or five adverse events. Adverse events from their treatment are summarized in Table 4.

DISCUSSION

In this study, we describe the OS and PFS of patients with gastroesophageal tumors that underwent LF-IGRT with liquid fiducials inserted through standard gastroscopy injection technique. This report is a follow-up to our initial study which first described this technique[20]. We believe this to be the largest observational cohort study of its kind, adding to the limited body of knowledge on the long-term outcomes of F-IGRT for gastrooesophageal tumors using liquid fiducials.

Table 2 Patient demographics and treatment details based on fiducial-based image-guided radiotherapy and standard image-guided radiotherapy

Variables		F-IGRT	S-IGRT
		Median/*n*, range/%	Median/*n*, range/%
	Age	77.4, 57.5-88.0	77.3, 64.8-85.4
	Male	19, 70.4	1, 25.0
Site of cancer	Oesophageal	18, 62.1	3, 75.0
	GOJ (Siewert I/II)	0, 0.0	1, 25.0
	GOJ (Siewert III)	2, 7.4	0, 0.0
	Gastric	7, 25.9	0, 0.0
Type of cancer	Oesophageal SCC	13, 48.1	3, 75.0
	Oesophageal adenocarcinoma	5, 18.5	1, 25.0
	Gastric adenocarcinoma	9, 33.3	0, 0.0
Endoscopy data	Patients with both proximal and distal fiducial placed	22, 81.5	2, 50.0
	Patients with solely proximal or distal fiducials placed	5, 18.5	2, 50.0
ECOG	0	11, 40.7	2, 50.0
	1	9, 33.3	2, 50.0
	2	7, 25.9	0, 0.0
Stages[1]	LN negative without distant metastasis	18, 66.7	4, 100.0
	LN positive without distant metastasis	6, 22.2	0, 0.0
	Distant metastasis present	3, 11.1	0, 0.0
Treatment details	Time to treatment (d)	19.0, 9.0-44	14 .0, 9.0-17.0
	Curative intent	20, 74.1	4, 100.0
	Palliative intent	7, 25.9	0, 0.0
	Dose (Grays)	50, 25.2-55	47.7, 41.4-54
	Fraction	25, 10-30	26.5, 23-30
	Duration (d)	30, 14-47	37, 30-39
	Chemotherapy	14, 51.9	4, 100.0

[1]Staging as per American Joint Committee on Cancer 8th edition. T-stage was not available for all patients as endoscopic ultrasound is not routinely performed at our center.
F-IGRT: Fiducial-based image-guided radiotherapy; S-IGRT: Standard image-guided radiotherapy; SCC: Squamous cell carcinoma; ECOG: Eastern Cooperative Oncology Group; GOJ: Gastro-oesophageal junction; LN: Lymph Node.

Our cohort consisted mainly of patients with locally advanced oesophageal cancer with SCC. The majority (90.3%) of our cohort received chemoradiotherapy or radiotherapy alone as palliative and definitive treatment. The median OS was 19.0 mo, with the longest OS of 87 mo. Our results, albeit a small cohort, compare favorably to what is available in the literature, the 5-year survival rates for oesophageal and gastric cancers were 42.8% and 55.6%, respectively[24]. In the context of gastric cancer in an inoperable population undergoing chemoradiotherapy, the reported median survival was 25.0 mo[25]. We recognize that this is possibly due to judicious patient selection, or the relatively small numbers of patients included compared with larger randomized trials.

Also, we acknowledge that in Australia access to EUS for endoscopic staging is variable, and at our center is not routinely performed. Thus, limiting our accurate reporting of tumor staging as per AJCC 8th Edition. This further adds to our argument that the use of EUS-guided solid fiducials for marking tumors has limitations of which most can be mitigated with the use of liquid fiducials[19,20]. Additionally, the technical aspects of the injection technique required for placement of liquid fiducials

Table 3 Subgroup analysis of overall survival, progression-free survival and 5-year survival

Subgroups		OS (mo)	PFS (mo)	5-yr survival (%)
		Median/*n*, range/%	Median/*n*, range/%	
Treatment	F-IGRT (*n* = 27)	21.0, 0-87	12.0, 0-74	47.2
	S-IGRT (*n* = 4)	17.0, 3-49	18.0, 2-32	0.0
Intent	Curative (*n* = 23)	22.0, 0-87	14.0, 0-74	51.5
	Palliative (*n* = 8)	8.0, 0-79	4, 0-13	30.0
Location	Oesophageal (*n* = 22)	18, 0-87	13, 0-74	42.8
	SCC (*n* = 16)	20, 1-87	14, 1-74	62.0
	AC (*n* = 6)	10, 0-54	7, 0-19	0.0
	Gastric (*n*=9)	25, 0-79	13, 0-53	55.6

F-IGRT: Fiducial-based image-guided radiotherapy; S-IGRT: Standard image-guided radiotherapy; SCC: Squamous cell carcinoma; AC: Adenocarcinoma; OS: Overall survival; PFS: Progression-free survival.

Table 4 Complications

	LF-IGRT only	LF-IGRT with chemotherapy	S-IGRT with chemotherapy	Overall
Hospitalization	3	4	2	9
Fatigue (G1/G2/G3)	1/2/0	3/1/0	2/0/0	6/3/0
Odynophagia G1/G2/G3	3/1/1	2/4/1	0/4/0	5/9/2
Dysphagia (G1/G2/G3)	2/0/0	0/1/0	0/1/1	2/2/1
Oesophageal stricture (G1/G2/G3)	1/0/0	0/0/1	0/0/1	1/0/2
Nausea (G1/G2/G3)	0/2/1	-	-	0/2/1
Diarrhoea (G1/G2/G3)	-	1/0/0	-	1/0/0
Lung infection (G2, G3)	-	0/1	-	0/1
Dermatitis (G1/G2/G3)	-	1/0/0	-	1/0/0
Febrile neutropenia (G3)	-	1	-	1
Dehydration (G1/G2/G3)	0/0/1	-	-	0/0/1

G1: Grade 1 complication; G2: Grade 2 complication; G3: Grade 3 complication; LF-IGRT: Liquid fiducial-based image-guided radiotherapy; S-IGRT: Standard image-guided radiotherapy.

is, in essence, an adaptation of a skillset that most gastroenterologists would already have in the management of gastrooesophageal variceal bleeding[26]. As such, this technique of liquid fiducials can be more easily adopted. Furthermore, our group has previous described the potential cost-saving of liquid fiducials amounting to approximately AU$1150 to AU$1750 per procedure when compared to EUS-guided guided solid fiducials insertion[20]. Since the description of lipiodol as a fiducial for gastroesophageal tumors, similar techniques have been described with similar technical success and safety profiles[27].

The use of F-IGRT has many potential benefits over S-IGRT, including facilitating a higher dose focused on the tumor with a lower dose delivered to cover the submucosal spread, and more accurate treatment delivery (matching to the tumor rather than surrounding bony structures)[17,28]. There are conflicting data regarding the efficacy of increased radiation dose in treating oesophageal cancer[29,30]. While some retrospective studies demonstrated a dose-response, recent randomized control trials failed to find a difference in outcomes[31-33]. The effect of dose escalation in optimizing cure rate may be more evident in those with early-stage SCC where the tumor is radiosensitive and the rate of distant metastasis is low[34]. Higher doses can be associated with increased normal tissue toxicity and hence focusing radiotherapy as much as possible to the tumor area is essential. F-IGRT allows a higher dose to be

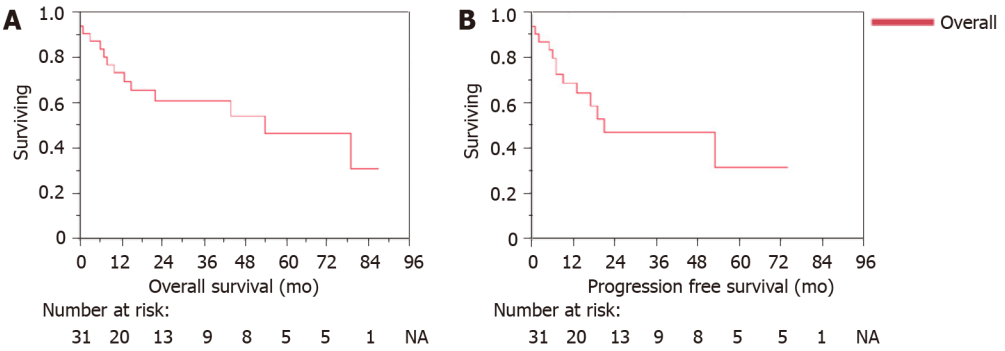


Figure 2 Kaplan Meier curve–cohort. A: The Kaplan-Meier curve for the cohort overall survival post-radiotherapy treatment; B: The Kaplan-Meier curve for the cohort progression free survival post-radiotherapy treatment. NA: Not applicable.

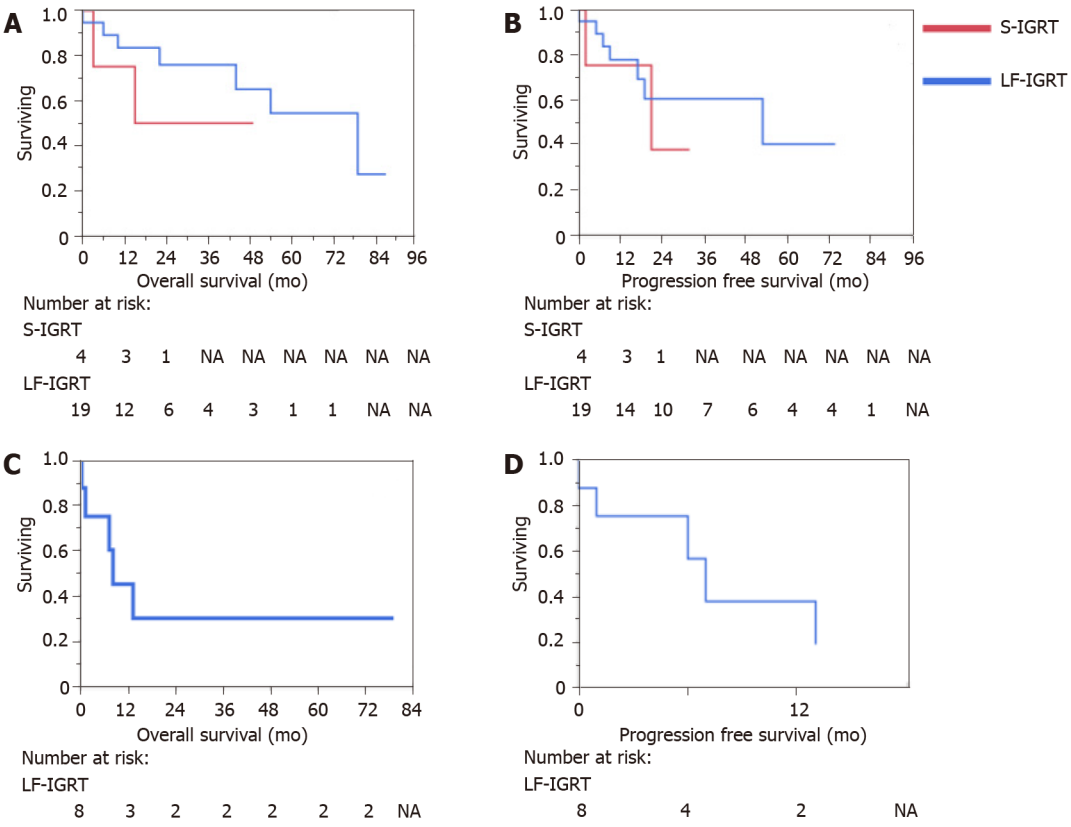


Figure 3 Kaplan Meier curve-treatment intent. A: The Kaplan-Meier curve for the overall survival (OS) of patients receiving curative intent standard image-guided radiotherapy (S-IGRT) and fiducial-based IGRT (F-IGRT) treatment; B: The Kaplan-Meier curve for the progression free survival (PFS) of patients receiving curative intent S-IGRT and F-IGRT treatment; C: The Kaplan-Meier curve for the OS of patients receiving palliative intent F-IGRT treatment; D: The Kaplan-Meier curve for the PFS of patients receiving palliative intent F-IGRT treatment, respectively. NA: Not applicable; S-IGRT: Standard image-guided radiotherapy; LF-IGRT: Liquid fiducial-based image-guided radiotherapy.

assigned to the tumor while simultaneously allowing for more confident identification of submucosal spread, and therefore facilitating lower doses to the adjacent esophagus.

The improvement in the delivery of radiotherapy for patients having endoscopically inserted liquid fiducials is illustrated in Figure 5.

In our cohort, 87.1% successfully underwent LF-IGRT after liquid fiducial placement. In two of our patients, the liquid fiducials were not visible at the time of radiation treatment planning. We hypothesis that this could be due to extravasation or diffusion of the submucosal bleb after the procedure. For two patients, the liquid fiducials were visible but were not sufficient for F-IGRT due to the fiducials not being reliably seen. This highlights a difference compared to metallic fiducials, in that liquid fiducials can have variable shapes and distribution. In addition, we consider our definition of technical success to be more clinically relevant relative to previous

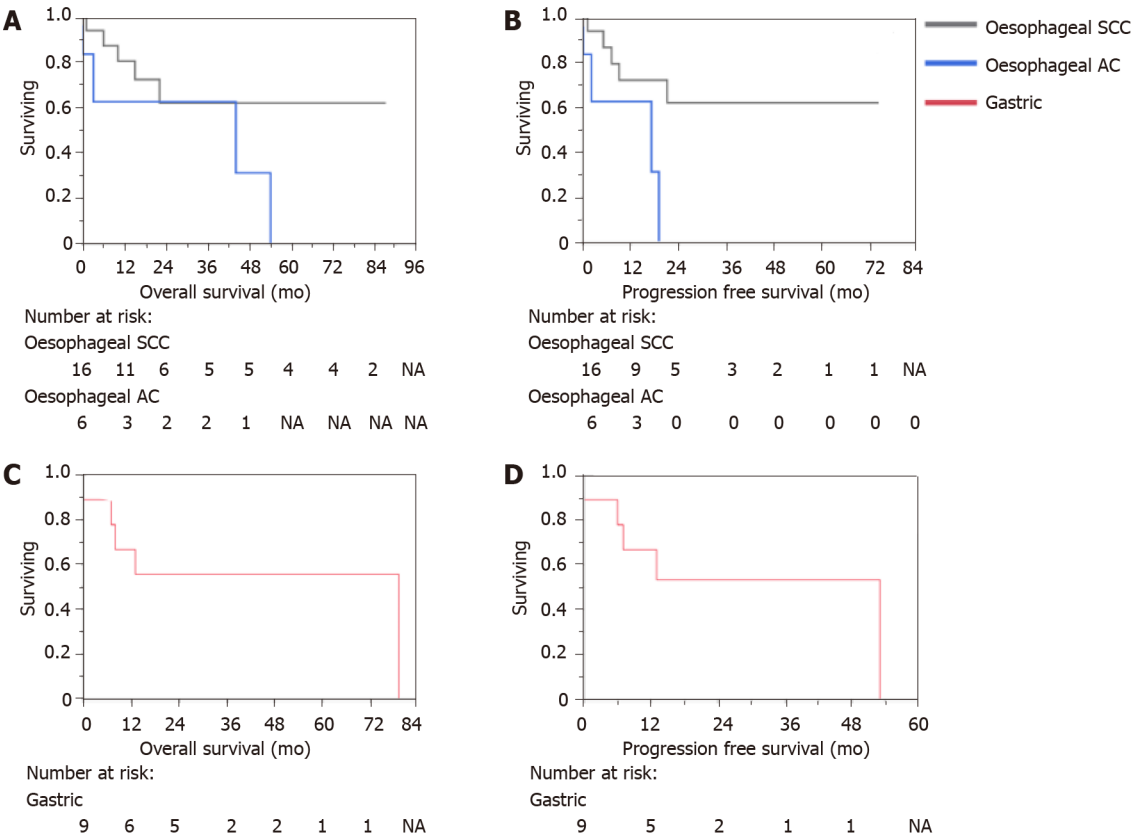


Figure 4 Kaplan Meier curve-site and histopathology. A: The Kaplan-Meier curve for the overall survival (OS) of patients with oesophageal squamous cell carcinoma (SCC) and adenocarcinoma (AC); B: The Kaplan-Meier curve for the progression free survival (PFS) of patients with oesophageal SCC and AC; C: The Kaplan-Meier curve for the OS of patients with gastric AC; D: The Kaplan-Meier curve for the PFS of patients with gastric AC. NA: Not applicable; SCC: Squamous cell carcinoma; AC: Adenocarcinoma.

definitions reported in the literature. This would account for our slightly lower success rates. However, applying the same technical definition, our rates of successful placement of liquid fiducials would be 98.1%, compared to 96.3%[18] and 98.0%[35].

Toxicity rates reported in the literature include grade 3 seen in 42% and grade 4 in 7%; mainly hematology, gastrointestinal and mouth ulceration[24]. Our lower complication rate from F-IGRT may be related to advances in treatment or the use of fiducials *per se*. Prospective randomized studies are needed to ascertain the utility of LF-IGRT in reducing complications.

Despite these potential benefits, prospective randomized trials are required as observational studies have failed to show differences in long-term outcomes such as OS for oesophageal cancers using IGRT[36]. Nevertheless, F-IGRT has been deemed by specialists as a promising IGRT modality for the future[37].

The limitations of our study are mainly the small numbers, the heterogeneity of the cohort, the retrospective design and the lack of a direct comparison with S-IGRT. Regarding the certainty of the delivered dose, one other study demonstrated that soft tissue (diaphragm) or bone matching on CBCT resulted in a larger margin to cover the tumor 95% of the time[38]. Direct soft tissue matching of locally advanced tumors would be possible if visible on CBCT but would be not feasible if the tumor is too small to be seen. Secondarily, although we present data on few patients, this is to date the largest cohort of F-IGRT for gastroesophageal tumors utilizing the liquid fiducial technique. The retrospective design and lack of a robust comparison between F- and S-IGRT could not be addressed in the present study but is a promising subject for future research. Furthermore, due to the small number of patients, a multivariate analysis was not performed to address potential bias in this study.

CONCLUSION

F-IGRT was considered feasible in 87.1% of patients undergoing liquid fiducial placement through standard gastroscopy injection technique. Our cohort had an OS of

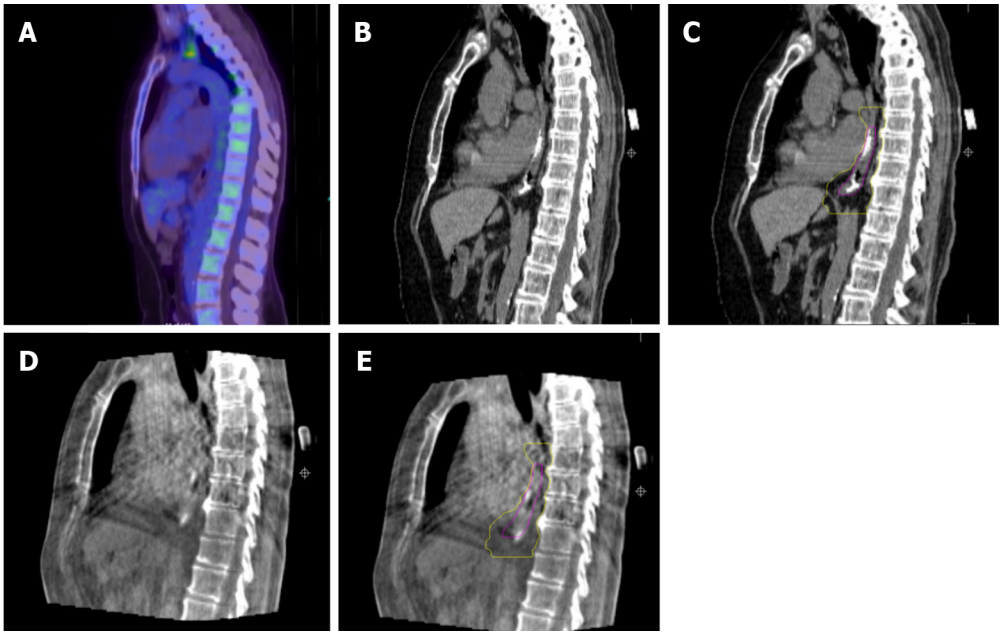


Figure 5 Endoscopically inserted liquid fiducials and liquid fiducial-based image-guided radiotherapy. A: An Fluorodeoxyglucose positron emission tomography of a patient with oesophageal cancer which demonstrates no uptake within the lesion; B: Planning computerized tomography (CTs) showing the fiducials without and with the high dose clinical target volume (CTV) (pink); C: Planning CTs showing the fiducials without and with the low dose CTV (yellow); D: Cone-beam CT (CBCT) images that are not labelled; E: CBCT images that are labelled.

19 mo and PFS of 13 mo. Further studies are warranted to determine the long-term outcomes of liquid-fiducial based IGRT.

ARTICLE HIGHLIGHTS

Research background

Further studies are warranted to determine the long-term outcomes of liquid fiducial-based image-guided radiotherapy (IGRT) in the treatment of oesophagogastric cancers.

Research motivation

Based on a cohort of 31 patients who had undergone lipiodol fiducial implantation through standard gastroscopy and received radiotherapy, fiducial-based IGRT was possible in 87.1%. Our cohort had an overall survival (OS) of 19 mo and progression-free survival (PFS) of 13 mo.

Research objectives

52 patients were referred for liquid fiducial placement within the study period. A total of 51 patients underwent liquid fiducial implantation. Of these a total of 31 patients received radiotherapy. Twenty-seven out of the 31 patients were able to have liquid fiducial-based IGRT (LF-IGRT) while four had standard-IGRT (S-IGRT). There were no complications after endoscopic implantation of liquid fiducials in our cohort. The cohort OS post-radiotherapy was 19 mo (range 0 to 87 mo). Whilst the PFS post-adiotherapy was 13 mo (range 0 to 74 mo).

Research methods

A retrospective cohort study of consecutive adults with oesophagogastric cancers referred for liquid fiducial placement before definitive/neo-adjuvant or palliative IGRT between 2013 and 2021 at a tertiary hospital in Melbourne, Australia was conducted. Up to four liquid fiducials were inserted per patient, each injection consisting of 0.2-0.5mL of a 1:1 mixture of iodized oil (Lipiodol; Aspen Pharmacare) and n-butyl 2-cyanoacrylate (Histoacryl®; B. Braun). A 23-gauge injector (Cook Medical) was used for the injection. All procedures were performed by or under the supervision of a gastroenterologist. LF-IGRT consisted of computer-assisted direct

matching of the fiducial region on cone-beam computerized tomography (CBCT) at the time of radiotherapy. Patients received S-IGRT if fiducial visibility was insufficient, consisting of bone match as a surrogate for tumor position. Radiotherapy was delivered to 54Gy in 30 fractions for curative patients and up to 45Gy in 15 fractions for palliative treatments.

Research results

To assess the long-term outcomes of liquid fiducial-guided IGRT in a cohort of oesophageal and gastric cancer patients.

Research conclusions

We believe this to be the largest observational cohort study of its kind, adding to the limited body of knowledge on the long-term outcomes of F-IGRT for gastrooesophageal tumors using liquid fiducials.

Research perspectives

IGRT has significantly improved the precision in which radiotherapy is delivered in cancer treatment. Typically, IGRT uses bony landmarks and key anatomical structures to locate the tumor. Recent studies have demonstrated the feasibility of peri-tumor fiducials in enabling even more accurate delineation of target and normal tissue. The use of gold coils as fiducials in gastrointestinal tumors has been extensively studied. However, placement requires expertise and specialized endoscopic ultrasound equipment. This article reports the long-term outcomes of using a standard gastroscopy to inject liquid fiducials for the treatment of oesophageal and gastric tumors with IGRT.

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7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
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ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2021 November 21; 27(43): 7402-7581



Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

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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: *Yan-Xia Xing*; **Production Department Director:** *Xiang Li*; **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, Subrata Ghosh

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

PUBLICATION DATE
November 21, 2021

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World Journal of Gastroenterology

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World J Gastroenterol 2021 November 21; 27(43): 7402-7422

DOI: 10.3748/wjg.v27.i43.7402

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

FRONTIER

Antimicrobial peptides and the gut microbiome in inflammatory bowel disease

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Author contributions: Gubatan J organized and led the literature review; Gubatan J, Holman DR, Puntasecca CJ, Polevoi D, Rubin SJS, and Rogalla S performed the primary literature and data extraction. Gubatan J reviewed literature search results; Gubatan J, Holman DR, Puntasecca CJ, and Polevoi D drafted the manuscript; Rogalla S provided critical review of the manuscript; All authors interpreted the results and contributed to critical review of the manuscript; Gubatan J had full access to the study data and takes responsibility for the integrity of the data and accuracy of the analysis.

Conflict-of-interest statement: The authors have no conflicts of interests or financial disclosures relevant to this manuscript.

Supported by Chan Zuckerberg Biohub Physician Scientist Scholar Award; and National Institutes of Health NIDDK Clinical Research Loan Repayment Program Award.

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Abstract

Antimicrobial peptides (AMP) are highly diverse and dynamic molecules that are expressed by specific intestinal epithelial cells, Paneth cells, as well as immune cells in the gastrointestinal (GI) tract. They play critical roles in maintaining tolerance to gut microbiota and protecting against enteric infections. Given that disruptions in tolerance to commensal microbiota and loss of barrier function play major roles in the pathogenesis of inflammatory bowel disease (IBD) and converge on the function of AMP, the significance of AMP as potential biomarkers and novel therapeutic targets in IBD have been increasingly recognized in recent years. In this frontier article, we discuss the function and mechanisms of AMP in the GI tract, examine the interaction of AMP with the gut microbiome, explore the role of AMP in the pathogenesis of IBD, and review translational applications of AMP in patients with IBD.

Key Words: Antimicrobial peptides; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Gut microbiome; Biomarkers

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Core Tip: Antimicrobial peptides (AMPs) play critical roles in protecting against

Country/Territory of origin: United States

Specialty type: Gastroenterology and hepatology

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

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

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Received: March 19, 2021
Peer-review started: March 19, 2021
First decision: May 1, 2021
Revised: May 13, 2021
Accepted: November 15, 2021
Article in press: November 15, 2021
Published online: November 21, 2021

P-Reviewer: Bruland T, Liagre B, Sassaki LY
S-Editor: Ma YJ
L-Editor: A
P-Editor: Ma YJ

infection while maintaining intestinal homeostasis to support commensalism with the gut microbiome. AMPs have broad spectrum antimicrobial activity with diverse mechanisms of action and regulate gut microbiome composition. Defects in endogenous AMP expression and function have been linked with animal models of inflammatory bowel disease (IBD). Exogenous delivery of AMPs such as defensins, cathelicidin, and elafin attenuates intestinal inflammation in murine models of IBD. AMPs such as calprotectin and lactoferrin are useful biomarkers for patients with IBD. Challenges with AMP stability, bioavailability, and selectivity are major barriers to their application as potential therapies.

Citation: Gubatan J, Holman DR, Puntasecca CJ, Polevoi D, Rubin SJ, Rogalla S. Antimicrobial peptides and the gut microbiome in inflammatory bowel disease. *World J Gastroenterol* 2021; 27(43): 7402-7422
URL: https://www.wjgnet.com/1007-9327/full/v27/i43/7402.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i43.7402

INTRODUCTION

The gastrointestinal (GI) tract is a highly complex and dynamic ecosystem consisting of a protective epithelial barrier in constant exposure to commensal microorganisms that are collectively known as the gut microbiome[1]. An intricate balance between tolerance to commensal microorganisms and protection against enteric pathogens is required to maintain intestinal homeostasis. A breakdown in this balance has been recognized to play a role in the pathogenesis of inflammatory disorders of the GI tract such as inflammatory bowel disease (IBD)[2]. Antimicrobial peptides (AMPs) are diverse and bioactive compounds that play critical roles in host defense and maintaining tolerance to commensal microorganisms[3,4]. Here we provide a comprehensive review of the significant AMP functions in the GI tract and the gut microbiome, potential roles of AMPs in the pathogenesis and treatment of IBD based on preclinical animal models, and translational applications of AMPs in patients with IBD.

ANTIMICROBIAL PEPTIDES IN THE GASTROINTESTINAL TRACT

Human defensins

Table 1 summarizes the major classes of AMPs in the GI tract. Defensins, which consist of small cationic peptides, protect against bacterial infections by directly disrupting bacterial membranes. The two major classes of defensins include α-defensins and β-defensins which differ structurally in their cysteine pairings[5]. Human α-defensins are also known as human neutrophil peptides (hNP). Human defensin 5 and 6 (HD5 and HD6) are the only α-defensins produced in the GI tract by Paneth cells, highly specialized secretory epithelial cells with antimicrobial function[6]. Known functions of HD5 include conferring resistance to oral challenge with enteric pathogens[7] and regulating the intestinal microbiota by reducing levels of segmented filamentous bacteria[8]. HD6 has been shown to restrict infection by limiting intestinal epithelial cell invasion[9]. β-defensins are expressed by enterocytes of the small and large intestine. The most relevant intestinal β-defensins include human β-defensins 1–4 (hBD-1, hBD-2, hBD-3, and hBD-4). hBD-2 and hBD-3 expression increases in response to infectious stimuli, whereas hBD-1 is constitutively expressed by the GI tract[10]. β-defensins hBD-2-4 have antimicrobial activity against *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa, Staphylococcus aureus, and Streptococcus pyogenes*, whereas hBD-1 only has activity against gram positive commensals[11-13].

Cathelicidin

Cathelicidin is another class of cationic peptides that mediates its bactericidal effects through direct disruption and lysis of bacterial membranes. Cathelicidin, also known as LL-37 or hCAP18, is an 18 kDa antimicrobial peptide involved in innate immune defenses and is encoded by the CAMP gene in humans[14]. Cathelicidin has a broad-

Table 1 Antimicrobial peptides in the gastrointestinal tract

Antimicrobial peptide class	Gene	Specific antimicrobial peptides	Tissue expression	Biologic function
α-defensins (human neutrophil peptides)[5-9]	*DEFA*	Human defensin 5 and 6 (HD5 and HD6)	Paneth cells	Confers resistance to oral challenge with enteric pathogens, regulates the intestinal microbiota by reducing levels of segmented filamentous bacteria, restricts infection by limiting intestinal epithelial cell invasion
β-defensins[5,10-13]	*DEFB*	Human β-defensins 1–4 (hBD-1, hBD-2, hBD-3, and hBD-4)	Intestinal epithelial cells	Antimicrobial activity (hBD-2-4) against bacterial pathogens including *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pyogenes*, antimicrobial activity (hBD-1) against gram-positive commensals
Cathelicidin[14-22]	*CAMP*	Cathelicidin (LL-37/hCAP18)	Colonic epithelial cells, neutrophils, monocytes, macrophages, mast cells	Cationic peptide that directly disrupts bacterial cell membranes, deficiency increases susceptibility to infection with enterohemorrhagic *E. coli (EHEC)*
Regenerating (Reg) protein [23-29]	*REG*	RegIII; Hepatocarcinoma-intestine pancreas (HIP)/pancreatitis-associated protein (PAP)	Paneth cells, intestinal epithelial cells	Regulates intestinal homeostasis by maintaining a physical separation between epithelial cells and the microbiota, selective for gram-positive bacteria through interaction with cell wall peptidoglycan
Lactoferrin[30]	*LTF*	Lactoferrin	Epithelial cells	Secreted iron binding protein, sequesters free iron required for bacterial growth
Lipocalin[31,129]	*LCN2*	Lipocalin-2 (neutrophil gelatinase-associated lipocalin, GAL)	Neutrophils, granulocytes, macrophages, epithelial cells	Binds to bacterial siderophore enterobactin and inhibits bacterial growth by sequestering iron
Calprotectin[32]	*S100A8, S100A9*	Calprotectin	Intestinal epithelial cells, neutrophils	Chelates and sequesters metal co-factors (manganese, zinc, iron) during infection and inhibits bacterial growth
Hepcidin[33]	*HAMP, LEAP1*	Hepcidin antimicrobial peptide	Intestinal epithelial cells	Regulates iron absorption and homeostasis, inhibits bacterial growth by limiting iron availability
Galectin[34,35]	*LGALS*	Galectin-3, Galectin-4, Galectin-8	Intestinal epithelial cells	Galectins has bactericidal activity against bacteria expressing blood group antigen, Gal-8 targets damaged vesicles for autophagy during bacteria invasion
Lysozyme[36]	*LYZ*	Lysozyme	Paneth cells	Enzymatic degradation of bacterial membranes, preference towards Gram-positive pathogens
Elafin[37]	*PI3*	Elafin (peptidase inhibitor 3)	Intestinal epithelial cells	Binds to bacterial lipopolysaccharide (LPS) and modulates innate immunity
Secretory Leukocyte Protease Inhibitor (SLPI)[38,39]	*SLPI*	SLPI	Intestinal epithelial cells, paneth cells, neutrophils, macrophages	Protease inhibitor binds to bacterial mRNA and DNA, dose-dependent bactericidal properties of SLPI against both Gram-positive and Gram-negative bacteria, has fungicidal properties

spectrum activity against bacteria, enveloped viruses, and fungi[15]. It is expressed by differentiated colonic epithelial cells as well as resident immune cells in the GI tract including neutrophils, monocytes, and macrophages, and mast cells[16,17]. Cathelicidin expression has been reported to be increased in inflamed and noninflamed mucosa in ulcerative colitis patients[18]. Butyrate[18] and vitamin D[19,20] are known inducers of cathelicidin expression on colonic epithelial cells and immune cells. Cathelicidin deficiency increases susceptibility to infection with enterohemorrhagic *E. coli* (EHEC)[21]. Vitamin D induction of cathelicidin in human colonic epithelial cells has been shown to inhibit *in vitro E. coli* growth[21]. Likewise, cathelicidin protects against colonization with epithelial adherent bacterial pathogens [22].

Regenerating protein

Another class of antimicrobial peptides expressed in the GI tract include the soluble lectins belonging to the regenerating (Reg) Protein family. RegIIIγ and its human counterpart RegIIIα, also known as Hepatocarcinoma-Intestine Pancreas/Pancreatitis-Associated Protein (HIP/PAP), are expressed by enterocytes and Paneth cells in response to microbial and inflammatory stimuli[23,24]. RegIIIα selectively binds to cell wall peptidoglycan in gram-positive bacteria to induce pore formation[25]. RegIIIβ interacts with surface Lipid A structures to target gram-negative bacteria[26]. In mice, RegIIIγ maintains physical separation between the gut microbiota and the intestinal epithelial surface and regulates bacterial colonization and intestinal immune responses by the microbiota[27]. In mice, RegIII is strongly induced in gut epithelial cells following bacterial reconstitution and colitis[28]. In human studies, Reg Iα, Reg Iβ, and Reg IV are overexpressed in colon mucosa with ulcerative colitis, whereas Reg IV is overexpressed in Crohn's disease[29].

Metal sequestering antimicrobial peptides

Some antimicrobial peptides function by sequestering metal micronutrients which are required as co-factors for microbial growth. Lactoferrin is a secreted iron binding protein that is expressed by intestinal epithelial cells. Lactoferrin mediates its antimicrobial activity by sequestering free iron required for bacteria growth[30]. Lipocalin-2 (neutrophil gelatinase-associated lipocalin, GAL) is expressed by intestinal epithelial cells after stimulation by proinflammatory cytokines IL-17 and IL-22. Lipocalin-2 sequesters the siderophore enterobactin which then prevents bacteria cells from binding iron[31]. Calprotectin, a heterodimer consisting of S100A8 and S100A9, is produced by intestinal epithelial cells and neutrophils. Calprotectin inhibits bacterial growth by sequestering zinc and manganese during infection[32]. The cationic peptide hepcidin plays a key role in regulating iron homeostasis through its binding to the iron exporter ferroportin. During infection and inflammation, hepcidin is upregulated and subsequently limits iron availability to bacterial pathogens. Hepcidin has antimicrobial activity against *E. coli*, *Pseudomonas aeruginosa*, and group A *Streptococcus*[33].

Antimicrobial peptides with different mechanisms of action

Other AMPs of various mechanisms of action have also been characterized. Galectins are β-galactoside-binding lectins that can bind to galactose-containing glycans on glycoproteins and glycolipids. They are highly expressed by intestinal epithelial cells and innate immune cells. Galectin-3, -4, and-8 recognize human blood group B antigen-like determinants on the surface of *E. coli* O86 and have bactericidal activity. Galectin-3 can bind to lipopolysaccharide (LPS) on gram-negative bacteria. Galectin-8 targets damaged vesicles for autophagy during bacteria invasion[34,35]. Another mechanism involves enzymatic degradation of bacterial membranes. Lysozyme which is secreted by Paneth cells preferentially binds to gram-positive bacteria and degrades bacterial membranes by hydrolyzing peptidoglycan linkages[36]. AMPs also function as protease inhibitors such as elafin and secretory leukocyte protease inhibitor (SLPI). Elafin is produced by epithelial cells of mucosal surfaces including the GI tract. Elafin mediates its antimicrobial activity by binding to LPS from gram-negative bacteria and modulating macrophages[37]. SLPI is a major serine proteinase inhibitor that is expressed and apically secreted by human intestinal epithelium as well as Paneth cells, neutrophils, and macrophages. SLPI has antimicrobial activity against the enteric pathogen *Salmonella typhimurium* as well as gram-positive and gram-negative bacteria and fungi[38,39].

ANTIMICROBIAL PEPTIDES AND THE GUT MICROBIOME

The appropriate maintenance of the gut microbiome is critical for health. In addition to offering competitive protection against pathogen growth, the microbiome regulates gut development[40] modulates digestion[41] and provides nutrients[42]. Thus, the microbiome must be carefully cultivated, without being permitted to proliferate excessively. However, the rapid renewal of epithelial layers, particularly in the gut where renewal rates are amongst the most rapid[43,44], poses a unique challenge for maintaining microbial composition and distribution. AMPs are a critical mechanism for regulating the microbiome, and act as part of a complex interplay between the gut microbiome, the innate immune system, and epithelium renewal. Reduced AMP production is associated with disorders such as IBD[45], which will be discussed in more depth in section III. In wounds or acute infections, multiple classes of AMPs are

rapidly upregulated, frequently through PAMP-dependent induction. Above threshold doses, they achieve rapid bacterial killing by synergistically targeting diverse yet critical microbial functions[46]. In contrast, direct interactions between AMPs and the gut microbiome occur at sub-lethal doses[47], though AMPs also act indirectly on the gut microbiome through the local modulation of immune response [48].

Evolutionary analysis of AMPs offers insights into AMP function

Across a wide array of species, the regional control of which AMP classes are expressed acts in concert with local environmental conditions to fine-tune both the microbiome's spatial heterogeneities as well as bacterial phenotype[49]. The requirements for broad-spectrum pathogen resistance, coupled with carefully tuned microbiome maintenance, lead to fascinating AMP evolutionary behavior. While genes associated with immune defense are associated with rapid evolution, AMP amino acid sequences evolve more slowly than the genome average. Indeed, they can be highly conserved across multiple species[50]. The relatively slow evolution rate of AMP amino acid sequences therefore suggests that pathogen control is likely a result of a complex AMP mixture, and that any individual AMP exerts minimal co-evolutionary pressure[51,52].

Given the importance of microbiome composition for health, and in light of the highly conserved AMP amino acid sequences, one might expect strict control over AMP copy number and regulation. Unexpectedly, this is not what has been observed. While AMP coding sequences are highly conserved within a species, there is substantial variability in both copy number and regulatory sequences, as reviewed in [53]. This is particularly intriguing given that there is a high evolutionary cost associated with AMPs; when model organisms are propagated in germ-free environments, AMPs are rapidly lost[54]. Together, these data strongly suggest that the regulatory variability that is observed within humans may be a function of geography, specifically long-term local diets, local pathogens, and/or candidate microbiome components.

Dynamics of AMP-microbiome interactions

AMP serve as key regulators of host-gut microbiota interactions in a bi-directional and highly dynamic process[55]. AMP can shape the composition of the gut microbiome. For example, sublethal doses of AMPs could prime *E. coli* to develop tolerance and increase persistence by production of curli or colonic acid[56]. Prior studies have demonstrated that species-specific AMP profiles in animals maintains species-specific bacterial communities. Loss-of-function experiments have also shown that antimicrobial peptide composition is a predictor of bacterial colonization[57]. Furthermore, AMP resistance patterns maintains the resilience of prominent gut commensals during perturbations such as inflammation[58]. Conversely, the gut microbiome produces a complex array of metabolites[59] that directly regulate AMP production and function [60,61]. For example, the microbiota metabolite short chain fatty acid promoted the production of the AMP RegIIIγ and β-defensins by intestinal epithelial cells[62].

Manipulation of gut microbiome composition has been shown to control AMP production and function. Cazorla *et al*[63] demonstrated that oral administration of probiotics in mice increased Paneth cell and intestinal antimicrobial activity. In addition, treatment of mice with VSL #3, a common probiotic used in patients with IBD, was associated with restoration of *AMP* gene expression in the small intestine and increased abundance of bacterial commensals in the gut[64]. Some probiotic strains produce AMP and has been proposed as a strategy to improve immune responses in immunocompromised patients[65]. Finally, fecal microbial transplant also modulates AMP expression in the GI tract. Teng *et al*[66] demonstrated that fecal microbial transplant of piglets resulted in increased expression in porcine beta-defensins in the jejunum and subsequent increased gut *Firmicutes* and decreased *Bacteroides*.

Gut microbiome effects of different antimicrobial peptides

Different locations and cellular origins of AMP production are superimposed along the GI tract. Defensins, the most abundant AMPs in the gut, are notable for their multiple disulfide bridges which confer substantial structural resistance to bacterial-derived peptidases[67]. Defensins exert antimicrobial activity through forming pores in target bacterial membranes. Above sufficient thresholds, this results in cell death. Although the effect of sub-lethal concentrations is still undergoing characterization in humans, it is notable that a similar strategy is used by plants[50]. Here, pore-forming AMPs are used to facilitate the release of endosymbiotic microbe-derived nutrients.

Local immune cell populations such as macrophages, T cells, and B cells[68] secrete both classes of defensins. The highly spatially restricted secretion of α-defensins, in comparison to the ubiquitous secretion of β-defensins, strongly suggests that their role is likely to prevent bacterial overgrowth[61]. Indeed, Paneth cells are positioned just beneath the actively proliferating epithelial stem cells which are critical for epithelium renewal. Single-crypt studies show that Paneth cell degranulation of α-defensins is induced by both gram-negative and gram-positive bacteria, regardless of whether they are alive or dead, as well as bacterial components such as lipopolysaccharide, lipoteichoic acid, lipid A, and muramyl dipeptide[69]. Furthermore, the antimicrobial products of Paneth cells are protective against *in vitro* microbial challenges many orders of magnitude (> 10^6) higher than those encountered *in vivo*. Notably, degranulation is not induced by eukaryotic pathogens, including live fungi and protozoa[69]. While α-defensin deficiencies in mouse models do not affect total bacterial load, they do result in reduced *Bacteroides* abundance and increased *Firmicutes* abundance[70].

β-defensins act in the gut as a two-layered, ubiquitous defense system. β-defensin-1 is constitutively expressed at low levels, even in the gut of germ-free models[71]. β-defensin-2 and β-defensin-3 can be further induced by the local microbiome, and additionally act as potent chemo-attractants for neutrophils and memory T cells[72]. In contrast to α-defensins, cell culture models suggest that gut β-defensin induction may rely on live bacteria; pre-incubation of Caco-2 epithelial cells with *Enterococcus faecium* reduced *Salmonella typhimurium* uptake, while pre-incubation with heat-killed *E. faecium* did not[72]. Unlike α-defensins, at least one (β-defensin-3) has anti-fungal activity[72].

Cathelicidins (in humans: LL-37) have broad anti-microbial and immunomodulatory function, and act to maintain epithelial barrier integrity[73,74]. Cathelicidins also have a two-tiered anti-microbial activity. While their primary mechanism of activity at high concentrations is to disrupt bacterial membranes, their immunomodulatory functions occur at substantially lower concentrations. Epithelial barrier integrity maintenance is accomplished primarily through increasing tight junction protein expression, as well as post-translational effects including the redistribution of tight junctions[75]. Together, this suggests that cathelicidins are primarily used when the epithelial barrier becomes compromised. Furthermore, LL-37 has also been shown to alter the composition of the gut microbiome in mice. Cathelicidin knockout mice had significantly more OTUs belonging to the phylum *Verrucomicrobia* and had lower amount of OTUs belonging to phylum *Proteobacteria* and the genus *Lactobacillus* than the other genotypes[76].

Reg III AMPs, primarily secreted by Paneth cells and epithelial cells[28,61], are soluble lectins that appear to primarily govern spatial relationships between the microbiome host tissues *via* the mucosa. In mice, Reg IIIβ/γ are co-regulated; Reg IIIα is the human ortholog[27,77]. Thinning of the mucosa driven by dietary restrictions in microbiota-accessible carbohydrates resulted in increased Reg IIIβ[78], as did increased mucosal inflammation[28]. Reg IIIγ-/- mice exhibited increased mucosal bacterial burden and impaired spatial relationships between bacteria and their host tissues[27].

FUNCTION AND MECHANISMS OF ANTIMICROBIAL PEPTIDES IN THE PATHOGENESIS OF IBD

Alpha defensins: HNP-1

Several prior studies have linked defects or alterations in GI tract AMPs with the pathogenesis of IBD. Table 2 summarizes studies exploring the function and mechanisms of AMPs in IBD. HNPs and their role in IBD continues to be investigated. Maeda *et al*[79] found that mild transgenic overexpression of HNP-1 reduces the susceptibility to murine dextran sulfate sodium (DSS) induced colitis. Not only did the colon of HNP-1 transgenic mice show less tissue damage, but mice also had significantly lower disease activity index (DAI) scores when compared to wild type mice. Additionally, the authors found intraperitoneal injection of low dose HNP-1 mitigates DSS-induced colitis and results in reduced expression of pro-inflammatory cytokines in the colon of mice. This improvement of colitis from low-dose HNP-1 could be from its antimicrobial activity[79].

Furthermore, Hashimoto *et al*[80] found that intraperitoneal injection of high concentrations of HNP-1 exacerbate DSS-induced colitis in pathogen free (BALB/c) mice and severe combined immunodeficient (SCID) mice. Clinically, HNP-1 treated BALB/c mice had significantly decreased weight and colon length as well as significantly increased DAI score, histologic score and myeloperoxidase (MPO)

Table 2 Antimicrobial peptides in preclinical models of inflammatory bowel disease

Ref.	Antimicrobial peptides (expression location)	Antimicrobial peptide delivery	Preclinical models (animal, human cell culture)	Key findings
Maeda *et al* [79]	Alpha defensins: Human neutrophil peptide-1 (HNP-1) murine colon	Genetic overexpression, intraperitoneal	Murine dextran sulfate sodium (DSS) colitis	Mild transgenic overexpression of HNP-1 reduces the susceptibility to DSS-induced colitis; Intraperitoneal injection of low-dose HNP-1 ameliorates DSS-induced colitis; The amelioration of colitis by low-dose HNP-1 may be explained by its indirect antimicrobial activity
Hashimoto *et al*[80]	Alpha defensins: Human neutrophil peptide-1 (HNP-1): Murine colon, human colon cells	Intraperitoneal	Murine dextran sulfate sodium (DSS) colitis, SCID mice, human colon cell cultures	Body weight and colon length significantly decreased, and the disease activity index score, histologic score, and myeloperoxidase activity significantly increased in HNP-1-treated mice compared with PBS-treated mice. High concentrations of HNP-1 aggravate DSS-induced colitis, including upregulated expression of such macrophage-derived cytokines as IL-1β
Han *et al*[82]	Porcine β-defensin (pBD)2: Murine colon	Intrarectal	Murine dextran sulfate sodium (DSS) colitis, human colon cell cultures	Administration of pBD2 effectively attenuated colonic inflammation in mice with DSS induced colitis. pBD2 reduced the increased serum and colon levels of TNF-a, IL-6 and IL-8 all caused by DSS. The effects of pBD2 appeared to be through upregulation of the expression of genes associated with tight junctions and mucins
Koeninger *et al*[81]	Beta defensins: human beta defensin 2 (HBD-2): Murine colon	Subcutaneous	Murine dextran sulfate sodium (DSS) colitis, 2,4,6-trinitrobenzenesulfonic acid (TNBS) colitis, T cell transfer colitis model	Treatment improved disease activity index and hindered colitis-induced body weight loss on par with anti-TNF-α and steroids. Mechanistically, hBD2 engaged with CCR2 on its DC target cell to decrease NF-κB, and increase CREB phosphorylation, hence curbing inflammation
Koon *et al*[73]	Cathelicidin (LL-37): Murine colon	Genetic knockouts	Murine dextran sulfate sodium (DSS) colitis	Increased expression of cathelicidin in the colon of DSS-exposed mice; Compared with WT mice, cathelicidin KO mice developed a more severe form of DSS-induced colitis; Cathelicidin protects against induction of colitis in mice; Increased expression of cathelicidin in monocytes and experimental models of colitis involves activation of TLR9-ERK signaling by bacterial DNA
Fabisiak *et al* [83]	Cathelicidin (LL-37) KR-12 (active fragment of LL-37): Murine colon	Intraperitoneal	Murine dextran sulfate sodium (DSS) colitis, 2,4,6-trinitrobenzenesulfonic acid (TNBS) colitis, T cell transfer colitis model	LL-37 and KR-12 (1 mg/kg, ip, twice daily) showed a decrease in macroscopic and ulcer scores in the acute TNBS-induced model of colitis. KR-12 (5 mg/kg, ip, twice daily) reduced microscopic and ulcer scores in the semi-chronic and chronic TNBS-induced models of colitis compared with inflamed mice
Yoo *et al*[84]	Cathelicidin (LL-37): Murine colon	Intracolonic, intravenous	2,4,6-trinitrobenzenesulfonic acid (TNBS) Colitis,	Intracolonic cathelicidin (mCRAMP peptide) administration or intravenous delivery of lentivirus-overexpressing cathelicidin gene significantly reduced colonic col1a2 mRNA expression in TNBS-exposed mice compared with vehicle administration. Cathelicidin can reverse intestinal fibrosis by directly inhibiting collagen synthesis in colonic fibroblasts
Tai *et al*[85]	Cathelicidin (LL-37): Murine colon	Genetic knockouts, intrarectal	Murine dextran sulfate sodium (DSS) colitis	Cathelicidin knockout mice had more severe symptoms and mucosal disruption than the wild-type mice in response to DSS colitis. Intrarectal administration of plasmids encoding cathelicidin reversed colitis in cathelicidin knockout mice
Gubatan *et al* [21]	Cathelicidin (LL-37): Murine colon, human colon cells	Intrarectal	Murine dextran sulfate sodium (DSS) colitis, human colon cell cultures	Vitamin D-induced cathelicidin in human colonic epithelial cells suppressed *Escherichia coli* growth. Intrarectal cathelicidin reduced the severity of DSS colitis but did not mitigate the impact of colitis on microbial composition
Motta *et al*[91]	Elafin: Murine colon	Transgenic expression, adenoviral delivery	Murine dextran sulfate sodium (DSS) colitis, 2,4,6-trinitrobenzenesulfonic acid (TNBS) colitis	In mice given TNBS or DSS, transgenic expression of elafin protected against the development of colitis. Similarly, adenoviral delivery of Elafin significantly inhibited inflammatory parameters. Elafin modulated a variety of inflammatory mediators *in vitro* and *in vivo* and strengthened intestinal epithelial barrier
Ogawa *et al* [28]	RegIII (HIP/PAP): Murine colon	Endogenous expression	Murine dextran sulfate sodium (DSS) colitis	Epithelial expression of Reg III or HIP/PAP was induced under mucosal inflammation initiated by exposure to commensal bacteria or DSS as well as inflamed IBD colon

Jiang *et al*[93]	Donkey milk lysozyme (DML): Murine colon	Oral	Murine dextran sulfate sodium (DSS) colitis	DML ameliorated weight loss, colon damage and mucosal inflammation in DSS colitis mice. DML improved mechanical barrier function and increased gut microbiota composition diversity, promoting growth of probiotics and inhibiting pernicious bacteria
Reardon *et al*[92]	Secretory leukocyte peptidase inhibitor (SLPI): Murine colon	Genetic SLPI deficiency, oral	Murine dextran sulfate sodium (DSS) colitis, T cell transfer colitis model	Tslp−/− mice lead to endogenous SLPI deficiency which exacerbated DSS colitis. Treatment with recombinant SLPI (rSLPI) reduced DSS-induced mortality in Tslp−/− mice
Togawa *et al*[95]	Lactoferrin: Rat colon	Oral	Rat dextran sulfate sodium (DSS) colitis	DSS-induced colitis was attenuated by oral administration of lactoferrin in a dose-dependent manner. Reduced inflammation in response to lactoferrin was correlated with the significant induction of the anti-inflammatory cytokines and with significant reductions in the pro-inflammatory cytokines
Shanmugam *et al*[96]	Hepcidin: Murine colon	Endogenous expression	Murine dextran sulfate sodium (DSS) colitis, T cell transfer Colitis model	TNFα inhibits hepcidin expression in two distinct types of innate colitis, with down-regulation of Smad1 protein playing an important role in this process

activity when compared to control mice. Furthermore, inflammatory cytokines IL-1β and TNF-α were significantly higher in colon of HNP-1 treated mice. In both murine models, an increased recruitment of F4/80-positive macrophages in the inflamed colonic mucosa after HNP-1 injection has been observed. This enhanced disease activity is thought to be due in part to HNP-1 induced cytokine production in macrophages.

Beta defensins: Porcine B-defensin and hBD-2

Beta defensins are epithelial cell derived AMPs that have immunomodulating properties. Koeninger *et al*[81] found that subcutaneous recombinant hBD-2 reduced intestinal inflammation in three distinct animal models of IBD: chemically induced mucosal injury (DSS), loss of mucosal tolerance (TNBS), and T cell transfer into immunodeficient recipient mice. Mice treated with hBD-2 had less weight loss, better stool score and improved DAI scores in comparison to the T cell colitis control group. Additionally, mice given hBD-2 had less mucosal damage and inflammation as they maintained crypt anatomy and had reduced colon weight.

In addition to the protective effects of hBD-2, Han *et al*[82] found that intrarectal administration of porcine beta-defensin 2 (pBD2) ameliorated colonic inflammation in mice during the induction of DSS-induced colitis. Mice in the pBD2 plus DSS group had less symptoms, including less weight loss, firmer and less bloody stools compared to the DSS-treated group. Mice treated with pBD2 plus DSS also had less evidence of macroscopic and histological colitis in addition to reduced production of TNF-a, IL-6 and IL-8 when compared to the DSS-treated group. Through colon cell culture, the effects of pBD2 seemed to occur *via* an upregulation of genes associated with tight junctions and mucins. This may explain how pBD2 can improve DSS-induced changes in the mucosa and paracellular permeability through possible activation of the NF-kB signaling.

Cathelicidin (LL-37)

Koon *et al*[73] demonstrated that genetic knockout of LL-37 in mice had more severe forms of DSS-induced colitis and that inflamed colon in wild type mice in DSS colitis

models had increased cathelicidin expression. The authors suggested that this upregulation of cathelicidin involves activation of TLR9-ERK signaling from bacterial DNA, which may play a role in the development of colitis. In addition to its protection against the induction of colitis, Fabisiak *et al*[83] showed that intraperitoneal injection of LL-37, and its shortest active metabolite, KR-12, decreases ulcer and macroscopic scores in DSS-induced and TNBS-induced models of colitis. The study showed that intraperitoneal injection of KR-12 altered the microbiomes of TNBS-induced colitis mice by reducing total and *E. coli* group bacteria.

In addition to the protective and antimicrobial properties of LL-37, Yoo *et al*[84] found that intracolonic cathelicidin or intravenous delivery of lentivirus-overexpressing cathelicidin gene significantly reduced colonic collagen deposition TNBS-induced colitis mice when compared to TNBS-induced mice not receiving LL-37. These results suggest that cathelicidin reverses fibrosis in the intestines *via* inhibition of collagen synthesis in colonic fibroblasts.

Another unique property of LL-37 was investigated by Tai *et al*[85], who describe that intrarectal administration of plasmids containing cathelicidin to DSS-induced colitis mice reestablished colonic mucus thickness *via* increased expression of mucin genes and reduced severe symptoms compared to cathelicidin knockout mice with DSS-induced colitis. This increase in mucin genes protected against mucosal damage and was linked to the activation of MAP kinase.

Gubatan *et al*[21] found that cathelicidin is a key mediator of the protective role of vitamin D in ulcerative colitis (UC). The authors found higher levels of 25(OH)D correlate with increased levels of both serum and colonic LL-37 in UC patients, and these higher levels are associated with decreased histologic inflammation and probability of clinical relapse. Intrarectal LL-37 reduced the severity of DSS-induced colitis in mice, but did not alter the intestinal microbial imbalance, whereas 25(OH)D-induced cathelicidin in human colonic epithelial cells suppressed *E.coli* growth. The study demonstrated that 25(OH)D is an independent predictor of cathelicidin in UC patients in remission and may protect against microbial associated gut inflammation.

Arachidonic acid and its metabolism also play a role in the regulation of antimicrobial peptides in inflammatory bowel disease. Arachidonic metabolites such as leukotrienes and are elevated in both animal models of colitis and patients with IBD [86]. Leukotrienes have been shown to trigger release of human cathelicidin from neutrophils[87], whereas prostaglandins suppress cathelicidin in human macrophages [88]. In addition, cyclooxygenase-2 (COX-2), an enzyme that metabolizes arachidonic acid, is also induced in colonic epithelial cells in IBD[89]. Cox-2 selective inhibitors have been shown to inhibit production of human beta defensins but not cathelicidin [90].

Elafin

Motta *et al*[91] showed that in TNBS or DSS-induced mouse models of colitis, transgenic expression of elafin or disruption of enzymes that elafin inhibits protected against development of colitis. Transgenic mice expressing elafin had reduced inflammation as measured by a reduction in macroscopic tissue damage and myeloperoxidase (MPO) activity when compared to TNBS or DSS-induced mice that were not expressing elafin. Authors showed that adenoviral delivered elafin inhibited inflammatory parameters. The authors demonstrated that elafin is involved in inflammatory mediators and its protective effect could in part be from a bolstering of epithelial and mucosal barriers.

SLPI

Reardon *et al*[92] reported that thymic stromal lymphopoietin-deficient (TSLP-/-) mice led to endogenous SLPI deficiency, which prevented recovery from DSS-induced colitis and resulted in death. The authors demonstrated that the mechanism by which the absence of SLPI prevents healing of the colon is from increased neutrophil elastase (NE) activity in TSLP-/- mice. When TSLP-/- mice were treated with oral recombinant SLPI (rSLPI) there was reduced DSS-induced mortality.

Reg III (HIP/PAP)

Ogawa *et al*[28] aimed to identify genes that were modulated by bacterial flora to better understand mucosal inflammation in IBD patients. The authors found that expression of Reg III (HIP/PAP) was increased in DSS-induced colitis. Furthermore, the upregulation of Reg III may be due to an increase in the acute phase reactant IL-6 that occurs during gut inflammation.

Donkey milk lysozyme

Donkey milk contains high lysozyme levels and was studied by Jiang *et al*[93] due to its antimicrobial properties. Authors found that mice given donkey milk lysozyme (DML) orally in a DSS-induced colitis model had improved symptoms of colitis measured by a reduction in weight loss, loose stools, rectal bleeding and mucosal inflammation**.** The authors showed that 50% DML treatment brought cytokines, TNF-a and IL-13, a pleiotropic cytokine that has proinflammatory effects on intestinal epithelial cells resulting in apoptosis and epithelial barrier dysfunction in intestinal inflammation[94] back to basal levels similar to control mice. They hypothesized that DML improves the intestinal barrier by increasing expression of tight junction proteins in the colon. They also presume that DML increases gut microbiota diversity and reduces detrimental bacteria thereby restoring the gut microflora.

Lactoferrin

Lactoferrin, a known immunomodulator, was studied by Togawa *et al*[95] and was found to reduce DSS-induced colitis in a dose-dependent manner after oral administration to rats. The DAI, shortening of colon length, histological/macroscopic damage score, tissue levels of MPO activity, WBC, and reduction in hemoglobin were decreased when DSS-induced colitis rats were treated with lactoferrin. The authors postulate that the protective properties of lactoferrin were tied to its modulation of the immune system by reducing pro-inflammatory cytokines TNF-a, IL-1B and IL-6 as well as the augmented levels of anti-inflammatory cytokines IL-4 and IL-10 in colonic tissue of DSS-induced colitis rats given lactoferrin.

Hepcidin

Hepcidin is regulator of iron metabolism and is upregulated during the inflammation in IBD, often resulting in anemia. Shanmugam *et al*[96] investigated the mechanisms that control hepcidin during periods of inflammation. They showed that the pro-inflammatory cytokine TNF-a inhibits hepcidin in both a DSS-induced colitis and T cell transfer colitis model in mice with downregulation of Smad1 protein mediating this effect.

TRANSLATIONAL APPLICATIONS OF ANTIMICROBIAL PEPTIDES AS BIOMARKERS IN PATIENTS WITH IBD

The diagnosis and long-term monitoring of IBD commonly involve invasive and costly endoscopy combined with histologic screening. Consequently, a biomarker that reflects the ongoing severity of disease is attractive as a non-invasive, cost-effective, and convenient alternative for diagnosing new IBD cases and identifying flares of disease. Given their involvement in disease pathophysiology, AMPs represent such potential markers, and several have been studied to determine their utility in differentiating CD and UC from other conditions, such as celiac disease and IBS, as well as active from quiescent disease states. In addition to reflecting ongoing severity of inflammation, several AMPs have shown promise as predictors of relapse, complication risk, and treatment response in the setting of IBD. Table 3 summarizes the application of AMPs as biomarkers in IBD.

Calprotectin

Among all known AMPs, calprotectin is the one most frequently used in the clinical diagnosis and monitoring of IBD. It has been known for decades that fecal calprotectin (FC) concentrations are markedly increased in the setting of both CD and UC[97-100]. Elevated FC is a highly sensitive marker and is thus a particularly useful tool in the initial diagnosis and discrimination of IBD from non-inflammatory causes of abdominal discomfort and bowel dysfunction like IBS[97-103]. Based on this diagnostic utility, current practice guidelines from the World Gastroenterology Organization support measuring FC in the initial work-up of suspected IBD in both adult and pediatric patients[101,102]. Recent research has supported using FC measurements for the early diagnosis of IBD in at-risk populations, such as patients with ankylosing spondylitis[104].

FC is also particularly useful in the evaluation of IBD severity and the early identification of disease flares[104-106]. Data suggest that FC concentrations positively correlate with histologic inflammation in IBD, and assays can be used to accurately classify inactive, mild, moderate, and severe disease[102,103]. Cut-off values of fecal

Table 3 Biomarker applications of antimicrobial peptides in patients with inflammatory bowel disease

Ref.	Antimicrobial peptides	Type of IBD	Biomarker application	Key findings
Holgersen *et al* [110]	Alpha defensins 5 and 6 (DEFA5/DEFA6)	UC	IBD diagnosis	Marked upregulation of DEFA5 and DEFA6 in terminal ileal biopsies of inflamed ulcerative colitis relative to normal controls
Wehkamp *et al* [111]	Alpha defensin (HD -5/6)	UC/CD	IBD diagnosis	HD-5/6 both decreased in ileal Crohn's, and this correlated with a decrease in transcription factor Tcf-4, a known regulator of Paneth cell differentiation. Normal levels were observed in UC and colonic Crohn's
Yamaguchi *et al* [112]	Alpha defensin (HNP1-3), beta-defensin (HBD-2)	UC/CD	Disease activity	HNP-1-3 all elevated in IBD patients, while HBD-2 levels normal; serum HNP1-3 levels correlated with disease severity for Crohn's
Kanmura *et al* [113]	Alpha defensin (HNP)	UC/CD	Disease activity	Fecal-HNP levels were markedly elevated in both UC and Crohn's, but slightly more so in Crohn's; F-HNP was significantly higher during flares of UC than remission. For UC, HNP levels correlated with Mayo endoscopic score
Cunliffe *et al* [114]	Alpha defensin (HNP 1-3)	UC/CD	Disease activity	Surface epithelial cells strongly immunoreactive for neutrophil defensins and lysozyme were seen in active ulcerative colitis and Crohn's disease (but not normal or inactive IBD) mucosal samples. Many of these cells coexpressed both antimicrobial proteins.
Tran *et al*[116]	Cathelicidin	UC/CD	Disease activity	Cathelicidin levels were significantly increased in IBD patients and were inversely correlated with CD activity. In moderate to severe IBD, higher cathelicidin levels before treatment correlated with better prognosis.
Krawiec *et al* [115]	Cathelicidin	UC/CD	IBD diagnosis	Cathelicidin was significantly increased in patients with ulcerative colitis (1073.39 ± 214.52 ng/mL) and Crohn's disease (1057.63 ± 176.03 ng/mL) patients compared to controls (890.56 ± 129.37 ng/mL) (P = 0.0003)
Gubatan *et al*[21]	Cathelicidin	UC	Disease activity, clinical relapse	In ulcerative colitis patients, serum 25(OH)D positively correlated with serum and colonic cathelicidin. Higher serum cathelicidin is associated with decreased risk of histologic inflammation and clinical relapse but not independent of 25(OH)D or baseline inflammation
Borkowska *et al* [118]	Lactoferrin	UC/CD	IBD diagnosis, disease activity	Fecal concentration of lactoferrin in children with IBD was significantly higher than in the controls. The sensitivity and specificity were 80.7% and 92.7%, respectively, and its positive and negative prognostic values were 96.8% and 63.3%, respectively
Sugi *et al*[119]	Lactoferrin, lysozyme	UC/CD	Disease activity	Lactoferrin and lysozyme were significantly increased in the active phases of CD and UC relative to inactive. They both correlated with fecal Hb concentration in UC, and with alpha 1-AT concentration in CD
Sidhu *et al*[120]	Lactoferrin	UC/CD	IBD diagnosis, disease activity	Lactoferrin levels were significantly higher in IBD patients compared with IBS/healthy controls (P < 0.001). The sensitivity, specificity, positive and negative predictive values of lactoferrin in distinguishing active IBD from IBS/healthy controls were 67% and 96%, 87% and 86.8% respectively
Wang *et al*[121]	Lactoferrin	UC/CD	IBD diagnosis	FL test has a high sensitivity (82%) and specificity (95%) for the discrimination of patients with IBD against non-IBD patients
Kane *et al*[122]	Lactoferrin	UC/CD	Disease activity	Fecal lactoferrin was 90% specific for identifying inflammation in patients with active IBD. Elevated fecal lactoferrin was 100% specific in ruling out IBS
Turner *et al*[123]	Lactoferrin	UC	IBD diagnosis	Lactoferrin levels significantly were elevated in pediatric UC patients, but were not responsive to change or predictive of response to corticosteroids
Wang *et al*[132]	Elafin	CD	Disease activity, intestinal strictures	High serum elafin levels were associated with a significantly elevated risk of intestinal stricture in CD patients. Serum elafin levels had weak positive correlations with clinical disease activity but not endoscopic disease activity
Zhang *et al*[133]	Elafin	UC/CD	Disease activity	The expression of elafin mRNA in peripheral blood in active IBD patients is decreased, which may be correlated with the activity of IBD, and negatively correlated with corresponding disease activity score
Motta *et al*[130]	Elafin	UC	Disease activity	Study identified a previously unrevealed production of elastase 2A (ELA2A) by colonic epithelial cells, which was enhanced in IBD patients.

				Study demonstrated that ELA2A hyperactivity is sufficient to lead to a leaky epithelial barrier and modified the cytokine gene expression profile with an increase of pro-inflammatory cytokine transcript
Schmid *et al*[134]	Elafin and SLPI	UC/CD	Disease activity	Levels of mRNA and immunostaining of the antiproteases elafin and SLPI were enhanced strongly in inflamed versus noninflamed UC
Frol'ová *et al*[124]	Galectin-3	UC/CD	Disease activity	Serum concentrations were significantly increased in specimen of patients with active and remission-stage ulcerative colitis and Crohn's disease (relative to healthy controls)
Yu *et al*[125]	Galectin-1, -3	UC/CD	IBD diagnosis	Serum level of galectin-1 and -3, but not galectins-2, -4, -7 and -8, were significantly higher in IBD patients than in healthy people. None of the galectins however were able to distinguish active disease from remission in UC or CD
Tibble *et al*[97]	Calprotectin	CD	IBD diagnosis	The cross-sectional study showed a sensitivity of 96% for calprotectin in discriminating between normal subjects and those with Crohn's disease. With a cutoff point of 30 mg/L fecal calprotectin has 100% sensitivity and 97% specificity in discriminating between active CD and irritable bowel syndrome
Moniuszko *et al*[100]	Calprotectin	UC/CD	Disease activity, progression	Rapid bedside FC test reliably detected disease flares in patients with both UC and CD. FC levels increased even with early signs of inflammations; values were lower in isolated small bowel disease for CD patients
Pous-Serrano *et al*[101]	Calprotectin	CD	Disease activity	FC was the only inflammatory marker significantly associated with the degree of histologic inflammation in surgical specimens
Scheopfer *et al*[102]	Calprotectin	CD	Disease activity	FC correlates more closely with endoscopic disease activity that CRP, blood leukocytes, and CDAI. It was the only marker that reliably discriminated inactive from mild, moderate, and highly active disease, underscoring its value in disease monitoring
Ferreiro-Iglesias *et al*[103]	Calprotectin	UC/CD	Relapse	In IBD patients under Infliximab maintenance therapy, high FC levels allow predicting relapse within the following 2 mo. Long-term remission is associated with low calprotectin levels
Klingberg *et al*[104]	Calprotectin	CD	IBD diagnosis, treatment monitoring	FC was a useful predictor of the development of CD in patients with ankylosing spondylitis; NSAIDs increase FC levels; FC levels drop following TNF-blocker treatments
Godny *et al*[109]	Calprotectin	CD	Treatment monitoring	FC decreases following successful diet-based treatment of active CD
Karaskova *et al*[126]	Hepcidin	UC/CD	IBD diagnosis	Serum hepcidin concentration was significantly decreased in IBD children compared with controls; levels did not differ significantly between patients with CD and UC
Martinelli *et al*[128]	Hepcidin	UC/CD	IBD diagnosis, iron deficiency Monitoring	Serum hepcidin was significantly higher in IBD patients with active disease versus healthy and celiac patients. Hepcidin levels corresponded with iron malabsorption and other inflammatory biomarkers like ESR
Aksan *et al*[129]	Hepcidin	UC/CD	Response to iron supplementation	Higher hepcidin and other inflammatory markers correlated with decreased iron absorption follow supplementation
Zollner *et al*[127]	Lipocalin	CD	Clinical and endoscopic activity	Fecal lipocalin-2 levels of 78.4 and 0.56 μg/g in Crohn's disease patients for clinical and endoscopic activity, respectively, corresponded well with fecal calprotectin levels in UC patients (R = 0.87, $P < 0.001$)

IBD: Inflammatory bowel disease: UC: Ulcerative colitis; CD: Crohn's disease.

calprotectin to differentiate active disease *vs* remission in patients with IBD have been previously evaluated[107]: A cutoff value of 50 mg/g had a pooled sensitivity of 0.92 and specificity of 0.60 (0.52–0.67), a cutoff value of 100 mg/g had a pooled sensitivity of 0.84 and specificity of 0.66, a cutoff value of 250 mg/g had a pooled sensitivity of 0.80 (0.76–0.84) and specificity of 0.82 (0.77–0.86). Decreased levels of FC after therapy are associated with clinical, endoscopic and histological improvement with a normal-

ization of FC (< 50 mg/g) signifying deeper remission[108].

Notably, FC has been found to correlate more strongly with IBD activity than other markers of inflammation, including C-reactive protein and blood leukocytes[104,105]. FC elevations are more pronounced in patients with pan-colonic CD than in those with isolated small bowel disease, indicating that concentrations may reflect disease location[105]. Rapid bedside and at-home FC assays are currently available as tools for monitoring IBD activity, with elevated concentrations detectable early in disease flares [104,109]. FC can be used to predict the risk of relapse for patients with quiescent CD and UC[105]. FC monitoring also plays a role in the treatment of IBD, as levels decrease following effective medical and diet-based management of disease[107,110].

Despite its clear clinical utility, FC remains an imperfect biomarker for the diagnosis and monitoring of IBD. Like many other inflammatory biomarkers, FC is not 100% specific for IBD. Other factors, including the use of NSAIDs, can also result in elevated FC, thereby introducing potential inaccuracy when using the biomarker to evaluate IBD[104,105].

Defensins

Previous studies have revealed increased defensin concentration at the intestinal surface epithelium in the setting of IBD, and dysregulation of defensin gene expression has been proposed as one pathogenic mechanism of disease[110,111]. Thus, defensins have been explored as potential biomarkers of IBD[112,113]. Among the 10 known human defensins, the alpha defensins HNP-1, HNP-2, and HNP-3 have been found to be significantly elevated in the sera of both UC and CD patients[114,115]. In CD, serum HNP-1-3 Levels have been shown to correlate with disease severity, as measured by Crohn's disease Activity Index (CDAI)[114]. In UC, these levels are significantly greater in active disease than in inactive disease, and serum HNP-1-3 Levels decrease following successful treatment with corticosteroids[113]. Notably, serum HNP-1-3 Levels do not decrease following corticosteroid administration in non-responders, signifying the potential use of defensins in the monitoring of treatment efficacy[114]. Fecal HNP-1-3 Levels are also significantly elevated in both CD and UC as well, with greater elevations measured during UC flares than in remission[113]. In the same study, fecal HNP-1-3 Levels correlated more closely with endoscopic severity than calprotectin. Results involving the ability to differentiate between UC and CD using defensin levels remain mixed[110-113].

Cathelicidin

Significantly elevated levels of serum LL-37 have been detected in both adult and pediatric IBD cohorts[115,116]. Multiple studies have indicated that cathelicidin can be used to reliably differentiate both CD and UC from healthy controls, reflecting the AMP's potential diagnostic utility[115,116]. While cathelicidin levels are increased in both active and remission-stage IBD patients relative to controls, these levels seem to inversely correlate with disease activity, histologic inflammation, and risk of clinical relapse[21,116,117]. In moderate to severe IBD, higher serum cathelicidin prior to treatment is associated with better prognosis and may therefore serve as a predictor of treatment response[21]. Cathelicidin may also be a useful indicator of complication risk, as reduced serum levels correlate with significantly increased risk of intestinal stricture in CD[117]. Serum levels positively correlate with 25(OH)D levels, and the apparent protective effect of elevated cathelicidin is likely at least partially dependent on this increase in vitamin D[21].

Lactoferrin

Lactoferrin is among the most thoroughly explored AMPs in the diagnosis and clinical evaluation of IBD. Fecal concentrations of lactoferrin are consistently elevated among both children and adults with IBD relative to healthy controls[118-123]. While estimates of fecal lactoferrin sensitivity in identifying CD and UC vary, several studies have confirmed the AMP's utility as a highly specific marker of IBD-related inflammation[120-122]. This specificity makes lactoferrin a particularly valuable biomarker for differentiating IBD from IBS, with studies indicating that lactoferrin levels can discriminate between the two conditions with a specificity at or near 100%[119-121]. Lactoferrin levels positively correlate with disease activity, with significantly higher fecal concentrations found in those with moderate to severe IBD relative to those with mild or inactive disease[122]. Unlike some of the other AMPs, lactoferrin has not been shown to predict responsiveness to corticosteroid treatment, and only insignificant concentration changes have been detected following both effective and ineffective treatment regimens[123].

Galectin

Many members of the galectin family of proteins have been studied as potential biomarkers of IBD. Though several galectins are known to be expressed by intestinal epithelial cells, only galectin-1 and -3 have been shown to be significantly elevated in the serum of IBD patients[124,125]. Unlike those of galectin-1 and -3, serum levels of galectins-2, -4, -7, and -8 have not been shown to differentiate IBD patients from healthy controls[125]. Of note, galectin-1 and -3 Levels cannot reliably distinguish active from remission-stage CD or UC, nor can they distinguish CD and UC from each other[125,126]. Evidence also suggests that galectin-1 is a slightly more sensitive marker of IBD than galectin-3[125]. Nevertheless, galectins-1 and -3 may have use as biomarkers either alone or when combined with other molecules, and their upregulation in the intestinal cells of IBD patients may indicate their potential as therapeutic targets[124,125].

Hepcidin

Data regarding the utility of hepcidin as a diagnostic biomarker remain mixed[126-129]. However, given hepcidin's crucial role in regulating iron absorption, the AMP may be useful in the monitoring of iron deficiency and related anemia, which are two common comorbidities seen in IBD patients[126,127]. These comorbidities are most frequently seen in pediatric IBD patients[126,127]. Consequently, multiple studies have aimed to elucidate the relationship between hepcidin expression and these comorbidities in pediatric IBD cohorts. In pediatric patients with IBD, elevated hepcidin levels negatively correlate with iron absorption and serum iron levels[125,126]. Elevated hepcidin corresponds with decreased response to iron supplementation in these patients, suggesting that the biomarker may serve a role in predicting response to oral iron supplementation in the setting of IBD[129].

Elafin

Elafin is known to be markedly upregulated in the intestinal mucosa of UC patients [130,131]. Intestinal expression seems to correlate closely with disease progression, as elevated concentrations are detectable in the right colon of patients with pan-colonic disease, but not those with exclusively left-sided disease[130]. This finding is further supported by enhanced colonic mRNA immunostaining in inflamed relative to non-inflamed UC samples[131]. While serum elafin levels are increased in UC patients relative to healthy controls, some evidence suggests an inverse correlation between serum elafin and disease severity within UC cohorts[131,132]. Among UC patients, significantly elevated serum elafin tends to correlate with decreased disease activity scores, with the highest elafin levels measured during disease remission[133,134]. Data involving elafin as a biomarker in CD remain mixed, with most results indicating only weak correlations between elafin and CD activity[132-134]. However, serum elafin measurements may play a role in the evaluation of complication risk in CD, as elevations are significantly associated with increased risk of intestinal stricture[132].

CONCLUSION

AMPs produced by innate immune cells of the GI tract and cells that support barrier function such intestinal epithelial cells and Paneth cells play critical roles in protecting against enteric pathogens while maintaining tolerance to support a complex ecosystem of commensal gut microbiota. These highly dynamic molecules have broad spectrum antimicrobial activity against bacteria, fungi, and enveloped viruses and mediate their protective effects through diverse mechanisms of action from disrupting cell membranes, binding microbial components such as LPS, and sequestering metal co-factors to limit microbial growth. AMPs also play major roles in regulating gut microbiome composition and spatial relationships between the microbiota and intestinal barrier.

Defects in endogenous AMP expression and function have been linked with intestinal inflammation in mice. Conversely, exogenous delivery of AMPs such as defensins, cathelicidin, and elafin have been shown to attenuate intestinal inflammation in murine models of IBD. AMPs such as calprotectin and lactoferrin have found clinical applications as biomarkers of intestinal inflammation in patients with IBD. Other AMPs including alpha- and beta-defensins, cathelicidin, and elafin may be useful biomarkers for disease activity and predicting clinical outcomes in patients with IBD. Although the protective effects of AMPs have been demonstrated in murine models of IBD, there are currently no AMP-based therapies approved or in clinical

trials for IBD. Future studies should focus on translation of AMPs as potential therapies in patients with IBD. Several challenges with AMPs including limited stability due to enzymatic degradation by endogenous proteases[135,136] and cross-reactivity of AMPs with host cells leading to cytotoxicity[137] pose major barriers to their application as therapies. Biochemical modifications to enhance AMP stability, selectivity, and delivery are being explored[46,137].

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 21; 27(43): 7423-7432

DOI: 10.3748/wjg.v27.i43.7423

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

FRONTIER

Idiopathic chronic pancreatitis: Beyond antioxidants

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Author contributions: Mehta RM, Pandol SJ, and Joshi PR were equally involved in writing the manuscript;Pandol SJ provided final approval of the manuscript; All authors meet the criteria for authorship established by the International Committee of Medical Journal Editors and verify the validity of the results reported.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

Country/Territory of origin: India

Specialty type: Gastroenterology and hepatology

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

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

Open-Access: This article is an open-accessarticle that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in

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Abstract

Chronic pancreatitis (CP) is a complex disease associated with gene-gene or gene-environment interactions. The incidence of idiopathic CP has shown an increasing trend, withits phenotypeshaving changed considerably in the last two decades. The diseaseitself can be regulated before it reaches the stage of established CP; however, the etiopathogenesis underlying idiopathic CP remains to be established, making the condition difficult to cure. Unfortunately, there also remains a lack of consensus regarding the beneficial effects of antioxidant therapiesfor CP. It is known that antioxidant therapy does not reduce inflammatory and fibrotic cytokines, making it unlikely that they could modulate the disease process. Although antioxidants are safe, very few studies to date have reported the long-term beneficial effects in patients with CP. Thus, studies are being performed to identify drugs that can improve symptoms and alter the natural history of CP. Statins, with their numerous pleiotropic effects, may play a role in the treatment of CP, butin 2006, their use was found to be associated with the undesirable side effect of promoting pancreatitis. Latter studies showed favourable effects of statins in CP, highlighting the particular benefits of lipophilic statins, such as lovastatin and simvastatin, over the hydrophilic statins, such as rosuvastatin. Ultimately, studies to repurpose N-acetylcysteine as a CP therapy areyielding very promising results.

Key Words: Chronic pancreatitis; Simvastatin; Antioxidants; Quality of life; N-acetylcysteine; Acute pancreatitis

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Received: March 30, 2021
Peer-review started: March 30, 2021
First decision: June 26, 2021
Revised: July 5, 2021
Accepted: October 27, 2021
Article in press: October 27, 2021
Published online: November 21, 2021

P-Reviewer: Xu ZL
S-Editor: Ma YJ
L-Editor: A
P-Editor: Ma YJ



Core Tip: The clinical management of a majority of chronic diseases has seen a paradigm shift over the last two decades. To date, however, a well-defined standard of care has not been established for patients with chronic pancreatitis (CP). Lack of sufficient scientific evidence regarding the use of antioxidant supplementation, in particular, provides opportunities to repurpose drugs and study their efficacy and safety in clinical trials. Statins and N-acetylcysteine represent two of the most promising molecules for the treatment of CP, today.

Citation: Mehta RM, Pandol SJ, Joshi PR. Idiopathic chronic pancreatitis: Beyond antioxidants. *World J Gastroenterol* 2021; 27(43): 7423-7432
URL: https://www.wjgnet.com/1007-9327/full/v27/i43/7423.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i43.7423

INTRODUCTION

Biography

Rajiv M Mehta, MD, is Professor and Head of the Department of Gastroenterology and Clinical Research at Surat Institute of Digestive Sciences (SIDS) Hospital and Research Centre in Surat, India. He received his undergraduate and postgraduate degrees from Baroda University in Vadodara, India, in 1996 and 2000, respectively. He received his advanced degree in gastroenterology from Amrita Institute of Medical Sciences (AIMS) in Kochi, India. Only 5 years later, in 2005, he was awarded the prestigious presidential gold medal from the National Board of Examination, New Delhi, in Gastroenterology. In his continued efforts to advance the overall field of gastroenterology, Dr. Mehta authored the very popular "Clinical Gastroenterology" book for undergraduate and post-graduate students, withits fourth edition published in December 2019 at the Asia Pacific Digestive Disease Week held in Kolkata, India. He has also published more than 50 articles in various prestigious journals, to date. Dr. Mehta has also been collaborating with Dr. Stephan Pandol and his team from Cedars-Sinai Medical Center in Los Angeles, CA, United States over the past 3 years, focusing on chronic pancreatitis (CP). Collectively, this group isinvolved in the development of novel biomarkers for the diagnosis of "early CP". Additionally, Dr. Mehta has investigated the effects of simvastatin on the histology of L-arginine-induced pancreatitis in mouse models, in association with the Jay Research Foundation (JRF) in Vapi, Gujarat, India. Defining the overall role of simvastatin and N-acetylcysteine (NAC) in CPtreatment remains Dr. Mehta's seminal work, while defining the role of genetic polymorphisms in patients with idiopathic CP is his focused area of interest. Further, he is diligently working towards the development of "New Chemical Entity" in pancreatic cancer.

Background

CP is a fibro-inflammatory disorder of the exocrine pancreas occurring in individuals with genetic, environmental and other risk factors who develop persistent pathological responses to parenchymal injury or stress[1]. CP is characterized by acinar cell damage, ductal dysfunction, persistent inflammation, atrophy, fibrosis, and neuroimmune responses. The clinical course of CP involves significant abdominal pain, exocrine function deficiency (manifested as maldigestion), and endocrine deficiency (manifested as diabetes). The causes of CP include alcohol intake, smoking, metabolic derangements, genetic disorders, autoimmune factors, obstructive mechanisms, and idiopathic aetiologies[2]. Unfortunately, it is difficult to determine the exact prevalence of CP, since the early diagnosis of CP continues to be challenging.

Alcoholic chronic pancreatitis (ACP) is most commonly observed in Western countries, whereas idiopathic chronic pancreatitis (ICP) is observed more frequently in developing countries, like India; reportedly, ICP accounts for 57.3%–69.6% of the cases of CP in India[3]. The cause of ICP remains unknown[4]. In genetically susceptible individuals, environmental factors initiate the fibroinflammatory process (gene-gene or gene-environment interplay), which leads to the development of CP[5]. The subtle pathophysiological changes pose challenges to the early diagnosis of CP, and the parameters for early diagnosis are ill-defined. Late-stage CP is characterized by variable fibrosis and calcification in the pancreatic gland, leading to parenchymal and

ductal changes[6].

Abdominal pain is a well-recognized and debilitating symptom, prompting patients with CP to seek medical assistance. Among all the complications of CP, abdominal pain was found to be strongest predictor of poor quality of life (QoL)[7]. Endoscopic and surgical treatments are often performed to relieve pain, but these methods are invasive and are beneficial only in a specific subgroup of patients with CP. The therapeutic strategies currently used for the management of CP include a combination of analgesics, pancreatic enzymes, adequate nutrition, and antioxidants[8]. However, the effect of antioxidants on providing sustained pain relief or reversing disease activity has not been established, thus far. As such, further studies are warranted to address this unmet need for an alternative therapeutic approach for CP.

PATHOPHYSIOLOGIC BASIS OF ANTIOXIDANT THERAPY

The use of antioxidant therapy is based on observations that CP tissues show marked oxidative changes.

Oxidative stress

Oxidative stress is caused by an imbalance between production and accumulation of reactive oxygen species (ROS) in cells and tissues and the ability of a biological system to detoxify these reactive products[9]. Xenobiotics, such as alcohol and smoking molecules, are detoxified in the body through phase I and phase II pathways. The phase I reaction involves cleaving the parent molecule by enzymes into a less toxic molecule. The phase II reaction adds an endogenous molecule to the compound at the end of phase I to make it more polar and excretable. Increased exposure to xenobiotics may overwhelm the capacity of phase I and phase II detoxification pathways and result in oxidative stress. Production of ROS is a particularly destructive aspect of oxidative stress. ROS are mainly produced by mitochondria, during both physiological and pathological conditions[10]. When the production of ROS increases, they exert harmful effects on important cellular structures, like proteins, lipids and nucleic acids, leading to destruction of the cell membrane, depletion of cellular antioxidants, and alteration in various signalling pathways. Previous studies have indicated that oxidative stress is involved, to a varying extent, in the onset and/or progression of several diseases[9].

Oxidative stress in CP

Pancreatic acinar cells are the main site for generation of oxidant stress and, therefore, are exposed to its detrimental effects. Intra-acinar oxidative stress leads to impairment of the transsulfuration pathway, which is required for zymogen exocytosis[11,12]. This leads to recurrent intra-acinar zymogen activation. Methionine and ascorbic acid appear to be important components in maintaining the transsulfuration pathway[13]. Several studies conducted in the 1990s showed that deficiency of essential antioxidants, such as vitamin A, ascorbic acid, methionine, vitamin E and selenium, is particularly prevalent among patients with ACP. Persistent exposure to xenobiotics *via* smoking and consumption of alcohol increase the levels of oxidative stress. Thus, the role of oxidative stress and micronutrient deficiency in patients with CP exposed to high levels of xenobiotics has been established. "Tropical pancreatitis" has been typically associated with protein and micronutrient deficiencies; however, the role of malnutrition in the etiopathogenesis of CP has been discarded[14].

Over the past one and a half decades, results of several studies conducted in India have shown that the phenotype of patients with ICP has changed significantly. A complex gene-environment interplay is now known to be involved in the development of ICP[5]. Our experience has revealed that the levels of antioxidant micronutrients are normal in patients with ICP who carry normal genetic polymorphisms (in press as of the writing of this paper).

ANTIOXIDANT SUPPLEMENTATION IN CP: THE STORY, SO FAR

In 1990, Uden *et al*[15] performed a double-blind, placebo controlled, crossover trial in 20 patients to determine the efficacy of the combination of 600 mcg of organic selenium, 9000 IU of b carotene, 0.54 g of vitamin C, 270 IU of vitamin E and 2 g of methionine. Results of this trial indicated that antioxidants were superior to placebo in

relieving pain. Several non-randomized studies in small patient populations have also shown the benefits of antioxidant treatments in patients with CP. In a study by Bharadwaj *et al*[16], 147 patients were randomized to an antioxidant therapy or placebo group, and the results indicated a beneficial effect of antioxidant therapy in reducing "painful days" and in improving the markers of oxidative stress. Although the study by Bharadwaj *et al*[16] was conducted in a large and heterogenous patient population (including patients with ACP as well as those with ICP), validated pain scores were not used and formal analysis of QoL was not performed. Results of the ANTICIPATE study showed that administration of antioxidants to patients with painful ACP does not reduce pain or improve QoL, despite a sustained increase in blood levels of antioxidants[17]. The results of a randomized controlled trial by Singh *et al*[18] showed that antioxidant supplementation increased the blood antioxidant levels but produced no additional benefit on endocrine and exocrine functions, markers of fibrosis, inflammation, nutritional status, pain or QoL.

Gooshe *et al*[19] performed a meta-analysis to determine the efficacy and adverse effects of antioxidant therapy in patients with acute pancreatitis (AP), CP and post-endoscopic retrograde cholangiopancreatography pancreatitis (commonly known as PEP). This meta-analysis provided evidence to support the efficacy of antioxidant therapy only in AP, whereas its effects in CP and PEP were less clear. The meta-analysis by Rustagi *et al*[20] demonstrated a benefit of antioxidants; however, the investigators did not control for a heterogenous study population and the use of different types of antioxidants among such. The most recent Cochrane Systematic Review of 18 studies concluded that antioxidants could result in a slight reduction in pain in patients with CP but there were no conclusive data reported for analgesic requirement and QoL[21]. Rupjoyti *et al*[22] showed that treatment with a methionine-containing antioxidant was associated with a significant increase in the number of pain-free patients and a trend towards decreased requirement for hospital visits or admissions. Thus, methionine may help to restore the transsulfuration pathway and decrease intrapancreatic inflammation. Rupjoyti *et al*[22] performed a meta-analysis based upon data from January 1980 to August 2014, encompassing eight studies (six randomized controlled trials and two non-randomized trials). Although the overall results supported the efficacy of methionine supplementation, when the two non-randomized studies (by Shah *et al*[23] and Castasno *et al*[24]) were excluded, the antioxidant combination was no longer statistically significant for decreasing the pain score.

A recent meta-analysis by Mohta *et al*[25] showed negative results for antioxidants' ability to reduce pain and improve QoL in patients with CP. These findings are important because all studies included in the meta-analysis had been performed using a similar type of antioxidant and were based on a combination of commercially-available antioxidants and those used in clinical practice; therefore, the findings of that meta-analysis are more relevant to clinical practice. However, the meta-analysis itself had some limitations. First, there was variation in the method of reporting of pain among the studies included and, as such, the analysis had to be performed with two different parameters, namely the visual analogue scale (VAS) score and pain-free participants. This resulted in a decrease in the number of patients that could be simultaneously included in the analysis. Second, limited information was available regarding the QoL.

Although the micronutrient antioxidant therapy was proposed for relieving the pain associated with CP more than three decades ago, this treatment has been used sporadically; moreover, the optimal formulation and duration of the antioxidant regimen has not been completely elucidated. Further, a majority of the clinical trials were not well-designed and did not include a homogenous study population. In view of the fluctuating nature of this disease, a well-defined method to determine the pain scores and measure the QoL was not developed and validated. Differences in the assessment and reporting of pain (*i.e.*, VAS score, numeric rating score, brief pain inventory scores, vocabulary score sheet, and "painful days") in various clinical studies make study comparisons and meta-analysis difficult. These featurescan be attributable to the inconsistent results obtained in the various meta-analysis[20-22,25,26].

The lack of alternative therapies for patients with CP warrants an urgent need for a well-designed study to evaluate the effect of antioxidant therapy in a clearly defined patient population.

STATINS: THE ROAD AHEAD

Antioxidant therapy has inconsistent efficacy in patients with CP. Therefore, a reliable, effective, safe and predictable agent represents an unmet (but required) need for the treatment of CP. A statin appears to be the most appropriate candidate.

Non-lipid-lowering effects of statins

Several primary and secondary prevention trials have shown the clinical benefits of statins in coronary artery disease[27]. An increasing number of studies indicate that statins have many non-lipid-lowering effects, known as pleotropic effects[28,29]. Some of the effects include anti-inflammatory actions and improvement of endothelial function by prevention of lipid peroxidation. Statins exert antioxidant effects by increasing the bioavailability of nitric oxide[30], decreasing the production of ROS[31] and inhibiting the distinct oxidation pathways[32]. Antiproliferative and immunomodulatory properties of statins suggest novel applications of statins in various diseases apart from dyslipidaemia[33].

Statins and pancreatitis

The relationship between statins and AP is controversial, considering simvastatin-induced AP was found in a previous study[34]. Statins are among the most widely prescribed medications worldwide for cardiovascular diseases. Thus, it is important to understand the relationship between statins and pancreatitis. A population-based study from Denmark found no association between the risk of AP and use of statins [35]. In contrast, a recent meta-analysis of 21 high-quality randomized controlled trials showed an overall decrease in the risk of pancreatitis among patients treated with statins compared with those treated with placebo[36]. However, since pancreatitis was not the primary outcome of the 21 trials included in that meta-analysis, these results may not be a true indicator of the protective effects. The protective effects of simvastatin and ezetimibe were shown in the Study of Heart and Renal Protection (SHARP) study[37]. The result of a recent study by Wu *et al*[38] also indicated that simvastatin and atorvastatin were associated with an overall decrease in the risk of AP. Moreover, subgroup analysis in the same study showed a decrease in the risk of pancreatitis in patients with chronic alcohol abuse, suggesting the possible role of simvastatin in preventing recurrent AP and subsequent progression to CP.

Statins and experimental studies

The effects of simvastatin pre-treatment on 10 Wistar rats was published by a group from Brazil in 2008[39]. They reported no beneficial effects on pancreatic inflammation but a trend towards improved survival rate in the simvastatin group. Of interest, the lovastatin treatment was found to successfully inhibit *invitro* activation of pancreatic stellate cells[40]. This is an important observation, as activated stellate cells mediate the fibroinflammatory response of CP. In a similar study of L-arginine-induced pancreatitis in rats, performed by Metalka *et al*[41], levels of malondialdehyde (commonly known as MDA) were significantly reduced in the pancreas tissues of the simvastatin treatment group.

Another considerable pathologic process that occurs in pancreatitis is impaired autophagy[42-44]. Autophagy is the process of removal of damaged cellular compounds, including dysfunctional mitochondria (mitophagy) during stress conditions[45]. Because dysfunctional mitochondria are a significant source of ROS generation, as indicated above, their dysfunction can be an important source of cellular stress that can promote pancreatitis. During autophagy, organelles such as mitochondria and cytoplasmic materials are sequestered by the autophagosomes (a double–membrane structure) and transported to the lysosome for digestion[46]. Autophagosome-lysosome fusion is impaired in pancreatitis, resulting in incomplete autophagy andmitophagy[47]. Piplani *et al*[48] showed that simvastatin restores autophagy and mitophagy, resulting in improvement in pancreatitis pathology in the cerulein-induced model of experimental AP.

Statins and pancreatic cancer

Autophagosome accumulation during pancreatitis can promote pancreatic cancer[49]. CP is the strongest identified risk factor for pancreatic cancer[50]. Intriguingly, the results of a study involving 250 patients with pancreatic cancer showed an improved survival among those patients with diabetes who were subject to statin treatment[51]. Based on these results, Jeon *et al*[52] retrospectively analysed a cohort of elderly patients with pancreatic cancer using the Surveillance, Epidemiology and End Results

(commonly known as SEER) database of patients in the United States. They studied the use of statins in patients after diagnosis of pancreatic ductal adenocarcinoma. Simvastatin was the most prescribed statin. The patients who used statins were found to live longer after their cancer diagnosis (median overall survival of 4.7 mo; interquartile range of 1.9-11.7 mo) as opposed to those who were not prescribed statins (median overall survival of 2.4 mo; interquartile range of 1.5-7.3 mo). There was a favourable impact of statin use on survival in those who had undergone pancreatectomy *vs* those who had undergone no surgery (HR = 0.80, 95%CI: 0.66, 0.97). Of interest, simvastatin treatment was associated with significantly lower hazard of death compared to no statin treatment (HR = 0.91, 95%CI: 0.84, 0.99). The results of that study were confirmed by another, showing the beneficial effect of statins in pancreatic cancer patients in a large health care system in Southern California, United States[53].

The mechanism of action of statins in pancreatic cancer remains poorly understood. Statins are known to decrease the expression of inflammatory cytokines and to modulate the expression of several genes involved in angiogenesis and inflammation, which may protect against carcinogenesis. Statins also inhibit protein prenylation. This prevents the proper functioning of guanosine triphosphatase proteins, such as Ras and Rho, thereby inhibiting downstream pathways that are involved in cell growth, proliferation, survival, motility and invasion, which leads to cell cycle arrest in G1. Furthermore, statins impair cancer cell proliferation by inhibiting the synthesis of cholesterol, which is essential for new membrane formation in rapidly proliferating cells[54].

OUR EXPERIENCE IN CP AND SIMVASTATIN

On the basis of the experimental and limited retrospective/population-based data, we performed two prospective studies to assess the role of simvastatin in patients with ICP. In the first prospective study, patients were assigned to receive either the standard antioxidant preparation (a tablet containing 3000 IU β carotene, 550 mg methionine, 200 μg selenium, 40 mg vitamin C, and 10 mg vitamin E administered thrice daily) or the combination of the standard antioxidant preparation and simvastatin (40 mg/d). Improvement in pain was assessed using the VAS. At the end of 12 mo, the decrease in the VAS score was significantly greaterin the simvastatin group (P = 0.032)[55]. In the other pilot study (under publication), health-related QoL was assessed using the European Organization for Research and Treatment of Cancer (referred to as EORTC) QLQ PAN 28 and QLQ C30 scoring. The results of the study showed that at 6 mo patients who received a combination of simvastatin and antioxidants showed a significant improvement in the pancreatic pain score compared to those who received antioxidants alone (P= 0.01) (Figure 1). Patients who received simvastatin and antioxidants also consumed fewer analgesics (P = 0.03) (Figure 2) and required less hospitalization (P = 0.04) than those who received antioxidants alone. Our findings indicate that, compared to antioxidants alone, simvastatin in combination with antioxidants significantly improves the overall QoL (Figure 3) (P = 0.01), which is consistent with the findings reported in several preclinical studies. Thus, simvastatin may emerge to modulate the disease process in CP.

Considering the complexity of CP, we have also identified NAC as another molecule that can be repurposed for CP. NAC exerts antioxidant and antifibrotic effects *via* inhibition of tissue growth factor signalling in fibrogenic cells[56]. In the environment of inflamed pancreatic tissue, activation of pancreatic stellate cells produces abundant extracellular matrix proteins, leading to fibrosis as well as inflammatory cytokines. Beneficial roles of the combination of simvastatin and NAC on the pathophysiology of inflammation and necrosis of the acinar cell involve inhibition of pancreatic stellate cells. Thus, we designed a prospective, randomized, open-labelled clinical trial entitled "The safety and efficacy of simvastatin plus standard of care or simvastatin plus NAC plus standard of care *vs* only standard of care in patients with idiopathic acute recurrent pancreatitis and chronic pancreatitis" (*i.e.*, the SNAPstudy). This study aims to assess not only the QoL but also various biomarkers for inflammation in CP.

CONCLUSION

There is a scarcity of scientific evidence to substantiate the use of antioxidants in the treatment of CP. Simvastatin and NAC appear to be promising candidates for the treatment of CP.

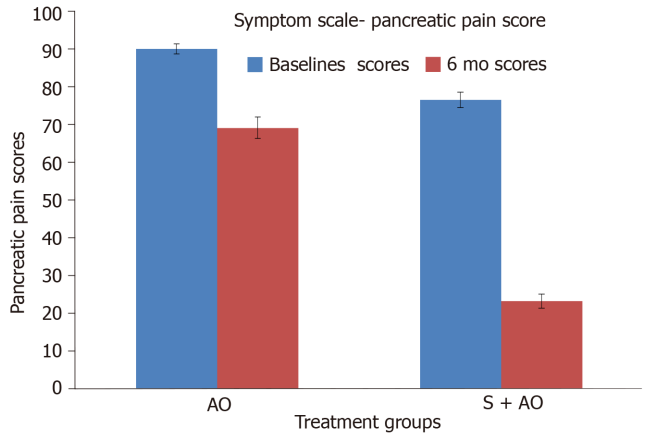


Figure 1 Reduction in pain score observed in both treatment groups. AO: Antioxidants; S: Simvastatin.

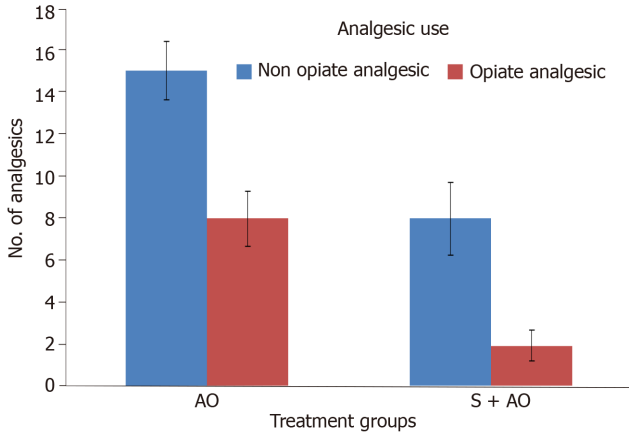


Figure 2 Reduction in analgesic requirement observed for both treatment groups. AO: Antioxidants; S: Simvastatin.

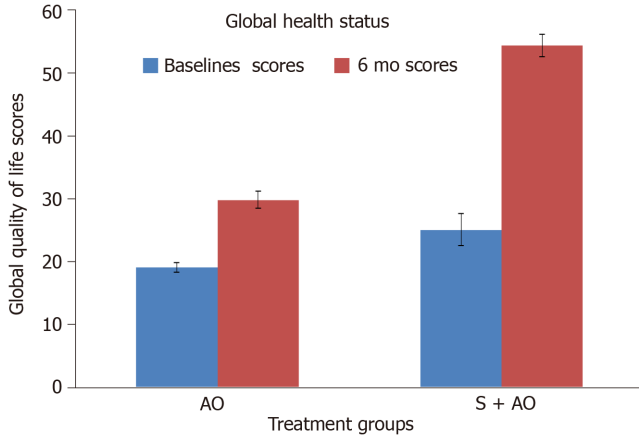


Figure 3 Global health status observed in both treatment groups. AO: Antioxidants; S: Simvastatin.

ACKNOWLEDGEMENTS

The authors would like to acknowledge their consultant team of Dr. Mayank Kabrawala, Dr. Pankaj Desai, and Dr. Subhash Nandwani for their generous support during patient care and clinical trials. The authors would also like to extend their gratitude to Dr. K. Rajagopalan for his editorial assistance.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 21; 27(43): 7433-7445

DOI: 10.3748/wjg.v27.i43.7433

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

OPINION REVIEW

COVID-19 as a trigger of irritable bowel syndrome: A review of potential mechanisms

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Author contributions: Gasbarrini A and Cammarota G conceived the idea for the manuscript, suggested the topics, provided the setting of the paper and supervised the entire work; Bibbò S wrote the introduction; Ponziani FR summarised the current literature about the gastrointestinal involvement of COVID-19; Ianiro G wrote the irritable bowel syndrome (IBS) section; Settanni CR analysed the hypothetical COVID-19 related factors which can promote the development of IBS and drew the conclusion; Segal JP revised the whole manuscript.

Conflict-of-interest statement: The authors declare no conflict of interests.

Country/Territory of origin: Italy

Specialty type: Gastroenterology and hepatology

Provenance and peer review:

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Abstract

In December 2019 a novel coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), started spreading from Wuhan city of Chinese Hubei province and rapidly became a global pandemic. Clinical symptoms of the disease range from paucisymptomatic disease to a much more severe disease. Typical symptoms of the initial phase include fever and cough, with possible progression to acute respiratory distress syndrome. Gastrointestinal manifestations such as diarrhoea, vomiting and abdominal pain are reported in a considerable number of affected individuals and may be due to the SARS-CoV-2 tropism for the peptidase angiotensin receptor 2. The intestinal homeostasis and microenvironment appear to play a major role in the pathogenesis of COVID-19 and in the enhancement of the systemic inflammatory responses. Long-term consequences of COVID-19 include respiratory disturbances and other disabling manifestations, such as fatigue and psychological impairment. To date, there is a paucity of data on the gastrointestinal sequelae of SARS-CoV-2 infection. Since COVID-19 can directly or indirectly affect the gut physiology in different ways, it is plausible that functional bowel diseases may occur after the recovery because of potential pathophysiological alterations (dysbiosis, disruption of the intestinal barrier, mucosal microinflammation, post-infectious states, immune dysregulation and psychological stress). In

Invited article; Externally peer reviewed.

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

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Received: March 4, 2021
Peer-review started: March 4, 2021
First decision: May 5, 2021
Revised: May 10, 2021
Accepted: November 15, 2021
Article in press: November 15, 2021
Published online: November 21, 2021

P-Reviewer: Lødrup AB, Rettura F, Lv L
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

this review we speculate that COVID-19 can trigger irritable bowel syndrome and we discuss the potential mechanisms.

Key Words: SARS-CoV-2; COVID-19; Irritable bowel syndrome; Microbiota; Dysbiosis; Gut-brain axis

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Core Tip: Coronavirus disease 2019 (COVID-19) is not only a respiratory tract illness, as it may involve other systems, including the gastrointestinal tract. Persistent symptoms after the resolution of the infection are described, but there is almost no mention on the possible consequences on bowel function. However, some aspects concerning COVID-19, its management, and psychological aspects, may contribute to trigger disorders of the gut-brain interaction, among which the irritable bowel syndrome is the most frequent.

Citation: Settanni CR, Ianiro G, Ponziani FR, Bibbò S, Segal JP, Cammarota G, Gasbarrini A. COVID-19 as a trigger of irritable bowel syndrome: A review of potential mechanisms. *World J Gastroenterol* 2021; 27(43): 7433-7445
URL: https://www.wjgnet.com/1007-9327/full/v27/i43/7433.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i43.7433

INTRODUCTION

In December 2019 a cluster of acute atypical respiratory infections were reported in the Wuhan city of Hubei province by the Chinese authorities to the World Health Organization (WHO). The responsible pathogen was identified as a new member of the family Coronaviridae, and it was called severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) due to its similarity to the SARS coronavirus, previously involved in the 2002-2003 pandemic. The SARS-CoV-2-related disease was named coronavirus disease 2019 (COVID-19) and rapidly spread worldwide. Indeed, COVID-19 became a public health emergency on January 30, 2020 and, subsequently, a pandemic state was declared on March 11, 2020 by the WHO[1].

SARS-CoV-2 is a positive-sense single-stranded RNA virus, whose genome encodes for four major structural proteins: Spike (S) protein, envelope protein, membrane protein and nucleocapsid protein. The S protein mediates the entering of SARS-CoV-2 in the host cells by binding to the peptidase angiotensin receptor 2 (ACE2)[2].

COVID-19 is a contagious and highly lethal illness, especially for individuals with chronic comorbidities (such as diabetes mellitus, hypertension, cardiorespiratory disorders, chronic hepatic and renal diseases), elderly, oncological and immunosuppressed patients[2]. The infection is predominately transmitted by person to person through respiratory droplets, although many other modes of potential transmission have been postulated, which include through faecal-oral transmission. The average incubation period for COVID-19 is 5.2 d, but it can last up to 15.5 d.

The infection can have an asymptomatic course or it can present with fever, malaise and dry cough in the initial phase, during the invasion and infection of the upper respiratory tract. Patients may also experience gastrointestinal symptoms such as abdominal pain, vomiting and diarrhoea, and signs of systemic involvement (mainly neurological, cardiological, renal, and hepatological manifestations). Subsequently, the disease can involve the lower respiratory tract in approximately 20% of the cases and, in most severe situations, it can culminate in acute respiratory distress syndrome. This condition is characterized by a surge in circulatory inflammatory cytokines [mainly interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF)-α], termed 'cytokine storm', which is responsible for the subsequent inflammation and lung injury[1,3].

Treatments for COVID-19 change according to the disease severity: They include symptomatic and supportive therapy (such as oxygen supplementation, fluid resuscitation and vasopressors in case of septic shock), broad-spectrum antibiotics for prevention/management of secondary bacterial infections or sepsis, steroids if respiratory failure occurs, and prophylactic low molecular weight heparin in patients

with moderate to severe disease because of the high risk of thromboembolism. The efficacy of antivirals (predominantly remdesivir and lopinavir/ritonavir combination), immunomodulatory drugs (including tocilizumab, chloroquine and hydroxychloroquine) and other treatments in reducing mortality and exacerbation of COVID-19 pneumonia is controversial and needs further evidence. However, these drugs are frequently used in clinical practice in the absence of any alternative[1].

As in other infectious diseases, recovered patients often continue to suffer from various long-term sequelae involving the respiratory system, as dyspnoea and cough, as well as less defined disabling manifestations; the latter include neuropsychiatric sequelae such as fatigue, anxiety, depression, post-traumatic stress disorder and insomnia[3-7]. It is still unknown whether these symptoms derive from the infection itself, from its general management (mainly medical therapies) or from the disease itself through mechanisms that have yet to be determined.

As COVID-19 affects also the gastrointestinal tract, some sequelae may derive from a disequilibrium of the intestinal homeostasis, but current evidence is almost absent[5,8].

In this review we hypothesised that the direct involvement of the gut and the one derived from COVID-19-related circumstantial conditions can predispose to the development of irritable bowel syndrome (IBS). To support this idea, we analysed the mechanism through which SARS-CoV-2 perturbs the intestinal physiology in infected individuals, went through the physiopathology of IBS and finally considered the possible factors that can subsequently trigger IBS after the COVID-19 recovery. For this aim, PubMed and Google Scholar were searched using various combinations of the terms "SARS-CoV-2", "COVID-19", "gastrointestinal", "gut", "symptoms", "irritable bowel syndrome", "microbiota", and "microbiome". Subsequently, we selected the most pertinent articles in support of reasonable common factors between COVID-19 and IBS enhancement and summarised current evidence.

GASTROINTESTINAL INVOLVEMENT OF COVID-19

Gastrointestinal manifestations of COVID-19 can be present with variable incidence (40%-50%), and include mainly diarrhoea, nausea, anorexia, vomiting, abdominal pain and belching. These symptoms may arise even in the absence of respiratory involvement or may appear after the onset of respiratory symptoms[2,9,10]. SARS-CoV-2 is also associated with other gastrointestinal symptoms. One of these include liver injury, which can manifest as increased serum aminotransferases, bilirubin and γ-glutamyl transferase[2,10]. Furthermore, elevated blood levels of amylase and lipase have been described, but a strict causality of pancreatic damage with SARS-CoV-2 infection has not been ascertained. Importantly, the drugs used to treat COVID-19 may also have impact on the gastrointestinal tract[11].

Gastrointestinal involvement in COVID-19 may be due to the capacity of SARS-CoV-2 to directly infect the intestinal tract: This hypothesis is supported by detection of the virus in enterocytes and in stool samples of affected patients, and also in faecal samples of individuals with negative nasopharyngeal tests[12-14]. As previously mentioned, SARS-CoV-2 attaches to the ACE2 to enter into human cells and to infect the host. This receptor exists in two forms: The full-length mACE2, which is located on cell membranes with a transmembrane anchor and an extracellular domain, and the sACE2, a soluble form released into blood circulation. The N-terminal domain of the mACE2 is the target of the S protein of SARS-CoV-2[15]. The S protein consists of two different subunits: The S1, which binds to the cell receptors of the host, and the S2, which mediates the fusion of the viral and cell membranes[16]. Two transmembrane protease serines, TMPRSS2 and TMPRSS4, are essential to cleave the S protein at S1/S2 and S2 sites, to enhance the S fusogenic activity, the entry and replication of the virus in mature small intestinal enterocytes[17,18].

The ACE2 is expressed in several tissues within the human body with specific localization on different cells, including enterocytes, renal tubules, gallbladder, cardiomyocytes, male reproductive cells, placental trophoblasts, ductal cells, eye, vasculature and others[15,19]. Concerning the digestive system, the expression of ACE2 gene is highest in the small intestine, but it is also present among other sites, such as colon, stomach, oesophagus, liver, biliary tract and pancreas[19-22]. Specifically, this receptor is expressed in the muscularis mucosa and mucosa of the intestine, including the epithelial cells, cholangiocytes, hepatocytes, pancreatic ductal, acinar and islet cells, and in the gastrointestinal vasculature[11,23,24]. ACE2 seems to play a key role in the intestinal homeostasis and functions. Indeed, it can regulate the

blood flow perfusion by increasing the vascular resistance (primarily the mesenteric vasculature). Moreover, it is possible that ACE2 is capable of enhancing the mucosal nitric oxide production, which regulates the properties of the epithelial barrier, and of modulating the ion transport and the paracellular permeability. It can also induce duodenal secretory responses of mucosal bicarbonate against the luminal acid from the stomach and stimulate sodium and water absorption. It seems plausible that ACE2 is involved in the relaxation of the gastrointestinal wall musculature. Nonetheless, current evidence suggests that ACE2 is involved in inflammation and immunomodulation, and in the pathophysiology of IBS for contributing to enhance low-grade inflammation in the enteric nerve plexa[24,25]. The ACE2 can also regulate the intestinal amino acid homeostasis and absorption, the production of antimicrobial peptides, the intestinal motility and the gut microbiota independently of the renin-angiotensin system[24,26]. It is also reported that the deficiency of this receptor in a murine model of colitis leads to an increased susceptibility to intestinal inflammation. This effect seems to be mediated by an impaired epithelial immunity and induced dysbiosis, defined as the impairment of the diversity and function of intestinal microbes. This is suggested by the increased propensity to develop severe colitis after the faecal microbiota transplantation of an impaired intestinal microbiota from mice with genetic inactivation of ACE2 into germ-free wild-type animals[26]. Moreover, preclinical evidence indicates that ACE2 can impair the electrophysiological and synaptic functions of the neurons of the enteric nervous system, thus influencing the gastrointestinal motility, sensitivity and the pathways of inflammation[27].

Overall, it is plausible that the impairment of bowel physiology by SARS-CoV-2 may derive from a dysregulation of all these ACE2-mediated functions due to a competitive mechanism of the virus on this receptor or from a downregulation of its anti-inflammatory activity. Moreover, the gastrointestinal manifestations may arise from a direct cytopathic effect of the virus on the mucous epithelium, from a malabsorption secondary to the invasion of enterocytes, or from the triggered inflammatory response with plasma cells and lymphocytes infiltration in the intestinal lamina propria[2,28]. Accordingly, SARS-CoV-2 infection can be associated with microscopic bowel inflammation with infiltrating plasma cells and lymphocytes, and with interstitial edema in the lamina propria, as well as overt acute haemorrhagic colitis with endoscopically confirmed mucosal injury[28,29]. The hypothesis of intestinal inflammation is supported by the detection of significantly increased levels of faecal cytokines, as IL-8, in COVID-19 patients when compared to uninfected controls[30]. Additionally, a significant number of patients (approximately 30%–75%), more frequently those with gastrointestinal manifestations, has elevated values of faecal calprotectin, a protein released by neutrophils of the intestinal mucosa[31]. The occurrence of diarrhoea seems also higher among patients with higher SARS-CoV-2 RNA loads in stool samples[30]. Finally, the presence of virus-specific immunoglobulin A (IgA) in faecal samples suggests that the gastrointestinal tract may be immunologically active during SARS-CoV-2 infection[30].

It is likely that the gut homeostasis and the intestinal immunity play a major role in the pathogenesis of COVID-19 and in the enhancement of the systemic inflammation triggered by the SARS-CoV-2 infection, which is characterized by significantly higher serum IL-6, IL-8, IL-10 and TNF-α in severe cases[30]. Indeed, it is described that higher levels of faecal IL-23 correlate with more severe COVID-19 disease, as well as the finding of intestinal virus-specific IgA responses[30]. Interestingly, gut microbial alterations in COVID-19 patients can contribute to regulate systemic inflammation, as suggested by the correlation between specific changes in genera and inflammation indices[32].

IBS

IBS is the most common chronic disorder of the gut-brain interaction, and it is characterized by mild to severe recurrent abdominal pain and bloating associated to alterations in bowel habits in the absence of organic disease or biochemical abnormalities[33]. IBS is also often accompanied by other comorbidities, like psychiatric conditions, pain syndromes, overactive bladder, migraine, and visceral sensitivity[34]. The debilitating symptoms of IBS impose a significant burden on the quality of life of affected individuals, since it is associated with depression and suicidal ideation, reduces work productivity and increases the accesses to medical care[35,36]. The prevalence of IBS varies substantially between countries due to the different diagnostic criteria and survey methods used in worldwide studies, ranging from less

than 1% to more than 25%, with a predominance in women in comparison to men (12.0% *vs* 8.6% respectively; odds ratio 1.46)[37]. Moreover, it is more frequent in lower socioeconomic groups and individuals younger than 50 years[36]. IBS is diagnosed according to the Rome criteria, a clinical classification which includes four types of IBS according to the predominant bowel habits: IBS with predominant constipation, IBS with predominant diarrhoea (IBS-D), IBS with mixed bowel habits (IBS-M) and unclassified IBS[38]. IBS-M and IBS-D are reported to be the most prevalent subtypes[37]. For an accurate diagnosis of IBS, organic underlying conditions must be excluded, with an accurate patient history, physical examination, laboratory tests and, if necessary, endoscopic assessment. Common conditions which should be ruled out include celiac disease, microscopic colitis, inflammatory bowel disease, bile acid malabsorption, colorectal cancer, and dyssynergic defecation[38,39].

The physiopathology of IBS is currently not fully understood, but it is considered a complex multifactorial disorder with a still unknown molecular pathophysiology. Indeed, it has been hypothesised that an impairment of different functions (such as central and autonomic neurophysiology, visceral nociception, bowel motility, secretory activity and psycho-somatic balance) due to perturbing factors (*i.e.*, stress exposure, psychosocial conditions, food antigens, antibiotics and infections of various origin) leads to physiological abnormalities, which may be involved in the development and perpetuating of IBS. These include intestinal dysbiosis, increased intestinal permeability, immune cell hyper-reactivity with impaired expression and release of mucosal and immune mediators, microinflammation with altered mucosal functions, hyper-sensitivity of the enteric nervous system, dysregulation of the hypothalamus-pituitary-adrenal (HPA) axis and of the enteric nervous system. Increased levels of faecal bile acids and predisposing inheritable susceptibility are recognised as co-occurring factors as well[34,39,40]. Increasing evidence suggests that all these affected pathways are part of the microbiota-gut-brain axis, a bidirectional crosstalk between the brain, the bowel and the gut microbiota which occurs through nervous signalling, immune mediators, microbial products, tryptophan metabolites and other hormones[39,41].

Accordingly, dysbiosis may contribute to IBS by triggering the gut immune system and enhancing low-grade inflammation in susceptible individuals. This hypothesis is supported by a higher prevalence of small intestinal bacterial overgrowth and imbalances of the gut microbiota composition in patients with IBS compared with healthy controls in many recent studies, and by the benefit from the use of non-absorbable antibiotics on related symptoms. A reduction of the diversity and stability of the gut microbiota in patients with IBS has been described[42]. Increased Enterobacteriaceae, which includes several harmful genera (as *Escherichia*, *Shigella*, *Campylobacter*, and *Salmonella*), and Lactobacillaceae families, together with high levels of *Bacteroides* genus, reduced *Faecalibacterium* and *Bifidobacterium* genera and uncultured Clostridiales I are reported in patients with IBS in comparison with controls in a recent systematic review including 24 studies[43].

In 6%-17% of the patients suffering with IBS the onset of the symptoms occurs after a recent episode of gastrointestinal infection, which can increase up to 6-fold the risk of developing IBS. This phenomenon is characterised by the persistence of IBS-like disturbances (mainly diarrhoea and abdominal discomfort) after the resolution of the infection, and it is known as post-infectious IBS (PI-IBS). The prevalence of PI-IBS is approximately 4%-36% in patients with previous infectious gastroenteritis and is higher in females, young people, patients who experienced severe infections and individuals with psychological comorbidities. Moreover, some pathogens seem more predisposing than others; indeed, bacterial infections (particularly by *Campylobacter*, *Shigella*, *Escherichia coli* and *Salmonella*) are more likely to enhance PI-IBS than viruses and other microorganisms[34,44,45]. The pathogenesis of this condition is poorly understood, but it is hypothesized that the responsible pathogenic microorganism may trigger an immunologic and inflammatory response with low-grade inflammation and mucosal injury, which causes the prolongation of IBS symptoms in predisposed individuals. Furthermore, it is described that patients with PI-IBS may have increased macrophages and T lymphocytes in intestinal samples, together with high expression of IL-1 in rectal biopsies and elevated blood level pro-inflammatory cytokines (such as TNF-α, IL-6, IL-8, IL-10 and IL-1β)[44,46]. As in IBS, it is likely that an altered intestinal permeability, an impairment of the gut eubiosis and of the neuromuscular function are involved in PI-IBS as well[47].

COVID-19 AND ITS MANAGEMENT: WHAT ARE THE POSSIBLE TRIGGERS OF IBS?

The plausible mechanisms involved in the development of IBS in individuals who experienced COVID-19 are summarised in Figure 1.

Gastrointestinal disturbances are often associated with respiratory infections or to secondary complications, and the gut-lung axis, a hypothetical bidirectional pathway which works *via* biochemical and immunologic systemic signalling molecules, is possibly involved in the pathophysiology. Among the perturbing factors of the gut microbial environment, respiratory viral infections, including COVID-19, can play a relevant role[48]. As previously mentioned, an impairment of the gut microbiota composition, which is frequently associated to a dysregulation of the overall intestinal homeostasis and gut-brain axis, can participate to the development and maintaining of IBS[39,41,42]. An imbalance of the gut microbiota is described in SARS-CoV-2-infected individuals. Gu *et al*[32] found a significant reduction in mean community richness and bacterial diversity in COVID-19 patients in comparison with healthy controls according to the Shannon diversity index and Chao diversity index. A significantly higher relative abundance of *Streptococcus*, *Rothia*, *Veillonella*, and *Actinomyces*, which are opportunistic pathogens, and a lower relative abundance of beneficial symbionts were reported. Moreover, *Fusicatenibacter*, *Romboutsia*, *Intestinibacter*, *Actinomyces* and *Erysipelatoclostridium* were identified as biomarkers to discriminate the COVID-19 patients from healthy individuals[32]. Zuo *et al*[49] reported that even antibiotics-unexposed patients with COVID-19 have a significantly changed intestinal microbiota during the hospitalization, with enrichment of opportunistic pathogens (including *Clostridium hathewayi*, *Actinomyces viscosus*, and *Bacteroides nordii*) and depletion of beneficial commensals when compared to healthy individuals. Moreover, a correlation between the disease severity and the baseline abundance of certain genera and strains was found, suggesting that the gut microbiota may contribute to the systemic involvement in the immune system responses; specifically, a positive relation was observed with *Coprobacillus*, *Clostridium ramosum*, and *Clostridium hathewayi*, while a negative association was described with *Faecalibacterium prausnitzii*. The loss of beneficial bacteria persisted even after a negative throat swab and the disease resolution, suggesting a persistent deleterious effect on the gut microbiota[49]. The same working group also observed that an active intestinal infection is present in approximately half of COVID-19 patients even without gastrointestinal manifestation, and persisted even after respiratory clearance of SARS-CoV-2. Of interest, stool specimen with a signature of high SARS-CoV-2 infectivity were characterised by an enrichment of opportunistic pathogens (including *Collinsella aerofaciens*, *Collinsella tanakaei*, *Streptococcus infantis* and *Morganella morganii*). On the other hand, faecal samples with a signature of "low-to-none" SARS-CoV-2 infectivity displayed higher concentration of *Parabacteroides merdae*, *Bacteroides stercoris* and *Lachnospiraceae bacterium 1_1_57FAA*. The latter are short-chain fatty acid producing bacteria, which play a crucial role in boosting host immunity. A longitudinal follow-up revealed relevant alterations of the faecal microbiota composition in a subset of patients[50].

More generally, intestinal and pulmonary dysbiosis are described in various acute and chronic pulmonary diseases. For example, pulmonary viral infections, such as the ones caused by influenza virus and respiratory syncytial virus, can even directly impair the gut microbiome[49]. Moreover, patients suffering from asthma have functional and structural impairment of the intestinal mucosa, and patients with chronic obstructive pulmonary disease often have leaky gut[51]. Apart from the acute COVID-19 phase, respiratory sequelae and radiological abnormalities (such as dyspnoea, chronic cough, fibrotic lung disease, bronchiectasis, and pulmonary vascular disease) may persist in recovered patients, and the optimal management is still undefined[5,52,53]. Thus, it might be plausible that an impairment of the gut homeostasis may occur in patients during the acute COVID-19 illness and persist after the disease resolution, even in those who did not experience gastrointestinal disturbances. This can be hypothetically explained by the communication between the two systems through the gut-lung axis. The existence of this connection is not entirely understood, but it is strengthened by the occurrence of lung diseases worsening as a consequence to intestinal microbial imbalances, gut inflammation and increased intestinal permeability[54]. Accordingly, it is reported that elevated values of faecal calprotectin are associated with a pathological chest X ray in COVID-19 patients[55]. Of interest, the enriched presence in faecal samples of patients with COVID-19 with high SARS-CoV-2 infectivity of *Streptococcus infantis*, an upper respiratory tract and oral cavity colonizer bacterial pathogen, may indicate a translocation or transmission

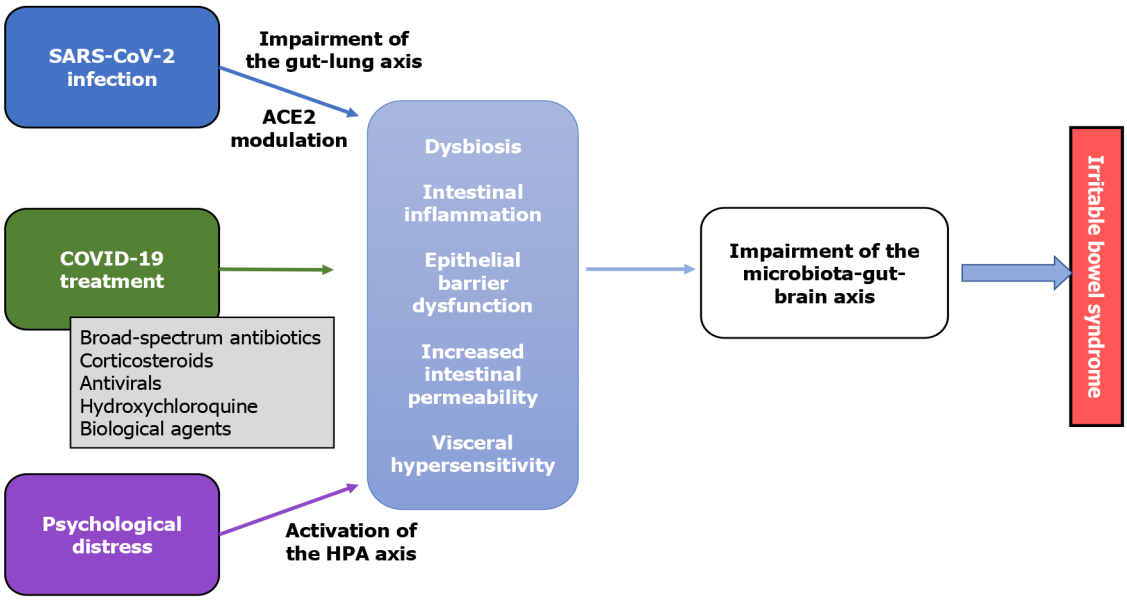


Figure 1 Possible pathophysiology of irritable bowel syndrome in coronavirus disease 2019 patients. ACE2: Peptidase angiotensin receptor 2; COVID-19: Coronavirus disease 2019; HPA: Hypothalamus–pituitary–adrenal; SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2.

of extraintestinal microbes into the gut during COVID-19[50]. Moreover, lung and gut are independent systems which originate from one common embryonic organ, the foregut[56]. The microbiota of these two systems develop almost simultaneously after birth and is influenced by common factors, such as diet[57]. Overall, it is possible that a COVID-19-induced dysregulation of the gut-lung axis may enhance predisposing circumstances for IBS. This is also supported by the increased occurrence of gut disturbances, like inflammatory bowel disease or IBS, in patients with chronic respiratory diseases. Moreover, a pulmonary involvement has been described in approximately 33% of patients with IBS[51].

As previously mentioned, viral enteritis is described as a risk factor for developing PI-IBS. This has been assessed for norovirus infections in particular. Porter *et al*[58] found a significant increase in the incidence of functional gastrointestinal disorders, including constipation, in individuals who experienced a gastroenteritis during a norovirus outbreak, suggesting that dysmotility-related disorders may arise from viral infections[58]. Previously, Marshall *et al*[59] described a significantly increased prevalence of PI-IBS in a small cohort of subjects after a large outbreak of acute gastroenteritis attributed to food-borne norovirus when compared to unexposed individuals (23.6% *vs* 3.4%, $P = 0.014$), with OR of 6.9 (95%CI: 1.0–48.7)[59]. Similarly, an Italian study assessed the incidence of PI-IBS and functional gastrointestinal disorders after a norovirus outbreak. At 12 mo follow up a significant greater proportion of the infected participants (13%, 40 of 186 adults) developed PI-IBS in comparison with unexposed controls (3 of 198 subjects). The mechanisms through which IBS is elicited by norovirus are unknown, but this micro-organism can lead to epithelial barrier dysfunction, increased intestinal permeability, reduction in villous surface area and villous height, and to a mucosal immune response with an increase of cytotoxic intra-epithelial T cells[47]. It is possible that these alterations may trigger a perpetual immune stimulation or a prolonged immune activation toward cross-reacting non-pathogenic antigens, which impairs the gut sensory-motor function[58]. Since SARS-CoV-2 can have an intestinal tropism and induce intestinal flogosis[13,30,31], it can be speculated that a mechanism similar to that of norovirus is involved in the enhancement of PI-IBS.

Other non-infective factors may possibly play a key role in IBS pathophysiology. To date, COVID-19 management has involved a wide range of medications, whose efficacy has yet to be rigorously proven or is still under evaluation, and that can enhance a dysbiotic state. Above all, empiric antimicrobial use is often part of the treatment of respiratory infections, including SARS-CoV-2[60]. It is well known that broad-spectrum antibiotics can cause a rapid and significant drop in taxonomic richness, diversity and evenness, that can persist even for years after the treatment interruption. Beyond taxonomical compositional alterations, the gene expression, protein activity and metabolism of the gut microbiota can be impaired by antibiotics.

Overall, these changes can predispose to intestinal infections, to overgrowth and pathogenic behaviour of resident opportunistic organisms, and to impairment of the immunological equilibrium with systemic and long-term consequences[61]. Alongside with gut dysbiosis, broad-spectrum antibiotics can induce a disruption of the intestinal barrier function by altering the tight junction protein expression and localization, enhancing a pro-inflammatory state by NLRP3 inflammasome activation and promoting autophagy[62]. Hospitalised patients affected by COVID-19 are frequently treated with broad spectrum antibiotics for 5-8 d, mainly to prevent or to treat pathogens causing atypical pneumonia and staphylococci[60]. For instance, azithromycin, which is largely prescribed to COVID-19 patients, can induce a decline in the microbial richness and diversity, as well as changes in microbiota composition, with a shift in the Actinobacteria phylum, a reduction in the relative abundance of Proteobacteria and Verrucomicrobia (including *Akkermansia muciniphila*) and a decrease of the levels of bifidobacteria[63,64]. It is also reported that COVID-19 patients treated with vancomycin and/or ceftriaxone went through significant compositional alteration with reduced diversity of the gut microbiota[30].

Additionally, it is described that the use of corticosteroids, which is considered a treatment option in severe COVID-19 cases, may induce dysbiosis and alter intestinal homeostasis as well[57,65]. This is supported by the influence of steroid hormones on the gut bacterial communities in animal studies. In example, gonadectomy can reduce the microbiota-related sex differences observed between male and female rats. Similarly, hormone replacement to rodent females from the birth can decreased the microbial diversity in adulthood by increasing the Firmicutes:Bacteroidetes ratio[66]. As to systemic glucocorticoid therapy, there is evidence that subcutaneous prednisolone administration can alter the gut microbiota of mice, inducing significant shifts in the relative abundance of bacteria (decrease in Verrucomicobiales and Bacteriodales and increase in Clostridiales) in comparison with controls[67]. Interestingly, it is reported that individuals with glucocorticoid-induced obesity, who received prednisolone for at least three months, have significant decrease in gut microbial diversity in comparison with healthy controls, alongside with increased Firmicutes and Actinobacteria levels, and depletion in Bacteroidetes. Taxonomic analysis revealed a significantly reduced relative abundances of *Bacteroides*, *Bifidobacterium*, and *Eubacterium* in treated patients, whereas *Streptococcus* and *Geobacillus* displayed higher abundances. Also the faecal content of short-chain fatty acids (propionate and butyrate), which are products of carbohydrate fermentation by the gut bacteria, tended to be remarkably lower when compared to healthy control[68].

Other treatment options for the pulmonary phase of COVID-19 include antiviral drugs, mainly the adenosine nucleotide analogue prodrug remdesivir and the protease inhibitor lopinavir in combination with ritonavir[69]. Multicentre randomized controlled trials to assess the efficacy in reducing inpatient mortality, ventilation rate, and duration of hospital stay are still undergoing, although preliminary results are overall not encouraging[70]. While remdesivir seems not to affect the gut microbiota composition, antiretrovirals may somehow have a modulatory activity. The knowledge of the impact of antiretroviral drugs on the microbiome is limited by little evidence and it is predominantly focused on human immunodeficiency virus (HIV) treatment. It is still unclear whether antiretroviral treatment can consistently restore gut health of HIV-infected individuals or not, but it is likely that the initiation and prosecution of these medications can promote dysbiotic states[71,72]. Importantly, there is evidence that this drug class can promote microbiome changes independently from those induced by HIV. Indeed, a decreased alpha diversity is reported among treated patients in comparison with untreated HIV-positive ones. Moreover, protease inhibitors can directly interfere with the *in vitro* adherence of *Candida albicans* to an epithelial cell layer, which can possibly contribute to the reduction of oral candidiasis in HIV treated individuals[73].

Even if current evidence discourages its use in the prevention or treatment of COVID-19, hydroxychloroquine has been largely administered to patients due to its in-vitro capability of inhibiting SARS-CoV-2 by interfering with membrane fusion between host cell and the virus, especially in the early phase of the pandemic[70,74]. Hydroxychloroquine was initially used as an antimalarial, but, subsequently, it has been used as a disease-modifying anti-rheumatic drug to treat rheumatic disorders due to its immunomodulatory properties[75]. The impact of this medication on the human gut microbiota has been assessed by Balmant *et al*[48] in patients affected by systemic lupus erythematosus. They reported that the use of this drug is associated to different degrees of dysbiosis with a dose-dependent effect[48].

Severe SARS-CoV-2 infection is associated to an aberrant immune response with a massive cytokine release, mainly the IL-6 and IL-8. The elevation of inflammatory markers, such as IL-6 and C-reactive protein, has been associated with mortality and severe disease with pulmonary involvement in comparison to moderate disease. Thus, the targeted blockade of systemic inflammation has been proposed as a strategy to treat advanced acute conditions with lung lesions when contrasting the virus alone might not be sufficient. Specifically, Tocilizumab, a monoclonal antibody that inhibits the IL-6 receptor, has been proposed in patients with advanced lung injury[69]. Little evidence about the effect of this biological agent on the intestinal microbiota is available. A study of patients with rheumatoid arthritis reported that biologics, including tocilizumab, significantly reduced the total bacterial count and led to a decrease of *Clostridium coccoides* group, *Bifidobacterium*, and *Lactobacillus plantarum* and *Lactobacillus gasseri* strains after 6 mo[76]. Furthermore, it has been hypothesized that the aetiology of tocilizumab-related intestinal perforation may lie in the compositional or functional microbial changes[8]. Possibly, another IL-6 receptor inhibitor, which is currently being tested for severe COVID-19, may induce similar changes to the intestinal microbiota, but no study with this objective has been performed to date[69].

A further significant imbalance in the commensal bacterial populations may also be caused by polypharmacotherapy (*e.g.* proton-pump inhibitors, laxatives and metformin), which is common especially in elderly comorbid COVID-19 patients, and by the use of commonly prescribed drugs to manage mild COVID-19, as nonsteroidal anti-inflammatory drugs[63].

Finally, another considerable element involved in the IBS onset, exacerbation and relapse, is the activation of the HPA axis consequential to the secretion of the corticotropin-releasing hormone due to acute or chronic stressful conditions. This signalling pathway affects the gut functions by regulating the stimulation of the sympathetic and parasympathetic activity, the release of catecholamines, the mucosal immunity, the intestinal barrier function, the splanchnic blood flow and the composition and growth of the gut microbiota. Immune activation and intestinal micro-inflammation are described in IBS and can increase the intestinal permeability, modulate the peripheral sensitization of mucosal neuronal afferents and the recruitment of "silent" nociceptors involved in the hypersensitivity. Similarly, stress-induced dysbiosis may modulate the neuro-immune-endocrine systems and interfere with the brain-gut axis. Accordingly, the prevalence of at least one psychiatric disorder (mainly depression and anxiety) in patients with IBS ranges from 40% to 60% approximately, and the severity of IBS manifestations is remarkably correlated with the intensity co-morbid psychiatric disturbs. Moreover, early adverse life events and major traumatic experiences are frequently described before the onset of IBS[77,78]. COVID-19 is having a significant impact on mental health worldwide, since various psychological stress-associated factors are linked to the pandemic. More than 40% of patients experiences psychological distress even when the disease is under control during the acute infection phase. A considerable role is also played by anxiety, panic and fear for the isolation environment and the uncertain sequelae following resolution of a new and dangerous disease. Long-term psychological consequences (as anxiety, depression, post-traumatic stress disorder, insomnia, irritability, memory impairment, fatigue, and traumatic memories) are frequently reported among those who suffered from COVID-19, especially hospitalized ones and individuals with previous emotional dysregulation[7,79]. Overall, COVID-19-related psychological disturbances are significant and can definitely contribute to IBS occurrence.

CONCLUSION

The COVID-19 pandemic is a threat to global public health. A wide spectrum of respiratory and systemic symptoms can occur during the acute disease with different degrees of severity, and some of them can persist over time after the recovery. A large body of evidence supports the gastrointestinal involvement of SARS-CoV-2 infection during the acute phase, possibly because the intestinal ACE2 is an additional target of viral infection. Importantly, little is known about the gastrointestinal sequelae; at present, there is no study reporting the occurrence of IBS in individuals recovered from COVID-19. However, a number of considerations may be made regarding the plausible role of COVID-19, its management and global setting in the enhancement of IBS. Specifically, it can be assumed that many factors contributing to promote a dysbiotic state, epithelial barrier impairment, intestinal inflammation and gut dysfunction (like antibiotics and other treatments of the acute phase, gut-lung axis

impairment, disease-related psychological stress, as well as the virus itself) can be involved in this process. Prospective cohort studies are necessary to confirm these hypotheses before clinical significance can be concluded.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 21; 27(43): 7446-7461

DOI: 10.3748/wjg.v27.i43.7446

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

REVIEW

Overview of the microbiota in the gut-liver axis in viral B and C hepatitis

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Author contributions: Catinean A and Neag MA contributed to conceptualization; Catinean A, Neag MA and Mitre AO contributed to methodology and formal analysis; Mitre AO, Neag MA contributed to data curation; Catinean A, Neag MA, Mitre AO and Buzoianu AD wrote the original draft, reviewed and edited the manuscript; Buzoianu AD, Neag MA and Catinean A contributed to supervision.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

Country/Territory of origin: Romania

Specialty type: Gastroenterology and hepatology

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

Peer-review report's scientific quality classification

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

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Abstract

Viral B and C hepatitis are a major current health issue, both diseases having a chronic damaging effect on the liver and its functions. Chronic liver disease can lead to even more severe and life-threatening conditions, such as liver cirrhosis and hepatocellular carcinoma. Recent years have uncovered an important interplay between the liver and the gut microbiome: the gut-liver axis. Hepatitis B and C infections often cause alterations in the gut microbiota by lowering the levels of 'protective' gut microorganisms and, by doing so, hinder the microbiota ability to boost the immune response. Treatments aimed at restoring the gut microbiota balance may provide a valuable addition to current practice therapies and may help limit the chronic changes observed in the liver of hepatitis B and C patients. This review aims to summarize the current knowledge on the anato-functional axis between the gut and liver and to highlight the influence that hepatitis B and C viruses have on the microbiota balance, as well as the influence of treatments aimed at restoring the gut microbiota on infected livers and disease progression.

Key Words: Viral B hepatitis; Viral C hepatitis; Gut-liver axis; Immunomodulation; Lipopolysaccharides; Short-chain fatty acids

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Grade E (Poor): 0

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Received: March 17, 2021
Peer-review started: March 17, 2021
First decision: August 9, 2021
Revised: August 13, 2021
Accepted: November 2, 2021
Article in press: November 2, 2021
Published online: November 21, 2021

P-Reviewer: Chi G, Sira AM, Wang L
S-Editor: Gao CC
L-Editor: Filipodia
P-Editor: Gao CC

Core Tip: We have provided an overview of the mechanisms involved in the immunomodulation of the gut-liver axis. We highlight the mechanisms by which hepatitis B virus and hepatitis C virus infections influence the microbiota and how in turn these changes affect the liver pathology. We have also looked at the current treatment options and their influence on the intestinal microflora.

Citation: Neag MA, Mitre AO, Catinean A, Buzoianu AD. Overview of the microbiota in the gut-liver axis in viral B and C hepatitis. *World J Gastroenterol* 2021; 27(43): 7446-7461
URL: https://www.wjgnet.com/1007-9327/full/v27/i43/7446.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i43.7446

INTRODUCTION

Viral B and C hepatitis are two types of infections with a high rate of morbidity and mortality[1]. Hepatitis B virus (HBV) is a DNA virus belonging to the Hepadna virus, and hepatitis C virus (HCV) is an RNA virus in the Flaviviridae family. These viruses have hepatic tropism, are non-cytopathic with the ability to cause chronic liver inflammation and even liver cirrhosis and hepatocellular carcinoma[2].

Both HBV and HCV may cause similar clinical manifestations. Some patients may be asymptomatic, while others may have mild signs and symptoms from general manifestations (fatigue, fever, loss of appetite) to gastrointestinal symptoms (abdominal pain, nausea, vomiting, jaundice)[3].

The microbiota represents the totality of microbes (bacteria, viruses, fungi, protozoans, and archaea) associated with the human microorganism, while the microbiome consists of all microbes and their genes[4]. The main part of the body colonized by microbes is the gastrointestinal tract, whereas other parts such as skin, airways, vaginal tract, *etc.* are also colonized, but to a lesser extent. Changes in the microbiota are continuous throughout our life and there are many influencing factors, from type of delivery and breastfeeding, to long-term dietary changes, frequent and prolonged antibiotic treatment or other medications, *etc.*[5]. There are six bacterial dominant phyla in the gut microbiota: *Firmicutes* and *Bacteroidetes* (90%), *Proteobacteria, Actinobacteria*, *Fusobacteria* and *Verrucomicrobia*[6]. The intestinal microbiota is a cornerstone in maintaining the homeostasis of the human body. Firstly, this "organ" provides nutrients and energy from ingested food and, secondly, it is able to produce important metabolites that play a role in maintaining the host's metabolism [7].

The liver can be considered the largest immune organ in the body with a high ability to select and activate immune cells in response to metabolic products in the gut or to signals sent by various pathogens[8]. Recent years have seen advances in our understanding of the human microbiome and its interaction with us as hosts. The gut-liver axis is part of these new discoveries, integrating the microbiome modifications and dysbiosis in hepatic pathologies.

Our review will discuss part of the mechanisms by which the microbiome influences host immunity, as well as the gut-liver axis, with an accent on viral hepatitis B and C.

MICROBIOTA AND THE IMMUNE SYSTEM

Through its products, the human microbiota can influence both the local, enteric, and the systemic immune system, dysbiosis being correlated with several autoimmune, metabolic and neurodegenerative diseases (inflammatory bowel disease progression, rheumatoid arthritis, diabetes, asthma and bones homeostasis)[9-15]. This shows that the microbiota is not only involved in intestinal, but also in systemic and organ specific pathologies. This relationship is bidirectional; systemic modifications can trigger intestinal changes, but also intestinal dysbiosis can trigger and maintain organ dysfunctions. Gut-associated lymphoid tissue (GALT) is an important "immunological organ" of the body that belongs to the gut-mucosal immune system. GALT consists of Peyer's patches, intraepithelial lymphocytes, lamina propria lymphocytes (including dendritic cells) and mesenteric lymph nodes. Activation of this system has the ability

to produce various mediators with immunostimulatory or immunosuppressive effect [16].

Some of the products by which the intestinal microflora communicates with the rest of our organism are lipopolysaccharides (LPS), bacterial DNA and RNA, flagellin, short-chain fatty acids (SCFA) such as acetate, propionate and butyrate, tryptophan (Trp) and it's metabolites, teichoic acid and peptidoglycans and secondary bile acids (BA)[9,17]. These bacterial components and products of the bacterial metabolism are recognized by pattern recognition receptors, which particularly include the toll-like receptors (TLR) family. TLRs are expressed on epithelial and immune cells and are capable of recognizing specific bacterial molecules, triggering specific local protective and immunomodulatory (both pro- and anti-inflammatory) responses[18,19]. TLR activation is an essential element of the innate immune systems fight against the HBV and HCV infections[20,21]. Not all of these pathways were studied directly in connection with HBV and HCV. Therefore, more studies are needed to determine the exact relationship between the bacterial products, the immune system and hepatitis.

We will briefly mention some of the most important of the microbial-produced products and their interaction with the immune system (Figure 1).

LPS

In Gram-negative bacteria, LPS are an important pathogen-associated molecular pattern and a well-studied microbial marker in connection with bacterial translocation and host systemic responses[22,23]. The outer membrane of gram-negative bacteria consists of LPS, which possess a hydrophobic endotoxin, called lipid A[24]. This component is recognized by TLR4 and *via* this mechanism it further activates nuclear factor kappa B (NF-κB) and elicits pro-inflammatory effects[25,26]. One type of LPS is *Escherichia coli* (*E. coli*) produced LPS. This stimulates TLR4 receptors and triggers the release of pro-inflammatory cytokines. *E.coli* LPS also increases endotoxin tolerance and decreases the autoimmune activity, protecting against autoimmune diabetes[27]. However, some bacterial species produce LPS molecules with underacylated lipid A that exhibit an immuno-inhibitory effect[28]. These LPS molecules are produced especially by members of the *Bacteroidales* order and instead of stimulating TLR receptors, they silence the TLR4 signaling and the inflammatory process[29]. LPS induces the upregulation of cluster of differentiation 14 protein (CD14) *via* the TLR4 pathway, which decreases the relative epithelial resistance and increases its permeability. Increased intestinal permeability allows for more LPS to reach the general circulation, aiding it in reaching different organs and exhibiting a pro-inflammatory effect[30]. This is also true in cases of dysbiosis with an increase in LPS production that is correlated with an increase in tumor necrosis factor alpha (TNF-α), interleukin (IL) 6 and C-reactive protein levels[31,32]. Intestinal dysbiosis caused an LPS-induced inflammatory response in a mice model, while unaltered host microbiota reduced the inflammatory response to LPS in the liver[33]. LPS-induced monocyte activation has been shown to be increased in patients with HBV or HCV[34].

This underlines the ability of LPS and gut lipid metabolism to modulate both intestinal and organ-specific inflammatory response.

SCFA

In the gut, non-digestible carbohydrates are transformed by the microbiota into SCFA such as acetate, propionate and butyrate[35]. Acetate and propionate are produced mainly by *Bacteroidetes*, while butyrate, the main source of energy for colonocytes, by *Firmicutes*. A small portion of SCFA that is not metabolized can reach the liver through the portal vein, being used as energy substrates for hepatocytes[36,37]. Certain bacteria such as *Butyricimonas* and *Prevotella* have the ability to generate butyrate and propionate, SCFAs with anti-inflammatory effect[38].

SCFA bind to the G-protein coupled free fatty acid receptors (FFA): GPR41 (FFA2) and GPR43 (FFA3)[39,40]. Enteroendocrine and pancreatic β-cells present both GPR41 and GPR43 receptors, while immune cells and adipocytes present mostly GPR41 and peripheral neurons GPR43[41]. This links SCFA production to a multitude of metabolic, neurological and inflammatory mechanisms. Thus, FFA receptors and SCFA production presents therapeutic targets in these diseases[41-43].

In immune cells (leukocytes and neutrophils) SCFA increase the intracellular calcium levels[39,44,45]. This reaction leads to an increased production of reactive oxygen species, as well as an increased neutrophil recruitment and a pro-inflammatory effect[46-48]. GPR41 activation by SCFAs in the gut promotes the function and size of regulatory T cells, protecting against intestinal inflammation[49]. Also, GPR43 was found to be a chemotactic receptor for neutrophils, stimulating their migration towards the source of SCFAs[50,51]. In a mouse model of gout, the intestinal

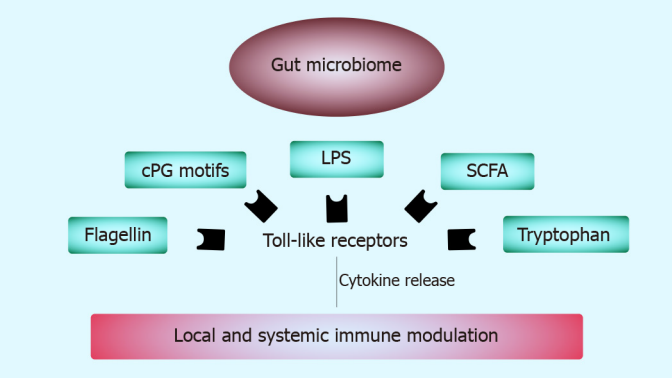


Figure 1 The mechanisms by which the gut microbiome influences the immune system. LPS: Lipopolysaccharides; SCFA: Short-chain fatty acids.

microbiota-produced SCFA determined inflammasome assembly, reactive oxygen species formation and IL-1b production and improved the inflammatory response[52]. Increased SCFA levels determined the production of macrophages and dendritic cells, protecting the lung against allergic inflammation[53]. Also, by activating another G-protein coupled receptor, GPR109A, the microbiota is involved in inflammatory suppression *via* the NF-κB pathway in normal and colon cancer cells[54].

Another SCFA mechanism involved the inhibition of histone deacetylases (HDAC). By non-competitively inhibiting the activity of HDAC 1 and 2, butyrate causes histone hyperacetylation. By this mechanism, butyrate and other SCFAs are thought to serve as a protective factor against colon cancer, dysbiosis being a risk factor for the development of this disease, as well as other chronic inflammatory diseases[55]. HDAC inhibition also promotes macrophage activity and CD8 T cells and improves anti-cancer therapy[56-59]. Furthermore, class 1 HDACs inhibition is proposed as a target in pulmonary inflammation, due to its contribution in the release of pro-inflammatory cytokines[60]. HDAC inhibition promotes effector and regulatory T-cell differentiation and the production of IL-17, interferon-γ (IFN-γ) and IL-10, contributing to an overall anti-inflammatory effect mediated by SCFAs[61,62].

By increasing acetyl-CoA activity and controlling gene expression, SCFA are involved in plasma B cells metabolism, activity, energy production boosting, and differentiation. During an infection, they support B cells antibody production, decreasing the host susceptibility to pathogens[63].

Therefore, SCFA present both a pro- and anti-inflammatory role[61]. There is still the need for more studies to fully understand the implications of SCFA in inflammatory and immune diseases and determine in which conditions they act as pro-inflammatory or as anti-inflammatory factors.

Trp

The microbiota is involved in the transformation of Trp in indole derivatives, serotonin (5-hydroxytryptamine) and kynurenine[64].

Lactobacilli species can metabolize Trp into indole-3-aldehyde, a ligand for the aryl hydrocarbon receptor (AhR) that is involved in intestinal immunity and the production of IL-22[65,66]. There are only a few species such as *Peptostreptococcus russellii* and *Lactobacillus spp.* with the ability to produce AhR ligands[64]. In high fat diets IL-22 can act as an antioxidant and anti-inflammatory agent, protecting the intestinal mucosa and epithelial cells from oxidative and inflammatory stressors[67]. Also, IL-22 is involved in the intestinal mucosa immune response against exterior pathogens[68,69]. However, in patients with inflammatory bowel disease, Il-22 is considered a "two-headed cytokine": it acts as a mucosal producing and healing agent, but in the chronic form of the disease it is also involved in tumorigenesis, promoting tumoral growth[70-72].

The Trp microbiota metabolite AhR regulates the activation and transcription of several other pathways, including IL-6, cytochrome P450 1A1 (CYP1A1), and 1B1 (CYP1B1), vascular endothelial growth factor A, and prostaglandin G/H synthase 2 and also stimulates innate lymphoid cells and intraepithelial lymphocytes development, mediating their anti-inflammatory effects[73,74]. Other bacteria that interfere with Trp metabolism are *E. coli*, *Lactobacilli* and *Clostridium sporogenes*. The first two possess tryptophanase which converts Trp to indole, while the latter decarboxylates Trp and increases tryptamine production[64].

The microbiota influence on Trp provides intestinal anti-inflammatory effects, but it also poses potential research directions regarding systemic inflammation[75,76].

Flagellin

The locomotive bacterial flagella contain flagellin, which is recognized by the host TLR5. Via the TLR pathways, flagellin is involved in several immunological mechanisms, both locally, in the gut, but also systemic, inducing the release of pro-inflammatory molecules[77]. In a study administering purified flagellin in mice, there was a decreased microbial dysbiosis, as well as an amelioration of IL-10 deficiency-induced colitis[78]. This shows that flagellin presenting bacterial species could pose a beneficial effect in chronic inflammatory diseases. However, in patients with inflammatory bowel diseases there have been observed higher concentrations of flagellin, putting into question its supposed protective role[79]. Also, flagellin has been observed to be a potent TLR5/NF-κB activator, promoting inflammation in intestinal epithelial cells[80]. *Via* the same TLR5/NF-κB mechanism, flagellin could also promote the attachment and development of viral molecules, supporting viral infections *via* the intestine[81].

Bacterial CpG motifs

Bacterial DNA contains unmethylated CpG dinucleotides that are recognized by the immune system and produce an immunostimulatory effect[82,83]. These bacterial CpG motifs are recognized by TLR9 receptors and, depending on their localization, they exhibit several effects. Apical TLR9 activation inhibits NF-κB activation, while basolateral receptors stimulate NF-κB activation and the subsequent inflammatory pathways[84].

INFLAMMATION AND B AND C HEPATITIS

Many extrahepatic changes (metabolic, cardiovascular, autoimmune, renal) have been correlated with chronic HCV infection. This statement is supported by a prospective cohort study in which patients with chronic HCV infection (with HCV RNA detected in the serum) had a high risk of death due to liver or non-liver disease (cardiovascular and renal disease) compared to uninfected patients (without serum HCV RNA) or with patients presenting HCV antibodies[85].

Inflammatory cytokines are normally released in response to various stimuli, including viral infection. This limits cellular stress and cell damage[86]. HCV infection is associated with an immune activation status that can further influence the levels of inflammatory markers (Il-6, TNF-α, iNOS, COX-2, IL-1), which are correlated with various extrahepatic diseases[87,88]. In HBV-infected patients there is an increase in Il-8, IL-29 and COX-2. Under normal conditions, adult hepatocytes do not express COX-2, but in chronic inflammatory diseases, the expression of this isoenzyme increases. Furthermore, IL-8 activates the extracellular signal-regulated kinase and c-Jun N-terminal kinase signaling pathways, which are also involved in inflammatory processes[86].

In infected hepatocytes with HCV, the production of type 1 and 3 interferons is blocked by the action of the viral NS3/4A protease. This protease may also influence the innate immune adaptor molecules mitochondrial antiviral signaling proteins with an effect on the intracellular antiviral defense system. In an experimental study on hepatic macrophages the first activated factor in liver macrophages with HCV infection has been shown to be TNF-α that further activates NF-κB and increases IL-1β. Adding to this, the HCV core protein also activates the NLRP3 inflammasome. The hepatic inflammatory environment is ensured by the activity of the NLRP3 inflammasome, phospholipase-C and IL-1β. Thus, NLRP3 inflammasome and IL-1β can be considered as target of treatment in HCV-induced liver disease[89].

THE GUT-LIVER AXIS

The gut microbiome can interact tightly with the liver *via* the so-called gut-liver axis. Blood from the intestine, rich in microbiota-derived molecules, reaches the liver *via* the portal vein. In the liver, these molecules are recognized by TLRs pattern recognition receptors, mediating their effect on the liver tissue[90]. Related to liver pathologies, the gut microbiota is particularly involved in liver fibrosis and cirrhosis, hepatic cancers,

alcoholic and non-alcoholic fatty liver disease, autoimmune hepatitis, primary sclerosing and primary biliary cholangitis as well as viral hepatitis[91-96]. Some of the most studied components that affect liver pathologies are represented by LPS and SCFAs.

LPS produced by the microbiota are scarcely found in the normal liver, being cleared by Kupffer cells and not causing any damage[97]. However, in alcoholic liver disease, because of an increase intestinal permeability, an increased amount of LPS reached the liver[96]. LPS binds to TLR4, causing an excessive release of pro-inflammatory cytokines IL-1 and TNF-α[33,98]. Also, LPS can upregulate the expression of the cluster of differentiation 14 (CD14) receptor on Kupffer cells[99]. This could potentially make the liver more sensitive to LPS toxicity, as CD14 is vital for Kupffer cells LPS activation[100]. Kupffer cells activation produces a pro-inflammatory state, increasing the levels of NF-κB, TNF-α and IL-1. This leads to liver injury and disease progression, dysbiosis favoring the chronic inflammatory state[101].

SCFA such as acetate, propionate and butyrate may have a protective effect on liver diseases progression. High levels of butyrate restore the intestinal microbiota in cases of dysbiosis, reducing the intestinal permeability and thus the levels of endotoxins reaching the liver *via* the portal circulation. This attenuated the histological aspect of steatohepatitis livers, reducing the levels of TNF-α, IL-1, IL-6 and IFN-γ pro-inflammatory cytokines, as well as the expression of TLR4 receptors[102]. In an experimental study by Endo *et al*[103], administering probiotics, aimed at increasing butyrate levels, significantly improved non-alcoholic fatty liver disease progression, reducing the inflammation and oxidative stress. This clearly shows that intestinal-produced metabolites can influence the immune and inflammatory state of the liver. Dysbiosis and an increased intestinal permeability allows for the gut-liver balance to change, causing a pro-inflammatory state of the liver and contributing to disease progression [104,105]. Pathogen-associated molecular patterns (bacterial antigens and products) such as LPS and viral RNAs activate TLR4 on Kupffer cells and other immune cells. Thus, the innate immune response is induced.

The liver is influenced by the intestine through the portal circulation, while the intestine is influenced by the liver through the released mediators and hepatic bile flow. It is known that increased intestinal permeability contributes to systemic inflammation and disease progression[106]. BA and other mediators such as immunoglobulin A (IgA) regulate the gut-liver axis. IgA influences the homeostasis of the intestinal microbiota, preventing bacterial translocation. BA modulate the intestinal barrier and have antimicrobial activity. Several enzymes involved in BA synthesis are regulated by the microbiota. However, some secondary BA (*e.g.*, deoxycholic acid) resulting from intestinal biotransformation produce microbial dysbiosis and increase the intestinal permeability[107].

TGR5 is a G-protein-coupled BA receptor involved in the anti-inflammatory immune response, energy homeostasis, metabolic pathways and in pathologies such as diabetes and obesity[108]. In the intestine, TGR5 is involved in regulating the colonic motility and the intestinal permeability *via* the farnesoid X receptor – cAMP pathway [109,110]. Moreover, TGR5 activation stimulates mucosal proliferation and protects against mucosal injuries[111]. In liver pathologies, the levels of BA are significantly decreased, leading to a reduced activation of TGR5 in the gut[112,113]. In a mouse model with TGR5 silencing, there was a significant reduction in gut epithelial cellularity, with histological abnormalities and distortions and an increased intestinal permeability[114]. BA and TGR5 activation are therefore necessary for a normal functioning of the intestine and the gut-blood barrier. BA administration is beneficial for viral hepatic diseases. In a HBV model, TGR5 agonists administration suppressed the infection[115]. BA and TGR5 agonists pose as potential treatment options for viral hepatitis[116].

Decreased BA quantities in virus hepatitis could be responsible for the increased intestinal permeability and the subsequent increase in LPS and other endotoxins. This in turn favors the progression of the liver pathology, creating a vicious circle where the liver pathology creates an environment that further promotes the liver pathology (Figure 2). Future studies should determine the exact mechanism by which liver diseases influence the intestinal permeability and lead to the production of dysbiosis.

THE GUT MICROBIOTA-VIRAL B AND C HEPATITIS

The presence of the HBV or HCV infection can lead to intestinal dysbiosis[117]. Some of the microbial changes present in patients with HBV and HCV-related liver diseases

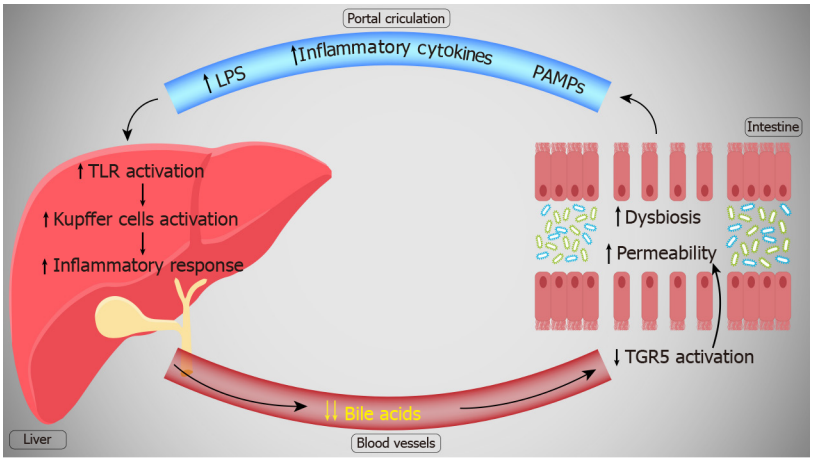


Figure 2 The gut-liver axis in liver diseases. TGR5: G-protein-coupled bile acid receptor; PAMPs: Pathogen-associated molecular patterns; LPS: Lipopolysaccharides; TLR: Toll-like receptor.

are shown in Table 1.

These studies showed significant differences in the composition of the intestinal microbiota between patients with B or C hepatitis with or without cirrhosis present. A healthy gut microbiota means a gut microbiota with great diversity and the ability to react to changes. Thus, B and C viruses can cause changes and can shape the gut microbiota in different directions[122].

Nowadays, the treatment of B and C hepatitis is well established by international guidelines[124-126]. The main question is: does the treatment of B or C hepatitis influence the diversity and abundance of the intestinal microbiota? And if so, are these changes helping in preventing or halting the evolution of the disease? A part of the studies looking into the microbial changes caused by HBV and HCV treatments are presented in Table 2.

Entecavir increases the abundance of the genus *Clostridium sensu stricto 1* which has been associated with large and extra-large HDL particles and also with a decreased risk of cardiovascular disease[131]. Increased lipid content in the liver and steatosis can result in the development of inflammation and, over time, cirrhosis, and can also increase oxidative stress[132]. Genus *Intestinibacter* along with genus *Escherichia, Shigella* can be considered as a major contributor to NAFLD progression. Increases in the abundance of *Intestinibacter* have been correlated with severe intestinal disorders in humans and are recognized as a biomarker of the onset of Crohn's disease[133].

In a study by Pérez-Matute *et al*[129], it was shown that the use of direct antiviral agents in patients with chronic HCV infection could only restore the intestinal bacterial changes in those patients with a lower degree of fibrosis (F0-1). The data highlight a strong relationship between the liver and the intestine and suggest that mild intestinal changes caused by liver damage could possibly be counteracted with the appropriate drugs.

Blautia, Coprococcus, Dorea, Lachnospira, Oribacterium, Roseburia and *L-Ruminococcus* were detected in the human intestine as the main genera belonging to the *Lachnospiraceae* family[134]. *Lachnospiraceae* is considered a "good" family of bacteria, having a beneficial role in host homeostasis. The bacteria belonging to this family can convert carbohydrates into SCFA in the gut[135]. Decreasing the abundance of *Lachnospiraceae* leads to decreased SCFA production and thus increases the pH of the colon. This change increases the production of ammonia and its absorption in the intestine[136].

Direct-acting antivirals (DAA) treatment in cirrhotic patients appears to have a positive impact on changes in the intestinal microbiota, as well as fibrosis and inflammation, but without a positive impact on the function of the intestinal barrier. DAA has greatly reduced the abundance of *Enterobacteriaceae, Staphylococcus*, and *Veillonellaceae*[130]. The abundance of the *Enterobacteriaceae* family, belonging to the *Proteobacteria* phylum, depends on the amount of oxygen that crosses the intestinal barrier. The abundance of *Enterobacteriaceae* is elevated after the oxygen level increases and can aggravate intestinal inflammation. Members of this family cannot degrade complex carbohydrates (as *Clostridia* and *Bacteroidia* do); they are only involved in the passive transport of oligosaccharides. This disadvantage may explain the lower abundance of *Enterobacteriaceae* compared to *Clostridia* and *Bacteroidia* in the healthy distal intestine [137]. *Veillonellaceae* belonging to *Firmicutes* phylum, is one of the main microbial taxa

Table 1 Microbiota changes in different studies regarding hepatic B and C virus

	Changes of gut microbiota in patients *vs* healthy subjects	Ref.
Type of HBV infection		
Chronic HBV infection	↓ *Bacteroidetes* and *Firmicutes*; ↑ *Proteobacteria* and *Actinobacteria*	Chen *et al* [117]
	↑ *Bifidobacterium dentium*; ↓ *Bifidobacterium catenulatum* and *longum*	Xu *et al* [118]
	↑ *Veillonellaceae*; ↓ *Lachnospiraceae, Rikenellaceae, Ruminococcaceae*	Wang *et al* [119]
HBV liver cirrhosis	↓↓↓ *Bacteroidetes* and *Firmicutes*; ↑↑↑ *Proteobacteria* and *Actinobacteria*	Chen *et al* [117]
Decompensated HBV cirrhosis	↓ *Bifidobacteria/Enterobacteriaceae ratio*; ↑ *Enterobacteriaceae*; ↓ *Firmicutes* (*F.prausnitzii, Clostridium clusters XI* and *XIVab, Bifidobacterium*); ↓ *Bacteroidetes*	Lu *et al* [120]
HBV related hepatocellular carcinoma	↓ *Proteobacteria*; ↑ *Prevotella, Phascolarctobacterium, Anaerotruncus*; ↑ *Proteus, Veillonella, Prevotella 2, Barnesiella* and *Ruminococcaceae spp.*	Liu *et al* [121]
Type of HCV infection		
Chronic HCV infection without cirrhosis	↑ *Veillonella spp., Lactobacillus spp., Streptococcus spp.* and *Alloprevotella spp.*; ↓ *Bilophila spp., Clostridium IV spp., Clostridium XIVb spp., Mitsuokella spp.* and *Vampirovibrio spp.*; No changes: *Akkermansia spp., Bifidobacterium spp., Escherichia/Shigella spp., Haemophilus spp., Micrococcus spp.* and *Weissella spp.*	Heidrich *et al*[122]
Chronic HCV infection with cirrhosis	↑↑↑ *Veillonella spp., Lactobacillus spp., Streptococcus spp.* and *Alloprevotella spp.*; ↓↓↓ *Bilophila spp., Clostridium IV spp., Clostridium XIVb spp., Mitsuokella spp.* and *Vampirovibrio spp.*; ↑↑↑ *Akkermansia spp., Bifidobacterium spp., Escherichia/Shigella spp., Haemophilus spp., Micrococcus spp.* and *Weissella spp.*	Heidrich *et al*[122]
Stage 4 HCV infection (cirrhosis)	↓ *Firmicutes*; ↑ *Prevotella, Faecalibacterium* (*F. prausnitzii*); ↑ *Acinetobacter*; ↑ *Veillonella*	Aly *et al* [123]

HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 2 Microbial changes as a result of several treatments in viral B and C hepatitis

Drug	Type of study	Changes in gut microbiota	Ref.
Entecavir	Experimental (mice)	↑ *Lachnospiraceae, Akkermansia, Alistipes, Escherichia, Shigella, Oscillibacter, Bilophila*	Li *et al*[127]
	Clinical	↑ *Clostridium sensu stricto 1, Erysipelotrichaceae UCG-007, Intestinibacter*; ↓ *Streptococcus, Atopobium*, and *Murdochiella*	Lu *et al*[128]
Direct antiviral agents in patients with HCV infection	Clinical	↑ *Phylum Firmicutes, genera Lachnospira*	Pérez-Matute *et al*[129]
Direct antiviral agents in patients with HCV-related liver cirrhosis	Clinical	↓ *Enterobacteriaceae, Staphylococcus* and *Veillonellaceae*	Ponziani *et al* [130]

HCV: Hepatitis C virus.

associated with the severity of fibrosis in non-obese patients. This family has the ability to produce propionate, one of the most important SCFAs and has been associated with chronic liver disease[138]. The LPS and SCFA metabolites produced by intestinal *Veillonella* stimulate the release of cytokines (Il-6, IL-10, TNF-α) in human peripheral blood mononuclear cells and thus have a negative impact on liver pathology and host inflammation[139].

GUT MICROBIOTA-TARGET OF TREATMENT

Although standard therapy for B and C viral hepatitis is well established and presented in clinical guidelines, many dietary supplements, including pre-, pro-, and symbiotic agents, are being studied to reduce the toxicity of standard therapy (side effects) or to increase their effect. Also, fecal microbiota transplantation (FMT) is one of the methods that can manipulate the composition of the intestinal microbiota. It has

the ability to strengthen the intestinal barrier, reduce intestinal permeability and also improve host immunity[140]. There are various routes of administration for FMT: nasogastric tube, upper endoscopy or colonoscopy, retention enema, *etc*. The route of administration depends on the characteristics of the disease. For example, good results have been obtained after duodenal administration in metabolic disease[141].

There are only a few studies that support the effect of certain probiotics in viral B or C hepatitis.

Oo *et al*[142] studied the long-term (36-mo) effect of probiotic heat-treated strain Enterococcus faecalis FK-23 in patients with HCV infection. This probiotic may change the microbiota in these patients and may have an important role of decreased ALT in serum.

In patients with HBV-induced liver cirrhosis, the role of a probiotic (*Clostridium butyricum* combined with *Bifidobacterium infantis*) has been studied in the treatment of minimal hepatic encephalopathy. The results claim that the probiotic modulates the intestinal barrier and thus can lower the level of ammonia and can improve cognition [143].

CONCLUSION

Most of the microbiota-derived components elicit an immunomodulatory effect, both pro- and anti-inflammatory. Alteration of the host microbiome produces an unbalance of these factors, leading to negative effects both locally in the intestine, as well as at distance in other organs. Therefore, we can conclude that by its factors, the host microbiota is an important determinant in the hosts immune response modulation. Future experimental and clinical studies are needed to determine the exact mechanisms of these changes, as well as the exact conditions in which the microbiota can serve as a protective factor.

Currently, the intestinal microbiota is a target of treatment for various diseases in humans. Future studies should focus on the effects and efficacy of treatments aimed at restoring the gut microbial environment (prebiotics, probiotics, symbiotics, fecal transplant) and their exact relationship with liver pathologies. By understanding the natural communication pathways between the liver and the gut, in both health and disease, we could potentially formulate better therapies aimed at reducing the effects of the chronic inflammatory response on the progression of liver diseases.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 21; 27(43): 7462-7479

DOI: 10.3748/wjg.v27.i43.7462

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

REVIEW

Hepatocellular carcinoma locoregional therapies: Outcomes and future horizons

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Author contributions: Makary MS, Ramsell S, Eric Miller E, Beal EW, and Dowell JD performed the literature review, wrote the manuscript, prepared the figures and tables, made the requested revisions, and provided final approval of the final version of the manuscript to be published.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

Country/Territory of origin: United States

Specialty type: Oncology

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

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 open-access article that was

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Abstract

Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver and has an overall five-year survival rate of less than twenty percent. For patients with unresectable disease, evolving liver-directed locoregional therapies provide efficacious treatment across the spectrum of disease stages and *via* a variety of catheter-directed and percutaneous techniques. Goals of locoregional therapies in HCC may include curative intent in early-stage disease, bridging or downstaging to surgical resection or transplantation for early or intermediate-stage disease, and local disease control and palliation in advanced-stage disease. This review explores the outcomes of chemoembolization, bland embolization, radioembolization, and percutaneous ablative therapies. Attention is also given to prognostic factors related to each of the respective techniques, as well as future directions of locoregional therapies for HCC.

Key Words: Hepatocellular carcinoma; Bland embolization; Chemoembolization; Radioembolization; Transarterial embolization; Thermal ablation; Locoregional therapy

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Core Tip: This article reviews prognostic factors and outcomes of current locoregional

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Received: April 23, 2021
Peer-review started: April 23, 2021
First decision: June 23, 2021
Revised: July 9, 2021
Accepted: October 27, 2021
Article in press: October 27, 2021
Published online: November 21, 2021

P-Reviewer: Levi Sandri GB
S-Editor: Wang JL
L-Editor: A
P-Editor: Wang JL

therapies for hepatocellular carcinoma, as well as future directions and promising new techniques. Therapies including transarterial bland embolization, chemoembolization, and radioembolization, as well as percutaneous ablation are reviewed. Prognostic considerations vary by indication but generally follow baseline disease staging and tumor quantification. Outcomes data reveal survival benefits in appropriately selected patients. New advances in precision medicine, combination therapy, and immunotherapy are being investigated.

Citation: Makary MS, Ramsell S, Miller E, Beal EW, Dowell JD. Hepatocellular carcinoma locoregional therapies: Outcomes and future horizons. *World J Gastroenterol* 2021; 27(43): 7462-7479
URL: https://www.wjgnet.com/1007-9327/full/v27/i43/7462.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i43.7462

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer globally[1] and the most common primary liver malignancy[2,3], comprising over 90% of liver tumors[4,5]. The overall prognosis of HCC involves a complex interplay of baseline clinical staging, underlying liver function, and demographic factors[6,7]. Nonetheless, the 5-year relative survival rate for primary liver cancer is estimated to be 19.6%[8], with a mean survival being reported between 6-20 mo[5]. The unfavorable prognosis of HCC highlights the importance of treatment innovation and improvement. Surgical therapy has been the traditional definitive management in eligible patients; however, fewer than 20% of HCC patients are candidates for surgical resection based on a variety of tumor and disease characteristics. For the remainder of HCC patients, liver-directed locoregional therapies form the mainstay of treatment.

Locoregional therapies play a vital role in HCC therapy across a vast range of disease stages[9]. Image-guided techniques with locoregional delivery of chemotherapeutic, radiotherapeutic, or ablative therapy are flourishing[10,11]. Minimally-invasive approaches, such as transarterial chemoembolization (TACE), transarterial embolization (TAE), transarterial radioembolization (TARE), and ablation may be indicated based on patient clinical status and tumor characteristics. Treatment goals may include bridging to or downstaging for transplant eligibility, inducing parenchymal hypertrophy to enhance function following resection, disease control and palliation, and, in some instances, cure[10]. In general, locoregional liver-directed treatments provide less morbidity than traditional surgical options while also improving outcomes compared to traditional systemic therapies[12]. This paper reviews prognostic factors and outcomes of locoregional therapies for HCC. We discuss how prognostic factors overlay the clinical staging systems most commonly used, the existent data regarding survival and treatment response, and future directions of locoregional HCC therapy.

TACE

TACE relies on a combination of targeted chemotherapeutic and embolic agents[13]. Transarterial therapies make use of a mismatch in blood flow between healthy liver parenchymal tissue and hepatocellular tumors. Unlike normal liver parenchyma, which derives most of its blood supply from the portal venous system, hepatocellular tumors are primarily perfused *via* the hepatic arterial system[14]. Thus, normal tissue is preferentially spared when therapies are targeted at tumor tissue through the hepatic arterial tree. Conventional TACE (cTACE) utilizes hepatic arterial administration of a chemotherapeutic agent emulsed with lipiodol oil to increase chemotherapeutic concentration and decrease pharmacologic washout. Chemotherapeutic and embolic agent administration *via* drug-eluting beads (DEB-TACE) has been shown to provide less systemic chemotherapy uptake, an increased ischemic effect, and a more homogenous drug distribution due to decreased variability in delivery technique[15-19]. Use of small drug-eluting microspheres (DEM-TACE) and balloon occlusion

catheters (B-TACE) represent newer approaches to chemoembolization, albeit with less comparative data in HCC treatment at this stage[20-22]. A summary of these TACE approaches, as well as approaches, clinical strengths, and risks of the other locoregional therapies discussed here can be seen in Table 1.

Prognostic factors

The staging of HCC is particularly complex due to the varying presence of accompanying liver dysfunction. Prognostic factors for HCC patients undergoing non-surgical treatment have coalesced into several existing clinical staging systems to predict survival and adverse events. Examples include the Okuda staging system, Cancer of the Liver Italian Program staging system, Hong Kong Liver Cancer staging system, and Barcelona Clinic Liver Cancer (BCLC) classification scheme[23-25]. Other clinical indices which must be considered for prognosis include Albumin-Bilirubin and Model for End-stage Liver Disease[26]. Overall survival (OS) in HCC patients is most strongly related to performance status, tumor burden, hepatic reserve, and extrahepatic spread[23,27,28].

The most widely used prognostic tool in HCC is the BCLC[29,30], which has garnered international consensus endorsement for patient treatment stratification[31]. A treatment schematic for HCC based on BCLC classification is shown in Figure 1. The BCLC staging system matches liver dysfunction, tumor burden, and performance status to a recommended therapy[27,32]. Specifically, the BCLC utilizes Child-Pugh score and Eastern Cooperative Oncology Group (ECOG) status in addition to indicators of tumor burden. BCLC stratifies patients into five stages, categorized from stage 0 for "very early detection" to stage D for the most advanced disease cases.

Patients in BCLC stage 0 and BCLC stage A should generally undergo surgical resection if they are otherwise strong surgical candidates. In certain circumstances, TACE may be used as a bridge to surgery or as primary therapy when patients in these stages are non-candidates for surgery or ablation[12,33]. TACE is a first-line therapy recommendation for intermediate, unresectable HCC (BCLC stage B)[34]. Advanced disease (BCLC stage C) patients typically require systemic therapy, traditionally in the form of sorafenib or Lenvatinib[35]. More recently, combination atezolizumab and bevacizumab has gained endorsement as first-line therapy in the American Society of Clinical Oncology's 2020 clinical practice guideline on systemic therapy[36]. When local disease control is needed for advanced disease, TACE may be indicated for use alone or in combination with systemic therapy. A current phase III study is evaluating TACE in combination with Lenvatinib and Pembrolizumab for advanced, non-metastatic disease[37]. In addition to being useful in stratifying patients to appropriate treatment, BCLC class is a useful tool in prognosticating survival following TACE, with many studies stratifying survival based on these categories.

Despite the prevalent adoption of BCLC as the gold standard staging and management decision tool for HCC, other staging tools have been investigated and are utilized in certain situations. The AFP, BCLC, Child-Pugh, and Response (ABCR) score also predict retreatment success. Specifically, ABCR uses a baseline AFP over 200, more advanced BCLC stage, increase in Child-Pugh score of at least 2 from baseline, and absence of radiologic response to create a score range of -3 to +6, correlating with survival post-TACE retreatment. A score greater than or equal to 4 prior to a second TACE treatment prognosticates poor outcomes.

Prognostic factors may also indicate risks related to post-procedural complications. Post-embolization syndrome (PES) consists of post-procedural fever in the absence of infection, transaminitis, right upper quadrant pain, and nausea or vomiting. PES is a risk common to each of the transarterial therapies. Up to 80% of patients may experience a component of PES following TACE, however rates of serious clinical sequela of PES are much lower[38]. Most cases of PES resolve within 72 h. Features predictive of an increased risk of PES following TACE are tumor > 5 cm, multiple tumors, and technical considerations relating to procedure performance[39]. Other complications of TACE include acute hepatic failure, abscess, biloma, iatrogenic dissection, and acute cholecystitis. These more serious complications are reported at a combined rate of approximately 5% of cases[40,41].

Outcomes

In addition to typical outcomes measures in oncology - such as OS, progression-free survival (PFS), and time-to-progression (TTP) - tumor response outcomes are especially important in HCC due to the complexity of such patients who often undergo multiple therapies that can confound long-term outcomes. The most widely used tool for measuring tumoral response to treatment in HCC is the 2010 modified Response Evaluation Criteria in Solid Tumors (mRECIST)[42,43]. The mRECIST

Table 1 Locoregional therapy techniques, benefits, and risks

Locoregional modality	Techniques	Clinical advantages	Clinical risks
TACE	Drug-eluting beads or conventional delivery	Provides both local embolic and chemotherapeutic effect	PES, biloma, liver abscess, liver failure
TAE	Particulate or other embolic agents	Avoids radio and chemotoxicity; less expensive than other embolotherapies	PES, biloma, liver abscess, liver failure
TARE	Y^{90} microspheres	May be used in early disease with curative intent; intermediate disease can be used to increase FLV to qualify for curative intent surgery; best QoL scores of all options	PRS, RILD, radiation-induced pneumonitis, biloma, liver abscess, liver failure
Ablation	Radiofrequency current, microwaves, or cryoablation	Efficacious as monotherapy for early-stage disease; less morbidity than transarterial therapies	PAS, iatrogenic injury, bleeding

TACE: Transarterial chemoembolization; PES: Post-embolization syndrome; TAE: Transarterial embolization; TARE: Transarterial radioembolization; FLV: Functional liver volume; QoL: Quality of life; PRS: Post-radioembolization syndrome; RILD: Radiation-induced liver disease; PAS: Post-ablation syndrome.

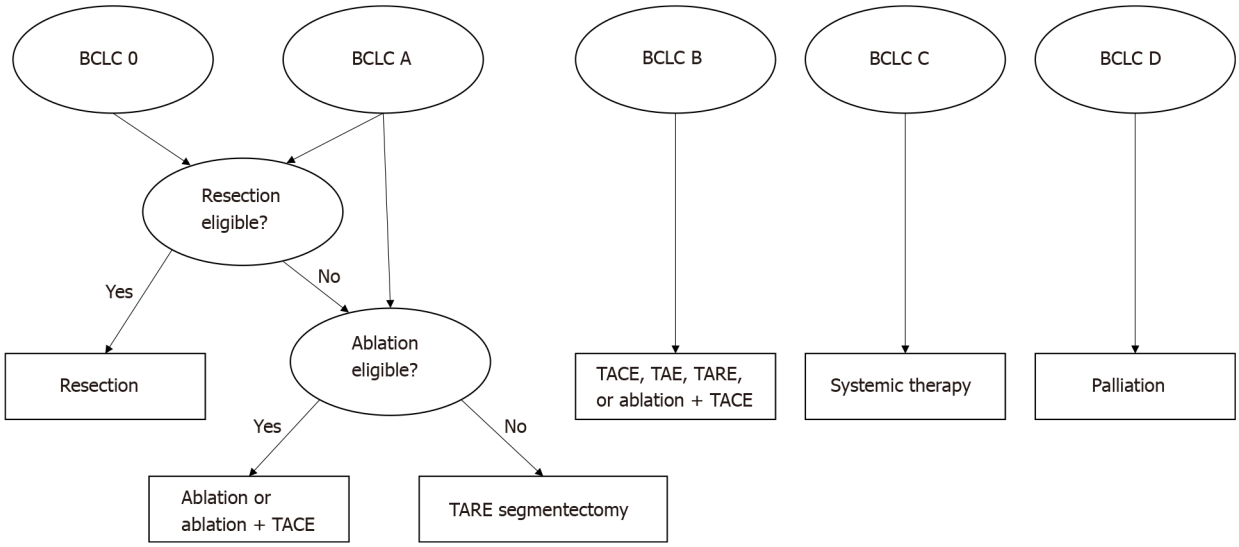


Figure 1 Hepatocellular carcinoma treatment algorithm based on Barcelona Clinic Liver Cancer-staging[13]. BCLC: Barcelona Clinic Liver Cancer; TARE: Transarterial radioembolization; TACE: Transarterial chemoembolization; TAE: Transarterial embolization.

treatment response tool builds on the traditional RECIST model of evaluating reduction in tumor size; however, because locoregional therapies induce devascularization and necrosis—and not always a reduction in size[44] - the American Association for the Study of Liver Diseases proposed mRECIST and the utilization of arterial enhancement measurements[45]. The effectiveness of these tools has been validated by a meta-analysis from Vincenzi *et al*[46]. More recently, three-dimensional imaging techniques have brought about more quantitative versions of these clinical response tools, namely volumetric RECIST and quantitative EASL (qEASL), which are being evaluated for efficacy and specific indications[47]. However, mRECIST remains the most widely used treatment response tool in practice.

Outcomes for both cTACE and DEB-TACE have consistently proven superior to conservative therapy[48,49]. Thus, as previously mentioned, TACE is to be considered for patients with advanced, unresectable disease who may not tolerate side effects of systemic therapy and who have acceptable hepatic function. This becomes especially important because systemic sorafenib has considerable toxicity, including diarrhea, weight loss, dermatitis, and hypophosphatemia[50]. Combination therapy using TACE with systemic sorafenib for both advanced and intermediate disease has been investigated. TACE induces ischemia which leads to the production of neoplastic angiogenic growth factors. The anti-angiogenic actions of sorafenib block these angiogenic factors. The GIDEON study[51] demonstrated through global observational data that patients given TACE concomitantly with sorafenib achieved better OS (21.6 mo) compared to

patients treated with sequential TACE then sorafenib (12.7 mo) and compared to patients treated with only sorafenib (9.7 mo). The concomitant treatment group's survival superiority was present across all BCLC stages. Importantly, the study calls attention to the need for further standardization of TACE technique as many centers reported logistical differences in treatment plans. The phase II SPACE trial[52] randomized intermediate stage HCC patients to DEB-TACE with sorafenib *vs* DEB-TACE with placebo treatment arms. Time to progression was similar in both treatment groups, and both options demonstrated adequate safety profiles. More recently, the phase II TACTICS trial showed a significant difference in PFS of TACE and sorafenib *vs* TACE alone (25.2 mo and 13.5 mo, respectively)[53]. Combination TACE and sorafenib also displayed significantly prolonged TTP.

TACE is also implicated in strategies for early-stage patients who need adjunctive procedures prior to surgery. In combination with portal vein embolization (PVE), TACE may induce contralateral liver hypertrophy to allow for tumor resection in patients with inadequate predicted future liver remnant (FLR). The utility of TACE in this setting is to decrease the risk of tumor progression during the period of time it takes PVE to induce FLR hypertrophy. A systematic review and meta-analysis concluded that TACE combined with PVE provided higher OS than PVE, portal vein ligation (PVL), or radioembolization of the portal vein alone[54]. Of patients receiving both TACE and PVE, 90% went on to receive resection. These strategies may be further explored to increase liver resection eligibility rates in the future. Bridging or downstaging patients for liver transplantation is another use for TACE in early-stage patients[5].

Several prospective studies comparing cTACE *vs* DEB-TACE have found no significant difference in OS, including one meta-analysis examining results from four randomized clinical trials and eight observational studies[48,49,55]. Beyond survival, the PRECISION-V trial demonstrated that in a subgroup of advanced HCC patients with Child-Pugh B, ECOG 1, bilobar, and recurrent disease, patients receiving DEB-TACE had higher rates of complete response, objective response, and disease control at 6 mo compared to patients receiving cTACE[48]. Concerning safety endpoints, DEB-TACE was originally theorized to provide fewer adverse events and a lower risk of post-embolization syndrome characteristics; however, the PRECISION-V trial found comparable 30-day adverse event incidence between the two groups[48]. Comparable safety profiles between cTACE and DEB-TACE were upheld through meta-analysis as well[55]. Observationally, DEB-TACE has displayed higher localized biliary injury rates and global hepatic damage[56]. In another randomized trial, Golfieri *et al*[49] found that DEB-TACE patients suffered less post-operative pain. The overall comparative safety and efficacy of cTACE *vs* DEB-TACE needs further exploration and likely has significant situational considerations which must be applied. Other areas of TACE outcomes that warrant further investigation include more rigorous standardization of cTACE protocols[57], the efficacy and considerations of TACE in portal vein thrombosis, and TACE for larger (> 5 cm) or multifocal lesions[58]. Primary outcomes for TACE, as well as the other forms of locoregional therapy discussed here, can be viewed in Table 2.

TAE

TAE is commonly referred to as "bland" embolization because the embolic particles lack additional chemical or radiation components. With this therapy, endovascular arterial occlusion induces hypoxia and subsequent death of tumor cells[59]. As with other transarterial therapies, optimal vascular catheter placement results in a maximally selective effect on tumor cells. Care must be taken to avoid arterial-venous shunts which could cause pulmonary arterial embolization with smaller particles. Embolic agents used have historically included gel foam, polyvinyl alcohol, and various drug-eluting beads[45]. The lack of chemotoxicity and radiotoxicity associated with this therapy means that tumoral response is primarily derived from a hypoxic cell death mechanism. It also means that the therapy may hold advantages in certain patients who have an especially high need to spare healthy liver parenchyma.

Prognostic factors

BCLC class B patients receive the most disease-control benefit from TAE, followed by class C patients[48]. Additionally, BCLC class A patients may undergo TAE to maintain eligibility for transplantation per the Milan criteria, a prognostic tool shown to improve 4-year OS in liver transplant when used for patient selection[60]. The Milan

Table 2 Summary of primary outcomes of locoregional therapies for hepatocellular carcinoma

Locoregional technique	Primary outcomes
TACE	TACE provides a survival benefit compared to supportive care in unresectable disease[34]. Concomitant TACE and sorafenib is superior to standalone therapy for unresectable disease[51-53]. Comparisons of DEB-TACE versus cTACE have yet to reveal significant differences in OS and short and long-term complication rates. Further studies are needed for considerations in more specific circumstances[48,49]. When combined with PVE, TACE provides more robust FLR increase and results in better survival compared to monotherapy strategies to enhance FLR[54].
TAE	TAE provides a survival benefit compared to supportive care in unresectable disease[34]. Early data of chemoembolization has shown little survival benefit over TAE, but superior proximate outcomes such as TTP and tumor response compared to TAE[63,64].
TARE	TARE shows similar complication and survival rates to TACE, while producing higher QoL scores and longer TTP[77,78]. TARE segmentectomy for early-stage disease (tumors < 3 cm) results in a 5-year survival of 75%, which is comparable to curative intent therapies such as transplantation and surgical resection[81]. TARE lobectomy provides a significant increase in FLR and is a safe mechanism to treat tumor while inducing contralateral hypertrophy[82-86].
Ablation	In early-stage patients, standalone percutaneous ablation produces comparable survival outcomes to surgical resection[113-116]. RFA and MWA techniques show similar outcomes in early-stage disease (tumor < 3 cm)[108,119]. Combination therapy using TACE and ablation (particularly MWA) provide the best outcomes for large tumors (tumor 3-5 cm)[120].

TACE: Transarterial chemoembolization; DEB-TACE: Drug-eluting bead chemoembolization; cTACE: Conventional transarterial chemoembolization; OS: Overall survival; PVE: Portal vein embolization; FLR: Future liver remnant; TAE: Transarterial embolization; TTP: Time-to-progression; TARE: Transarterial embolization; QoL: Quality of life; RFA: Radiofrequency ablation; MWA: Microwave ablation.

criteria consists of one lesion less than 5 cm or up to three lesions less than 3 cm, no evidence of extrahepatic manifestation, and no evidence of vascular invasion. Similarly, TAE is an option for downstaging of BCLC class B patients for transplantation. TAE is contraindicated in patients with severe comorbidities or with poorly preserved liver function as evidenced by Child-Pugh scores of B8 or higher, untreated esophageal varices, elevated markers of liver function, creatinine clearance < 30 mL/min, and high tumor burden[61].

Prognostically, a key advantage of TAE relative to other transarterial options is its gentler impact on short-term adverse events, possibly due to the avoidance of chemotherapy toxicity. As with other embolotherapies, bland embolization poses risk for PES. Agrawal *et al*[62] reported a higher incidence of PES among patients undergoing TACE (74.7%) compared to TAE (68.7%). PES following TACE resulted in a significantly longer hospital stay than PES following TAE (1.47 d *vs* 1.12 d). This observational study further identified that, in addition to the PES risk factors mentioned in the previous section, more patients who were female or who had alcohol-related HCC developed PES.

Outcomes

Like other embolotherapies, TAE offers a survival benefit compared to conservative treatment. Llovet *et al*[34] found that compared to best supportive care, repeated administration of either TACE or TAE showed a survival benefit in patients with unresectable HCC. While TACE provided even higher survival probabilities than TAE in the study, both therapies performed better than conservative treatment. Comparison of bland embolization and chemoembolization is an ongoing focus of research. Despite some data suggesting TACE's superiority to TAE, multiple studies investigating bland embolization compared to either cTACE or DEB-TACE have failed to demonstrate significant differences in OS between the two[63-65]. Importantly, much of the early data accumulated on the comparative efficacy of TACE and TAE was collected during a period of evolving indications and chemotherapeutic protocols for TACE. Furthermore, the development of DEB-TACE may continue to influence overall comparisons of bland embolization and chemoembolization.

Some studies have shown benefits to TACE compared to TAE in more proximate outcomes such as TTP, tumor recurrence, and local response. For example, a trial by Meyer *et al*[65] found insignificant differences in median OS and PFS among TAE and cTACE, but found a significantly greater mRECIST treatment response in cTACE *vs* TAE (47.3% *vs* 67.4% respectively). However, because several studies have shown TAE performing comparably to TACE in terms of survival, and because TAE lacks utilization of chemotherapy particles, TAE may be better tolerated in HCC patients with borderline liver function[45].

TARE

TARE, also referred to as selective internal radiotherapy (SIRT) and Y^{90}, uses a radioisotope form of yttrium to selectively target tumor cells *via* the hepatic arterial tree[66]. The Y^{90} radioisotope is delivered using microspheres and, once reaching target tissue, undergoes beta decay to locally irradiate the tumor in a continuous, low-dose fashion over a fourteen-day period. TARE is considered a two-stage treatment process because a planning arteriography must be performed one to two weeks before the radiation-delivering procedure[67]. This planning stage helps to differentiate tumor and hepatic arterial supply, isolate the future path of radiation delivery *via* embolization of extrahepatic vessels at risk of nontarget microsphere delivery, and identify the degree of hepatopulmonary shunting[68]. Technetium-99m labeled macroaggregated albumin is combined with single photon emission computed tomography imaging technology to provide imaging for this stage[69]. After TARE, there is a longer wait time compared to other embolization techniques until the treatment effect is fully realized, with therapy response imaging taking place 3-6 mo following the procedure[70].

Prognostic factors

Characteristics most predictive of post-TARE prognosis are extrahepatic disease, baseline BCLC stage, ECOG performance status, and tumor burden[71]. In a multicenter study, Sangro *et al*[72] analyzed a cohort of 325 patients undergoing TARE. Median OS was strongly influenced by BCLC staging (BCLC A 24.4 mo; BCLC B 16.9 mo; BCLC C 10.0 mo). Other significant predictors of superior survival following TARE were ECOG performance status (ECOG 0), Child-Pugh class (A), absence of ascites, baseline total bilirubin (< 1.5), number of tumor nodules (< 5), alpha-fetoprotein level (< 400), patent portal vein, single lobe disease, and absence of extrahepatic disease.

Understanding the influence of the pre-procedural disease stage on post-TARE survival is important because TARE maintains indications across the spectrum of HCC severity. Patients with advanced disease may benefit from the local tumor control and palliative effects of TARE. TARE is an acceptable treatment alternative to TACE for first-line therapy for BCLC class B patients[73]. Early-stage patients in BCLC classes 0 and A may benefit from TARE radiation segmentectomy. Early-stage patients may also benefit from TARE lobectomy in an attempt to either downstage for transplantation or induce contralateral hypertrophy for future resection.

Common complications of TARE are well characterized. Radiation-induced liver disease (RILD) is an adverse event unique to TARE among the locoregional therapies. RILD involves an extensive array of local vascular, fibroblastic, and parenchymal change[74]. Risk of RILD may be increased by gemcitabine, which must be held for four weeks prior to the procedure[75]. Padia *et al*[76] report the overall risk of RILD following TARE to be 1%-4%. They also report rates of other common adverse events, including GI ulcers (0%-5%), PES requiring extended hospitalization or readmission (1%-2%), iatrogenic dissection (1%), and death within 30 d (2%). There was a less than 1% reported rate for radiation-induced skin-injury, radiation pneumonitis, radiation-induced pulmonary fibrosis, biloma requiring drainage, and abscess. A hepatopulmonary fraction above 20% predicts an increased likelihood of radiation pneumonitis. Relative contraindications to TARE include an elevated baseline bilirubin level (> 2 mg/dL), an elevated hepatopulmonary fraction (> 20%), Child-Pugh class C, ECOG score over 2, significant transaminitis (ALT or AST > 5x upper limit of normal), and total tumor burden over 70% of the liver or total tumor burden over 50% with a high number of nodules[12]. Of these variables, elevated baseline bilirubin and increased tumor burden have been shown to decrease OS[18].

Outcomes

TARE appears to have comparable complication and survival rates to TACE. The SIRTACE trial compared TARE *vs* TACE in unresectable HCC and found that a single TARE session was as safe and produced a better quality of life (QoL) change than multiple TACE sessions[77]. More recently, the PREMIERE trial revealed a significantly longer median TTP for patients receiving TARE (> 26 mo) compared to cTACE (6.8 mo). A smaller randomized trial compared QoL measures between TACE and TARE, finding that patients treated with TARE had improvements in QoL despite being treated for more severe disease than the TACE cohort. In contrast, the TACE cohort had worsened QoL post-procedurally[78]. Both TACE and TARE are being investigated to identify the optimal transarterial therapy for downstaging tumors for

transplantation eligibility. Lewandowski *et al*[70] compared triple-drug cTACE to TARE in their ability to downstage tumor size from UNOS T3 to UNOS T2 to achieve eligibility. TARE significantly outperformed cTACE in rates of T2 achievement, event-free survival, and OS.

In addition to sustaining or achieving transplantation eligibility, TARE is also useful in early-stage disease for its ability to act as primary therapy in certain circumstances. TARE's effectiveness in early-stage disease is in part due to its evolution into more selective indications for earlier tumors *via* radiation segmentectomy[79]. First described in 2011[80], TARE segmentectomy is an alternative option in non-surgical candidates whose tumor anatomy discourages ablative techniques due to nearby high-risk structures[33]. For example, in a retrospective study, Lewandowski *et al*[81] analyzed 70 patients with similar inclusion criteria with the additional exclusion of patients who received secondary surgery. Median OS in this cohort was found to be 6.7 years with a median TTP of 2.4 years. The cohort had comparable five-year OS (75%) and response rates to other curative-intent treatments like ablation, resection, and transplantation.

Similar to segmentectomy, radiation lobectomy is a relatively novel application of TARE. Its primary use is to treat the tumor-occupied lobe while inducing hypertrophy of the contralateral lobe, thus increasing the FLR in patients who were deemed unresectable due to low FLR[12]. Scarring of the treated lobe slowly creates a shunting of blood to the contralateral portal vein and, over time, induces hypertrophy of that lobe[82]. Multiple observational studies show that TARE lobectomy increases the FLR by an approximate average of 30% from baseline[83-86]. In contrast to TACE treatment to increase FLR, TARE lobectomy does not require concomitant PVE. A comparison of standalone PVE and TARE lobectomy by Garlipp *et al*[84] has proven that, while both display significant increases in FLR, PVE does it more effectively at the 6-wk mark (61.5% *vs* 29%). Issues have been raised with this measurement, however, as PVE has been shown to increase FLR quicker than radiation lobectomy[86], and as some evidence suggests that PVE may actually induce mild growth of existing tumor tissue [87]. The safety of using radiation lobectomy as a strategy to qualify for and subsequently undergo resection was demonstrated in a prospective cohort studied by Gabr *et al*[88]. Among 25 patients receiving major hepatic resection and 6 patients receiving partial hepatectomy, a range of perioperative outcomes following resection were comparable to resection-only outcomes. Survival rates at one and three years was reported at 96% and 86%, respectively.

TARE is also being compared to sorafenib as monotherapy in advanced disease. The phase III SARAH trial[89] randomized 467 patients with intermediate-stage, unresectable HCC to either sorafenib or TARE. Median OS and median PFS were comparable; however, TARE showed significantly fewer treatment-related adverse events, higher QoL scores, and a higher treatment response rate than sorafenib. The SIRveNIB trial[90] was another phase III study that failed to show significant differences in survival between TARE and sorafenib, but which also demonstrated the improved toxicity profile of TARE. Further trials are needed to better power subgroup analyses of TARE *vs* sorafenib and define specific patients who may see the improved tumor response of TARE translated into improved survival over sorafenib[91]. TARE is also useful in advanced disease because it has relatively less embolic activity compared to other transarterial therapies. This becomes useful in the setting of portal vein thrombus (PVT) of unresectable HCC patients. A retrospective study of HCC patients with PVT compared OS between those treated with TARE and those treated with sorafenib. TARE led to significantly longer median OS (26.2 mo) than sorafenib (8.7 mo)[92].

TARE and sorafenib combination therapy for patients ineligible for TACE but with BCLC classes B and C disease was investigated in the SORAMIC trial[93]. Again, no significant differences in median OS were found; however, survival benefit was found with combination therapy among patients without cirrhosis, with cirrhosis of nonalcoholic etiology, and in patients younger than 65 years of age. The phase III STOP-HCC trial is a larger study. It is expected to evaluate further what specific subset of patients may benefit most from combination therapy with TARE and systemic therapy. It is expected to be complete in September 2022[94]. Further, with the development of additional effective systemic therapies for HCC including immuno-therapy and most recently, atezolizumab/bevacizumab, the combination of TARE with these agents is a potential area of synergy and an active area of clinical investigation[95,96].

ABLATION

Generally, ablation is recommended for early-stage, small tumors (up to 3 cm) in patients who would otherwise qualify for resection but are considered unsuitable candidates for surgery[73]. Percutaneous ablative techniques were originally centered around ethanol injection (PEI), however this has fallen out of favor when a patient is a strong candidate for more contemporary ablative techniques[10]. Today, commonly used ablative techniques in the setting of HCC include microwave ablation (MWA), radiofrequency ablation (RFA), and cryoablation (CA). RFA utilizes a radiofrequency electrode to deliver an alternating electric current (460-500kHz) to the target lesion. Frictional heating, necrosis, and cell death ensue. MWA utilizes a common final cell death pathway involving local heating and eventual cell death, however MWA heats tissue *via* an oscillating microwave field (915/2450 MHz). The properties of microwaves result in reduced heat sink effect compared to RFA. RFA has the ability to decrease unwanted energy delivery to nearby structures compared to MWA[97]. However, due to less heat-sink, MWA may perform better near large vessels, in patients with comparatively larger tumors (between 3 cm and 5 cm), and in patients with multiple nodule disease[98]. CA relies on argon and helium gasses to rapidly alternate freezing and thawing of local tissue and vascular structures[99]. CA is not as commonly used due to the complication profile[100]. Laser ablation and irreversible electroporation are two examples of newer therapies still under investigation[10].

Prognostic factors

Prognostic factors for ablative therapy follow general prognostic patterns for HCC. Across multiple studies examining prognostic factors of RFA, survival has been consistently and independently predicted by Child-Pugh classification, tumor size, and tumor number[101-104]. Long-term survival following MWA is predicted by similar factors. Three-year PFS following MWA can range from 27% to 91.7%, with heavy influence from the above clinical characteristics[105]. Prognostic factors for combination therapy of MWA with TACE were well characterized by Ni *et al*[106] Predictably, adjusted prognostic factors associated with better OS rates of MWA with TACE combination therapy were earlier BCLC stage, smaller tumor size, lack of portal vein thrombus, MWA therapy times, and targeted drug usage.

Complication risk of biloma following percutaneous ablation may be predicted by comparatively large lesions situated closer to major bile ducts or near the hilum[107]. Additional characteristics prognostic of increased complication risk include tumor volume, ablated tissue volume, multiple tumors, and Child-Pugh class B or above [108]. In general, however, ablation carries less morbidity than other curative therapies due to its less invasive nature, coagulative properties related to heating tissue, liver preservation, and shorter hospital stay[109,110]. Bertot *et al*[111] found a pooled major complication rate of 3.29% for RFA, PEI, and MWA across 34 randomized trials and observational studies.

A complication unique to ablation among the locoregional therapies is post-ablation syndrome (PAS). PAS is a transient, flu-like illness which may occur about three days following ablation and lasts an average of five days. PAS occurs in roughly 25%-35% of patients undergoing ablation and is correlated with the volume of liver tissue treated. Pre-procedural tumor volume and post-procedural rise in AST are predictive of an increased likelihood of PAS[112].

Outcomes

Studies have demonstrated comparable survival *via* ablation *vs* surgery in early-stage HCC, despite ablative patients usually having poorer baseline hepatic function. In 2006, percutaneous thermal ablation was compared to resection in a randomized trial of 105 patients[113]. Results showed nonsignificant differences in complete tumor elimination rates, time to first recurrence, and disease-free and OS rates at 1, 2, and 3-year follow-ups. More recently, Fang *et al*[114] showed through a randomized trial of 120 patients that RFA had a similar complete remission rate (95%) to surgical resection (96.7%) and similar disease-free and OS rates at years 1, 2, and 3 follow-ups. In addition, RFA demonstrated significantly better hepatic function at day-7 post-treatment and fewer post-operative complications. The trend of comparable ablative survivability does not always extend to patients meeting Milan criteria. A trial that randomized HCC patients meeting Milan criteria to either RFA or surgical resection found significantly lower OS and recurrence-free survival along with higher overall recurrence among RFA patients[115]. As shown by the STORM trial, curative-intent ablation is best as a standalone therapy, without the addition of adjuvant sorafenib

following the procedure[116]. CA has largely fallen out of favor due to the severity of complications despite similar performance to other ablative techniques. A meta-analysis including a total of 433 total HCC patients revealed significantly fewer complications and less local tumor recurrence in RFA compared to CA[117].

As mentioned previously, the two ablative techniques most common in current practice are RFA and MWA, which are a source of ongoing outcomes comparison[118] and which feature similar curative-intent indications in early-stage disease (up to three tumor nodules smaller than 3 cm with the absence of extrahepatic disease) and similar complication rates[108]. A meta-analysis looking at seven studies comparing RFA and MWA found comparable rates of complete response, local recurrence, major complications, and 3-year survival[119].

HCC tumors between 3-5 cm fall outside the purview of curative-intent ablation but may still be addressed by combination therapy with TACE and ablation[9]. Comparison of the best combination therapies for specific indications in this population is ongoing. For example, Sheta *et al*[120] compared cTACE alone, combined MWA with cTACE, and combined RFA with cTACE in a clinical trial of 50 patients with nonresectable, single-lesion HCC greater than 4 cm. They found the highest success rates in the combined MWA with the cTACE group and the lowest success rates in the cTACE alone group. Whereas combination therapy of TACE with MWA may be indicated for 3-5 cm HCC, combination therapy of TACE with RFA may serve a role in the treatment of early-stage HCC. Kim *et al*[121] found that combined cTACE with RFA provided decreased local tumor progression and better PFS at 1, 3, and 5-year follow-ups. OS at follow-up intervals, however, was similar.

FUTURE DIRECTIONS

The future of HCC therapy will likely rely on a combination of the current proven standards of care and several other promising areas of innovation. One such promising area of growing evidence for HCC is immunotherapy. In addition to its use as a second-line monotherapy agent, immunotherapy may augment the effects of sorafenib and locoregional therapy in HCC. Locoregional therapies produce an immune response that can be augmented *via* immune checkpoint inhibition. Given that prognosis in HCC is correlated with T-cell tumoral infiltration[122], potentiation of both tumoral and locoregional therapy-induced T-cell response could improve outcomes. In an early-phase trial, Duffy *et al*[123] safely treated HCC with tremelimumab (anti-CTLA4) combined with ablation and showed that the post-procedural immune response could be enhanced. The effects of combined anti-PD1 inhibitors and TARE or TACE are also being evaluated[124]. The ongoing phase II DEMAND trial evaluates first-line combination therapy of systemic anti-angiogenic and immunotherapy while reserving TACE as second-line therapy[125].

As with immunotherapy, precision medicine has potential to create a paradigm shift in the way HCC patients are treated. By relying on big data and genomics to personalize clinical care, precision medicine will allow further customization of HCC treatment plans across the spectrum of therapeutic modalities, based on an individual's genetic mutations, local tumor environment, and further stratification of many clinical factors already in use today. Precision medicine will likely feature small molecule inhibitors, epigenetic regulators, and monoclonal antibodies specific to an individual's disease. A number of these agents are currently being evaluated for both safety and efficacy in advanced disease[7]. The success of these agents in treating HCC is reliant upon accurate characterization of multiple carcinogenic molecular pathways - including mutations to TERT, Wnt/ß-catenin, P53, Akt/mTOR, VEGFR, and EGFR genetic pathways. Ideally, molecular therapy will target multiple genetic pathways within the same patient and will be combined with other therapies such as locoregional treatment to optimize OS.

As treatments such as immunotherapy and molecular therapy evolve and become integrated with current standards of care, further prognostic sophistication is a priority for immediate improvement of care. This growth is already underway. In some cases, pre-procedural evaluation of inflammatory markers may provide prognostic information for HCC patients treated with locoregional therapy. A meta-analysis of 22 studies showed poorer OS following TACE in HCC patients with higher C-reactive protein levels, neutrophil to lymphocyte ratio, and platelet to lymphocyte ratio[126]. This is in line with the general understanding of inflammation being tumor-protective. More research is needed to routinely integrate inflammatory markers into the larger prognostic landscape of clinical staging systems for locoregional HCC

treatments.

With the numerous advancements within HCC treatment, outcomes research must continue to be robust. With the need for increased outcomes research related to locoregional therapies comes a call for increased reporting of randomized controlled trial data. Grégory *et al*[127] found that nearly two-thirds of RCTs conducted regarding HCC treatment with TACE did not yield public results. This highlights the importance of increased data reporting as evidence and indications behind various locoregional therapies for HCC continue to mature. For the care of HCC patients to continue to improve, and for future directions of care such as personalized medicine and immunotherapy to flourish, high-quality outcomes data must be generated and distributed throughout the field.

CONCLUSION

HCC is the most common primary liver malignancy[2] and carries a 5-year survival rate under 20%. Organ transplant availability and eligibility is limited, and fewer than 20% of HCC patients are candidates for surgical resection. For the remainder of patients with HCC, liver-directed, locoregional therapies serve a growing purpose across a spectrum of disease stages. Transarterial and ablative procedures are involved in treatment for curative-intent, disease control, bridging to surgery, downstaging for future treatment, and palliation. In addition to bland embolization, TAE techniques with locoregional delivery of radioactive or chemotherapeutic microspheres offer survival benefits in appropriately selected patients. Microwave and radiofrequency ablative techniques offer comparatively less morbidity and curative results in select early-stage patients. Multiple indications exist for various locoregional therapies in the adjunctive realm of transplantation, resection, and systemic therapy. Prognostic considerations for locoregional therapies vary by indication but generally follow baseline disease staging and tumor quantification. Outcomes data reveal that locoregional therapies provide survival benefits in appropriately selected patients. New advances in precision medicine, combination therapy, and immunotherapy are being investigated and have potential to augment available treatment strategies.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 21; 27(43): 7480-7496

DOI: 10.3748/wjg.v27.i43.7480

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

MINIREVIEWS

Recent advances in artificial intelligence for pancreatic ductal adenocarcinoma

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Author contributions: Hayashi H and Baba H conducted the topic investigated in this paper; Uemura N, Matsumura K, Zhao L, Sato H, Shiraishi Y and Yamashita YI assisted in the useful discussions and wrote the manuscript; all authors contributed to the article and approved the submitted version.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

Country/Territory of origin: Japan

Specialty type: Gastroenterology and hepatology

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

Peer-review report's scientific quality classification

Grade A (Excellent): 0

Grade B (Very good): B, B, B

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) remains the most lethal type of cancer. The 5-year survival rate for patients with early-stage diagnosis can be as high as 20%, suggesting that early diagnosis plays a pivotal role in the prognostic improvement of PDAC cases. In the medical field, the broad availability of biomedical data has led to the advent of the "big data" era. To overcome this deadly disease, how to fully exploit big data is a new challenge in the era of precision medicine. Artificial intelligence (AI) is the ability of a machine to learn and display intelligence to solve problems. AI can help to transform big data into clinically actionable insights more efficiently, reduce inevitable errors to improve diagnostic accuracy, and make real-time predictions. AI-based omics analyses will become the next alterative approach to overcome this poor-prognostic disease by discovering biomarkers for early detection, providing molecular/genomic subtyping, offering treatment guidance, and predicting recurrence and survival. Advances in AI may therefore improve PDAC survival outcomes in the near future. The present review mainly focuses on recent advances of AI in PDAC for clinicians. We believe that breakthroughs will soon emerge to fight this deadly disease using AI-navigated precision medicine.

Key Words: Pancreatic cancer; Pancreatic ductal adenocarcinoma; Artificial intelligence; Machine learning; Precision medicine

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Core Tip: Pancreatic ductal adenocarcinoma (PDAC) remains the most lethal type of cancer. Artificial intelligence (AI) is the ability of a machine to learn and display

Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

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Received: April 3, 2021
Peer-review started: April 3, 2021
First decision: July 3, 2021
Revised: August 2, 2021
Accepted: November 15, 2021
Article in press: November 15, 2021
Published online: November 21, 2021

P-Reviewer: Hameed MM, Moldogazieva NT, Santos-García G, Surlin VM
S-Editor: Gao CC
L-Editor: A
P-Editor: Gao CC



intelligence to solve problems. AI can help to transform big data into clinically actionable insights more efficiently, reduce inevitable errors to improve diagnostic accuracy, and make real-time predictions. AI-based omics analyses should be the next alternative approach to improve survival outcomes in PDAC by discovering biomarkers for early detection, molecular/genomic subtyping, treatment guidance, and predicting recurrence and survival. The present review mainly focuses on recent advances of AI in PDAC for clinicians.

Citation: Hayashi H, Uemura N, Matsumura K, Zhao L, Sato H, Shiraishi Y, Yamashita YI, Baba H. Recent advances in artificial intelligence for pancreatic ductal adenocarcinoma. *World J Gastroenterol* 2021; 27(43): 7480-7496
URL: https://www.wjgnet.com/1007-9327/full/v27/i43/7480.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i43.7480

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) stands the most life-threatenning type of cancer[1]. The recent 5-year survival rate for PDAC in all stages is 8.5% according to American Cancer Society statistics 2017. In patients with early-stage diagnosis, the 5-year survival rate for can be as high as 20%. During the past ten years, median overall survival (OS) has improved from 22.1 mo to 35 mo in resectable PDAC, considerably owing to improvements in adjuvant therapies[2-5]. These findings suggest that early diagnosis plays a pivotal role in the prognostic improvement of PDAC cases. Furthermore, the high recurrence rate, even in patients who have undergone curative resection, and chemoresistance to the current systemic chemotherapies (FOLFIRINOX: 5-fluorouracil, folinic acid, irinotecan, and oxaliplatin; and GnP: Gemcitabine plus nab-paclitaxel)[6,7] are major issues. Based on recent advances in genetic analysis, PDACs have been divided into several molecular subtypes[8-11], which is a prelude of precision medicine. Genetic and molecular profiling researches have revealed that up to 25% (range 12%-25%) of PDACs maintained actionable molecular alterations. Actually, matching to relevant molecular-specific treatments improves the OS compared to that of those without actionable mutations or those who do not receive molecular-specific therapy[12]. The comprehensive biomedical data has led to the dawn of the "big data" era in the medical field[13].

To overcome this deadly disease, how to well utilize big data is a next step for physicians and researchers physicians in the era of precision medicine. The main issue for physicians has shifted from gathering data to competently analyzing it. Artificial intelligence (AI) is the ability of a machine to learn and display intelligence to solve problems (Figure 1)[14]. An artificial neural network (ANN) can imitate the human neural meridian system. It is divided into three parts: Input layer, hidden layer, and output layer. "Deep learning" refers to an ANN with multiple hidden layers. Machine learning helps researchers spend less time on data processing. The processess for employing machine learning generally contain the following: Gathering the basic data, separating the data into an experimental group and a verification group, buildinging a screening and processing model, inputting the experimental group data into the model, accounting the output results, and confirming the model's workability using the verification group. The verification group can be employed to examine the sensitivity and specificity of the experimental group, while the experimental group can fabricate more intelligent model. An overview of types in AI is provided in the Supplementary material. Chen *et al*[15] developed a survival prediction model of non-small cell lung cancer patients through the use of ANN. AI has also been applied to tackle and manage the recent coronavirus disease 2019 crisis in many areas, including screening, diagnosis, severity stratification, mortality prediction, and epidemiology controls[16].

In the age of precision medicine, AI can support to convert big data into clinically actionable perception more conveniently, reduce the inevitable errors to improve diagnostic accuracy, and make real-time predictions[17,18]. Due to latest breakthroughs, the demand of AI in cancer treatment has been swiftly increasing, including for PDACs[19-21]. Recent studies have demonstrated considerable potential for AI application in PDAC care (Table 1). Advances in AI for PDACs may thus be the

Table 1 Comprehensive list of artificial intelligence-based investigations in pancreatic ductal adenocarcinoma

Ref.	Modality	Type of algorithm	Sensitivity (%)	Specificity (%)	ROC-AUC (or accuracy %)
PDAC risk prediction					
Boursi *et al*[25], 2021	7 clinical variables	Logistic regression	66.53	54.91	0.71
Appelbaum *et al*[29], 2021	18 risk factors	Logistic regression	NA	NA	0.71
Muhammad *et al*[30], 2018	Personal health data (18 features)	ANN	80.7	80.7	0.85
Hsieh *et al*[28], 2018	ICD-9 code	Logistic regression	NA	NA	0.727
Boursi *et al*[26], 2017	10 clinical variables	Logistic regression	44.7	94	0.82
Cai *et al*[27], 2011	5 clinical variables	Logistic regression	NA	NA	0.72
Early diagnosis of PDAC					
Zhang *et al*[34], 2020	Nine-gene signature	Support vector machine	98.65	100	93.3
Zhang *et al*[83], 2020	CT	DCNN	83.76	91.79	0.9455
Si *et al*[42], 2021	CT	Fully end-to-end deep learning	86.8	69.5	0.871
Liu *et al*[54], 2020	CT	CNN	79 (United States)	97.6 (United States)	0.920 (United States)
Ma *et al*[84], 2020	CT	CNN	98.2	91.6	95
Chu *et al*[85], 2019	CT	Deep learning (details are NA)	94.1	98.5	NA
Liu *et al*[53], 2019	CT	CNN	NA	NA	0.9632
Tonozuka *et al*[86], 2021	EUS	CNN	90.2	74.9	0.924
Ozkan *et al*[87], 2016	EUS	ANN	83.3	93.3	87.5
Săftoiu *et al*[88], 2015	EUS	ANN	94.64	94.44	NA
Zhu *et al*[63], 2013	EUS	Support vector machine	92.52	93.03	NA
Zhang *et al*[62], 2010	EUS	Support vector machine	94.32	99.45	NA
Das *et al*[61], 2008	EUS	ANN	93	92	0.93
Săftoiu *et al*[89] 2008	EUS elastography	NN	91.4	87.9	89.7
Norton *et al*[60], 2001	EUS	NN	73	NA	83
Alizadeh Savareh *et al*[40], 2020	Circulating microRNA signatures	PSO + ANN + NCA	93	92	93
Urman *et al*[90], 2020	Bile juice	NN	88	100	0.98
Pancreatic fistula after pancreaticoduodenectomy					
Kambakamba *et al*[71], 2020	CT	*k*-NN, random forest classifier, etc	96	98	0.95
Mu *et al*[72], 2020	CT	CNN	86.7	87.3	0.89
Pathological tumor response to neoadjuvant chemotherapy					
Watson *et al*[80], 2020	CT and CA19-9	CNN	NA	NA	0.785
Survival model					
Zhang *et al*[77], 2020	CT	CNN	NA	NA	11.81% in IPA
Alizadeh Savareh *et al*[40], 2020	Circulating microRNA signatures	PSO + ANN + NCA	NA	NA	NA
Kaissis *et al*[66], 2019	MRI	Random forest	87	80	0.90
Walczak *et al*[79], 2017	14 clinical variables	ANN	91	38	0.6576

Molecular subtype					
Kaissis *et al*[68], 2020	CT	Random forest	84	92	0.93
Tumor subtype (QM *vs* non-QM)					
Kaissis *et al*[67], 2019	MRI	Gradient boosting decision tree	90	92	0.93
Molecular subtype (KRT81 positive *vs* negative)					
Microsatellite instability status					
Li *et al*[19], 2020	PreMSIm (15-gene signature)	*k*-NN	85	97	95

AI: Artificial intelligence; PDAC: Pancreatic ductal adenocarcinoma; NA: Not available; ROC-AUC: Area under the receiver operating characteristic curve; ICD-9: International Classification of Diseases 9th Revision; ANN: Artificial neural network; CT: Computed tomography; DCNN: Deep convolutional neural network; EUS: Endoscopic ultrasound; NN: Neural network; CA19-9: Carbohydrate antigen 19-9; IPA: Index of prediction accuracy; MRI: Magnetic resonance imaging; QM: Quasi-mesenchymal; PSO: Particle swarm optimization; NCA: Neighborhood components analysis; *k*-NN: *k*-Nearest neighbor.

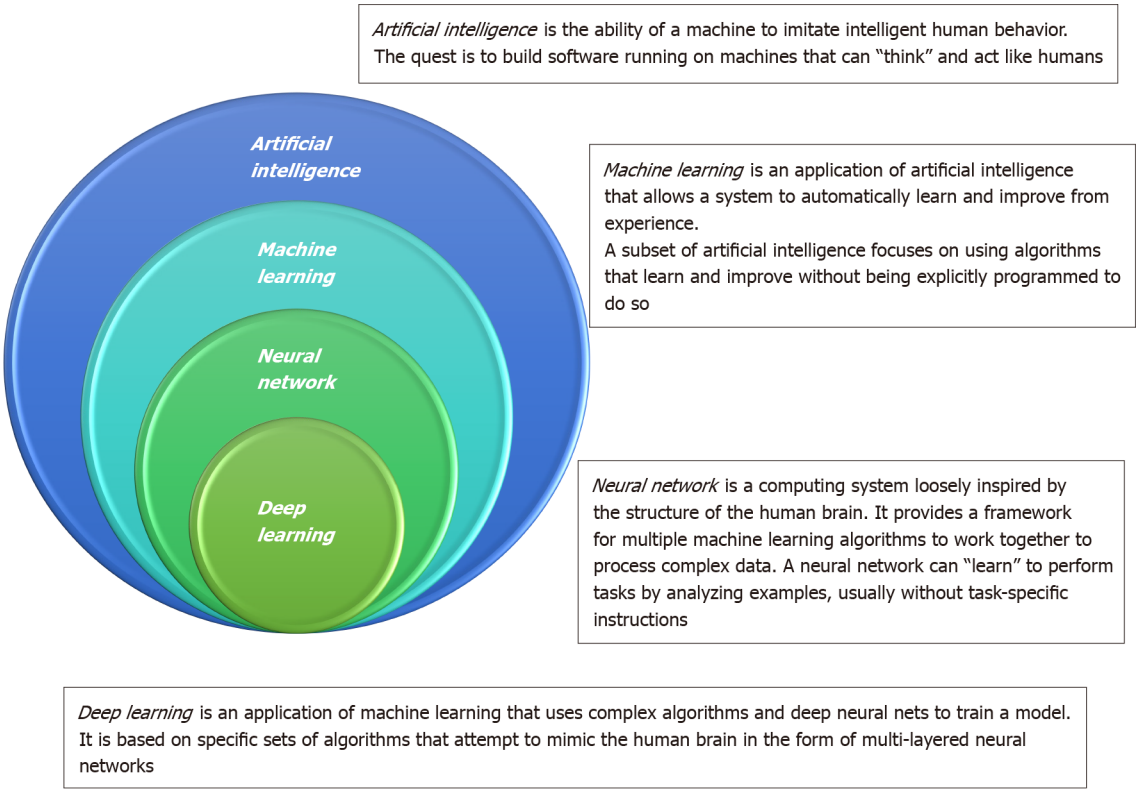


Figure 1 Differences among artificial intelligence, machine learning, neural network, and deep learning.

alternative stream to improve survival outcomes for this deadly disease. The present review mainly focuses on recent advances of AI in PDAC care for clinicians.

PDAC RISK PREDICTION BY AI

The radiographic traits of unoperability and the appearance of symptoms of PDAC occur concurrently[22]. At the time of diagnosis, only a small part of patients (< 15%) have surgically resectable state[22]. In addition, identification of individuals at high risk for PDAC or with early stage is hard due to the absence of trusty screening tools, the lack of clinically relevant biomarkers, and low prevalence[22]. No established screening strategy has been introduced for sporadic PDAC. It is estimated that symptoms manifest about 6 mo after PDAC becomes unresectable[22]. Identifying individuals at high risk but asymptomatic is crucial for finding PDAC while it is still

resectable.

Approximately 50% of all patients with PDAC develop diabetes mellitus prior to their diagnosis[23,24]. Screening patients with new-onset diabetes may enable earlier diagnosis of PDAC. In pre-diabetic and new-onset diabetic patients, an AI-based prediction model of PDAC risk has been developed[25,26]. In a pre-diabetic study, 245 of 138232 patients with impaired fasting glucose were thereafter diagnosed as having PDAC within 3 years of impaired fasting glucose detection. The AI (logistic regression model)-based prediction model consisted of age, body mass index, PPIs, total cholesterol, low-density lipoprotein, alanine transaminase, and alkaline phosphatase [25]. This model achieved an area under the curve (AUC) of 0.71. Furthermore, by analyzing 109,385 new-onset diabetic patients including 390 PDAC cases, a multivariable prediction (logistic regression) model that included age, smoking, body mass index, change in body mass index, usage of proton pump inhibitors and anti-diabetic medications (insulin, oral hypoglycemic except metformin, and metformin), as well as levels of hemoglobin, hemoglobin A1C, creatinine, cholesterol, and alkaline phosphatase, was established (AUC, 0.82)[26]. Among these diabetic patients, 390 (0.4%) were diagnosed with PDAC within 3 years. If the predicted risk threshold for definitive PDAC screening was set at 1% over 3 years, only 6.19% of the new-onset diabetes cases would undergo definitive screening, which could identify PDAC cases with 94.0% specificity, 44.7% sensitivity, and a positive predictive value of 2.6%[26].

Cai *et al*[27] established a PDAC risk prediction model by analyzing 138 chronic pancreatitis patients with focal mass lesions. A scoring method based logistic regression was employed to build the prediction model, which included five variables: sex, mass number, mass location, bilirubin, and carbohydrate antigen 19-9 (CA19-9) (AUC, 0.72). Hsieh *et al*[28] predicted PDAC in patients with type 2 diabetes using ICD-9 code data by logistic regression and ANN models. The AUCs achieved by these models were 0.72[27] and 0.73[28], respectively.

Appelbaum *et al*[29] used a logistic regression model and developed a prediction model of PDAC using electronic health record data. A total of 18 risk factors (*i.e.*, age, gender, race, abdominal pain, angina pectoris, asthma, atherosclerotic heart disease, calculus gallbladder, chest pain, chronic pancreatitis, coronary heart disease, diabetes mellitus, emphysema, essential hypertension, family history pancreatic cancer, jaundice, stroke, and ulcer) were used to weigh the risk factors, and their prediction model displayed an AUC of 0.71. Their risk model based on patients' prior diagnoses derived from electronic health record data would predict PDAC 6-12 mo before an eventual diagnosis date. Such a risk score could be employed as an initial screening prior to additional biomarkers or genetic testing, to pick out individuals from the general population for closer surveillance.

Muhammad *et al*[30] used the ANN model to focus on the early prediction and stratification of PDAC risk based on personal health data (800114 answers in the National Health Interview Survey and Pancreatic, Lung, Colorectal, and Ovarian cancer datasets, including 898 cases diagnosed with pancreatic cancer) before symptoms appear. The prediction model using 18 personal health features produced a specificity of 80.7%, a sensitivity of 80.7%, and an AUC of 0.85 to predict PDAC[30]. Furthermore, the model based solely on personal health data was able to divide individuals into low, medium, and high cancer risk. Identification of high-risk individuals who would benefit from tailored screening may increase the probability of detecting early PDAC. While logistic regression was used to develop risk prediction models in many previous studies, Muhammad *et al*[30] employed an ANN model based on personal health big data and produced the highest AUC in the prediction model of PDAC risk.

Such prediction models using AI will be beneficial for clinicians to estimate the PDAC risk of their patients easily after inputting their data. These models can be combined into an electronic medical record system or be available on portable devices such as tablets and mobile phones. They may also be useful for primary care physicians to stratify individuals into various risk categories. By such PDAC risk stratification, higher-risk individuals can be referred to a diagnostic department for more intensive and tailored assessments. More data and testing will be required to refine the performance of the AI-based prediction model of PDAC in order to facilitate its application in the clinical setting. An AI-based prediction model using clinical variables is non-invasive, cost-effective, and easy for early diagnosis of PDAC. Using AI to recognize signs in early PDAC and precancerous lesions is one of the key strategies to improving survival.

DETECTION OF EARLY PDAC BY BIOMARKERS USING AI

It is highly desirable to identify an effective PDAC diagnostic biomarker. Currently, the most widely employed biomarker for early PDAC detection is CA19-9, however it is not an perfect because of its comparatively low level of specificity and sensitivity (70% with a 5% error rate, for diagnosis of PDAC)[31,32]. Several molecular elements such as CA19-9, CEA, DUPAN, and Span-1 have been employed as biomarkers for diagnosis of pancreatic tumors[33], but none of them are sufficiently specific and sensitive to clearly distinguish cancer from healthy or benign diseases. Therefore, a solid tool with sufficient specificity and sensitivity is required to enable early PDAC diagnosis. Zhang *et al*[34] designed a novel AI (support vector machine) method based on relative gene expression ranking within tissue samples using the microarray gene expression data and RNA-seq data collected from two databases, GEO and TCGA. Zhang *et al*[34] then identified a qualitative diagnostic signature comprising 9 gene pairs (16 genes), that could distinguish PDAC (using expression profiles from PDAC and adjacent normal tissues) patients from non-PDAC (pancreatitis and normal tissues) and was a useful biomarker for early detection of PDAC. Seven genes in the nine-gene-pair signature, namely CTSE, HOXB7, LAMC2, ONECUT1, RRM2, SERPINB5, and UBE2C, had previously been known to be associated with PDAC. Thus, AI-based tissue biomarker analysis identified a multiple-gene expression signature for detection of early PDAC.

MicroRNAs (miRNAs) have been proposed as promising biomarkers for diagnosis of PDAC[35]. miRNAs are a group of short non-coding RNA molecules with 19-25 nucleotides that have been considered as candidate biomarkers for early cancer diagnosis and precise prognosis[36]. Recently, miRNA-used liquid biopsy has become a promising approach for early detection of cancers. Several miRNAs in plasma of PDAC patients are abundantly expressed, supporting that circulating miRNAs could be helpful for PDAC detection[37]. Ganepola *et al*[38] employed three circulating miRNAs (miR-22, miR-642b-3p, and miR-885-5) for detection of PDAC, and the AUC value was 0.97 for discrimination of the PDAC cases. Liu *et al*[39] utilized a serum panel including miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, and miR-191 for diagnosis of PDAC at different stages, and the AUC value was 0.99. Alizadeh Savareh *et al*[40] assessed the value of top miRNAs using a machine learning method (particle swarm optimization + ANN + neighborhood components analysis) to assist early diagnosis of PDAC. They identified a number of serum miRNAs that were significantly differentially expressed in 671 microarray PDAC expression profiles, using bioinformatics techniques Their final model comprised the most promising miRNAs (miR-92a-2-5p, miR-125b-3p, miR-532e5p, miR-663a, and miR-1469) with the high performance (accuracy, 0.93; sensitivity, 0.93; and specificity, 0.92) in differentiation of PDAC from controls.

Early detection of PDAC using tissue and/or blood biomarkers in conjunction with AI is an alternative approach to improving survival.

DETECTION OF EARLY PDAC BY RADIOMICS

Nowadays, computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound, endoscopic ultrasound are the most popular imaging modalities for PDAC detection. However, these modalities are often employed in patients with symptoms, which results in delayed detection of PDACs in most cases. A promising application of AI technology is in the earlier detection of PDAC from radiological findings. CT is the most frequently used modality for the initial assessment of suspected PDAC, and its sensitivity of detection ranges from 76%-96%[41]. CT imaging can collect information about tumor location, size, and morphology. The pancreas is considerably different in size, shape, and location among the individuals and possesses only a very small part of the entire CT image, or about 1.3% of each CT image in a CT dataset[42]. Furthermore, a tumor shows high similarity to the surrounding tissues. Therefore, visual diagnosis demands doctors with enough clinical experience, because the quality of CT images varies between different CT scanners and operators, and pathological texture features are hard to distinguish. Actually, 19% of patients with pancreatic cancer who underwent a review of submitted outside imaging and repeat imaging at a tertiary referral center received major changes in diagnosis and/or disease stage[43].

The features of early PDAC can be delicate and retrospectively ascertained up to 34 mo before the diagnosis of PDAC[44]. In a tertiary medical center, 7.1% of PDACs were missed even by radiologist assessment. This fact emphasizes the limitations in

the conventional CT approach for PDAC. Prognostic outcome in patients with PDACs considerably deteriorates when tumor size exceeds 2 cm[45], however tumors smaller than 2 cm are frequently invisible on CT images and so about 40% of small PDACs are missed[46]. An AI-based diagnostic tool might minimize such oversight. Therefore, there is a growing need to develop AI-based algorithms for accurate pancreatic tumor detection. Although deep learning has been investigated for the diagnosis of pancreatic cystic neoplasms[47], neuroendocrine tumors[48] and segmentation of the pancreas[49-52], the usefulness of AI in the detection of PDAC has not yet been widely explored. AI can analyze thousands of images on a pixel-by-pixel level and is not susceptible to mistakes due to human error. Another strength of AI is automatic diagnosis, which takes no more than approximately 20 s per case from inputting the original CT image to obtaining a diagnosis.

Liu *et al*[53] reported that the AUC of an AI [convolutional neural network (CNN)] platform for CT-assisted diagnosis of PDAC was 0.963. Furthermore, the time of the CT-assisted diagnosis was 20 s/case, which is remarkably shorter than the duration required for diagnosis by radiologists, indicating AI has good clinical feasibility. In a deep learning (fully end-to-end deep learning) model for diagnosing pancreatic tumors, Si *et al*[42] reported that the average test time per case was 18.6 s, compared with at least 8 min for manual reviewing. Thus, the AI diagnosis system was more efficient than the conventional diagnostic approach.

Liu *et al*[54] showed that CNN-based analysis could precisely discriminate cases with and without PDAC in portal venous CT. The CNN-based analysis achieved an accuracy approaching 99% and missed fewer tumors than did radiologists. In this study, CNN-based analysis provided higher sensitivity compared to radiologists (0.983 *vs* 0.929, respectively)[54]. CNN missed three (1.7%) of 176 PDACs (1.1-1.2 cm). Radiologists missed 12 (7%) of 168 PDACs (1.0-3.3 cm), of which 11 (92%) were correctly classified by CNN. The sensitivity of CNN for tumors smaller than 2 cm was 92.1% in local test sets and 63.1% in an external (US) test set. Although the latter sensitivity for tumors smaller than 2 cm initially seemed unsatisfactory, DeWitt *et al* [46] reported that the sensitivity of CT by radiologist assessment was 53% for PDACs smaller than 2.5 cm[46]. Consequently, the sensitivity of the CNN-based analysis was equivalent to radiologist assessment. The lower sensitivity of the CNN in the external test set compared with local test sets might be attributed to differences in patients' ethnicity and race, and protocols or scanners, between the training and external test sets, which could present greater challenges for small tumors. An important factor that affects the imaging features of the pancreas is fat content. Higher fat content decreases the density of the pancreas on CT images, and several studies reported marked differences in pancreatic fat content between ethnicities and races[55,56].

Radiologists were given with important clinical information from the clinicians when they assessed the CT images, whereas the CNN was provided with no information except CT images. Therefore, the major utility of the CNN was to support radiologists in judging whether a lesion or suspicious area in the pancreas harbored pancreatic cancer. For example, patients present with obstructive jaundice which is a typical sign of pancreatic cancer in the pancreatic head. Nevertheless, the CT findings are negative or equivocal. In such a situation, occult pancreatic cancer should be highly suspected even if no apparent mass is noted on CT image, given that about 40% of PDACs smaller than 2 cm are missed on CT image due to undefined borders with surrounding tissue[46,57].

With the wide application of endoscopic ultrasonography (EUS) and EUS-fine needle aspiration (FNA) have become the important diagnostic modalities for PDAC; these modalities provide diagnostic accuracies up to 85%, which are remarkably greater than the 50% accuracy in CT-assisted diagnosis. The sensitivity of diagnosis of pancreatic tumors 3 cm in diameter was reported to be 93% for EUS, which was greater than that of CT (53%) and MRI (67%)[58]. A meta-analysis revealed that CT and EUS were comparable in determining the resectability of PDAC, with high sensitivity and specificity[59].

However, based on EUS for early diagnosis of PDAC, the experience and subjective factors affect on the accuracy, especially in the presence of chronic pancreatitis. Additionally, the availability of the EUS-FNA is restricted in community hospitals. Even when the EUS-FNA is utilized, the diagnosis can be also influenced by the operator's experience and the location of the needle insertion. In 2001, Norton *et al*[60] reported the usefulness of neural network analysis of EUS images to distinguish between PDAC and chronic pancreatitis using 4 different image parameters. Although they provided a high sensitivity, the specificity was only 50%. In 2008, Das *et al*[61] applied techniques of digital image analysis to EUS images of the pancreas to develop a classification model that could differentiate PDAC from non-neoplastic tissue using

ANN. The model accurately classified PDAC, with an AUC of 0.93 and a 93% sensitivity rate[61]. Digital analysis of EUS images is useful in differentiating PDAC from normal tissue and chronic inflammation. Given the possibility of real-time application, digital image analysis may become a helpful diagnostic modality in pancreatic diseases and may sometimes evade EUS-guided FNA. In another study, Zhang *et al*[62] differentiated between PDAC and normal tissue on EUS images. Regions of interest were selected from 216 images obtained from 153 cancer and 63 non-cancer patients, and a 97.98% sensitivity rate was obtained from the 29 features that were identified[62]. Zhu *et al*[63] conducted a computer-aided diagnosis utilizing EUS images of 262 PDAC and 126 chronic pancreatitis patients, from which 105 features were extracted. Sixteen of these features were selected for classification by a support vector machine and a 94% sensitivity rate was obtained[63].

EUS imaging is a common imaging method for diagnosing PDAC, and is often applied with FNA in distinguishing benign and malignant tumors. However, FNA is not available in all health centers. AI-assisted diagnosis *via* EUS images should guide physicians toward more accurate and easier diagnosis.

Collectively, AI can supplement radiologists to reduce miss rates, rather than replace them. The AI stands as a diagnostic tool to assist clinicians and radiologists in diagnosing PDAC. The application of AI in the diagnosis of PDAC has made substantial advances and is certainly improving.

AI IN MOLECULAR/GENETIC SUBTYPE CLASSIFICATION

Recent advances in biotechnology enable us to execute comprehensive genomic, transcriptomic, proteomic, and metabolomic analyses rapidly and cheaply. Such inclusive gene expression studies have uncovered subtypes of PDAC with biological and prognostic relevance. Collisson *et al*[9] proposed the categorization of PDACs into three subtypes: classical, quasi-mesenchymal (QM), and exocrine-like. The prognostic outcome of PDAC patients following operation and conventional medical treatment was notably better in the classical subtype than in patients with the QM subtype; patientss with the exocrine-like subtype displayed intermediate prognostic outcome between the two other subtypes[9]. Muckenhuber *et al*[64] subsequently reported that the most of PDAC can be categorized into two distinct subtypes based on transcriptome profiling and on immunohistochemical staining of cytokeratin-81 (KRT81) and hepatocyte nuclear factor-1A (HNF1a). The epithelial KRT81-/HNF1a- (double-negative) subtype (the so-called classical subtype) showed better survival and response to chemotherapy, notably to the FOLFIRINOX regimen, but not to a gemcitabine-based regimen. On the other hand, the epithelial KRT81+/HNF1a- subtype (the so-called QM subtype) has worse OS. But, the QM subtype displays a better response to the gemcitabine-based regimen compared to the non-QM subtype [65]. These features encourage precision medicine based on individual molecular features.

Recent developments in AI using medical image analysis such as radiomics reveal promising models of molecular phenotyping from imaging data. The radiomics approach can perform whole-tumor analytics without invasiveness. Kaissis *et al*[66,67] have reported on machine learning algorithms to preoperatively predict molecular subtypes and survival risk in PDAC patients from MRI. However, the restricted availability of MRI data, overall decreased image quality, and the less-quantitative and unstandardized nature of MRI render obstacles to algorithm development and generalization. To reinfoce clinical application, Kaissis *et al*[68] extended their previous results to CT by training and validating an algorithm (random forest) capable of discriminating between the QM and the non-QM subtypes of PDAC. The advantages of CT comprise broad availability, fewer motion artifacts, and high standardization. Their retrospective study assessed baseline CT from 207 PDAC cases. By immunohistochemical staining for KRT81 and HNF1a, the molecular subtype was determined as QM *vs* non-QM. The random forest algorithm was used to predict the molecular subtype from the radiomic features. Then, the algorithm was applied to an independent cohort of histopathologically unclassifiable tumors. The classification algorithm achieved sensitivity, specificity, and AUC of 0.84, 0.92, and 0.93, respectively. The median OS for predicted QM and non-QM tumors was 16.1 and 20.9 mo, respectively. The application of the algorithm to histopathologically unclassifiable tumors showed two groups with remarkably different survival (8.9 and 39.8 mo). Thus, the machine learning-based analysis of CT imaging provided the possibility of the prediction of molecular subtypes that is clinically relevant for prognostic outcome,

permitting pre-operative stratification for precision medicine. This approach is encouraged by the fact that histopathological approachs are by default a significant underrepresentation of the tumor, since they are derived from a small sub-section of the tissue, and regions of differing molecular subtype are likely to coexist within the same tumor[69]. On the other hand, the radiomic approach enables whole-tumor assessment, providing better information required for precision therapy.

Microsatellite instability (MSI) is a genomic property of cancers with defective DNA mismatch repair. Notably, MSI has been recognized as a biomarker for the favorable immune checkpoint blockade therapy response in cancer[70]. Most standard methods for examing MSI are based on DNA sequencing data and a few are based on mRNA expression data. Using RNA-Seq pan-cancer datasets for three cancer cohorts (colon, endometrial, and gastric cancers) from TCGA program, Li *et al*[19] established an algorithm called PreMSIm (Predicting MSI from mRNA) to predict MSI in cancer from the expression profiling of a 15-gene panel. A benefit of mRNA-based over DNA-based MSI prediction algorithms is that mRNA data are closer to protein and phenotype than DNA data. Pathway analysis revealed that these genes were mainly involved in DNA damage repair (MLH1 and MSH4), gene expression (MLH1, HENMT1, and RPL22L1), cell cycle regulation (MLH1, MSH4, and HENMT1), and metabolism (NHLRC1 and RPL22L1). Gene ontology analysis showed that these genes were involved in the biological processes of DNA repair (MLH1, MSH4, and RTF2), gene expression regulation (NHLRC1 and HENMT1), cell cycle (MLH1, MSH4, RPL22L1, and RTF2), biogenesis (DDX27, EPM2AIP1, NHLRC1, and RNLS), metabolic process (HENMT1, LYG1, NHLRC1, and SMAP1), and cell and organism development (SMAP1, SHROOM4, and TTC30A). The PreMSIm algorithm provided high performance in predicting MSI using both RNA-Seq and microarray gene expression datasets[19]. Furthermore, PreMSIm showed superior or comparable performance *vs* other DNA- or mRNA-based methods. Li *et al*[19] comment that PreMSIm can be an alternative approach for identifying MSI. The introduction of machine learning algorithms such as this as a clinical decision support tool should be beneficial to predict molecular/genetic signatures that may help to stratify patients in clinical routines.

AI IN RISK ASSESSMENT FOR PANCREATIC SURGERY

In the pancreatic fields, the availability of AI in surgery is still very limited. An AI-based risk prediction model of postoperative complication has been reported[71,72]. Postoperative pancreatic fistula (POPF) is a serious complication after pancreatoduodenectomy (PD). The fistula risk score (FRS), which consists of four variables — soft pancreas, small main pancreatic duct, high-risk pathology (PDAC or chronic pancreatitis), and massive intraoperative blood loss — is useful to predict clinically relevant POPF development after PD[73,74]. However, the score contains subjective factors related to surgeons. Therefore, an accurate and easy-to-use preoperative index is desired. Kambakamba *et al*[71] examined whether quantitative analysis of plain CT with five types of machine learning algorithms (*k*-nearest neighbors, sequential minimum optimization, multilayer perceptron, random forest, and C5.0) could predict clinically relevant POPF in 110 patients from a single institution, and found that machine learning-based CT analysis provided an magnificent AUC of 0.95 in predicting clinically relevant POPF[71]. Mu *et al*[72] tried to predict clinically relevant POPF after PD using a deep learning (CNN model) score derived from preoperative CT. The deep learning score offered significantly greater predictability compared to FRS in training (0.85 *vs* 0.78 in AUC, respectively), validation (0.81 *vs* 0.76 in AUC, respectively) and test (0.89 *vs* 0.73 in AUC, respectively) cohorts. In particular, in patients of intermediate risk (FRS 3-6), the deep learning score achieved remarkably higher accuracy compared to FRS (test: 92.1% *vs* 65.8%, respectively). Interestingly, the deep learning score was independently associated with pancreatic fibrosis, diameter of main pancreatic duct and remnant volume in multivariate linear regression analysis. The automated scores reflected histomorphological features related to pancreatic duct, parenchymal fibrosis, and remnant pancreatic tissue volume. Thus, an AI model using preoperative CT represents a novel tool to predict clinically relevant POPF after PD, especially at intermediate risk levels. Such an AI system helps surgeons to optimize preoperative strategy.

AI IN SURVIVAL PREDICTION AND RESPONSE TO CHEMOTHERAPY

The potential of radiomics in prediction of clinically relevant conditions, such as expected OS or response to a specific therapy, has been reported in recent studies[75,76]: For instance, CT-derived radiomic features were useful to predict local disease control and OS in PDAC[75,76]. Entropy-related and cluster tendency features were described as predictive of OS in PDAC[76]. Zhang *et al*[77] proposed that a CNN-based survival model outperforms a Cox proportional hazard model-based radiomics pipeline in PDAC prognosis. This model provides a better fit for survival patterns based on CT images and overcomes the limitations of conventional survival models. Kaissis *et al*[66] reported that a machine learning algorithm (random forest) using MRI achieved 87% sensitivity, 80% specificity, and AUC 0.9 for the prediction of above- *vs* below-median OS in the independent validation cohort. Alizadeh Savareh *et al*[40] have identified several circulating miRNAs as a diagnosis model in PDAC patients by analyzing microarray miRNA expression profiles from the Gene Expression Omnibus database. Three (hsa-mir-1469, hsa-mir-663a and hsa-mir-532) of five miRNAs with a high rank in the final model were comprehensively associated with the OS of patients with PDAC based on their up- or down-regulated expression patterns[40].

Late diagnosis of PDAC can cause to lose the chance of surgical treatment and lead to a high mortality rate[78]. On the other hand, surgical treatments for PDAC can have a high morbidity and mortality rate. Therefore, the clinicians must weigh the potential survival advantage of the invasive treatment, the complications due to invasive treatment, and the impacts on the patient's quality of life with and without treatment.

Walczak and Velanovich[79] established ANN models that could accurately predict the 7-mo survival of PDAC patients using 14 clinical variables including eight SF-36 domain values, both with and without surgical resection, at 91% sensitivity and 38% specificity. The ANN model to predict 7-mo survival consisted of age, sex, the eight domains of quality of life measurements from the SF-36, the stage of the cancer, whether or not a resection had taken place, if any adjuvant therapy had been given, and time in months since diagnosis. The quality of life domains from the SF-36 are bodily pain, vitality, physical functioning, social functioning, role-physical, role-emotional, general health, and mental health. Such an ANN model for predicting the survival of PDAC patients helps physicians and patients to reduce anticipated regret from treatment decisions including observation. This information may be useful for patients and surgeons in determining invasive treatment plans to minimize regret and improve the patients' quality of life.

Neoadjuvant therapy may provide improved survival of PDAC patients; but, determining the efficacy is difficult. Watson *et al*[80] hypothesized that a deep learning (CNN) model could predict the tumor response to neoadjuvant therapy using CT and CA19-9. A total of 81 cases were divided between partial responder (333 images) and non-responder (443 images). The model using only the deep learning model had an AUC of 0.738, whereas a hybrid model incorporating a decrease in CA19-9 of 10% in addition to the deep learning model had an AUC of 0.785. CA19-9 reduction alone was not an effective predictor of the response to neoadjuvant therapy, with an AUC of 0.564. A deep learning model can predict the pathological response to neoadjuvant therapy for PDAC patients, and the model is amended with the incorporation of decreases in serum CA19-9. Abraham *et al*[81] investigated the clinical relevance of a machine learning-derived signature in predicting the responses from first-line oxaliplatin-based chemotherapy in PDAC and advanced colorectal cancer. The machine learning-derived signature was effective for metastatic colorectal cancer, but not for PDAC. AI has already been applied to match biological information with chemical properties of specific drugs to predict the response to these specific agents in cancers[82].

In the near future, the combined analysis of clinical variables, less-invasive biological samples, and radiological features through machine learning should be able to simulate responses to chemotherapy and patient survival. On the other hand, radiological features and biological tissue can be variable in response to the treatment including chemotherapy and radiotherapy. In particular, PDAC has a high potential for acquired drug resistance. During sequential treatment, good communication and the accumulation of knowledge from various fields such as gastroenterology, radiology, oncology, computer science, and pathology must will be required to fight this deadly disease.

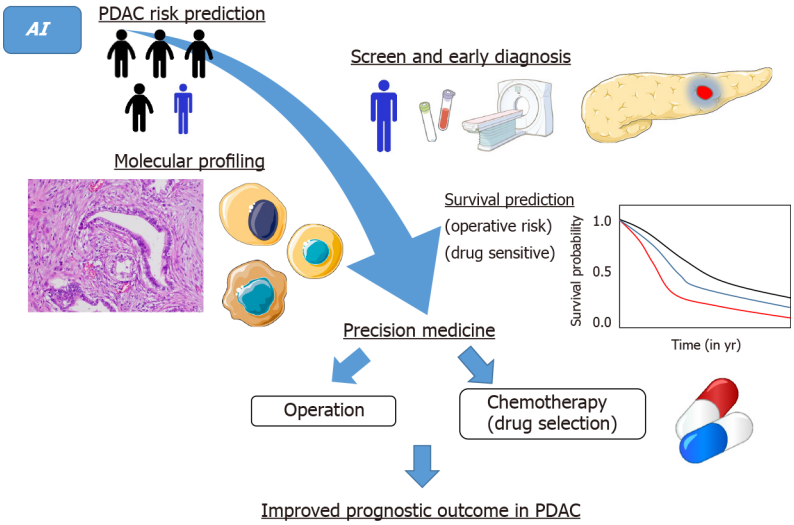


Figure 2 Future perspectives in the management of pancreatic ductal adenocarcinoma by artificial intelligence. AI: Artificial intelligence; PDAC: Pancreatic ductal adenocarcinoma.

LIMITATIONS AND FUTURE PERSPECTIVES

A major limitation is the lack of adequate standardization. Universal and uniform protocols for data collection, data quality, storage, processing, reproduction, and analysis must be established. For instance, ANNs can be trained to appropriately categorize histologic slides of pancreatic biopsies. However, the trained ANNs may underperform, or not perform at all, when the prepared slides are fixed and stained in a different manner. Development of universal and uniform protocols during data and sample processing will be required for medical AI to be feasible. Further improvement of the technology is also essential for medical AI in clinical practice.

AI in the medical field should become an indispensable tool to reduce human error. Because of human limitations, we cannot achieve zero errors. Furthermore, it is time-consuming to train professional radiologists, gastroenterologists, oncologists, and pathologists. Combined work by experts from multiple fields will be needed to establish feasible medical AI systems in clinical practice. With further research, AI must have a great impact on the diagnosis and treatment of PDAC in near future. Ultimately, a sequential approach involving risk prediction, diagnosis, treatment, and survival prediction using IA will realize timely and consecutive precision medicine and lead to improved prognosis in PDAC (Figure 2). Dr. William Osler stated: "Medicine is a science of uncertainty and an art of probability." This is still true in medical AI, which is also "a science of uncertainty and an art of probability." However, the degree of uncertainty and probability will consistently shrink with the advance of AI technology and cooperation among various experts. While AI applications in PDAC are still in the early stage of development, further research must lead to great advances in screening, early diagnosis, and treatment.

CONCLUSION

Here we summarize the current advances of AI in PDAC. AI-based omics analyses are likely to be the next alternative approach to overcome this poor-prognostic disease by the discovery of biomarkers for early detection, molecular/genomic subtyping, and treatment guidance, and by the improved prediction of recurrence and survival. How to entirely utilize "big data" is a new challenge for physicians and researchers in the era of precision medicine. On the other hand, AI will not entirely act for doctors — human beings and machines working harmoniously together is the ideal state that results in excellent performance. Although AI data reveal that the diagnostic accuracy of deep learning models is better than that of radiologists, the aim in this field is to develop a helpful tool to aid radiologists in making effective and accurate diagnoses, not to be a replacement for doctors. To facilitate AI-based omics analyses, multidisciplinary collaboration between physicians, basic scientists, radiologists, statisticians, and engineers is mandatory. To further validate the clinical relevance of AI systems, next

step is to conduct a prospective study based on multicenter clinical data. We believe that breakthroughs will soon emerge to fight this deadly disease using AI-navigated precision medicine.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 November 21; 27(43): 7497-7508

DOI: 10.3748/wjg.v27.i43.7497

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

MINIREVIEWS

Hepatitis B: Who should be treated?-managing patients with chronic hepatitis B during the immune-tolerant and immunoactive phases

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Conflict-of-interest statement: Authors declare no conflict of interest regarding publication of this manuscript.

Country/Territory of origin: Japan

Specialty type: Gastroenterology and hepatology

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

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

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Abstract

New hepatitis B virus (HBV) infections are decreasing owing to improved antiviral therapy and increased HBV vaccination worldwide; however, the number of HBV infections remains a major cause of liver carcinogenesis. HBV triggers cytotoxic immunity to eliminate HBV-infected cells. Therefore, the HBV pathophysiology changes in persistently infected individuals depending on host immune responses and HBV DNA proliferation state. To prevent liver cirrhosis and carcinogenesis caused by HBV, it is important to treat HBV infection at an early stage. Active treatment is recommended for the immunoactive hepatitis B surface-antigen-positive and -negative phase, but not during the immune-inactive phase or immune-tolerant phase; instead, follow-up is recommended. However, these patients should be monitored through regular blood tests to accurately diagnose the immune-inactive or -tolerant phases. The treatment regimen should be determined based on the age, sex, family history of liver cancer, and liver fibrosis status of patients. Early treatment is often recommended due to various problems during the immune-tolerant phase. This review compares the four major international practice guidelines, including those from the Japanese Society of Hepatology, and discusses strategies for chronic hepatitis B treatment during the immune-tolerant, immune-inactive, and resolved phases. Finally, recommended hepatitis B antiviral therapy and follow-up protocols are discussed.

Key Words: Hepatitis B; Immune tolerance; Immune-inactive; Anti-viral therapy; Hepatocellular carcinoma; Cirrhosis

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Core Tip: Hepatitis B virus (HBV) is a global health problem that causes acute and chronic infections and often leads to liver cirrhosis and hepatocellular carcinoma.

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Received: April 23, 2021
Peer-review started: April 23, 2021
First decision: June 13, 2021
Revised: July 31, 2021
Accepted: October 31, 2021
Article in press: October 31, 2021
Published online: November 21, 2021

P-Reviewer: Kamal H, Sahin Y
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

Treatment of HBV is recommended for patients in the immunoactive hepatitis B surface-antigen-positive and -negative phases. Follow-up is recommended only for patients in the immune-inactive phase and the immune-tolerant phase, but opinion on this recommendation remain divided. This review discusses the major international guidelines for the treatment of chronic hepatitis B and highlights the importance of clinical factors for making decisions regarding the management of patients with HBV infection.

Citation: Kawanaka M, Nishino K, Kawamoto H, Haruma K. Hepatitis B: Who should be treated?-managing patients with chronic hepatitis B during the immune-tolerant and immunoactive phases. *World J Gastroenterol* 2021; 27(43): 7497-7508
URL: https://www.wjgnet.com/1007-9327/full/v27/i43/7497.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i43.7497

INTRODUCTION

Chronic hepatitis B (CHB) is the leading cause of hepatocellular carcinoma (HCC), and it is estimated that 240 million individuals are persistently infected by the hepatitis B virus (HBV)[1-4]. The prevalence of hepatitis B surface-antigen (HBsAg) is approximately 3%-4% worldwide; in addition, more than 880000 individuals die each year from HBV-derived liver damage[1-4]. The high prevalence of hepatitis B in Asia and Africa is accompanied by high mortality, and infections in the Western Pacific region account for approximately 50% of chronic HBV infections worldwide[1,5,6]. Therefore, prevention, diagnosis, evaluation, indication of treatment, and management of co-infected patients are important in these areas. HBV may exploit the immature neonatal immune system to establish a persistent infection. Following vertical or perinatal transmission, 90% of neonates develop CHB, whereas children infected between 1 and 5 years of age have a 30% chance of developing chronic infection. Studies on the natural history of HBV infection have shown that the timing of hepatitis B e-antigen (HBeAg) seroconversion varies from childhood to adulthood, with HBeAg seroconversion occurring primarily between 15 and 35 years of age[7-9]. Natural HBeAg seroconversion has been reported to be 4.6%, 7.1%, and 28% for those under 6, between 6 and 12, and above 12 years of age, respectively[9]. Most patients with HBV can resolve their infections, but approximately 10% continue to have viral activity, progress to cirrhosis at an annual rate of 2% and develop HCC or liver failure[5,10-14]. Since its discovery in 1965, the gene structure and replication mechanism of HBV, its infection route, natural course, and pathophysiology have been clarified, and treatment methods have been advancing continuously. The timeline of HBV infection is complex and comprises various overlapping immune phases[2,3,10,13].

The European Association for the Study of Liver Disease (EASL), American Association for the Study of Liver Disease (AASLD), Asian-Pacific Association for the Study of the Liver (APASL), and the Japanese Society of Hepatology (JSG) have defined HBsAg loss as a CHB treatment goal[15-18]. HBsAg loss is associated with improved clinical outcomes such as prevention of HCC and survival[19]. However, this endpoint is difficult to achieve with the existing antiviral therapies that include long-term nucleos(t)ide analog (NA) and pegylated interferon (Peg-IFN) therapies[]. A recent study reported that a higher portion of patients showed HBsAg loss in response to combined treatment with tenofovir disoproxil fumarate (TDF) and Peg-IFN α-2a for 48 wk than to TDF or Peg-IFN α-2a monotherapy[20]. Therefore, NA and Peg-IFN combination therapies are being constantly used and developed to prevent cirrhosis and HCC[20-22]. Several factors, such as the immune phase, genotype, race, degree of liver fibrosis, HCC family history[23-25], age, and sex should be considered to determine the most efficient treatment for CHB. Moreover, the appropriate drug type and timing of drug administration are crucial[19,26]. CHB immune phases can be divided into five categories, including the immunotolerant phase, immunoactive HBeAg-positive phase, immune-inactive phase, immunoactive HBeAg-negative phase, and resolved CHB phase; each phase is identified according to immunological features, virology, biochemistry, and histology specific to the infection[3,5,7,13]. Among these phases, phases 1, 3, and 5 are often not indicated for treatment, and instead, follow-up is often recommended[15-18]. In any case, it remains uncertain

whether follow-up is sufficient, especially for the immunotolerant phase.

CURRENT TREATMENT CONSIDERATIONS FOR HEPATITIS B PATIENTS

Indicators for initiating HBV treatment

The CHB treatment goals are to reduce liver disease mortality, improve survival, and enhance quality of life by preventing liver disease progression from fibrosis to cirrhosis and HCC. All global guidelines recommend initiating treatment based on the presence of HBV DNA and serum alanine aminotransferase (ALT) levels. Patients in either the immunoactive HBeAg-positive or -negative phase are likely to progress to cirrhosis and liver carcinogenesis. Considering the risk of developing cirrhosis and HCC associated with them, these phases are used as the main characteristics for initiating antiviral therapy[15-18]. The following host indicators are also considered prior to treatment: Sex (male), age > 40 years, family history of HCC, place of birth (sub-Saharan Africa or Asia), presence of virus- or disease-related cirrhosis, HBV-DNA > 2000 IU/mL and elevated ALT levels, presence of HBeAg, and genotype C, which causes delayed HBeAg seroconversion[23-28]. In patients treated using NA, HBsAg and HB core-related antigen (HBcrAg) levels are associated with HCC carcinogenesis, even at low levels of HBV DNA levels[29-32]. Recently, modified PAGE-B scores, which are determined based on patient age, sex, baseline platelet count, and serum albumin levels were shown to predict HCC in patients receiving NA treatment[33].

The AASLD guidelines propose different HBV-DNA levels for antiviral therapy, depending on the HBeAg status[17]. According to the APASL guidelines, the HBV DNA levels considered for starting treatment depend on whether the patients are HBeAg-positive or -negative, regardless of the ALT levels[16]. In contrast, the EASL guidelines state that treatment determination should be based upon HBV-DNA and ALT levels, regardless of HBeAg status[15]. Furthermore, according to the JSG guidelines, an ALT level value of 31 or higher, which exceeds normal values in Japan, and HBV DNA levels ≥ 2000 IU/mL are indicated for antiviral treatment, regardless of the HBeAg status[18]. For HBV cirrhosis, antiviral treatment is recommended by all the global guidelines[15-18] (Table 1).

Treatment indications for patients in the immune-tolerant phase

The first phase of CHB, the immune-tolerant phase, is characterized by minimal or no necroinflammatory activity, during which the risk of disease progression is minimal[8, 34]; moreover, 90% of patients infected in early childhood undergo seroconversion from HBeAg-positive to -negative at a young age, and the disease stabilizes in most cases[7-9]. Thus, most clinical practice guidelines do not recommend antiviral therapy for these patients, and follow-up is recommended[15-18]. Among these, the 2017 EASL guidelines[15] set the immune-tolerant phase as HBeAg-positive chronic HBV infection, and the treatment indications for the immune-tolerant phase are expanded compared to other guidelines. Furthermore, serum HBV DNA and HBsAg levels are associated with increased HCC risk and disease progression at serum ALT levels with or without HBeAg[26,27]. Early HBV treatment decreases mortality, improve transplantation outcomes, and decreases the risk of HCC[19]. Therefore, therapeutic intervention should be considered in cases of immune tolerance.

The HBV immune-tolerant phase exhibits high HBsAg and HBeAg titers, indicating high viral replication, and either normal or minimally elevated serum ALT levels. In this case, HBV proliferation is active but 'tolerated' as the host immune system does not recognize the viral antigen.

Table 2 presents the definition of immune tolerance and treatment guidelines[15-18]. The ALT cutoff value during the immune tolerance phase depends on the global guidelines. The AASLD guidelines indicate cutoff values of 35 IU/L for men and 25 IU/L for women. The APASL and EASL guidelines set the cutoff at 40 IU/L, whereas the JSG guidelines recommend no treatment for ALT < 30 IU/L. High HBV DNA level cutoffs are important to distinguish immune-tolerant CHB from other phases. The HBV DNA criteria during the immune-tolerant phase differs between the EASL (≥ 2000 IU/mL), and the AASLD and APASL (≥ 20000 IU/mL for both). Moreover, the age limit for considering a liver biopsy or treatment in the immune-tolerant phase also depends on the guidelines: > 40 years old (both AASLD and JSG), > 35 years old (APASL), or > 30 years old (EASL). Furthermore, antiviral therapy is considered when ALT levels increase during monitoring. According to the AASLD guidelines, treatment is indicated for patients with liver fibrosis stage F2 or higher, especially for patients over 40 years of age. The EASL guidelines indicate treatment for patients with liver

Table 1 Summary of treatment criteria for chronic hepatitis B

	HBeAg+; HBV DNA (IU/mL)	HBeAg+; ALT (IU/L)	HBeAg–; HBV DNA (IU/mL)	HBeAg–; ALT (IU/L)	Cirrhosis
EASL[15]	≥ 2000	> ULN and/or at least moderate liver necro-inflammation or fibrosis	≥ 2000	> × ULN or significant histological disease	HBV-DNA detectable
	≥ 20000	> 2 × ULN or irrespective of fibrosis	≥ 20,000	> 2 × ULN irrespective of fibrosis	
APASL[16]	≥ 20000	> 2 × ULN or significant histological disease	≥ 2000	> 2 × ULN or significant histological disease	HBV-DNA detectable
AASLD[17]	> 20000	> 2 × ULN or significant histological disease	≥ 2000	> 2 × ULN or significant histological disease	HBV-DNA detectable
JSG[18]	≥ 2000	> ULN	≥ 2000	> ULN	HBV-DNA detectable

AASLD: American Association for the Study of Liver Disease; ALT: Alanine aminotransferase; APASL: Asian-Pacific Association for the Study of the Liver; EASL: European Association for the Study of Liver Disease; HBeAg: Hepatitis B e-antigen; HBV: Hepatitis B virus; JSG: Japanese Society of Hepatology; ULN: Upper limit of normal.

Table 2 Treatment indications for patients with hepatitis B e-antigen positive, alanine aminotransferase < upper limit of normal for chronic hepatitis B

	Monitor criteria	Consideration for anti-viral therapy
EASL[15]	Normal ALT (< 40 IU/L) and high HBV DNA (≥ 2000 IU/mL) levels. Monitor ALT and HBV DNA levels every 3-6 mo	Age > 30 yr, family history of HCC or cirrhosis and extrahepatic manifestations. Consider liver biopsy or non-invasive test if: ALT level is elevated; excluding other causes
APASL[16]	Normal ALT (< 40 IU/L) and high HBV DNA (≥ 20000 IU/mL) levels Monitor ALT and HBV DNA levels every 3 mo	Age > 35 yr, liver biopsy showing F2/A2, significant fibrosis by non-invasive tests, stiffness ≥ 8 kPa, persistently elevated ALT, family history of HCC/cirrhosis
AASLD[17]	Normal ALT [< 35 IU/L (male), < 25 IU/L (female)] and high HBV DNA (≥ 20000 IU/mL) levels. Monitor ALT and HBV DNA levels every 3-6 mo	Liver biopsy or non-invasive test shows ≥ F2 or F3, persistently elevated ALT level; exclude other causes, especially age > 40 yr
JSG[18]	Normal ALT (≤ 30 IU/L) level	Consider liver biopsy or non-invasive test if Age > 40 yr, high HBV DNA or platelet counts < 15×10^4/uL, family history of HCC

A2: Activity score 2; AASLD: American Association for the Study of Liver Disease; ALT: Alanine aminotransferase; APASL: Asian-Pacific Association for the Study of the Liver; EASL: European Association for the Study of Liver Disease; F2/3: Fibrosis score 2/3; HBeAg: Hepatitis B e-antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; JSG: Japanese Society of Hepatology.

fibrosis stage F2/A2 or higher (as determined using elastography and/or liver biopsy), over 30 years old, and who have liver stiffness of ≥ 12 kPa. The APASL guidelines simply recommend that patients with F2/A2 or a higher score be considered for treatment. According to the JSG guidelines, determination of liver fibrosis (by liver biopsy or a non-invasive procedure) is recommended if the ALT level is intermittently elevated, HBV DNA is high, platelet counts are less than 15×10^4/μL, and the patient is older than 40 years of age.

To summarize the global guidelines: Age, family history of developing HCC or cirrhosis, and liver fibrosis are important factors for deciding the best therapeutic strategy during the immune-tolerant phase.

Arguments against treatment during the immune-tolerant phase

There are various opinions regarding the need to treat patients in the HBV immune-tolerant phase. This phase is characterized by high viral replication, the presence of HBeAg, and normal or minimally elevated serum ALT and/or aspartate aminotransferase levels[3,5,10,13]. The immune-tolerant phase is usually identified in patients below 30 years of age. Very mild non-specific hepatitis has also been reported in patients in immune-tolerant phase who are between the ages of 10 and 12 years, although the 5 years progression of liver damage is minimal among patients who remain in the immune-tolerant phase[8,34].

Immune-active HBeAg-positive CHB that occurs in adolescence is associated with continued hepatitis activity. Some patients develop fibrosis or cirrhosis during the HBeAg-positive phase, but most show decreased serum HBV DNA levels and HBeAg seroconversion, resulting in an immunoactive HBeAg-negative CHB phase. This phase is characterized by persistent normal serum ALT levels and low HBV DNA load. Some patients show spontaneous HBeAg antibody positivity and may not require antiviral therapy. Patients who spontaneously show HBeAg seroconversion before the age of 30 years usually have a good prognosis[35,36]. In fact, approximately 90% of patients will either be cured, or the hepatitis will not progress into adulthood. For instance, Tada *et al*[35] examined 408 HBV carriers who did not receive NA and found that individuals under the age of 40 with HBeAg seroconversion had a better prognosis than those without HBeAg seroconversion, even when matched by sex and age.

Furthermore, the HBeAg seroconversion rate is very low even if antiviral treatment is administered during the immune-tolerant phase[37-39]. Patients in the immune-tolerant phase requires continuous NA treatment due to high HBsAg levels; moreover, the HBeAg seroconversion rate is low, and serum clearance of HBsAg is rarely achieved[19,39].

In another example, Chan *et al*[39] administered TDF or a combination of TDF and entecavir (ETV) to 126 HBeAg-positive patients with normal ALT levels, who were close to the immune-tolerant phase and found that only 5% of patients receiving treatment showed HBeAg seroconversion. Although none of these cases were in the immune-tolerant phase, other reports have shown that HBeAg seroconversion is high in cases with elevated ALT levels[20]. In general, the higher the ALT level, the better the seroconversion. As ALT levels are naturally low during the immune-tolerant phase, HBeAg seroconversion is not expected.

Several patients in the HBV immune-tolerant phase are young and the NA treatment period is extensive. Treatment is also complicated by several factors, including the development of viral resistance, treatment cost, and long-term safety issues. Andreani *et al*[8] also showed that patients with normal ALT levels and high HBV DNA (10^7 copies/mL) do not require liver biopsies as liver tissue-related changes are minimal during the immune-tolerant phase. In conclusion, there is little evidence to support that treatment alters the clinical outcome in these patients, though some guidelines recommend follow-up[40].

Recommendations for treating patients in the HBV immune-tolerant phase

Earlier, most clinical practice guidelines did not recommend antiviral therapy for patients in the immune-tolerant phase. However, it was recently reported that antiviral treatment during the immune-tolerant phase reduces the risk of HCC, liver transplantation, and death. Thus, some evidence supports antiviral therapy for patients in the immune-tolerant phase[41]. Studies have reported that increase in HBV DNA levels is a risk factor for cirrhosis and HCC[26-28]; moreover, HBV infection itself may lead to HCC. Patients infected with HBV treated with antiviral NA therapy can achieve remarkable viral suppression[39,42]. Further, combined Peg-IFN and NA therapy is more effective in children who are in immune-tolerant phase than in adults [43-45]. A randomized control study evaluated the usefulness of IFN with lamivudine combined therapy for naive HBV infection for patients in immune-tolerant phase, aged 1–16 years, and the results showed reduced HBV DNA load, improved HBeAg seroconversion, and improved rate of HBsAg loss[45]. Management of hepatitis B in children has also been recently reported[46,47]. In fact, hepatitis B vaccination for infants and young children has markedly reduced HBV infections in the vaccinated younger generation, but a significant number of children are still infected with HBV. Most HBV infections in children are in a phase of immune tolerance, and therefore, many are followed up. Most chronically HBV-infected children have mild disease, but a small number may develop undetected fibrosis, cirrhosis, or HCC[47]. There are insufficient data to identify high-risk groups for HCC among children. In fact, children with HBV-associated HCC do not have cirrhosis and have normal alfa-fetoprotein levels, which should be considered for the duration of follow-up and indications for treatment[48].

In recent years, advances in understanding the immunopathogenicity of CHB have questioned whether treatment should be administered at an early stage of CHB regardless of ALT level or severity of liver disease. The immune-tolerant phase is not associated with immunological tolerance, and the results question whether good follow-up as benign is good[49].

Antiviral therapy may also suppress the risk of disease spread from patients with hepatitis B to other individuals. Indeed, antiviral therapy reduces the risk of horizontal transfer of HBV infection from immunotolerant patients with very high viral load, as

well as vertical HBV transmission by mothers with high viral load. Cases without hepatitis B immune globulin and a vaccine occur almost exclusively in HBeAg-positive women with high HBV DNA levels (> 200000 IU/mL) and HBsAg levels exceeding 4–4.5 logs/mL. Mother-to-child HBV transmission rates (MTCTs) were 0% in HBsAg-positive pregnant patients showing high HBV DNA levels (> 200000 IU/mL), who were treated with TDF at 28 gestational weeks[50]. In a meta-analysis based on 595 articles, the administration of antiviral drugs during pregnancy, especially TDF, was deemed safe in helping prevent MTCTs[51,52]. In addition to TDF, tenofovir alafenamide fumarate is administered to pregnant women at 24–35 wk gestation, and the safety of the pregnant women and infants as well as the prevention of MTCT were reported recently[53].

The WHO advocates administration of TDF to infected pregnant women with high HBV load (≥ 5.3 log 10 IU/mL; or ≥ 200000 IU/mL) from the 28th week of pregnancy till delivery. This treatment regimen is recommended to prevent MTCT. The WHO also suggests three hepatitis B vaccinations for newborns, including one dose at birth [54].

Standard follow-up of the immune-tolerant phase may not identify the transition to the immunoactive phase. Thus, evaluating the progression of liver fibrosis and liver carcinogenesis during follow-up is important.

Challenges associated with immune-tolerant phase-diagnosis, liver fibrosis, and liver carcinogenesis

Liver damage caused by HBV causes hepatocellular regeneration associated with chronic necrotizing inflammation, which leads to HCC. The onset of HCC in HBV may arise from immunopathogenic factors[11,55]. In addition, adult serum ALT levels and HBV DNA levels are associated with liver carcinogenesis. Active HBV DNA replication is strongly associated with HCC development and cirrhosis, regardless of ALT levels[26-28,56]. The cumulative incidences of HCC and liver-related diseases in patients who have been in the immune-tolerant phase for over 10 years is 2.7% and 12.7%, respectively[41]. Lee *et al*[57] examined the cumulative HCC risk over a 10-year period in immunologically active patients who achieved a virological response with antivirals in comparison with that in untreated patients in immune-tolerant phase. Surprisingly, the HCC risk was similar between these two groups of patients. In this study, the immune-tolerant phase was diagnosed by regular blood chemistry tests and serum HBV DNA tests every 3–6 mo. Additionally, liver stiffness measured by transient elastography was used to strictly determine whether patients were in the immune-tolerant phase. Thus, this report indicates that HCC carcinogenesis is as high during the immune tolerance period as in the immunologically active phase.

To clarify these points, it is important to determine whether the subject is truly in the immune-tolerant phase. One study found significant fibrosis in 60% of patients with a high viral load and normal or slightly elevated serum ALT for at least 12 mo [58]. Another study found that 37% of HBeAg-positive patients aged 35 years or above, with ALT greater than 0.5 × ULN, had progressive fibrosis, as assessed by transient elastography[59]. Therefore, patients with normal ALT levels and high HBV DNA levels are more likely to be immune-tolerant, subject to the status of their liver fibrosis status[60].

Patients with ALT and HBV DNA greater than 10,000 copies/mL should be carefully evaluated and monitored, even if they appear normal. Necrotic inflammation of the liver and/or fibrosis is observed or progresses unnoticed in certain cases, even in patients with persistently normal ALT levels[56,59-61]. Serum ALT levels cannot be used as a surrogate marker for hepatocyte damage to assess the severity of hepatitis activity.

For this reason, the 2017 EASL guidelines renamed this phase to "HBeAg-positive chronic HBV infection" instead of the "immune tolerance phase," to avoid confusion and the need for early treatment[15]. The AASLD guidelines suggest that ALT levels should be tested every 6 mo at least, even if the patient is in the "immune-tolerant phase," to monitor the potential for progression to "immunoactive or immune-inactive phase"[17]. Various global guidelines recommend regular evaluation of ALT level and HBV DNA load every 3–6 mo in patients who are in the immune-tolerant phase (Table 3). A previous study showed that 4.6% children (up to 12 years of age) progress from the immune tolerance to the immunoclearance phase; therefore, it may be helpful to evaluate ALT levels and HBV DNA load once every 6–12 mo in such children[9].

To diagnose the immune tolerance period accurately, constant monitoring of HBsAg levels, HBeAg levels, and HBV DNA, and ALT levels is required. Chan *et al*[62] performed transient elastography on 161 patients with HBV infection and determined liver stiffness cutoffs associated with normal and elevated ALT levels (> 1–5-fold

Table 3 Treatment indications for patients in the hepatitis B e-antigen-negative immune-inactive phase

	Monitor criteria	Consideration for anti-viral therapy
EASL[15]	Normal ALT (< 40 IU/L) and HBV DNA (< 2000 IU/mL) levels. Monitor ALT and HBV DNA levels (< 2000 IU/mL)every 6-12 mo, (≥ 2000 IU/mL)every 3-6 mo	Age > 30 yr, family history of HCC or cirrhosis and extrahepatic manifestations
APASL[16]	Normal ALT (< 40 IU/L) and HBV DNA (< 2000 IU/mL) levels. Monitor ALT and HBV DNA levels every 3-6 mo	Age > 35 yr, liver biopsy showing F2 or A2, significant fibrosis by non-invasive tests, stiffness ≥ 8 kPa, persistently elevated ALT, family history of HCC/cirrhosis
AASLD[17]	Normal ALT [< 35 IU/L (male), < 25 IU/L (female)] and HBV DNA (< 2000 IU/mL) levels. Monitor ALT and HBV DNA levels every 3 mo for 1 yr, then every 6 mo	Liver biopsy or non-invasive test shows ≥ F2 or F3, persistently elevated ALT level; exclude other causes, especially age > 40 yr
JSG[18]	Normal ALT level (≤ 30 IU/L) and HBV DNA (< 2000 IU/mL) levels	Consider liver biopsy or non-invasive test if age > 40 yr, high HBV DNA or platelet counts < 15 × 10^4/uL, family history of HCC

A2: Activity score 2; AASLD: American Association for the Study of Liver Disease; ALT: Alanine aminotransferase; APASL: Asian-Pacific Association for the Study of the Liver; EASL: European Association for the Study of Liver Disease; F2/3: Fibrosis score 2/3; HBeAg: Hepatitis B e-antigen; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; JSG: Japanese Society of Hepatology.

ULN). These patients were divided into reassurance, observation, liver biopsy, and treatment consideration groups; notably, 58% and 62% of patients with normal and elevated ALT levels (> 1–5 times ULN), respectively, did not require a liver biopsy. In addition to measuring HBsAg, ALT levels, HBV DNA load, and liver fibrosis should be constantly monitored to diagnose true immune tolerance. Recently, non-invasive diagnostic methods for diagnosing fibrosis, such as transient elastography, and markers for liver fibrosis have been developed[63,64]. These methods must be used to accurately diagnose HBV and develop future policies for clinical management of patients with HBV infection.

Antiviral treatment during the immune-inactive phase

The third phase of CHB, which is the immune-inactive phase, is characterized by low HBV DNA load (usually < 2000 IU/mL) and ALT levels within the normal range. HBeAg seroconversion often reduces hepatitis symptoms, however, in 20%–30% of these patients, HBV re-proliferates, hepatitis relapses, and the third immunoactive HBeAg phase transitions into the immune-negative phase. When HBV does not repopulate, it leads to a decrease in HBV DNA load as well as reduction in HBsAg; then, CHB progresses to the HBsAg-negative resolved phase[2,5,7,13]. JSG guidelines define the immune-inactive phase (phase 3) when the patient is HBeAg negative at least three times over 1-year follow-up period, with HBV DNA levels < 2000 IU/mL, and ALT ≤ 30 IU/L. This guideline is based on the fact that histologically favorable liver disease is rare when HBeAg is negative three or more times during follow-up for 1 year or more. In this case, HBV DNA is usually < 2000 IU/mL and ALT is typically < 40 IU/L. Although liver biopsy is not required at this time, lifelong monitoring is indeed required. However, even under these conditions, patients with advanced fibrosis are at increased risk of liver carcinogenesis. Thus, treatment should be considered if liver fibrosis is suspected as recommended *per* the AASLD, EASL, and APASL guidelines. During this phase, antiviral treatment is recommended if the patient has a family history of HCC or cirrhosis or if significant histological findings are noted on the liver biopsy. Thus, in this phase, age, family history of cirrhosis and HCC, and fibrosis progression are important factors for consideration.

Loss of HBsAg is an ideal endpoint and antiviral therapy during the immune-inactive phase may promote HBsAg clearance and lead to low HBsAg levels. The annual incidence of HCC and liver-related deaths among patients in this phase are higher and this trend may be reduced by antiviral treatment. However, treatment should be carefully considered, as chances of HBsAg loss can still increase naturally during the immune stage compared to other stages. It is also believed that antiviral treatment is not necessary and should only be considered in exceptional circumstance. If antiviral treatment is provided, the administration time during the immune-inactive period will be shorter than that during the immune tolerance period.

Need for treatment in the resolved phase of CHB

In the resolved phase of CHB, the patient tests negative for HBsAg, HBV DNA is not detected, ALT level is normalized, and liver inflammation disappears. This condition

is the goal of hepatitis B treatment and no longer requires NA therapy. However, in cases where treatment is initiated with NA(s), AASLD guidelines recommend that patients consider discontinuing treatment when HBsAg is negative, and cirrhosis is absent[20]. The EASL guidelines may consider long-term (*i.e.*, 3 years or longer) NA treatment after the loss of HBsAg, with or without HBsAg positivity. However, the EASL guidelines do not consider cirrhosis at this stage[15]. The APASL guidelines recommend treatment discontinuation if HBsAg level decreases, antibody reversal is observed, and HBV DNA is not detected for at least two years at three separate follow-up visits every six months. Nevertheless, it is recommended that lifelong NA therapy be continued for patients with cirrhosis[16]. Although the JSG guidelines also set criteria for discontinuing NA therapy based on HBsAg levels and HBcrAg[18], treatment discontinuation in the resolved CHB phase is not specified. However, for liver cirrhosis, relapse after discontinuation of NA treatment has a risk of inducing liver failure; therefore, treatment is generally continued lifelong.

If HBsAg is absent, treatment discontinuation remains an option; however, this is not recommended since results on the long-term prognosis of patients with discontinued treatment are currently unavailable. Thus, further therapy is not required if the HBsAg test yields negative results; however, indefinite treatment is recommended for patients with liver cirrhosis.

CONCLUSION

In summary, hepatitis B should be followed up or treated according to the hepatitis B disease course. Toward that end, the treatment selection, as well as mode and timing of drug administration are important for improving HBV prognosis. It is necessary to determine whether patients are in the immune-tolerant or immune-inactive phase to recommend appropriate follow-up and assess their need for antiviral therapy. Furthermore, a major consideration during the course of HBV treatment is to determine the true extent of immune inactivity and immune resistance, for which it is necessary to devise non-invasive evaluation of continuous ALT and HBV DNA level changes, and liver fibrosis. In addition, it is important to consider clinically relevant factors such as age, sex, and genotype during the treatment decision-making process. The option of early treatment also needs to be discussed during the immune tolerance stage. As these factors are not yet clarified in the global guidelines, future research is warranted to elucidate treatment options and prognosis according to the cirrhosis and HCC risk profiles of HBV-infected patients.

ACKNOWLEDGEMENTS

Thanks for Masayuki Kurosaki at Misasino Red Cross Hospital, Tokyo, Japan.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 November 21; 27(43): 7509-7529

DOI: 10.3748/wjg.v27.i43.7509

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

ORIGINAL ARTICLE

Basic Study

Liver injury changes the biological characters of serum small extracellular vesicles and reprograms hepatic macrophages in mice

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Abstract

BACKGROUND

Serum small extracellular vesicles (sEVs) and their small RNA (sRNA) cargoes could be promising biomarkers for the diagnosis of liver injury. However, the dynamic changes in serum sEVs and their sRNA components during liver injury have not been well characterized. Given that hepatic macrophages can quickly

clear intravenously injected sEVs, the effect of liver injury-related serum sEVs on hepatic macrophages deserves to be explored.

AIM

To identify the characteristics of serum sEVs and the sRNAs during liver injury and explore their effects on hepatic macrophages.

METHODS

To identify serum sEV biomarkers for liver injury, we established a CCL_4-induced mouse liver injury model in C57BL/6 mice to simulate acute liver injury (ALI), chronic liver injury (CLI) and recovery. Serum sEVs were obtained and characterized by transmission electron microscopy and nanoparticle tracking analysis. Serum sEV sRNAs were profiled by sRNA sequencing. Differentially expressed microRNAs (miRNAs) were compared to mouse liver-enriched miRNAs and previously reported circulating miRNAs related to human liver diseases. The biological significance was evaluated by Ingenuity Pathway Analysis of altered sEV miRNAs and conditioned cultures of ALI serum sEVs with primary hepatic macrophages.

RESULTS

We found that both ALI and CLI changed the concentration and morphology of serum sEVs. The proportion of serum sEV miRNAs increased upon liver injury, with the liver as the primary contributor. The altered serum sEV miRNAs based on mouse studies were consistent with human liver disease-related circulating miRNAs. We established serum sEV miRNA signatures for ALI and CLI and a panel of miRNAs (miR-122-5p, miR-192-5p, and miR-22-3p) as a common marker for liver injury. The differential serum sEV miRNAs in ALI contributed mainly to liver steatosis and inflammation, while those in CLI contributed primarily to hepatocellular carcinoma and hyperplasia. ALI serum sEVs decreased both CD86 and CD206 expression in monocyte-derived macrophages but increased CD206 expression in resident macrophages *in vitro*.

CONCLUSION

Serum sEVs acquired different concentrations, sizes, morphologies and sRNA contents upon liver injury and could change the phenotype of liver macrophages. Serum sEVs therefore have good diagnostic and therapeutic potential for liver injury.

Key Words: MicroRNA; Small RNA sequencing; Biomarker; Monocyte-derived macrophage; Resident macrophage

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Core Tip: The liver injury changed the concentration, morphology and small RNA contents of serum small extracellular vesicles (sEVs). Altered serum sEV microRNAs (miRNAs) based on mouse studies were highly consistent with the circulating miRNAs reported in human liver diseases. Serum sEV miRNA signatures for acute liver injury and chronic liver injury and a panel of miRNAs that can be used as a common marker for liver injury were established. Acute liver injury serum sEVs depolarized monocyte-derived macrophages and educated resident liver macrophages to transform into M2-like cells. Serum sEVs have good diagnostic and therapeutic potential for liver injury.

Citation: Lv XF, Zhang AQ, Liu WQ, Zhao M, Li J, He L, Cheng L, Sun YF, Qin G, Lu P, Ji YH, Ji JL. Liver injury changes the biological characters of serum small extracellular vesicles and reprograms hepatic macrophages in mice. *World J Gastroenterol* 2021; 27(43): 7509-7529
URL: https://www.wjgnet.com/1007-9327/full/v27/i43/7509.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i43.7509

reviewed and approved by the Nantong University Medical School Ethics Committee (License No. NTUMEC2017-20).

Institutional animal care and use committee statement: All experimental protocols were approved by the Animal Ethics Committee of Nantong University (License No. S20170102-011). The animal care and experiments were performed in accordance with the relevant guidelines and regulations.

Conflict-of-interest statement: The authors declare no competing interests.

Data sharing statement: All data used for the present study are presented in the main manuscript and its supplementary information files.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Supported by National Natural Science Foundation of China, No. 81761128018, No. 81572871 and No. 81272027; Natural Science Foundation of Jiangsu Province, No. BK20151277; and Science and Technology Project of Nantong City, No. MS12020010.

Country/Territory of origin: China

Specialty type: Gastroenterology and hepatology

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

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

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Received: April 21, 2021
Peer-review started: April 21, 2021
First decision: July 14, 2021
Revised: July 21, 2021
Accepted: September 14, 2021
Article in press: September 14, 2021
Published online: November 21, 2021

P-Reviewer: Li WJ
S-Editor: Gao CC
L-Editor: Filipodia
P-Editor: Li JH

INTRODUCTION

Because of its strategic location and biological functions, the liver is particularly susceptible to various pathogenic factors, including drugs, alcohol and viruses. The initial insult induces acute liver injury (ALI) or even liver failure. Repeated or persistent insults will cause chronic liver injury (CLI), resulting in liver fibrosis and finally fatal cirrhosis[1,2]. Therefore, it is important to identify individuals with liver injury. However, liver injury does not always cause noticeable signs and symptoms. Aside from the widely used blood liver function tests on serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which are not always restricted to liver injury[3], there is still a need to explore specific and sensitive biomarkers. With the rapid progress in medical research, it is now possible and necessary to search for new biomarkers from serum small extracellular vesicles (sEVs).

Recently, serum sEVs have attracted tremendous interest due to their essential roles in intercellular communication and to their diagnostic and therapeutic potential[4]. The term sEVs refers to extracellular vesicles released by cells that are of relatively small size (< 200 nm) and were previously regarded as exosomes[5]. The cargoes carried by sEVs represent a snapshot of the parental cells at the time of release and change depending on the physiological and pathological states[6,7]. In the liver, sEVs are released from both hepatocytes and nonparenchymal cells into the extracellular space and circulation. Several studies have reported that circulating sEV RNAs or proteins are abnormally expressed in the contexts of drug-induced liver injury (DILI), steatohepatitis, viral hepatitis and hepatocellular carcinoma (HCC)[6,8].

MicroRNAs (miRNAs) are 22-24 nt small noncoding RNAs involved in posttranscriptional regulation and various biological processes[9]. Tissue-specific distribution is a key feature of miRNAs, making miRNAs good candidates as biomarkers or therapeutic targets for particular types of tissue injury[10-12]. Serum miRNAs have been studied in a variety of liver diseases[13]. However, compared to serum miRNAs, serum sEV miRNAs are well protected from RNA enzymes. Thus, serum sEV can serve as a more reliable miRNA pool[14]. We hypothesized that serum sEVs and their miRNA cargoes might reflect liver damage upon injury and could be promising biomarkers.

In the present study, we tried to determine the effects of liver injury on serum sEVs and the small RNAs (sRNAs) they transport; we were also interested in determining if there is any difference between acute and chronic injury. A study in this regard will aid in identification of potential serum sEV miRNA biomarkers. The dynamic changes in the number and morphology of serum sEVs and the sRNA components of serum sEVs were examined. The profiles of deregulated serum sEV miRNAs were obtained and compared to those of mouse liver enriched miRNAs and previously reported circulating miRNAs related to human liver diseases (HLD). To further evaluate the biological significance of serum sEVs upon liver injury, conditioned cultures of ALI serum sEVs and primary hepatic macrophages were carried out.

MATERIALS AND METHODS

Animal studies

Male C57BL/6 mice (8 wk old) were purchased from the Shanghai Medical Laboratory Animal Center (Shanghai, China) and housed in the animal facility of Nantong University with temperature 25 ± 2 °C and 12 h light/dark cycle controls. All experimental protocols were approved by the Animal Ethics Committee of Nantong University. The animal care and experiments were performed in accordance with the relevant guidelines and regulations. For ALI, mice were treated with a single dose of CCL_4 (0.5 mL/kg intraperitoneal injection) dissolved in olive oil (1:9). The mice were sacrificed at 2 d or 7 d. For CLI, mice were treated with CCL_4 (0.5 mL/kg) or vehicle twice a week for 8 wk[15]. The mice were sacrificed 48 h after the last injection at 8 wk or at 12 wk (Figure 1A). Mice treated with the same volume of olive oil served as the controls for the ALI and CLI models, and 10-12 mice were used in each group. Blood or livers were collected from each group for further analyses.

Liver function test and histopathologic examination

Blood was collected by left ventricular puncture from mice and was left undisturbed for 1 h at 37 °C and 2 h at 4 °C. Afterward, the samples were centrifuged at 1000 × *g* for 10 min at 4 °C; the clear upper fractions were aliquoted and stored at -80 °C. Serum ALT and AST levels were measured on an ADVIA 1800 autoanalyzer (Siemens

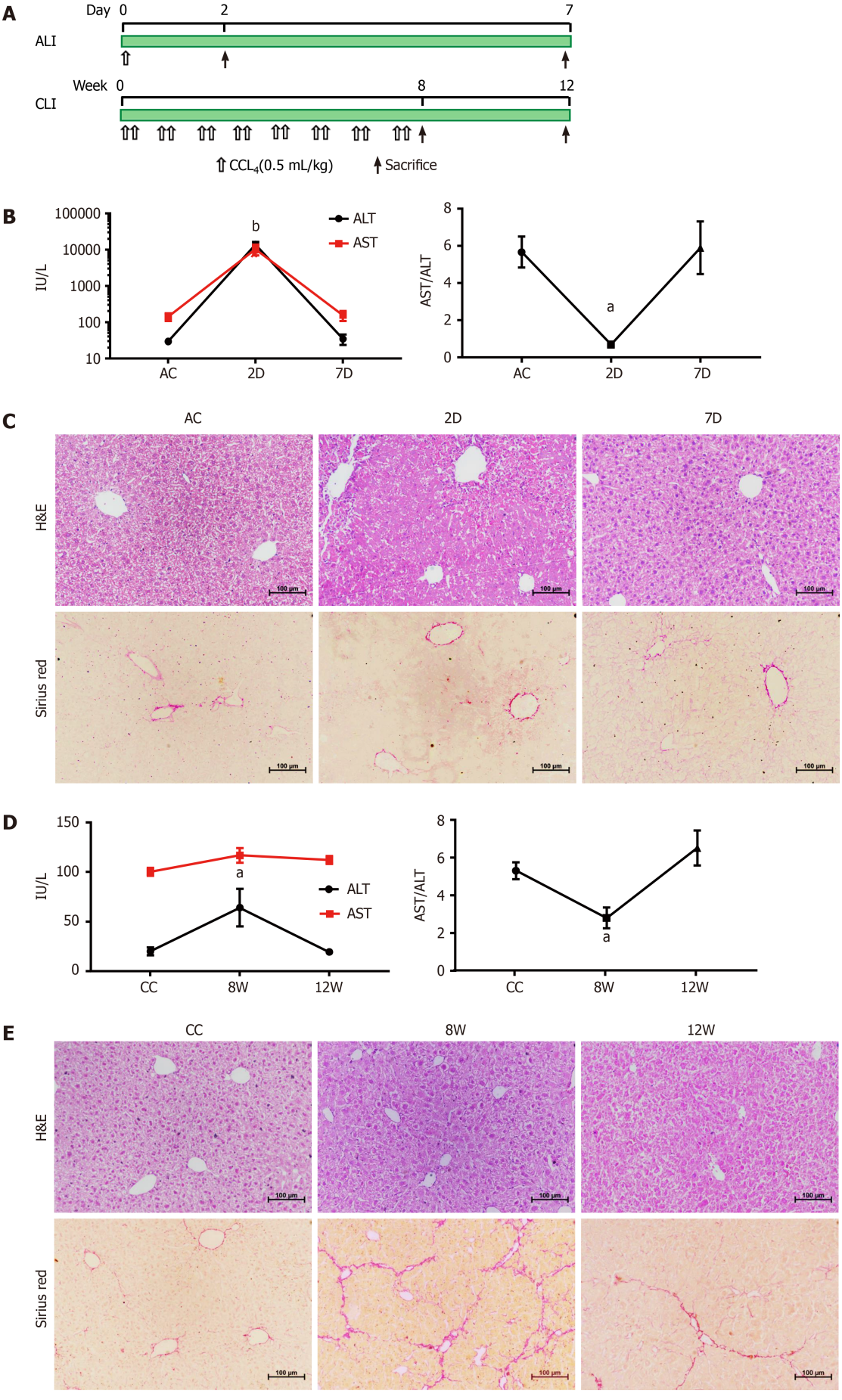


Figure 1 Establishment and validation of CCL_4-induced acute liver injury and chronic liver injury in mice. A: Workflow for the establishment of

the acute liver injury (ALI) and chronic liver injury (CLI) mouse models; B: Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in ALI mice. Compared with the ALI control (AC) group, $^{a}P < 0.05$, $^{b}P < 0.01$; C: Hematoxylin-eosin (H&E) and Sirius Red staining of liver sections from AC, 2 d and 7 d mice; D: Serum ALT and AST levels in CLI mice. Compared with the CC group, $^{a}P < 0.05$; E: H&E and Sirius Red staining of liver sections from CLI control (CC), 8 wk and 12 wk mice. Scale bar = 100 μm. D: Day; W: Week.

Healthcare Diagnostics, Deerfield, IL, United States). The livers were preserved in 4% paraformaldehyde, paraffin-embedded and sectioned. The liver tissue sections were stained with hematoxylin and eosin (Beyotime Biotechnology, Shanghai, China) for routine histology and 0.1% Sirius Red (Sigma-Aldrich, St. Louis, MO, United States) for collagen evaluation.

Mouse serum sEV isolation and characterization

Exosome-enriched serum sEV fractions were precipitated using ultracentrifugation and an ExoQuick precipitation kit (System Biosciences Inc., Mountain View, CA, United States)[16]. The sizes and particle concentrations of the isolated serum sEVs were measured by nanoparticle tracking analysis (NTA, NanoSight NS300, Malvern, United Kingdom). Serum sEVs were visualized using transmission electron microscopy (TEM, HT7700, Hitachi Ltd., Tokyo, Japan). The expression of exosomal protein markers was determined by Western blot analysis. The details are provided in the Supplementary material, Supporting Information.

sRNA library construction and deep sequencing

Serum sEV sRNA sequencing (RNA-seq) was conducted by BMK Biotech Co., Ltd. (Beijing, China) with biological replicates for each group.

Sequencing data analysis and bioinformatic analysis

The raw data were processed as described previously[16]. The trimmed sequencing reads were deposited in the European Nucleotide Archive (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-9462). Bioinformatic analysis of the differentially expressed serum sEV miRNAs was performed using Ingenuity Pathways Analysis (Qiagen, Valencia, CA, United States). The significance of enrichment for genes with particular biologically relevant functions was determined with a one-sided Fisher's exact test.

Systematic review of abnormally expressed circulating miRNAs in HLDs

The detailed procedure is provided in the Supplementary material, Supporting Information.

Isolation and culture of mouse hepatic macrophages

Primary mouse hepatic macrophages were isolated from male C57BL/6 mouse livers by Percoll (GE Healthcare, Princeton, NJ, United States) density gradient centrifugation. Incubation of liver macrophages with mouse serum sEVs and subsequent multiple-color flow cytometric analysis were carried out. The details are provided in the Supplementary material, Supporting Information.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA, United States). Quantitative data were reported as the mean ± standard deviation. Comparisons between groups were made by Student's *t*-test or one-way analysis of variance. All *P* values were two-sided, and statistical significance was accepted for a value less than 0.05. Except for the data from sRNA-seq experiments, which included two biological replicates for each group, the data provided in the present study were from three or more independent experiments.

Additional methods and details are provided in the Supplementary material, Supporting Information.

RESULTS

Establishment and validation of CCL_4-induced ALI and CLI in mice

The mouse CCL_4-induced ALI and recovery model and the mouse CCL_4-induced CLI and recovery model were established and validated (Figure 1).

In acutely injured mice (at 2 d; 2D group), serum ALT and AST levels were increased, and hepatocyte necrosis and inflammatory cell infiltration were observed around the lobular central vein. After 5 d of recovery (at 7 d; 7D group), the elevated ALT and AST levels had returned to the baseline of the ALI control group, and the histological changes were also reversed (Figure 1B and C). For CLI, repeated CCL_4 treatment induced a slight elevation in serum ALT (at 8 wk; 8W group), but the level returned to the baseline of the CLI control (CC) group by 4 wk after cessation of CCL_4 treatment (at 12 wk; 12W group) (Figure 1D). Although the ALT level change in CLI at 8W was not as prominent as those in ALI at 2D, the change was comparable to the ALT level changes reported by other study groups using the same mice CLI model[17, 18]. Damaged hepatocytes and centrilobular contracture were observed in the livers of the CLI mice (8W), with mild inflammatory cell infiltration. Sirius Red staining showed obvious collagen deposition and pseudolobule formation in 8W CLI mice. These morphological changes were alleviated in recovered mice (12W) (Figure 1E).

Identification and characterization of serum sEVs from ALI and CLI mice

The isolated particles were spherical or cup-shaped, as observed by TEM (Figure 2A). Exosomal protein markers, including CD63, CD81 and CD9, were all highly expressed (Figure 2B), as determined by Western blot analysis.

NTA showed that the mean diameter of the particles ranged from 90.2 nm to 127.8 nm. The number of particles was higher in the 2D group but lower in the 7D group than in the control group, and the particle diameters were smaller in the 2D group but larger in the 7D group than in the control group. We also noticed that the size distribution of sEVs widened and that multiple peaks were present in the 7D group (Figure 2C). For CLI mice, the particle concentrations in the 8W and 12W groups tended to be lower than those in the control group, although there were no significant differences. The size distribution of sEVs was expanded with multiple peaks in both the 8W and 12W groups, and the particle diameters were larger in both the 8W and 12W groups (Figure 2D). TEM examination revealed that the multiple peaks reflected the aggregation or fusion of mouse serum sEVs present in 7D, 8W and 12W samples (Figure 2C and D).

These findings suggested that both ALI and CLI changed the number and morphology of mouse serum sEVs, and even when the visible histological changes of the liver had recovered in the 7D and 12W groups, the changes in particle number and morphology of serum sEVs persisted.

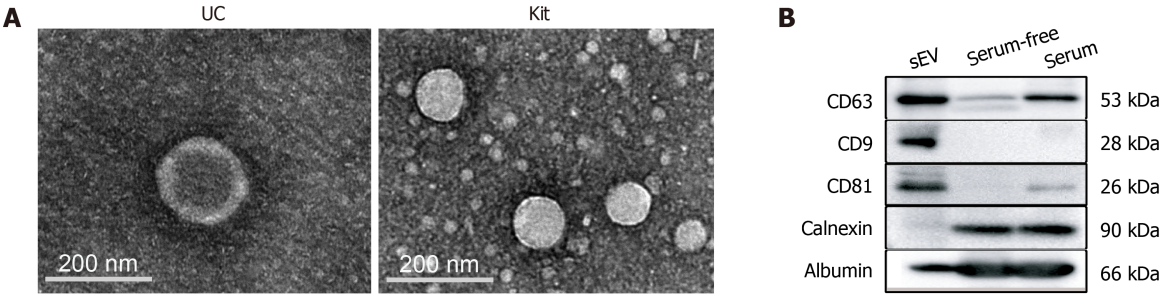
Comparison of the sRNA components in serum sEVs from ALI and CLI mice

Dramatic increases in both total RNA and sRNA levels in serum sEVs were observed upon ALI (2D) (Figure 3A and B). For CLI, there was no significant difference in either sEV RNA or sRNA content among the groups. RNA-seq and annotation revealed that each pair of biological repeats had at least 94.97% common sequences in clean reads (Supplementary Figure 1). In the control groups (ALI control and CC), tRNA was the dominant sRNA species in serum sEVs, followed by rRNA and miRNA (Figure 3C). The most remarkable change in serum sEV sRNAs was the increase in miRNA proportion in both ALI and CLI mice. Compared to the control condition, ALI increased the proportion of miRNAs by more than four-fold, but the proportion returned to baseline by 7 d; the proportion of miRNAs increased by almost three-fold in CLI mice but was partially restored by 12 wk. With the increase in miRNA, the proportion of tRNA decreased (Figure 3D).

miRNA expression profiles of serum sEVs from ALI and CLI mice

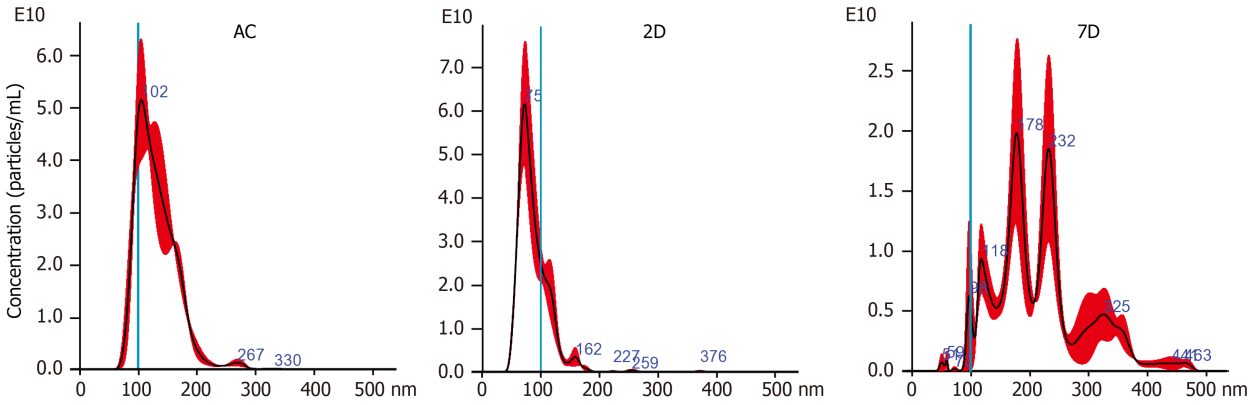
For the ALI and CLI groups, 467 and 488 detectable sEVs (transcripts per million reads ≥ 5.0) miRNAs were obtained, respectively. The biological replications were highly correlated in each group (Figure 4A). The RNA-seq data were further validated by quantitative real-time polymerase chain reaction. Differentially expressed miRNAs with different abundances were selected for validation (Figure 4B).

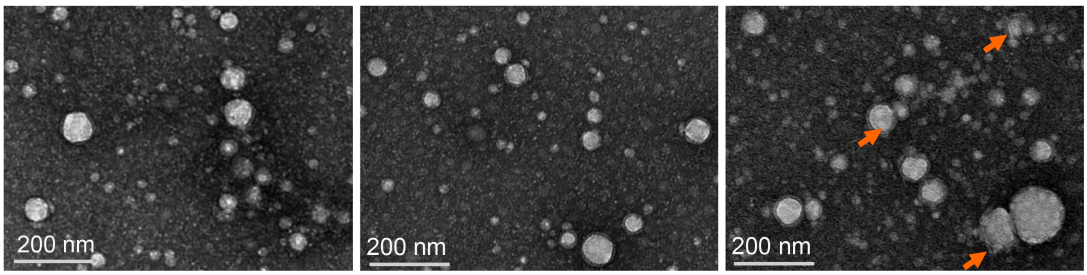
In total, 91 miRNAs were upregulated and 85 miRNAs were downregulated (fold change ≥ 2.0, $P < 0.05$) in the 2D group compared with the ALI control group (Figure 4C and Supplementary Table 1). The levels of most of these miRNAs had recovered to baseline levels in the 7D group, in which only 8 upregulated miRNAs and 11 downregulated miRNAs were detected (fold change ≥ 2.0, $P < 0.05$) (Figure 4C and Supplementary Table 2). The cumulative distribution frequency was calculated by adding each proportion of miRNAs from most to least abundant. The plot showed that the top five upregulated miRNAs (miR-148a-3p, miR-122-5p, miR-192-5p, miR-22-3p and miR-21a-5p) in the 2D group accounted for up to 84.27% of all detectable miRNAs



C

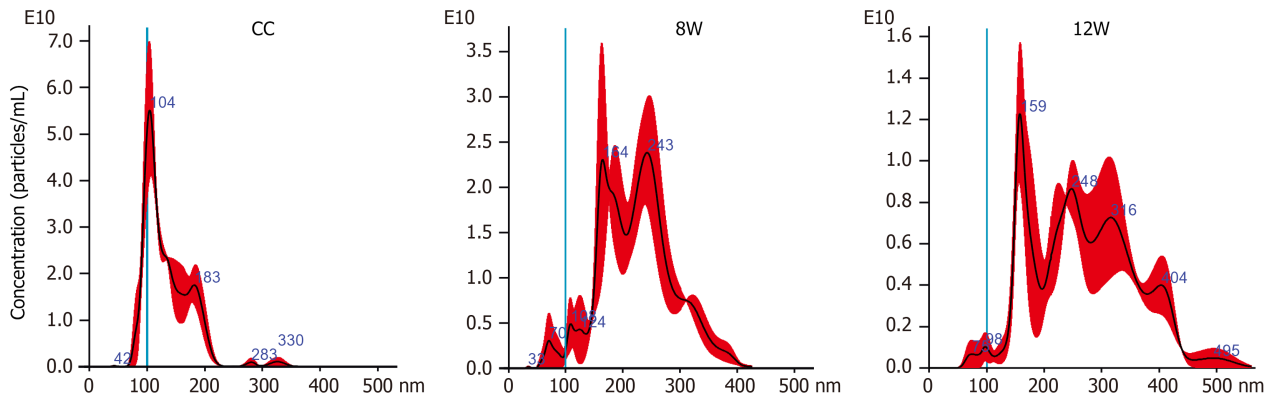
Group	Concentration (particles/mL)	Diameter (nm)
AC	4.80 ± 0.14 × 10^{12}	151.5 ± 11.23
2D	1.86 ± 0.44 × 10^{13a}	106.7 ± 11.98[a]
7D	1.50 ± 0.05 × 10^{12}	252.7 ± 21.34[b]





D

Group	Concentration (particles/mL)	Diameter (nm)
CC	4.91 ± 0.92 × 10^{12}	135.2 ± 2.23
8W	1.83 ± 0.76 × 10^{12}	258.9 ± 19.96[b]
12W	1.47 ± 0.31 × 10^{12}	268.2 ± 6.65[b]



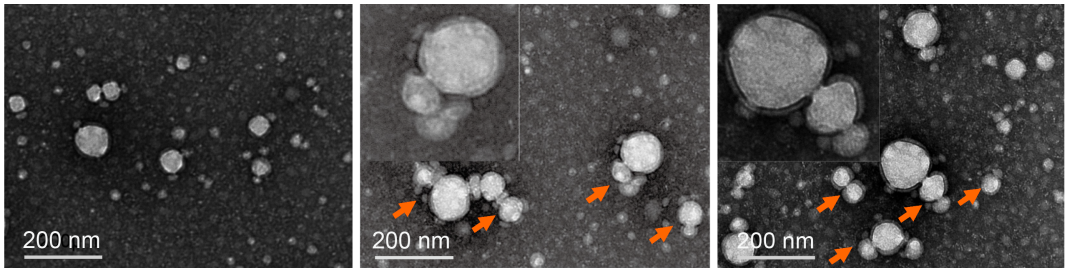


Figure 2 Characterization of isolated mouse serum small extracellular vesicles. A: Transmission electron microscopy (TEM) images of the particles isolated using ultracentrifugation and an ExoQuick precipitation kit; B: Representative Western blotting bands for CD63, CD81, CD9, calnexin and albumin; C: Nanoparticle tracking analysis (NTA) plots for the size distribution and concentration of the isolated particles in each group from ALI mice and the corresponding TEM images. Compared with the ALI control group, $^{a}P < 0.05$, $^{b}P < 0.01$; D: NTA plots for the size distribution and concentration of the isolated particles in each group from CLI mice and the corresponding TEM images. Compared with the CLI control group, $^{b}P < 0.01$. The red arrow indicates aggregated particles; representative particles were amplified and were exemplified in the top left corner. Scale bar = 200 nm. UC: Ultracentrifuge; D: Day; W: Week; AC: Acute liver injury control; CC: Chronic liver injury control; sEVs: Small extracellular vesicles.

(Figure 4C).

Only 13 miRNAs were upregulated and six miRNAs were downregulated (fold change ≥ 2.0, $P < 0.05$) in the 8W group compared with the CC group (Figure 4D and Supplementary Table 3); in addition, 8 miRNAs were upregulated and 3 miRNAs were downregulated (fold change ≥ 2.0, $P < 0.05$) in the 12W group compared with the CC group (Figure 4D and Supplementary Table 4). The cumulative distribution frequency analysis showed that the top three upregulated miRNAs (miR-122-5p, miR-192-5p, and miR-22-3p) in the 8W group constituted up to 43.48% of all detectable miRNAs (Supplementary Figure 2). These findings suggested that both ALI and CLI induced changes in serum sEV miRNA composition. The changes were caused by the differential expression of a small number of miRNAs with high abundance.

The biological significance of these differentially expressed serum sEV miRNAs in liver injury was explored by Ingenuity Pathway Analysis (www.qiagen.com/ingenuity). For the 176 differentially expressed serum sEV miRNAs upon ALI, hepatic steatosis was the most significant hepatotoxic effect, followed by liver inflammation. For the 19 differentially expressed serum sEV miRNAs upon CLI, HCC was the most significant, followed by liver hyperplasia (Figure 4E).

The liver is the main contributor to the differentially expressed serum sEV miRNAs during ALI and CLI

We were interested in determining the contribution of liver cells to the changes in serum sEV miRNAs upon ALI and CLI. First, the liver miRNA expression profile for wild-type male C57BL/6 mice was established using BRB-Array Tool v4.6.0 (https://brb.nci.nih.gov) based on the RNA-seq data from GSE78792 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE78792)[19] (Supplementary Table 5). Differentially expressed serum sEV miRNAs from ALI and CLI mice were compared to the liver miRNA expression profile. Among the top ten most abundant liver miRNAs, seven were also among the top ten increased miRNAs in serum sEVs upon ALI (Table 1) and constituted up to 84.70% of the increased serum sEV miRNAs. For CLI, the levels of three were raised in serum sEVs. These three miRNAs were the top three miRNAs that increased upon CLI (Table 1) and constituted up to 60.56% of the total increased serum sEV miRNAs. These findings suggested the liver as the primary contributor to the upregulated serum sEV miRNAs during ALI and CLI and confirmed that the serum sEV miRNA test could be a reliable and sensitive way to monitor either ALI or CLI.

ALI and CLI signatures based on serum sEV miRNA profiling

To identify serum sEV miRNA signatures for liver injury, we compared the differentially expressed serum sEV miRNAs in various stages of ALI and CLI. Compared to the levels in the vehicle control samples, eight miRNAs were upregulated and two miRNAs were downregulated significantly during the acute injury stage, and these changes were sustained through the chronic phase (fold change ≥ 2.0, $P < 0.05$); thus, they can serve as common liver injury signatures (Figure 5A). In addition, the levels of 166 miRNAs changed significantly during acute injury, while those of nine miRNAs changed dramatically during chronic injury. These miRNAs with high abundance (transcripts per million reads _mean > 1000) have the potential to be ALI or CLI

Table 1 Liver-enriched microRNAs ranked highly among serum small extracellular vesicle microRNAs from acute liver injury and chronic liver injury model mice

GSE78792 liver	Rank	ALI	ALI rank	CLI	CLI rank
miR-192	1	miR-192-5p	3	miR-192-5p	2
miR-22	2	miR-22-3p	4	miR-22-3p	3
miR-30a	3	miR-30a-5p	7	N/A	N/A
miR-148a	4	miR-148a-3p	1	N/A	N/A
miR-21a	5	miR-21a-5p	5	N/A	N/A
miR-26a-2	6	miR-26a-5p	105	N/A	N/A
miR-122	7	miR-122-5p	2	miR-122-5p	1
miR-10a	8	N/A	N/A	N/A	N/A
miR-143	9	miR-143-3p	92	N/A	N/A
miR-27b	10	miR-27b-3p	10	N/A	N/A

N/A: Not applicable; ALI: Acute liver injury; CLI: Chronic liver injury.

signatures. The complete lists of these potential ALI and CLI serum sEV miRNA signatures are provided in Supplementary Table 6. According to their abundance, up to the top 20 miRNAs are listed in Figure 5. For the recovery stage, 18 miRNAs were changed significantly in the ALI group, and 11 miRNAs were changed significantly in the CLI group (Figure 5B). Some of these miRNAs overlapped with differentially expressed serum sEV miRNAs in corresponding acute or chronic injury stages.

Comparison of the potential ALI and CLI serum sEV miRNA signatures to HLD-related circulating miRNAs

To explore the biological significance of the ALI and CLI serum sEV miRNA signatures in HLDs, we performed a systematic review of abnormally expressed circulating miRNAs reported in various HLDs. In total, 299 and 257 studies were identified from PubMed (https://pubmed.ncbi.nlm.nih.gov) and Web of Science (http://apps.webofknowledge.com/) databases, respectively (Figure 6A). Data were retrieved from 14 studies, including drug-induced liver injury[20], chronic hepatitis B [21-25], chronic hepatitis C[21,25-27], nonalcoholic fatty liver disease[28], nonalcoholic steatohepatitis[23], liver cirrhosis[24,29,30] and HCC[21-24,28-33] studies. Details on the 14 articles are summarized in Supplementary Table 7.

In total, 269 nonredundant abnormally expressed circulating miRNAs related to HLDs were extracted, and those that appeared ≥ four times were defined as high-frequency miRNAs (Figure 6A). Of the 30 high-frequency miRNAs (Supplementary Table 7), 23 miRNAs were detected in ALI and CLI serum sEVs (Figure 6B), 14 miRNAs overlapped with ALI and CLI serum sEV signatures, and 12 miRNAs showed the same expression trend. Of the 12 miRNAs, three miRNAs (miR-122-5p, miR-192-5p, and miR-22-3p) were identified as being increased in both ALI and CLI mice and thus have the potential to serve as common signatures for either ALI or CLI. The other 9 miRNAs were identified as ALI signatures (miR-21a-5p, miR-92a-3p, miR-194-5p, miR-17-5p and miR-19b-3p were increased; miR-451a, miR-27a-3p, miR-26a-5p, and miR-223-3p were decreased) and may reflect acute or active liver injury (Figure 6C). In addition, it was noteworthy that the four high-frequency circulating miRNAs reported in HLDs with decreased levels all exhibited decreased levels in serum sEVs upon ALI.

Serum sEVs from liver injury mice induced hepatic macrophage reprogramming

Primary mouse hepatic macrophages were isolated, purified and incubated with SYTO-labeled serum sEVs. After 24 h, green fluorescence was observed in most macrophages in both the control and ALI (2D) serum sEV incubation groups (Figure 7A). These observations indicated that serum sEVs could be taken up by hepatic macrophages. Serum sEVs from ALI mice (2D) accelerated the adhesion of hepatic macrophages (Figure 7B). As determined by quantitative real-time polymerase chain reaction, normal serum sEVs increased M1-like gene (*IL-1B* and *TNFA)*

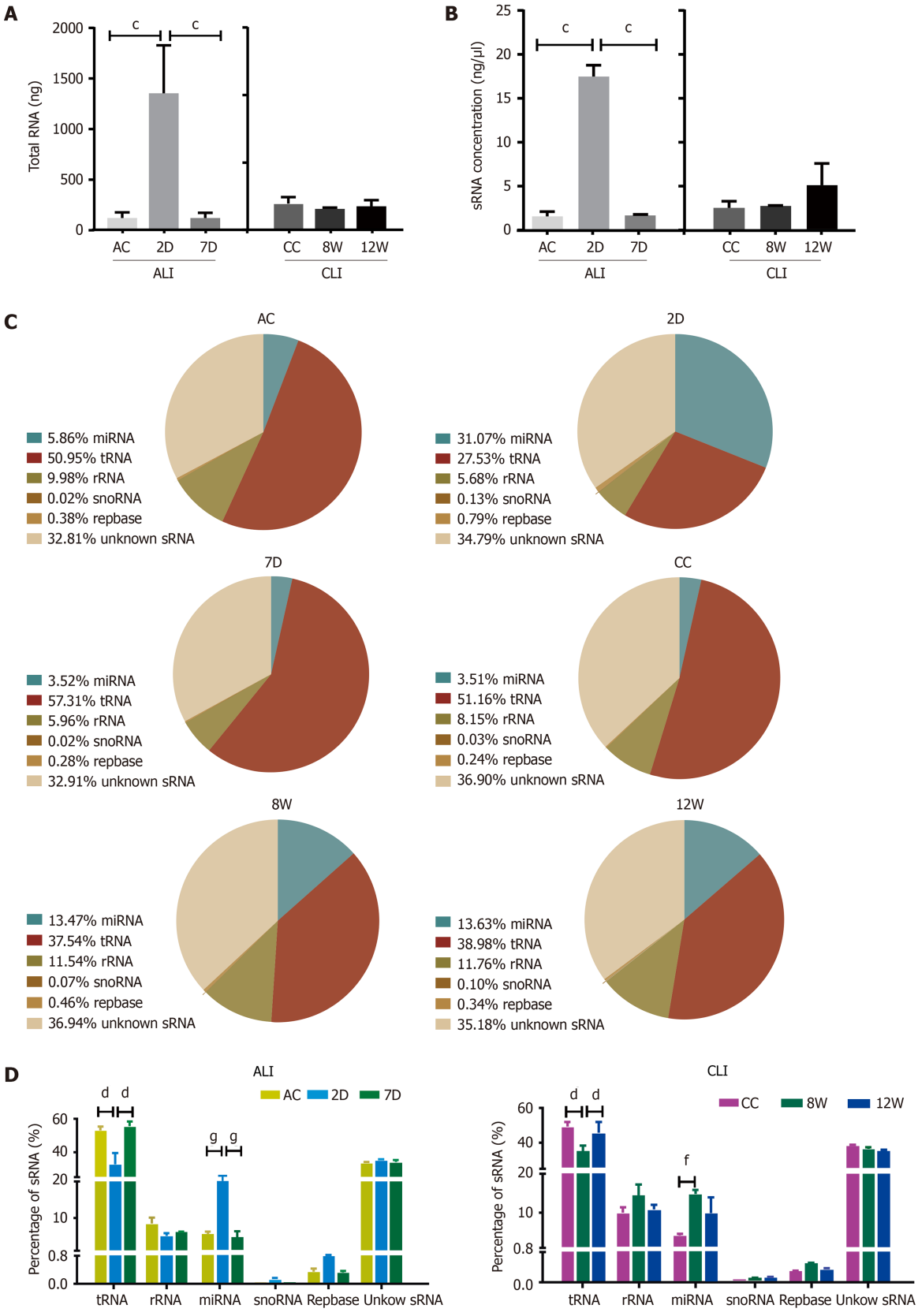
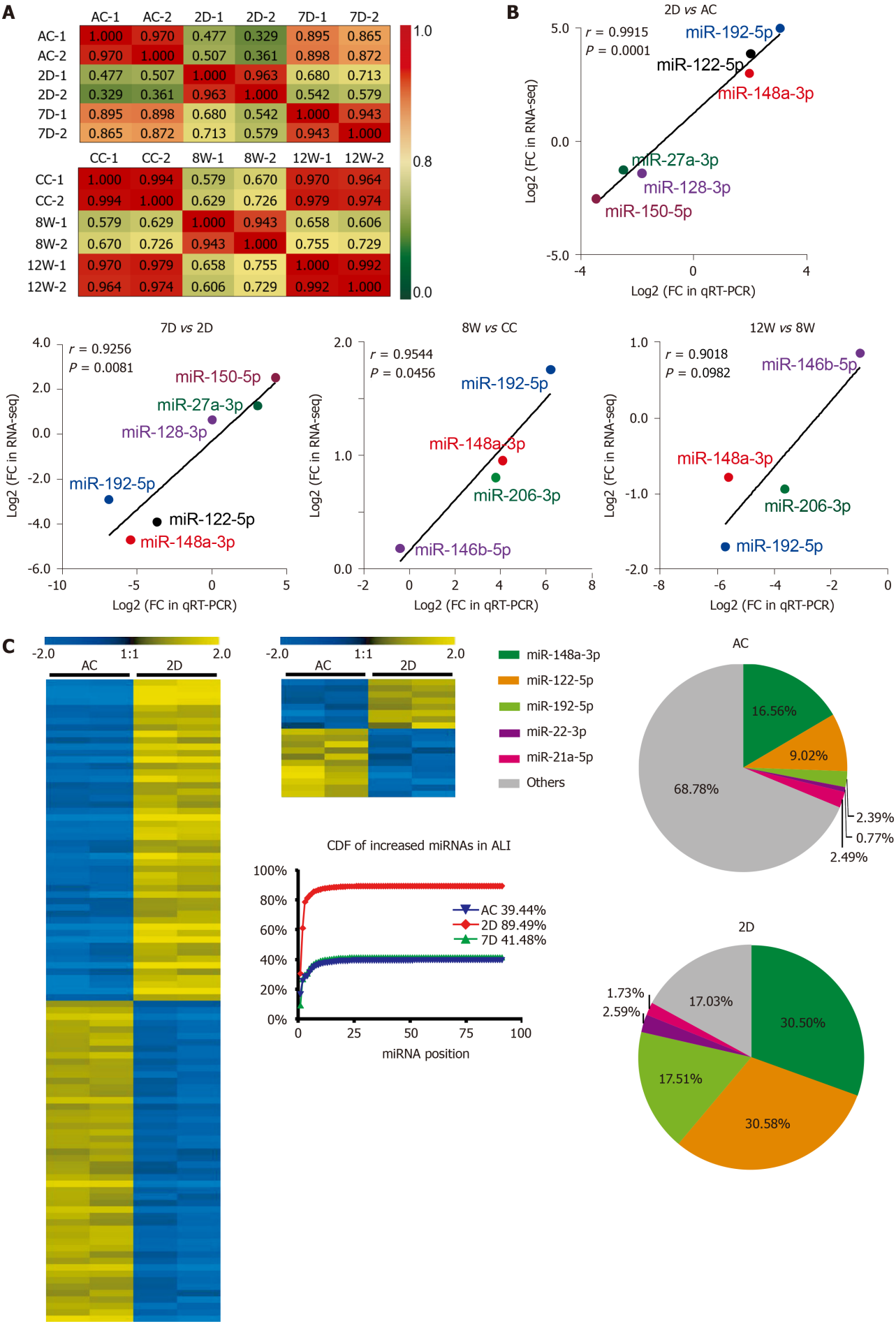


Figure 3 Annotations of small RNA in serum small extracellular vesicles from acute liver injury and chronic liver injury mice. A: Total RNA in serum small extracellular vesicles (sEVs) compared with the 2D group, $^{c}P < 0.001$; B: Small RNA (sRNA) concentrations in serum sEVs compared with the 2 d group, $^{c}P < 0.001$; C: Representative pie charts depicting the annotated sRNA species and their proportions in the different groups; D: Statistical analysis of sRNA species in sEVs. Compared with the 2 d group, $^{d}P < 0.0001$; compared with the 8 wk group, $^{f}P < 0.01$, $^{g}P < 0.0001$. D: Day; W: Week; AC: Acute liver injury control; CC: Chronic liver injury control; ALI: Acute liver injury; CLI: Chronic liver injury; miRNA: MicroRNA; snoRNA: Small nucleolar RNA.


A
AC-1 AC-2 2D-1 2D-2 7D-1 7D-2
AC-1 1.000 0.970 0.477 0.329 0.895 0.865
AC-2 0.970 1.000 0.507 0.361 0.898 0.872
2D-1 0.477 0.507 1.000 0.963 0.680 0.713
2D-2 0.329 0.361 0.963 1.000 0.542 0.579
7D-1 0.895 0.898 0.680 0.542 1.000 0.943
7D-2 0.865 0.872 0.713 0.579 0.943 1.000
CC-1 CC-2 8W-1 8W-2 12W-1 12W-2
CC-1 1.000 0.994 0.579 0.670 0.970 0.964
CC-2 0.994 1.000 0.629 0.726 0.979 0.974
8W-1 0.579 0.629 1.000 0.943 0.658 0.606
8W-2 0.670 0.726 0.943 1.000 0.755 0.729
12W-1 0.970 0.979 0.658 0.755 1.000 0.992
12W-2 0.964 0.974 0.606 0.729 0.992 1.000
1.0
0.8
0.0
B
2D vs AC
r = 0.9915
P = 0.0001
miR-192-5p
miR-122-5p
miR-148a-3p
miR-27a-3p
miR-128-3p
miR-150-5p
Log2 (FC in RNA-seq)
Log2 (FC in qRT-PCR)
7D vs 2D
r = 0.9256
P = 0.0081
miR-150-5p
miR-27a-3p
miR-128-3p
miR-192-5p
miR-122-5p
miR-148a-3p
8W vs CC
r = 0.9544
P = 0.0456
miR-192-5p
miR-148a-3p
miR-206-3p
miR-146b-5p
12W vs 8W
r = 0.9018
P = 0.0982
miR-146b-5p
miR-148a-3p
miR-206-3p
miR-192-5p
C
-2.0 1:1 2.0
AC 2D
miR-148a-3p
miR-122-5p
miR-192-5p
miR-22-3p
miR-21a-5p
Others
CDF of increased miRNAs in ALI
AC 39.44%
2D 89.49%
7D 41.48%
miRNA position
AC
16.56%
9.02%
68.78%
2.39%
0.77%
2.49%
2D
1.73%
2.59%
17.03%
30.50%
17.51%
30.58%

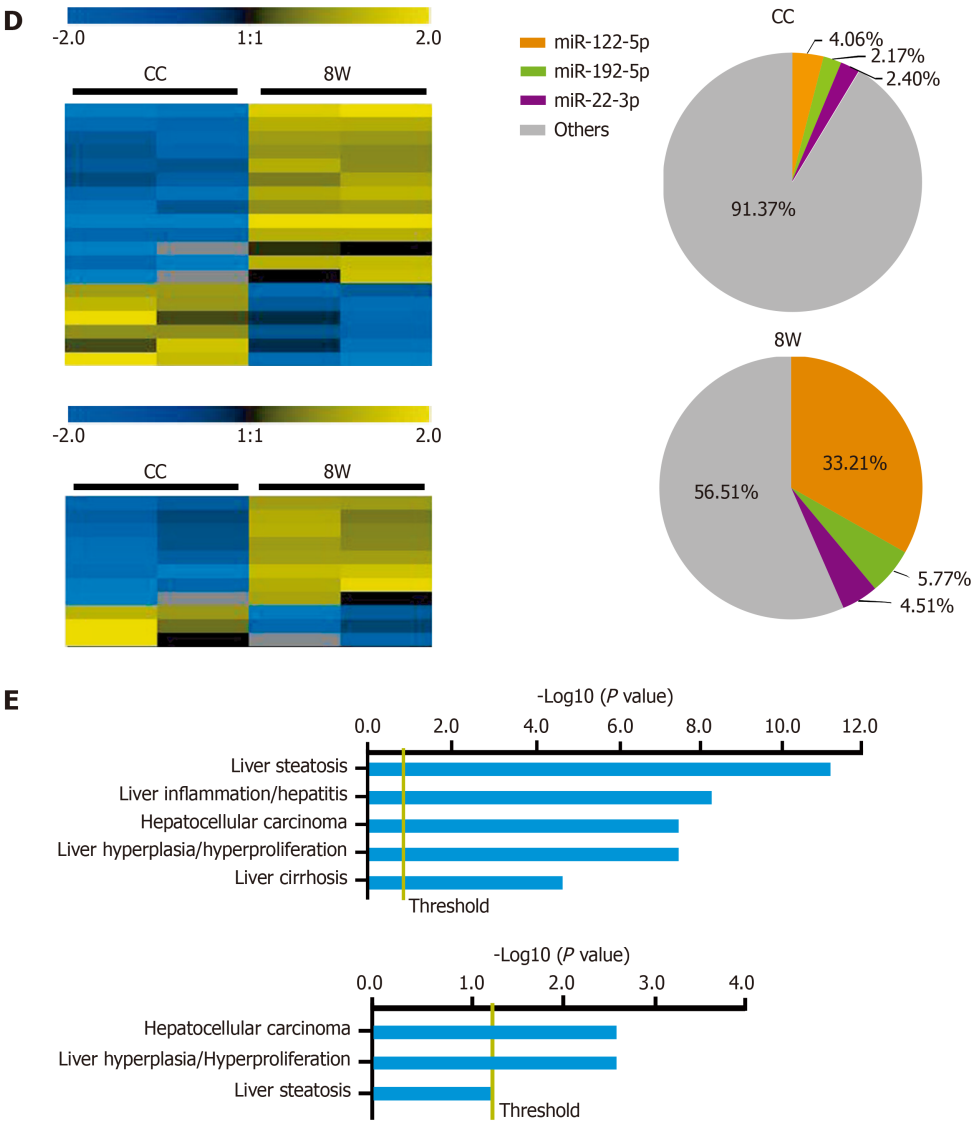


Figure 4 Differentially expressed microRNAs in serum small extracellular vesicles from acute liver injury and chronic liver injury mice and their biological significance. A: Pearson correlation coefficients between samples from acute liver injury (ALI) and chronic liver injury (CLI). The correlation coefficient values are labeled in the heat map; red or green represents high or low correlation, respectively; B: Validation of RNA sequencing data by quantitative real-time polymerase chain reaction for microRNAs (miRNAs) from ALI and CLI; C: Heatmap for the differentially expressed miRNAs in the ALI injury stage (2 d *vs* ALI control) and recovery stage (7 d *vs* ALI control), a cumulative distribution frequency plot for the increased miRNAs in ALI, and pie charts illustrating the proportions of the top five upregulated miRNAs in the ALI control and 2 d groups; D: Heatmap for the differentially expressed miRNAs in the CLI injury stage (8 wk *vs* CLI control) and recovery stage (12 wk *vs* CLI control). The pie charts illustrate the proportions of the top three upregulated miRNAs in the CLI control and 8 wk groups; E: The top hepatotoxicity processes related to the differentially expressed serum sEV miRNAs in ALI (top) and the differentially expressed serum sEV miRNAs in CLI (bottom) cataloged by Ingenuity Pathway Analysis-Tox function analysis. D: Day; W: Week; AC: Acute liver injury control; CC: Chronic liver injury control; ALI: Acute liver injury; CDF: Cumulative distribution frequency; qRT-PCR: Quantitative real-time polymerase chain reaction; FC: Fold change; RNA-seq: RNA sequencing; miRNA: MicroRNA.

expression and decreased the expression of some M2-like genes (*IL-10RB*, *CD163*, *ARG1* and *CD206*); ALI serum sEVs showed similar effects. However, compared to normal serum sEVs, ALI serum sEVs tended to decrease M1-like *IL-1B* but increase M2-like *CD163* gene expression (Figure 7C).

In order to further dissect the effects of ALI serum sEVs on monocyte-derived and resident hepatic macrophage subgroups, multiple-color flow cytometric analyses were performed to assess the expression of M1-like CD86 and M2-like CD206 in $CD11b^{+}F4/80^{Low}$ monocyte-derived and $CD11b^{+}F4/80^{High}$ resident macrophages (Tacke and Zimmermann[34] and our unpublished data). ALI serum sEVs decreased both CD86 and CD206 expression in the $CD11b^{+}F4/80^{Low}$ subgroup but increased CD206 expression in the $CD11b^{+}F4/80^{High}$ subgroup (Figure 7D and E). These findings indicated that ALI serum sEVs might induce depolarization of $CD11b^{+}F4/80^{Low}$ monocyte-derived macrophages but M2 differentiation of $CD11b^{+}F4/80^{High}$ resident macrophages.

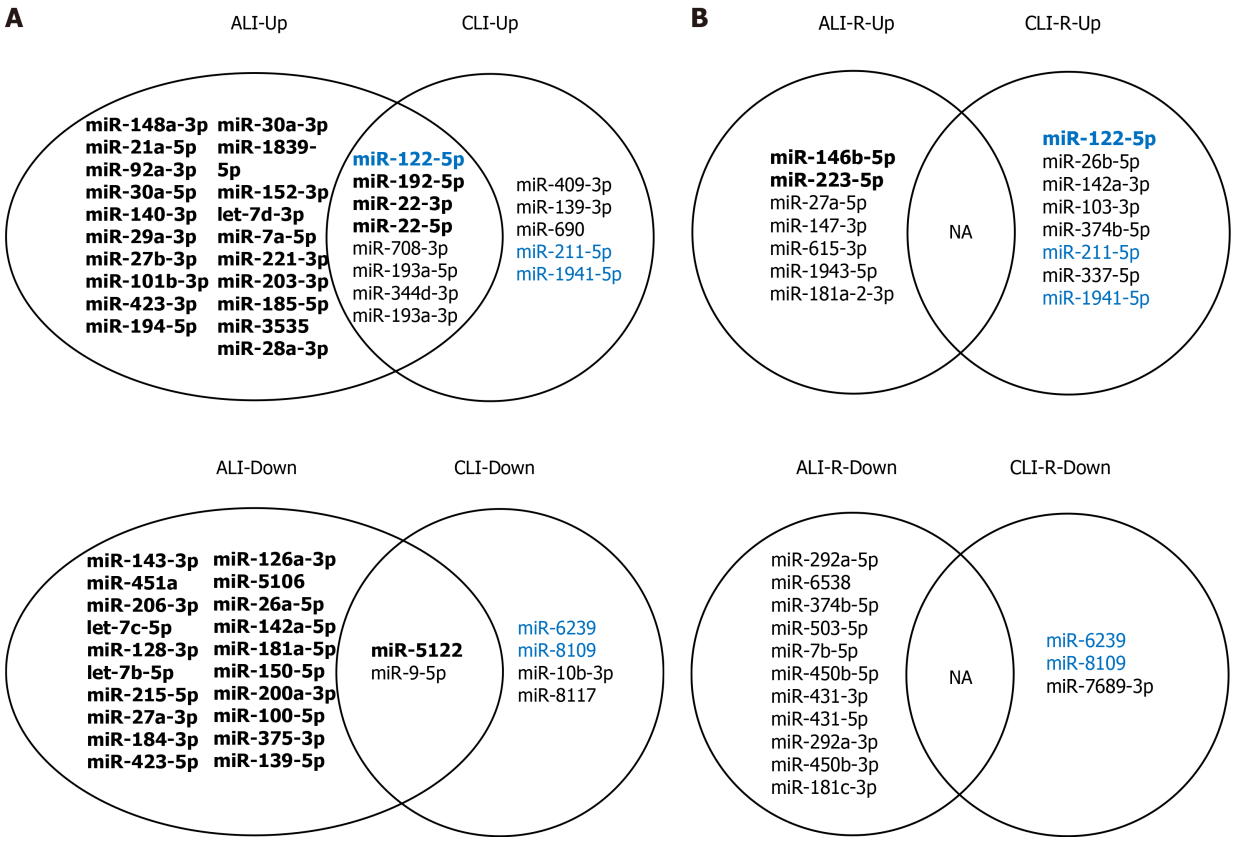


Figure 5 Common and specific microRNAs for acute and chronic liver injury and recovery in mouse models. A: Common and specific microRNAs (miRNAs) for acute and chronic liver injury; B: Common and specific miRNAs for the recovery stages of acute and chronic liver injury. Bold font: Transcripts per million reads Mean > 1000; Blue font: Overlapping microRNAs in injury and recovery; NA: Not applicable; ALI: Acute liver injury; CLI: Chronic liver injury; ALI-R: Acute liver injury recovery; CLI-R: Chronic liver injury recovery.

DISCUSSION

A growing number of studies have suggested the diagnostic value of serum sEV content for liver injury, especially miRNAs[6,8]. To explore potential serum sEV miRNA biomarkers for liver injury, we simulated the complex processes of liver injury and recovery in CCL_4-induced ALI and CLI mouse models. The 2D group and 8W group represented ALI and CLI, respectively, while the 7D group and 12W group represented the recovery stage of ALI and CLI, respectively. The ALT and AST levels elevated in the 2D group and 8W group and returned to the baseline of the control groups (Figure 1B and D). Moreover, the histological changes also reversed in the 7D and 12W recovery groups (Figure 1C and E), which indicated that the ALI and CLI and recovery models were well established.

Unexpectedly, aside from differentially expressed miRNAs, we found that the concentration, size and morphology of serum sEVs might be essential features in liver injury (Figure 2). The number of serum sEVs increased upon ALI, which has been reported in human alcoholic hepatitis and alcoholic liver injury mouse models[35-37]. Furthermore, we found sustained decreases in serum sEV number during the chronic injury stage and the recovery stage for both ALI and CLI. Moreover, serum sEVs became smaller upon ALI but enlarged during ALI recovery and the CLI stage, and the increased size persisted through the CLI recovery stage, at which time there were multiple peaks, as observed by NTA. Interestingly, aggregation of serum sEV particles was observed in samples from the ALI recovery stage, CLI stage and CLI recovery stage by TEM, which explained the multiple peaks and increased particle size found by NTA. We propose that the aggregation of sEVs might reflect membrane damage of extracellular vesicles following liver injury. Thus, changes in serum sEV concentration, size, and morphology are well connected to a particular stage of liver injury and could provide diagnostic clues. In addition, the changes in serum sEVs persisted even when liver function and visible histopathological changes were restored; thus, they could be useful to trace recent liver injury.

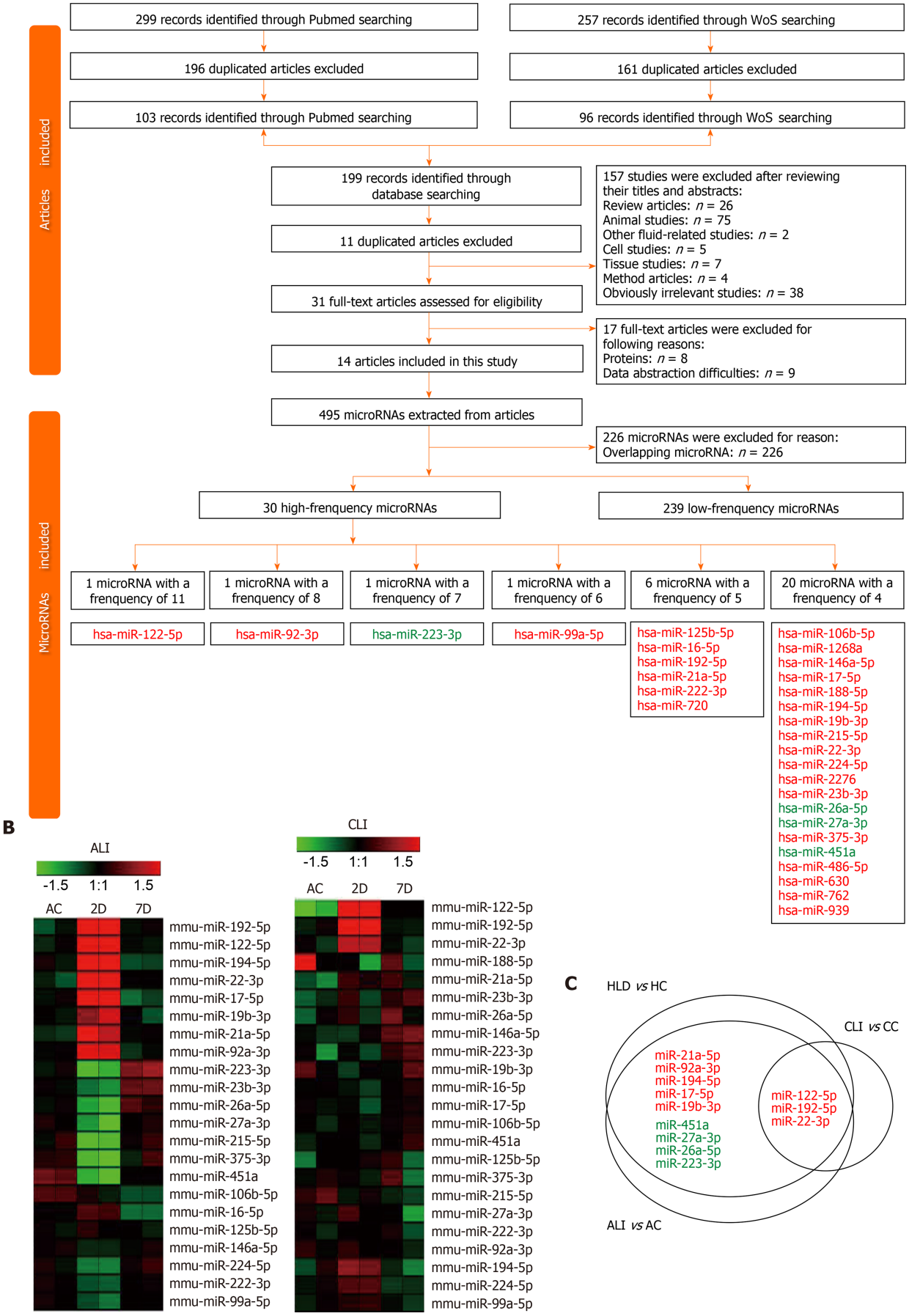


Figure 6 Differentially expressed circulating microRNAs in human liver disease and common microRNAs in mice. A: Flow chart of study selection and microRNA (miRNA) screening; B: Heatmap for the expression of high-frequency human liver disease (HLD)-related circulating miRNAs in acute liver

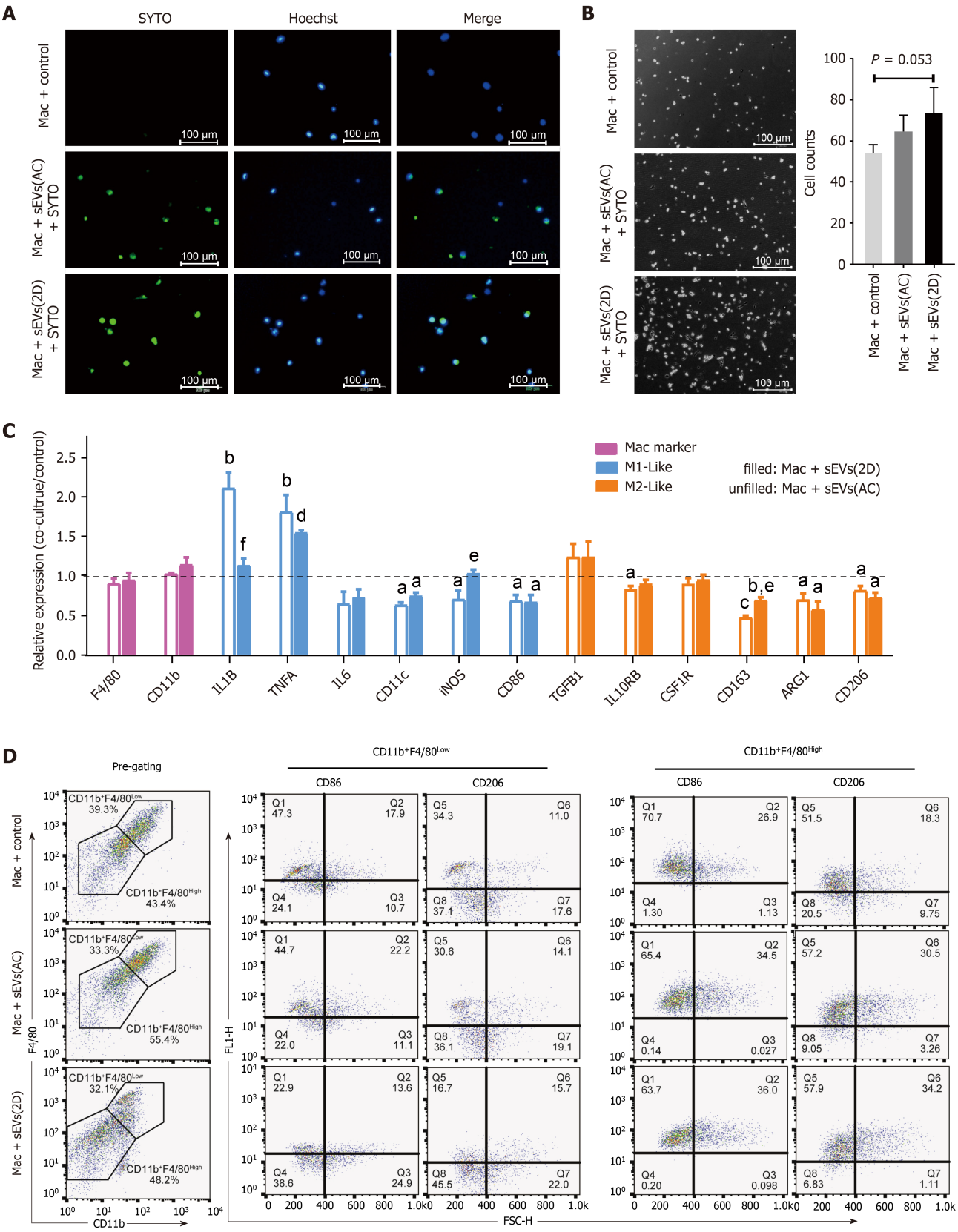
injury (ALI) and chronic liver injury (CLI) serum small extracellular vesicles (sEVs); C: Common miRNAs in the serum sEVs from mice with ALI and CLI and in the circulation in the context of HLDs. Red font: Upregulated miRNAs; Green font: Downregulated miRNAs; HC: Healthy control; AC: Acute liver injury control; CC: Chronic liver injury control; WoS: Web of Science; D: Day; W: Week.

Then, we showed that ALI and CLI altered the sRNA levels and components in serum sEVs. For ALI, both the total RNA and sRNA levels of serum sEVs increased significantly, and the proportion of miRNAs in sRNA also increased. Although there were no significant increases in RNA or sRNA levels for CLI, the proportion of miRNAs increased significantly. Compared to CLI, ALI significantly changed more miRNA species (176 *vs* 19, fold change ≥ 2.0, $P < 0.05$). The increased proportion of miRNAs upon liver injury was mainly attributable to a few highly abundant miRNAs. To traceback the primary source of the increased serum sEV miRNAs, the highly abundant miRNAs detected in serum sEVs from ALI and CLI mice were evaluated in the livers of normal male C57BL/6 mice. The miRNAs with the highest abundance were all liver-enriched miRNAs (Table 1). We propose that the liver is the main contributor to the elevations in miRNAs in serum sEVs for both ALI and CLI. Thus, serum sEVs carry the miRNA messages released from the injured liver, and examining the serum sEV miRNAs could be a reliable way to monitor either ALI or CLI.

By comparing the expression profiles of serum sEV miRNAs in various stages of ALI and CLI, we obtained a list of miRNAs that can be used as common liver injury signatures as well as the miRNA signatures for ALI, CLI and the recovery stages. However, these signatures were obtained from mouse models and need to be validated in human patients. Hence, we carried out a systematic review of previously published studies and obtained 30 miRNAs that were highly correlated with HLD, including drug-induced liver injury, chronic hepatitis B, chronic hepatitis C, nonalcoholic fatty liver disease, nonalcoholic steatohepatitis and liver cirrhosis (Figure 6). Of these 30 miRNAs, 3 miRNAs (miR-122-5p, miR-192-5p and miR-22-3p) were identified as common injury signatures that were increased in both ALI and CLI mice. Although these three miRNAs have been studied separately[24,36,38,39], here, for the first time, they were combined as a universal signature for either ALI or CLI. Nine miRNAs were identified as ALI signatures. Along with the five increased miRNAs (miR-21a-5p, miR-92a-3p, miR-194-5p, miR-17-5p and miR-19b-3p), four miRNAs were decreased (miR-451a, miR-27a-3p, miR-26a-5p and miR-223-3p): the same four miRNAs that are frequently reported to be decreased in HLD (Figure 6). Although the human data were mostly from patients with CLI, the overlapping ALI signatures might reflect active lesions. Based on serum sEV miRNAs, we established common signatures for liver injury and specific signatures for acute/active liver injury.

The biological significance of the alterations in sEV miRNAs upon liver injury was explored by Ingenuity Pathway Analysis. The top hepatotoxicity-related functions of the altered serum sEV miRNAs in ALI were hepatic steatosis and liver inflammation, while the altered miRNAs carried by serum sEVs from CLI were linked primarily to HCC, followed by liver hyperplasia. These findings are consistent with the clinical findings that ALI mainly causes inflammation and that sustained damage increases the risk of carcinoma[40,41]. Hepatic steatosis reflects fatty degeneration that is typically caused by CCL_4[42]. These findings reveal the pathological importance of serum sEVs during the processes of ALI and CLI.

It has been reported that hepatic macrophages can take up serum sEVs and play essential roles in the clearance of intravenously injected sEVs from the systemic circulation[43,44]. We were interested in determining the effects of liver injury-related serum sEVs on hepatic macrophages and whether these effects could aggravate liver damage or play a protective role. We found that *in vitro*, ALI serum sEVs could be taken up by hepatic macrophages and promote macrophage adhesion. Furthermore, ALI serum sEVs tended to decrease M1-like gene expression, such as *IL-1B* and *TNFA* expression, and increase M2-like gene expression, including *CD163* expression. However, the trends were not consistent. Considering that hepatic macrophages are heterogeneous populations composed of two subgroups, including resident macrophages and circulating monocyte-derived macrophages[34], we propose that macrophages of different origins might react inconsistently to ALI serum sEVs. The expression of M1 (CD86) and M2 (CD206) polarization signatures on the two subgroups of hepatic macrophages was examined by flow cytometry. ALI serum sEVs induced depolarization of $CD11b^{+}F4/80^{Low}$ monocyte-derived macrophages but induced M2 differentiation of $CD11b^{+}F4/80^{High}$ resident macrophages. We propose that the changes in serum sEVs upon ALI might alleviate liver damage by depolarizing


A
SYTO
Hoechst
Merge
Mac + control
Mac + sEVs(AC) + SYTO
Mac + sEVs(2D) + SYTO
100 μm
B
Mac + control
Mac + sEVs(AC) + SYTO
Mac + sEVs(2D) + SYTO
100 μm
P = 0.053
Cell counts
100
80
60
40
20
0
Mac + control
Mac + sEVs(AC)
Mac + sEVs(2D)
C
Relative expression (co-cultrue/control)
2.5
2.0
1.5
1.0
0.5
0.0
Mac marker
M1-Like
M2-Like
filled: Mac + sEVs(2D)
unfilled: Mac + sEVs(AC)
F4/80
CD11b
IL1B
TNFA
IL6
CD11c
iNOS
CD86
TGFB1
IL10RB
CSF1R
CD163
ARG1
CD206
D
Pre-gating
CD11b+F4/80Low
CD11b+F4/80High
CD86
CD206
Mac + control
Mac + sEVs(AC)
Mac + sEVs(2D)
F4/80
CD11b
FL1-H
FSC-H
CD11b+F4/80Low 39.3%
CD11b+F4/80High 43.4%
CD11b+F4/80Low 33.3%
CD11b+F4/80High 55.4%
CD11b+F4/80Low 32.1%
CD11b+F4/80High 48.2%
Q1 47.3
Q2 17.9
Q4 24.1
Q3 10.7
Q5 34.3
Q6 11.0
Q8 37.1
Q7 17.6
Q1 44.7
Q2 22.2
Q4 22.0
Q3 11.1
Q5 30.6
Q6 14.1
Q8 36.1
Q7 19.1
Q1 22.9
Q2 13.6
Q4 38.6
Q3 24.9
Q5 16.7
Q6 15.7
Q8 45.5
Q7 22.0
Q1 70.7
Q2 26.9
Q4 1.30
Q3 1.13
Q5 51.5
Q6 18.3
Q8 20.5
Q7 9.75
Q1 65.4
Q2 34.5
Q4 0.14
Q3 0.027
Q5 57.2
Q6 30.5
Q8 9.05
Q7 3.26
Q1 63.7
Q2 36.0
Q4 0.20
Q3 0.098
Q5 57.9
Q6 34.2
Q8 6.83
Q7 1.11

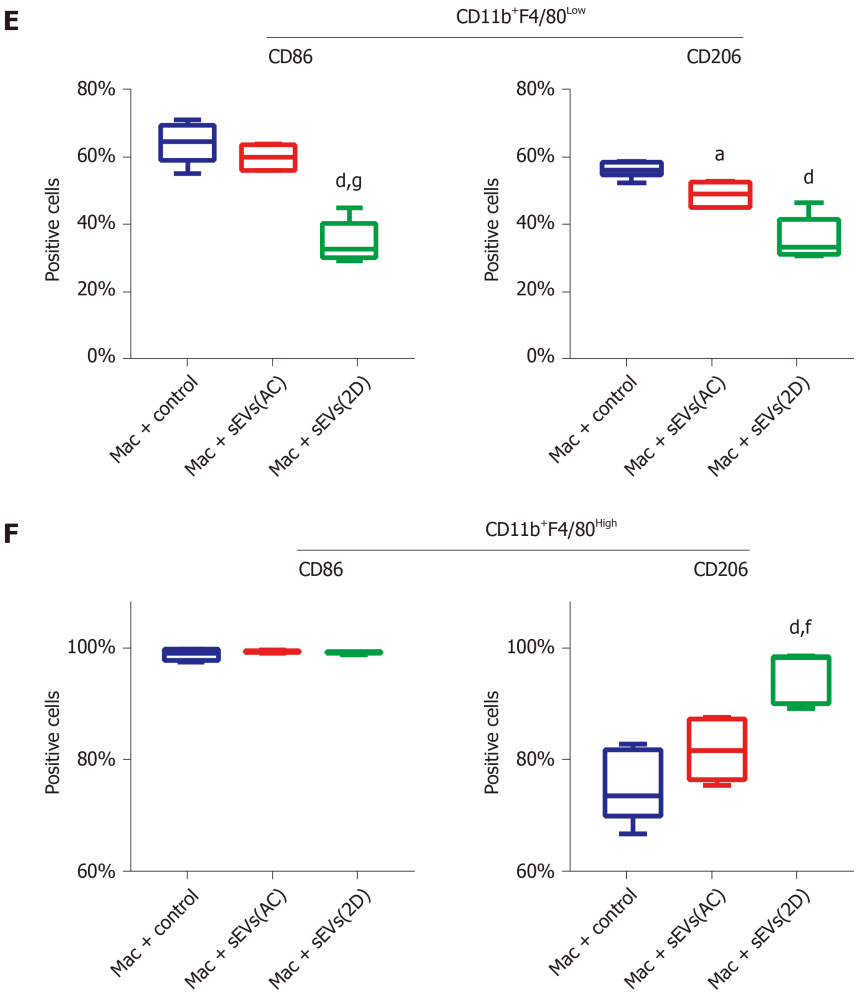


Figure 7 Uptake of serum small extracellular vesicles by hepatic macrophages and subsequent reprogramming. A: Uptake of SYTO-labeled serum small extracellular vesicles (sEVs) from normal (AC) or acute liver injury (ALI) (2D) mice by primary hepatic macrophages; B: Hepatic macrophages were incubated with AC or 2D serum sEVs for 24 h. The number of attached cells per 200 × field is shown; C: Expression of M1- and M2-like cell surface markers and cytokines in hepatic macrophages incubated with mice serum sEVs. The unfilled column represents macrophages incubated with AC sEVs, and the filled column represents macrophages incubated with 2D sEVs. Compared with the untreated control group, $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$; compared with the AC sEV treatment group, $^{e}P < 0.05$, $^{f}P < 0.01$; D: Macrophages were defined as $CD11b^{+}F4/80^{Low}$ and $CD11b^{+}F4/80^{High}$ subgroups. The representative images show the percentage of CD86-and CD206-positive cells in each subgroup subjected to the control, AC sEV and 2D sEV treatments; E: CD86- and CD206-positive cells in each subgroup. Compared with the control group, $^{a}P < 0.05$, $^{d}P < 0.0001$; compared with the AC sEV treatment group, $^{f}P < 0.01$, $^{g}P < 0.0001$. Scale bar = 100 μm. Mac: Macrophage; D: Day; AC: Acute liver injury control; sEVs: Small extracellular vesicles.

monocyte-derived macrophages and educating resident hepatic macrophages to transform into M2-like cells.

CONCLUSION

In conclusion, we found that the concentration, size and morphology of serum sEV particles were essential features for liver injury. We established specific serum sEV miRNA signatures for different liver injury stages and created a list of miRNAs that can be used as common liver injury biomarkers. The altered ALI and CLI serum sEV miRNAs were connected to diverse liver pathological processes. ALI serum sEVs reprogrammed hepatic macrophage subgroups differently. Serum sEVs not only have good diagnostic potential but also could be used to ameliorate liver injury. However, the diagnostic and therapeutic potential of these altered serum sEVs upon liver injury deserves further study.

ARTICLE HIGHLIGHTS

Research background

Both acute liver injury (ALI) and chronic liver injury (CLI) do not always cause noticeable signs and symptoms. Serum small extracellular vesicles (sEVs) have attracted tremendous interest due to their essential roles in intercellular communication and their diagnostic and therapeutic potential. The cargoes carried by sEVs represent a snapshot of the parental cells and change depending on the physiological and pathological states.

Research motivation

Serum sEVs and their small RNA (sRNA) cargoes could be promising biomarkers for the diagnosis of liver injury.

Research objectives

The present study aimed to characterize the dynamic changes of serum sEVs and their sRNA components during liver injury and to explore the effect of liver injury-related serum sEVs on hepatic macrophages.

Research methods

Male C57BL/6 mice were treated with CCL_4 to establish a mouse liver injury model for simulating ALI, CLI and recovery. Serum sEVs were obtained and characterized by transmission electron microscopy and nanoparticle tracking analysis. Serum sEV sRNAs were profiled by sRNA sequencing. Differentially expressed microRNAs (miRNAs) were compared to mouse liver-enriched miRNAs and previously reported circulating miRNAs related to human liver diseases. The biological significance was evaluated by Ingenuity Pathway Analysis of the altered sEV miRNAs and conditioned cultures of ALI serum sEVs with primary hepatic macrophages.

Research results

Both ALI and CLI changed the concentration and morphology of serum sEVs. The proportion of serum sEV miRNAs increased upon liver injury, with the liver as the primary contributor. The altered serum sEV miRNAs based on mouse study were consistent with human liver disease-related circulating miRNAs. We established serum sEV miRNA signatures for ALI and CLI and a panel of miRNAs (miR-122-5p, miR-192-5p, and miR-22-3p) as a common marker for liver injury. ALI serum sEVs decreased both CD86 and CD206 expression in monocyte-derived macrophages but increased CD206 expression in resident macrophages *in vitro*.

Research conclusions

Serum sEVs acquired different concentrations, sizes, morphologies and sRNA contents upon diverse liver injured pathological processes. ALI serum sEVs reprogrammed hepatic macrophage subgroups differently.

Research perspectives

Serum sEVs have good diagnostic and therapeutic potential for liver injury.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Shen Y for the statistical review.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 21; 27(43): 7530-7545

DOI: 10.3748/wjg.v27.i43.7530

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

ORIGINAL ARTICLE

Basic Study

Genome-wide map of N6-methyladenosine circular RNAs identified in mice model of severe acute pancreatitis

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Author contributions: Wu J and Yuan XH contributed equally to this work; Tang LJ and Sun HY participated in the study conception and design; Wu J and Yuan XH participated in the writing of the main manuscript; Wu J, Yuan XH, Jiang W and Lu YC participated in the performance of the experiments; Huang QL and Yang Y participated in statistical data analysis and interpretation; Qie HJ and Liu JT participated in the preparation of figures; Tang LJ and Sun HY participated in the revision of the manuscript and final approval.

Institutional review board statement: The study was reviewed and approved by the Institutional Ethics Committee at the General Hospital of Western Theater Command (Chengdu, China), No. A20190252005.

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Abstract

BACKGROUND

Severe acute pancreatitis (SAP) is a deadly inflammatory disease with complex pathogenesis and lack of effective therapeutic options. N6-methyladenosine (m6A) modification of circRNAs plays important roles in physiological and pathological processes. However, the roles of m6A circRNA in the pathological process of SAP remains unknown.

AIM

To identify transcriptome-wide map of m6A circRNAs and to determine their biological significance and potential mechanisms in SAP.

METHODS

The SAP in C57BL/6 mice was induced using 4% sodium taurocholate salt. The transcriptome-wide map of m6A circRNAs was identified by m6A-modified RNA immunoprecipitation sequencing. The biological significance of circRNAs with differentially expressed m6A peaks was evaluated through gene ontology and Kyoto Encyclopedia of Genes and Genomes analysis. The underlying mechanism of m6A circRNAs in SAP was analyzed by constructing of m6A circRNA-microRNA networks. The expression of demethylases was determined by

Institutional animal care and use committee statement: The experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the General Hospital of Western Theater Command (Chengdu, China), and were conducted in accordance with the established International Guiding Principles for Animal Research.

Conflict-of-interest statement: The authors declare that there is no conflict of interest related to this study.

Data sharing statement: We had submitted the data to the online repository, which can be found at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE173298.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Supported by the National Natural Science Foundation of China, No. 81772001; and the National Clinical Key Subject of China, No. 41732113.

Country/Territory of origin: China

Specialty type: Gastroenterology and hepatology

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

Peer-review report's scientific quality classification

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

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quantitative polymerase chain reaction and western blot to deduce the possible mechanism of reversible m^{6}A process in SAP.

RESULTS

Fifty-seven circRNAs with differentially expressed m^{6}A peaks were identified by m^{6}A-modified RNA immunoprecipitation sequencing, of which 32 were upregulated and 25 downregulated. Functional analysis of these m^{6}A circRNAs in SAP found some important pathways involved in the pathogenesis of SAP, such as regulation of autophagy and protein digestion. In m^{6}A circRNA–miRNA networks, several important miRNAs participated in the occurrence and progression of SAP were found to bind to these m^{6}A circRNAs, such as miR-24-3p, miR-26a, miR-92b, miR-216b, miR-324-5p and miR-762. Notably, the total m^{6}A level of circRNAs was reduced, while the demethylase alkylation repair homolog 5 was upregulated in SAP.

CONCLUSION

m^{6}A modification of circRNAs may be involved in the pathogenesis of SAP. Our findings may provide novel insights to explore the possible pathogenetic mechanism of SAP and seek new potential therapeutic targets for SAP.

Key Words: Severe acute pancreatitis; Circular RNAs; N^6-methyladenosine; MeRIP-seq; Epigenetic analysis

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Core Tip: We identified a transcriptome-wide map of N^6-methyladenosine (m^{6}A) circRNAs and determined their biological significance and potential mechanisms in severe acute pancreatitis (SAP). The main findings were: (1) Function analysis found that circRNAs with differentially expressed m^{6}A peaks were involved in the key process of SAP; (2) m^{6}A may affect the interplays of circRNAs and microRNAs to participate in the pathogenesis of SAP; and (3) Demethylase alkylation repair homolog 5 may play key roles in dynamic process of m^{6}A to downregulate the total m^{6}A level of circRNAs in SAP. We provided novel insights to explore the possible pathophysiological mechanism of SAP and seek new potential therapeutic targets.

Citation: Wu J, Yuan XH, Jiang W, Lu YC, Huang QL, Yang Y, Qie HJ, Liu JT, Sun HY, Tang LJ. Genome-wide map of N^6-methyladenosine circular RNAs identified in mice model of severe acute pancreatitis. *World J Gastroenterol* 2021; 27(43): 7530-7545
URL: https://www.wjgnet.com/1007-9327/full/v27/i43/7530.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i43.7530

INTRODUCTION

Acute pancreatitis (AP) is a pancreatic inflammatory disorder that is associated with substantial morbidity and mortality[1]. Approximately 20% of patients with AP develop into severe AP (SAP)[2]. Due to the extensive pancreatic necrosis, subsequent infection, systemic inflammatory response syndrome and multiple organ failure, the mortality of SAP is up to 30%[2,3]. Previous studies have suggested that some important pathological mechanisms, including premature trypsinogen activation in the acinar cells and macrophages, mitochondrial dysfunction, pathological calcium signaling, endoplasmic reticulum (ER) stress, and impaired autophagy, are involved in the initiation and development of SAP[1]. However, the pathophysiology of SAP is complex and remains unclear, especially the level of gene regulation.

CircRNAs were discovered in the 1970s[4] and were identified as single-stranded covalently closed RNA molecules that lack 5′ caps and 3′ tails[5]. Long after, they were thought to be the byproducts of splicing[6]. In recent years, as high-throughput sequencing developed, thousands of circRNAs were found to be expressed in a wide range of mammalian tissues[7,8], including the pancreas[9], and accumulating studies have demonstrated that circRNAs play vital roles in the whole process and prognosis

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Received: May 19, 2021
Peer-review started: May 19, 2021
First decision: June 22, 2021
Revised: June 23, 2021
Accepted: September 15, 2021
Article in press: September 15, 2021
Published online: November 21, 2021

P-Reviewer: Kontos CK, Surbatovic M
S-Editor: Wu YXJ
L-Editor: Filipodia
P-Editor: Xing YX

of many diseases, including cardiovascular diseases[8], cancer[10], neurodevelopmental processes[11], immune responses and immune diseases[12]. The main mechanisms of circRNAs participated in the initiation and development of diseases include the following functions[6,8,10,12]: interplay with RNA-binding proteins, microRNA (miRNA) sponges, regulating the stability of mRNAs, modulating the transcription of parental gene and the templates for protein synthesis. However, the post-transcription modification of circRNAs remains unclear.

N^6-methyladenosine (m^{6}A) is the most prevalent internal modification of RNA in eukaryotic cells[13]. In 2017, Zhou *et al*[14] reported that the m^{6}A modification is widespread in circRNAs and m^{6}A modifications are read and written by the same complexes in circRNAs and mRNAs. The regulatory role of m^{6}A is mainly performed by three homologous factors, namely so-called "writers", "erasers" and "readers"[13-15]. The writers mainly include methyltransferase-like 3 and 14 proteins (METTL3 and METTL14) and their cofactor WT1-associated protein (WTAP). They form a methyltransferase complex to catalyze the installation of m^{6}A. The erasers, including alkylation repair homolog 5 (ALKBH5) and fat mass and obesity related protein (FTO), can catalyze the oxidative demethylation of N-alkylated nucleic acid bases. The readers are mainly YT521-B homology (YTH) domain containing proteins family, including YTHDC1, YTHDC2, YTHDF1, YTHDF2 and YTHDF3. They can specifically recognize m^{6}A and regulate splicing, localization, degradation and translation of RNAs. Recently, it has been found that the m^{6}A modification of circRNAs plays a key role in innate immunity and tumors though regulating the metabolism and function of circRNAs[15]. In human embryonic stem cells and HeLa cells, m^{6}A circRNAs display cell-type-specific methylation patterns[14]. In colorectal carcinoma, the m^{6}A modification can modulate cytoplasmic export of circNSUN2 and stabilize HMGA2, ultimately enhancing the colorectal liver metastasis[16]. However, the roles of m^{6}A circRNAs in SAP are still unknown.

Here, we investigated the expression profile of m^{6}A circRNAs in SAP through m^{6}A-modified RNA immunoprecipitation sequencing (MeRIP-seq). We evaluated the biological significance of circRNAs with differentially expressed m^{6}A peaks though gene ontology (GO) analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and explored their underlying mechanism by construction of m^{6}A circRNA–miRNA networks. In addition, we determined the expression of demethyltransferase, ALKBH5 and FTO, to deduce the possible mechanism of reversible m^{6}A process in SAP.

MATERIALS AND METHODS

Animals and preparation of SAP model

Male C57BL/6 mice weighing 22-25 g were provided by Chengdu Dashuo Experimental Animal Technology Co. Ltd. All the mice were housed in ventilated plastic cage system and fed with the same food and water for 7 d to adapt to the environment. The entire research protocol was approved by the Institutional Animal Care and Use Committee at the General Hospital of Western Theater Command.

Before the operation, the mice were divided into SAP and control groups randomly (3 mice per group) and fasted for 12 h but had free access to water. Isoflurane (5%) was used to anesthetize mice by induction box prior to surgery. Then, the SAP was induced through 4% sodium taurocholate salt that was slowly retrogradely injected into the biliopancreatic duct with a microinfusion pump. All mice were killed 24 h after the establishment of model, and the blood samples and pancreatic tissues were collected for further analysis.

Pancreatic histological analysis

Pancreatic tissue (0.4 cm × 0.4 cm) was fixed in 4% paraformaldehyde solution. After dehydrating with ethanol, the tissue samples were embedded in paraffin. Then, the samples were cut into about 4-μm-thick sections, and the sections were stained with hematoxylin and eosin. The light microscopy at × 200 magnification was used to examine the slide. The scoring system described previously was used to evaluate the degree of pancreatic injury[17]. The scores were averaged for five different slides that were selected randomly from each pancreas.

Amylase and lipase measurement

The concentrations of lipase and amylase in serum were determined using Lipase Assay kit and Amylase Assay kit (Nanjing Jiancheng Bioengineering Institute,

Nanjing, China) according to the instructions.

RNA isolation and RNA quality control

TRIzol reagent (Invitrogen, Carlsbad, CA, United States) was used to extract total RNA from the homogenized pancreatic tissues of the control and SAP groups. The concentration of extracted RNA was measured at OD260 and 280 by NanoDrop ND-2000 instrument (Thermo Fisher Scientific, Waltham, MA, United States). We assessed the integrity of RNA through denaturing agarose gel electrophoresis. The OD A260/A280 ratio between 1.8 and 2.0 was set as the RNA purity standard.

Library preparation and MeRIP-seq

rRNAs in total RNA were removed using Ribo-Zero rRNA Removal Kits (Illumina, San Diego, CA, United States). The removal efficiency of rRNA by the residual determination of 28S and 18S of rRNA using quantitative polymerase chain reaction (qPCR). The fragmented RNA was incubated with the anti-m^{6}A antibody at 4 °C for 2 h in IPP buffer. Then, the mixture was immunoprecipitated by incubation with protein-A beads (Thermo Fisher Scientific) for 2 h at 4 °C. The bound RNA was eluted from the beads with m^{6}A (Berry & Associates) in IPP buffer and then extracted with TRIzol reagent (Thermo Fisher Scientific). The immunoprecipitated RNA and input RNA were used to construct the library using NEBNext® Ultra™ RNA Library Prep Kit and double-ended 150-bp sequencing of the m^{6}A-IP and input samples was performed on an Illumina HiSeq sequencer (performed by Cloudseq Biotech Inc., Shanghai, China).

Analysis of MeRIP-Seq data

Paired-end reads were harvested from the Illumina HiSeq 4000 sequencer, and were quality controlled by Q30. To obtain high quality clean reads, 3′ adaptor-trimming and low-quality reads were removed by cutadapt software. The clean reads with high quality of the input library were aligned to the mouse reference genome (UCSC MM10) with STAR software. DCC software was used for detecting and identifying the circRNAs. The identified circRNAs were annotated using the circBase database and Circ2Traits database. For all samples, raw junction reads were normalized to the number of total mapped reads and log2 transformed. The read alignments on the genome were visualized using the tool integrative genomics viewer. The adapter-removal reads were aligned to the reference genome using Hisat2 software. The methylated sites in each sample were identified using MACS software. Differentially methylated sites were identified using diffReps software.

GO and KEGG analysis

The parent genes of circRNAs with differential m^{6}A peaks were selected to analyze their potential biological roles through GO and KEGG pathway analysis. GO analysis included three parts, namely, biological process (BP) analysis, molecular function (MF) analysis, and cell component (CC) analysis[18]. GO analysis was performed by R topGO package. Fisher's exact test in Matlab MCR software was applied to calculate the enrichment of each pathway. The bubble plots and column plots were generated using the ggplot2 in R package (https://ggplot2.tidyverse.org).

Construction of circRNA–miRNA networks

circRNA containing miRNA-binding sites can bind to miRNA response elements competitively, further regulating the target mRNAs[19]. The top 10 upregulated and top 10 downregulated circRNAs according to the level of m^{6}A were selected to construct circRNA–miRNA networks. The m^{6}A circRNA–miRNA networks were constructed using TargetScan software and miRanda software and the circRNA–miRNA interactions were visualized by Cytoscape.

Conservation analysis

The top 10 upregulated and top 10 downregulated circRNAs were selected to analyze their homology with human circRNAs. The sequence of human circRNAs was downloaded from circBase database and the sequence of each selected m^{6}A circRNA was blasted against the human circRNAs sequence by the blastn function of Blast software.

Western blotting

The whole pancreatic tissues from SAP and control groups were placed in RIPA lysate buffer with protease inhibitor, phosphatase inhibitor and phenylmethylsulfonyl fluoride inside (Total Protein Extraction Kit; Beijing Solarbio Science and Technology

Inc., Beijing, China), and the tissues were homogenized with homogenizer. The tissue homogenate was centrifuged at 12000 g for 30 min at 4 °C, and the supernatant was collected. After protein concentration was measured by BCA Protein Assay Kit (Beyotime Biotechnology, Jiangsu, China), the supernatant was mixed with loading buffer (Beijing Solarbio Science and Technology), boiled at 100 °C for 10 min for protein denaturation, and stored at -80 °C after separation. The target proteins were separated by SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membrane (0.45 μm, IPVH00010; Millipore, Billerica, MA, United States), blocked in 5% nonfat milk for 1 h at room temperature (22 ± 3 °C), and then incubated with primary antibody, FTO (1:1000, D2V1I; Cell Signaling Technology, Danvers, MA, United States), ALKBH5 (1:2000, 16837-1-AP; Proteintech, Rosemont, IL, United States), GAPDH (1:5000, 10494-1-AP; Proteintech) at 4 °C overnight. The membranes were washed with Tris-buffered saline with Tween-20 (TBST) (Beijing Solarbio Science and Technology) three times and incubated with secondary antibody (1:10000, 15015; Proteintech) at room temperature for 1 h. After being washed three times with TBST, the protein bands were visualized by enhanced chemiluminescence (Immobilon Western Chemilum HRP Substrate; Millipore) in a biological imaging system.

qPCR

The total RNA was extracted from SAP and control groups as described above. qPCR was performed using One Step SYBR® PrimeScript™ RT-PCR kit II (Takara Biotechnology Co., Ltd., Dalian, China) and the primers (ALKBH5: forward 5′-GGCGGTCATCATTCTCAGGAAGAC-3′ and reverse 5′-CTGACAGGCGATCTGAAGCATAGC-3′; FTO: forward 5′-CTCACAGCC TCGGTTTAGTTCCAC-3′ and reverse 5′–CGTCGCCATCGTCTGAGTCATT G-3′; GAPDH: forward 5′-GGTGAAGGTCGGTGTGAACG-3′ and reverse 5′-CTCGCTCCTGGAAGATGGTG-3′) were synthesized by Shanghai Sangon Biotech Co., Ltd.. The outcomes were analyzed by means of $2^{-\Delta\Delta CT}$ through normalizing the quantity of GAPDH.

Data analysis

GraphPad Prism 8 (La Jolla, CA, United States) and SPSS 22.0 (IBM Corp., Armonk, NY, United States) were used for performing statistical analyses. Student's *t* test was used for estimating statistically significance between two groups. The results were evaluated through Spearman's correlation coefficient test. All values are shown as mean ± SE of the mean; $P < 0.05$ was regarded as statistically significant.

RESULTS

Evaluation of mouse model of SAP

Twenty-four hours after treatment with sodium taurocholate salt, the staining of hematoxylin and eosin on the pancreatic tissues from the SAP group showed typical histopathological changes, including pancreatic lobular edema, extensive acinar cell necrosis, focal expansion of the pancreatic interlobular septum and granulocyte infiltration (Figure 1A). By contrast, under light microscopy, the pancreases from the control group had a complete normal structure. Figure 1B showed the corresponding histopathological scores. At the same time, considering that the levels of serum lipase and amylase are as one of the diagnostic criteria of AP[20], we determined their concentrations in serum. As a result, the serum lipase and amylase levels in the SAP group were also markedly higher than those in the control group ($P < 0.05$; Figure 1C and 1D). These results confirmed the successful establishment of the SAP mice model.

Overview of m^{6}A circRNAs in SAP

We used MeRIP-seq to investigate the expression of m^{6}A circRNAs in pancreatic tissues from the control and SAP groups. We had submitted the data to the online repository, which can be found at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE173298. Before performing MeRIP-seq, the residual determination of 28S and 18S of rRNA showed that the rRNAs in total RNA were removed effectively (Supplementary Figure 1). In general, a total of 409 m^{6}A circRNAs were identified in all chromosomes (Figure 2A). Among these, 178 were specifically expressed in the SAP group, 107 in the control group, and 124 were shared in both groups (Figure 2B). m^{6}A level in total circRNAs from the SAP group was lower than that from the control group (Figure 2C). Besides, > 80% of circRNAs contained only one m^{6}A peak in both SAP and control groups (Figure 2D).

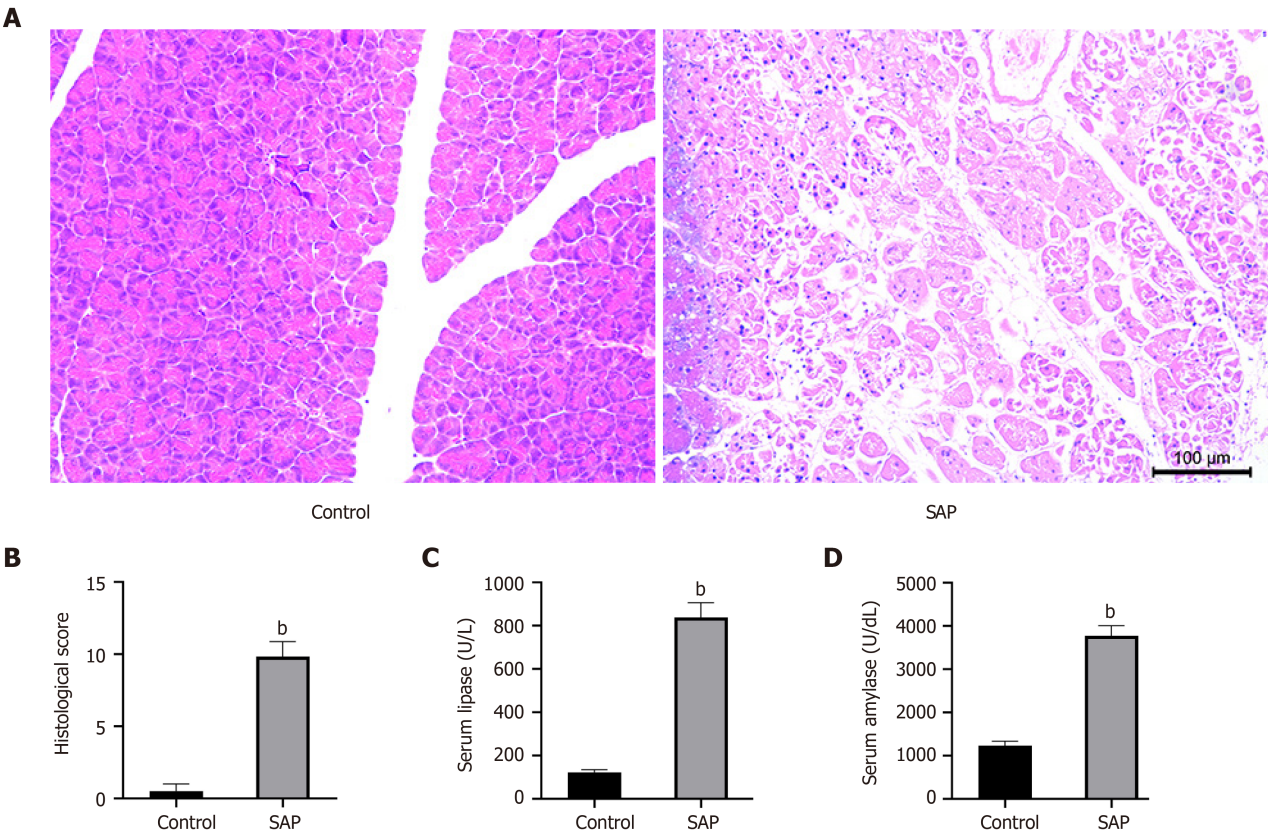


Figure 1 Evaluation of mouse model of severe acute pancreatitis. A: Representative images of pancreatic tissues stained with hematoxylin from control (left) and severe acute pancreatitis (SAP) (right) groups (× 100 magnification); B: Histological score of pancreatic tissues in control and SAP groups; C and D: Levels of serum lipase and amylase, respectively. $^bP < 0.01$ *vs* control group, $n = 3$ per group.

Differential m^{6}A modification of circRNAs in SAP

To understand the biological role of m^{6}A modification of circRNAs in SAP, the circRNAs with differentially expressed (DE) m^{6}A peaks were further analyzed. Significant differential expression was defined as fold-change > 2 and $P < 0.05$. Compared with the control group, 57 circRNAs with DE m^{6}A peaks were identified; 32 were upregulated and 25 downregulated in the SAP group. Table 1 presents the top 10 methylated m^{6}A sites that were up- and downregulated within circRNAs. Figure 3A shows the m^{6}A circRNAs expression profile in the SAP and control groups though hierarchical cluster analysis. The scatter plot exhibits the variation of DE m^{6}A circRNAs between the SAP and control groups (Figure 3B). The volcano plot depicted DE m^{6}A circRNAs between the two groups (Figure 3C).

Distribution of m^{6}A sites in SAP and control groups

We identified 903 m^{6}A peaks distributed on 781 circRNAs and it is reported that circRNAs can be generated from any region of the genome[21]. Therefore, we firstly analyzed the genomic distribution of m^{6}A and non-m^{6}A circRNAs according to their genomic origins to explore their distribution features. As a results, in non-m^{6}A circRNAs, 45.33% were sense overlapping, 21.15% exonic, 26.71% intronic, 4.94% intergenic and a few antisense; in m^{6}A circRNAs, 42.78% were sense overlapping, 30.32% exonic, 21.27% intronic, 3.42% intergenic and a few antisense (Figure 4A). These results indicated that the majority of m^{6}A and non-m^{6}A circRNAs were commonly encoded by sense overlapping sequences and the number of circRNAs that generated from protein-coding genes in m^{6}A circRNAs was more than those in non-m^{6}A circRNAs.

We further analyzed the distribution of circRNAs with DE m^{6}A peaks. The length of DE m^{6}A circRNAs was mainly enriched in 1–10 000 base pairs (Figure 4B). Although the host genes of m^{6}A circRNAs located in all chromosomes, the dysregulated parts mostly located in chromosomes 4, 9 and 11 (Figure 4C). A previous study reported that most circRNAs that derived from protein-coding genes spanned two or three exons [14]. In this study, the majority of circRNAs from protein-coding genes spanned one or two exons (Figure 4D). Similarly, the majority of m^{6}A circRNAs and non-m^{6}A

Table 1 Top 20 differently expressed N6-methyladenosine peaks compared with control group

	PeakStart	PeakEnd	circRNA	Regulation	Fold-change	*P* value
chr15	98658229	98658320	chr15:98656602-98658435-	Up	187.2	3.67392E-09
chr11	74929241	74929540	chr11:74928993-74990215+	Up	172.8	3.92116E-09
chr9	108248361	108248660	chr9:108207543-108263690-	Up	120.034482	3.70238E-08
chr2	153763381	153763760	chr2:153756037-153769786+	Up	106.330434	2.09239E-08
chr18	30281961	30282053	chr18:30276981-30282053+	Up	50.9545454	7.61781E-09
chr19	40346381	40346760	chr19:40314443-40373578-	Up	42.4	0.026929988
chr16	94641481	94641740	chr16:94611419-94694141+	Up	37.6772727	7.9549E-08
chr10	60144412	60144720	chr10:60144413-60144723-	Up	24.9	0.014047401
chr11	44652781	44652825	chr11:44651797-44652825+	Up	23.9	0.007108941
chr7	63895821	63896100	chr7:63891679-63938495-	Up	21.1	0.034908475
chr9	107852341	107852720	chr9:107847268-107860459-	Down	302.6	3.63198E-09
chr8	104143561	104143760	chr8:104143031-104143793+	Down	160.728571	1.15908E-08
chr1	150426881	150427260	chr1:150413021-150442180+	Down	52.095238	1.98141E-08
chr1	13312381	13312680	chr1:13298706-13325802-	Down	51.7	6.86061E-05
chr6	119970581	119970800	chr6:119951703-120038640-	Down	47.4	9.37286E-05
chr11	23271132	23271205	chr11:23261835-23271205+	Down	40.6	0.000442849
chr4	108499346	108499398	chr4:108486454-108508433+	Down	27.79	3.27262E-08
chr9	102619691	102619760	chr9:102618811-102619760-	Down	24.25	4.83147E-07
chr11	32296401	32296600	chr11:32283981-32297161+	Down	24.1	4.96449E-07
chr9	69414201	69414580	chr9:69408311-69432615+	Down	22.6	0.00943617

circRNAs were more commonly encoded by a single or two exons (Figure 4E).

Functional analysis of circRNAs with DE m^{6}A peaks

To explore the function of m^{6}A circRNAs in SAP, GO analysis and KEGG pathway analysis of circRNAs with the DE m^{6}A peaks were performed. Figure 5A presented the top 10 GO terms of circRNAs with upregulated m^{6}A peaks from the three aspects: BP, CC and MF. For BP, the most enriched and meaningful GO terms were cellular component organization, macromolecule metabolic process and regulation of developmental process. For CC, the top three terms were focal adhesion, cell–substrate junction and anchoring junction. For MF, the main represented GO terms were C2H2 zinc finger domain binding and protein binding. The top 10 pathways from KEGG pathway analysis for circRNAs with upregulated m^{6}A peaks were selected and presented in a bubble chart (Figure 5B). Among them, protein digestion and absorption and regulation of autophagy were the major signaling pathways associated with the SAP progression.

The GO terms of circRNAs with downregulated m^{6}A peaks are presented in Figure 5C. For BP, protein-containing complex localization, RNA transport and macromolecule metabolic process were the most enriched and meaningful GO terms. For CC, nucleus, dendrite and dendritic tree were the top three terms. For MF, the main represented GO terms were channel regulator activity, RNA, enzyme and protein binding. As for the KEGG pathway analysis of circRNAs with downregulated m^{6}A peaks, RNA transport was the main pathway (Figure 5D).

Relationship between m^{6}A level and expression of circRNAs in SAP

To explore whether m^{6}A modification could affect the expression of circRNAs, we analyzed the expression of m^{6}A circRNAs. The expression level of these circRNAs with DE m^{6}A peaks did not have significant differences (fold-change < 2 or $P > 0.05$; Supplementary Table 1), indicating that m^{6}A modification of circRNAs did not influence the expression of circRNAs. To verify this result further, we analyzed the cumulative distribution of circRNA expression between the control and SAP groups

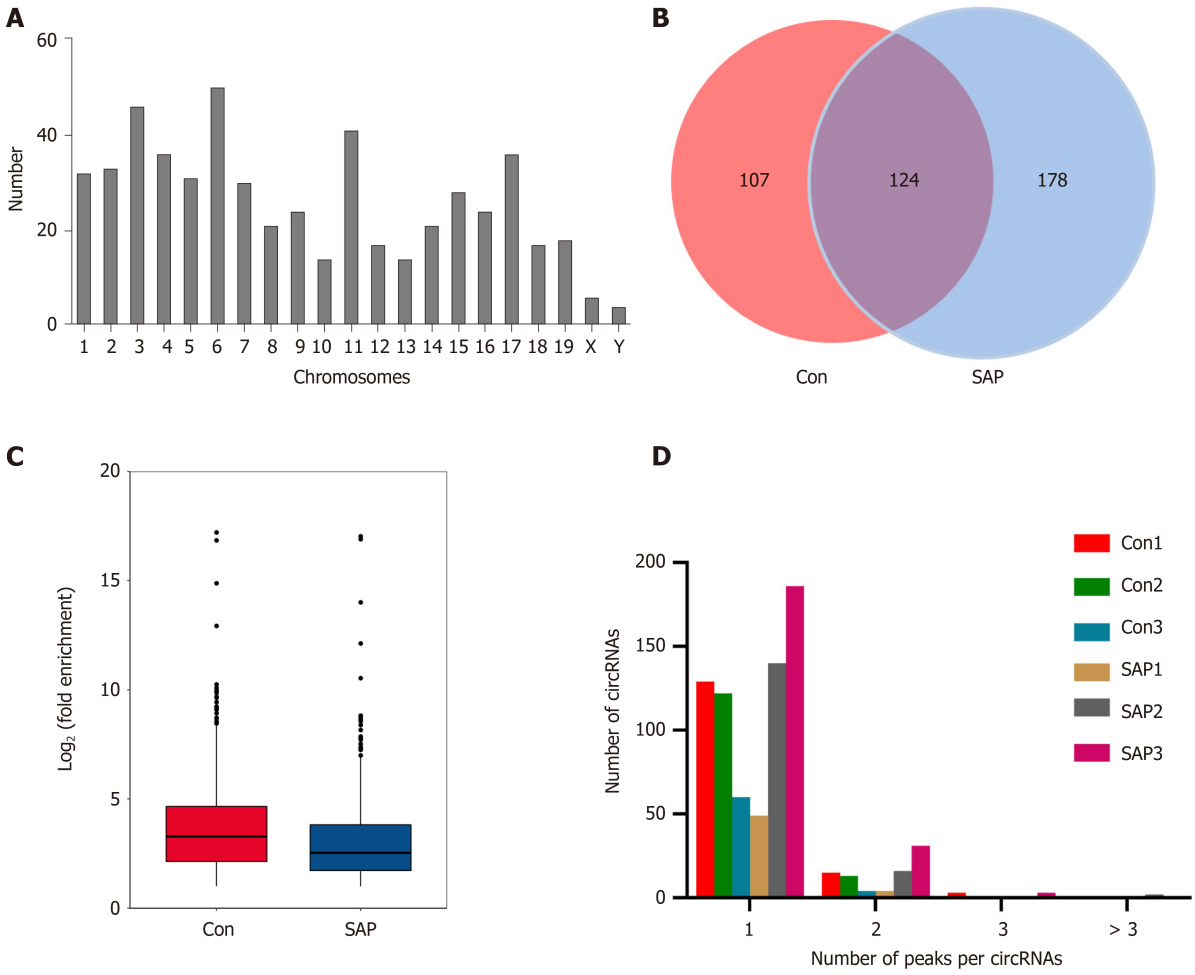


Figure 2 Overview of N^6-methyladenosine circRNAs in severe acute pancreatitis. A: Number of identified N6-methyladenosine (m^{6}A) circRNAs according to distribution on chromosomes; B: Venn diagram exhibiting number of common and specific m^{6}A circRNAs between control and severe acute pancreatitis (SAP) groups; C: Box plot showing level of m^{6}A peaks enrichment in circRNAs in control and SAP groups; D: Number of circRNAs containing variant numbers of m^{6}A peaks.

for m^{6}A and non-m^{6}A circRNAs (Figure 6). This was consistent with the above result.

Construction of m^{6}A circRNA–miRNA networks in SAP

Given the importance of circRNA–miRNA interaction[22] and to further explore the underlying mechanism of these circRNAs with DE m^{6}A peaks, the top 10 upregulated and top 10 downregulated circRNAs according to the level of m^{6}A were selected to construct circRNA–miRNA networks. In this network map, several important miRNAs participated in the occurrence and development of SAP were found to bind to these m^{6}A circRNAs (Figure 7), such as miR-24-3p, miR-26a, miR-92b, miR-216b, miR-324-5p and miR-762. These data suggest that these circRNAs with DE m^{6}A peaks might play a role in the pathological process of SAP.

Conservation analysis of identified m^{6}A circRNAs with human circRNAs

To explore whether the circRNAs with DE m^{6}A peaks identified in mouse SAP may have similar roles in human SAP, we performed the conservation analysis of the sequence of the top 10 upregulated and top 10 downregulated circRNAs preliminarily. Through aligning with the sequence of human circRNAs that downloaded from circBase database, we found that 15/20 of the selected circRNAs that have highly similar sequences to human circRNAs (sequence identity > 80%), as shown in the Table 2. These results suggested that these circRNAs may have similar roles in human SAP.

Expression of demethyltransferase in SAP

Given that the total m^{6}A level of circRNAs was reduced in SAP and to explore how the m^{6}A level was regulated in SAP, we detected the protein and mRNA expression of two

Table 2 The conservation analysis of the sequence between the selected circRNAs and human circRNAs

	Human circRNA				
Mouse circRNA	Human circRNA	Hg19 location	Transcript	Parent gene	Sequence identity, %
chr15:98656602-98658435-	hsa_circ_0026065	chr12:49223538-49245957-	NM_004818	*DDX23*	88.75
chr11:74928993-74990215+	hsa_circ_0041387	chr17:2139785-2203958-	NM_001170957	*SMG6*	86.32
chr9:108207543-108263690-	hsa_circ_0124055	chr3:49514281-49548252+	NM_001177634	*DAG1*	85.36
chr2:153756037-153769786+	hsa_circ_0059811	chr20:31436477-31438211+	NM_012325	*MAPRE1*	84.18
chr19:40314443-40373578-	hsa_circ_0094611	chr10:97110965-97114724-	ENST00000371247.2	*SORBS1*	93.52
chr16:94611419-94694141+	hsa_circ_0115989	chr21:38792600-38888974+	ENST00000338785.3	*DYRK1A*	91.34
chr7:63891679-63938495-	hsa_circ_0034321	chr15:31619082-31670102+	NM_015995	*KLF13*	85.89
chr9:107847268-107860459-	hsa_circ_0065768	chr3:50000008-50114685+	NM_005777	*RBM6*	90.84
chr1:150413021-150442180+	hsa_circ_0111511	chr1:186294895-186325581-	NM_003292	*TPR*	87.91
chr1:13298706-13325802-	hsa_circ_0113369	chr1:42166586-42254891-	ENST00000247584.5	*HIVEP3*	91.30
chr6:119951703-120038640-	hsa_circ_0024963	chr12:939168-990955+	NM_001184985	*WNK1*	92.02
chr11:23261835-23271205+	hsa_circ_0120688	chr2:61749745-61764803-	ENST00000404992.2	*XPO1*	95.11
chr4:108486454-108508433+	hsa_circ_0012539	chr1:52927184-53018762-	NM_001009881	*ZCCHC11*	92.38
chr11:32283981-32297161+	hsa_circ_0118668	chr2:202780266-202790202-	None	*None*	91.48
chr9:69408311-69432615+	hsa_circ_0035568	chr15:60720627-60748993-	NM_024611	*NARG2*	87.08

demethyltransferases (ALKBH5 and FTO). FTO was reduced at the level of protein, but ALKBH5 was increased in SAP at both the level of mRNA and protein (Figure 8). These results indicated that ALKBH5 might be related to the dynamic process of m^{6}A in SAP.

DISCUSSION

In the present study, we identified transcriptome-wide map of m^{6}A circRNAs and determined their biological significance and potential mechanisms for the first time in SAP. The main findings are: (1) We identified 57 circRNAs with DE m^{6}A peaks and found these DE m^{6}A circRNAs were involved in the key process of SAP by GO and KEGG analysis, such as protein digestion and regulation of autophagy; (2) In m^{6}A circRNA-miRNA networks, several important miRNAs participated in the initiation and development of SAP were found to bind to these m^{6}A circRNAs potentially, suggesting that m^{6}A may affect the interplays with miRNAs; and (3) The total m^{6}A level was reduced in SAP, and the demethylase ALKBH5 was found to be upregulated in SAP, indicating that ALKBH5 may be related to dynamic process of m^{6}A in SAP. These results suggested that m^{6}A modification on circRNAs may be involved in the pathophysiology of SAP, which may provide novel insights to explore the possible pathophysiological mechanism of SAP and seek new potential therapeutic targets.

To find effective therapeutic targets for SAP, many studies have explored the underlying molecular mechanisms of SAP. Our previous study found that many circRNAs are expressed in mice with SAP[9] and these circRNAs play an important role in the pathogenetic mechanism of SAP[9,23]. In recent years, m^{6}A modification of circRNAs was found to be widespread[14] and gained widespread attention in epigenetics. Several important studies have investigated the roles of m^{6}A modification in circRNA metabolism and found that m^{6}A circRNAs play key roles in some diseases [16,24-28]. In circRNA metabolism, m^{6}A modifications can regulate its translation through recognition by YTHDF3 and eIF4G2, and this progress of translation can be enhanced by METTL3/14 and inhibited by FTO[24,25]. In addition, m^{6}A circRNAs associate with YTHDF2 in an HRSP12-dependent manner and are selectively downregulated by RNase P/MRP[26]. In innate immunity, Chen *et al*[27] found that unmodified circRNA adjuvant induces antigen-specific T and B cell responses, but m^{6}A modification could abrogate circRNA immunity though YTHDF2-mediated suppression. In male germ cells, the back splicing tends to occur mainly at m^{6}A-

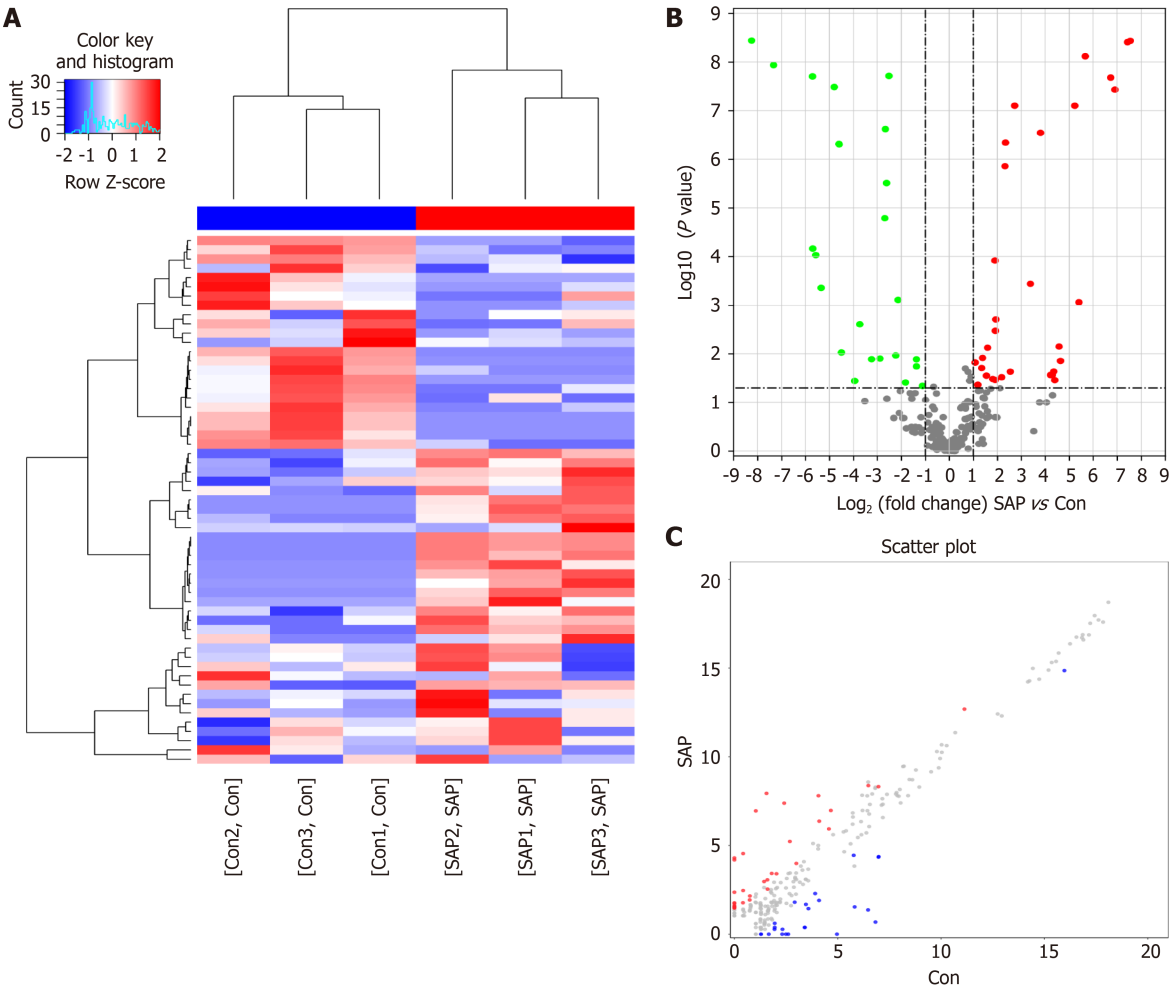


Figure 3 Differential N^6-methyladenosine modification of circRNAs in severe acute pancreatitis. A: Hierarchical clustering graph exhibiting differential N^6-methyladenosine (m^{6}A) modification of circRNAs in control and severe acute pancreatitis (SAP) groups. Higher expression is presented in red and lower expression in blue; B and C: Volcano and scatter plot showing the circRNAs with significant differentially expressed m^{6}A peaks.

enriched sites, which are usually located around the start and stop codons in linear mRNAs, resulting in about half of circRNAs containing large open reading frames. This potential mechanism could ensure long-lasting and stable protein production for specific physiological processes when lacking the corresponding linear mRNAs[28]. These findings showed the important roles of m^{6}A in circRNAs during disease progress. Therefore, it is essential to explore the roles of m^{6}A circRNAs in SAP.

In the present study, the function analysis of DE m^{6}A circRNAs in SAP found that two important pathways were involved in the pathogenesis of SAP, including protein digestion and regulation of autophagy. As an important pathological cellular event, the activation of premature trypsinogen can result in acinar cell necrosis[1]. Many pancreatic injury factors, such as trauma, obstruction of the pancreatic duct and alcohol, can initiate the fusion of lysosomes with zymogen in acinar cells, leading to the activation of trypsinogen through cathepsin B to trypsin. Once trypsin is released, it can cause self-digestion in and outside the acinar cells, and the release of cathepsin B can cause necroptosis. As a cytoprotective mechanism, autophagy can process and recycle various aged, defective or damaged cytoplasmic contents[29]. Selective macroautophagy is a biological process during which specific damaged organelles and misfolded proteins are processed and recycled. Autophagy is accomplished *via* a series of steps, which start with the enucleation of cytoplasmic inclusions in the open double membrane formed by the ER, Golgi apparatus and plasma membrane[30]. Knocking out *ATG7* genes (which are important to form autophagosome) and *LAMP* genes could lead to pancreatitis with extensive inflammation in mice[29,31]. Importantly, impaired autophagy leads to trypsinogen activation, ER stress and mitochondrial dysfunction. These events can together make acinar cells become more susceptible to other insults and cellular death[1]. In addition, RNA transport is enriched in GO terms of note, and Chen *et al*[16] found that m^{6}A modification can modulate the export of circNSUN2 to

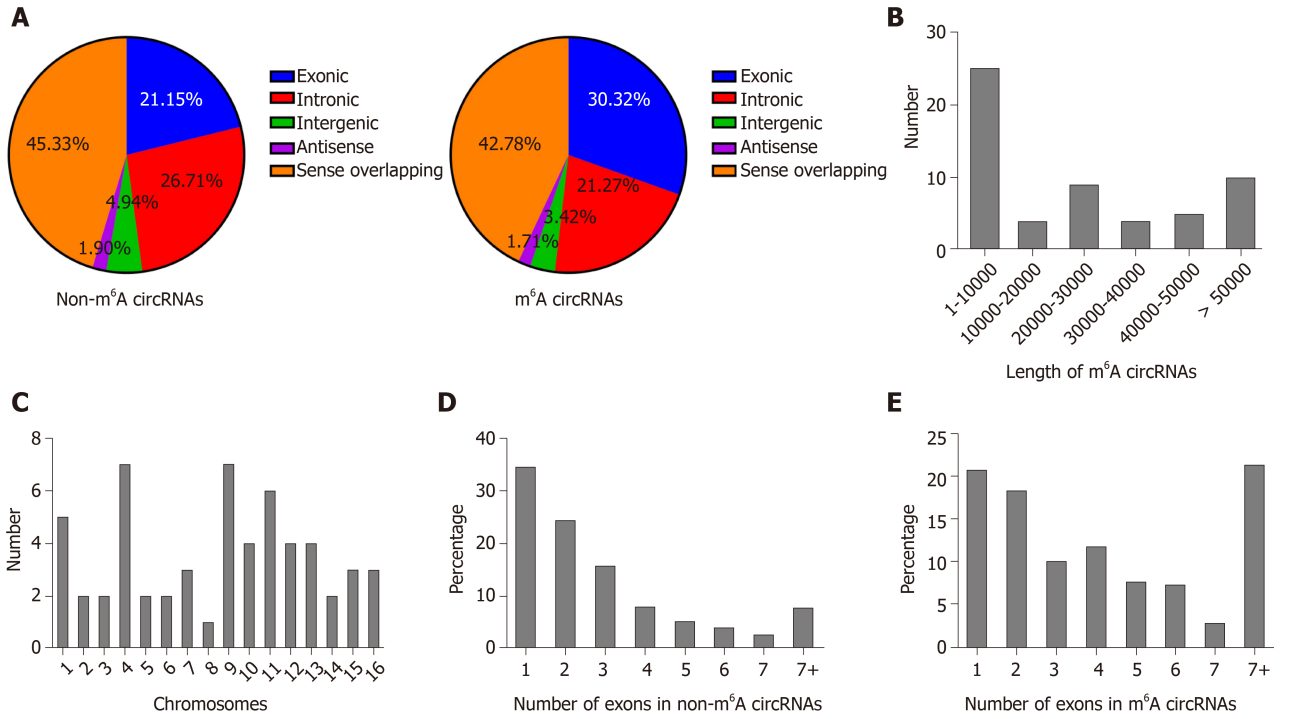


Figure 4 Distribution of N^6-methyladenosine sites in severe acute pancreatitis and control groups. A: Distribution of genomic origins of non-N6-methyladenosine (m^{6}A) circRNAs (left) and m^{6}A circRNAs (right); B: Number of circRNAs with differentially expressed m^{6}A peaks based on the distribution of length; C: Chromosomal distribution of all differential m^{6}A sites within circRNAs; D and E: Distribution of non-m^{6}A and m^{6}A circRNAs based on the number of exons in each circRNA.

the cytoplasm, suggesting that m^{6}A modification regulates transport of circRNAs in SAP. These results were consisted with the hypothesis that m^{6}A modification of circRNAs participated in the progression of SAP.

m^{6}A modification of mRNA can influence its expression by regulating transcription, splicing and degradation[32]. In circRNAs, Zhou *et al*[14] and Su *et al*[33] reported that m^{6}A levels are correlated with expression levels of circRNAs in HeLa cells and a rat model of hypoxia-mediated pulmonary hypertension. However, in SAP, we found m^{6}A modification in circRNAs was not associated with expression of circRNAs, suggesting that m^{6}A circRNAs function in SAP though other mechanisms, such as miRNA sponges. It is worth mentioning that more direct evidence is currently needed to support that m^{6}A can affect circRNA expression.

miRNA sponges is an important function of circRNAs. Cytoplasmic circRNAs can prevent miRNAs from binding to target mRNAs by competitive binding to miRNA response elements, further playing a key role in diseases[8,34]. For instance, in lung squamous cell carcinoma, circTP63 can competitively bind to miR-873-3p and prevent miR-873-3p from decreasing the level of FOXM1. The FOXM1 can upregulate the expression of CENPA and CENPB, ultimately facilitating cell cycle progression[35]. In SAP, circHIPK3 can enhance pyroptosis *via* regulating the miR-193a-5p/GSDMD axis in acinar cells, ultimately aggravating this disease[36]. In our previous study, we found that circZFP644 could sponge miR-21-3p, thereby participating in the pathogenesis of SAP[9]. Recently, Su *et al*[33] found that m^{6}A modification of circRNAs could influence the interactions between circRNAs and miRNAs. Therefore, analysis of m^{6}A circRNA–miRNA networks was performed in this study. Several important miRNAs participated in the pathological process of SAP were found to bind to these m^{6}A circRNAs, such as miR-24-3p, miR-26a, miR-92b, miR-216b, miR-324-5p and miR-762. For example, in caerulein-stimulated AR42J cells, expression of miR-92b-3p was decreased, while overexpression of miR-92b-3p could downregulate the expression of TRAF3 and inhibit the MKK3-p38 pathway, attenuating inflammatory response and autophagy[37]. These results suggest that m^{6}A modification of circRNAs functions by influencing the interactions between circRNAs and miRNAs.

m^{6}A modification is a reversible process that occurs by methyltransferase complex consisting of METTL3, METTL14 and WTAP, and is "erased" by ALKBH5 and FTO [13,15]. In pancreatic cancer, ALKBH5 could regulate the post-transcriptional activation of PER1 through m^{6}A abolishment, thereby inhibiting the cancer[38]. In

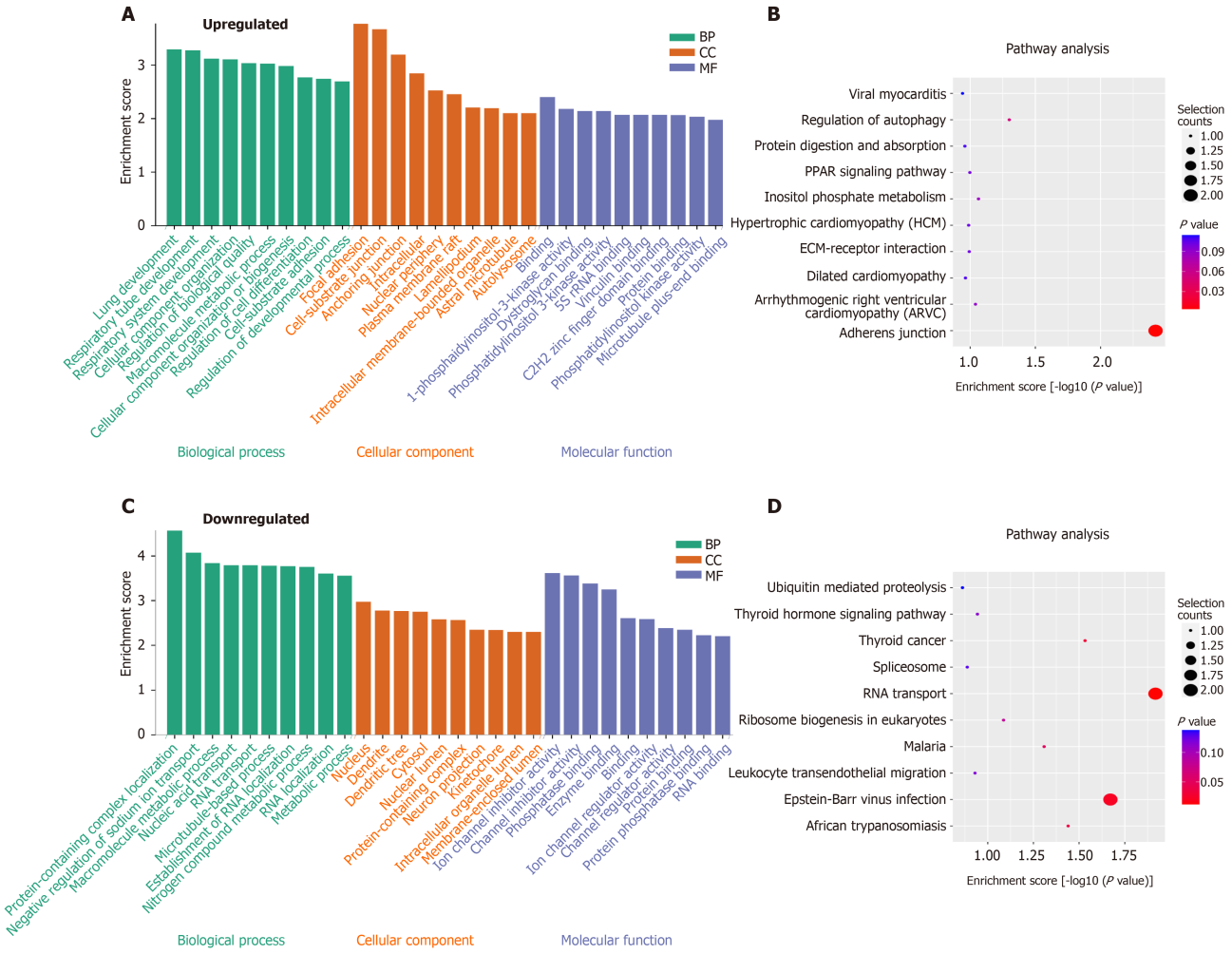


Figure 5 Functional analysis of circRNAs with differentially expressed N^6-methyladenosine peaks though gene ontology and Kyoto Encyclopedia of Genes and Genomes analysis. A and B: Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of circRNAs with upregulated N6-methyladenosine (m^{6}A) peaks; C and D: GO and KEGG analysis of circRNAs with downregulated m^{6}A peaks. GO analysis include biological process (BP) analysis, cellular component (CC) analysis, and molecular function (MF) analysis.

hepatocellular carcinoma, ALKBH5 could attenuate expression of LYPD1 by an m^{6}A-dependent manner and act as a tumor suppressor[39]. Overall, this evidence has suggested that ALKBH5 plays an essential role in m^{6}A modification. In this study, we found that expression level of ALKBH5 was upregulated in SAP. Consistent with this result, total m^{6}A level of circRNAs in SAP was reduced, indicating that ALKBH5 may play a role in the dynamic process of m^{6}A in SAP.

However, there are still limitations in our study. Firstly, further *in vivo* and *in vitro* experiments are needed to further explore the m^{6}A circRNA-mediated precise regulatory mechanisms in SAP. Secondly, the conservation analysis of the m^{6}A circRNAs showed that these circRNAs may have similar roles in human SAP. However, their clinical significance and the results should be investigated further in SAP patients. Additionally, the precise mechanism of ALKBH5 in m^{6}A circRNAs during SAP needs to be studied. Actually, these are in our next plans to explore the roles of m^{6}A circRNAs in SAP.

CONCLUSION

In conclusion, our study identified the transcriptome-wide profiling of m^{6}A circRNAs in SAP and predicted their biological significance and possible potential mechanisms, providing new insights to explore the possible pathophysiological mechanism of SAP and seek new potential therapeutic targets.

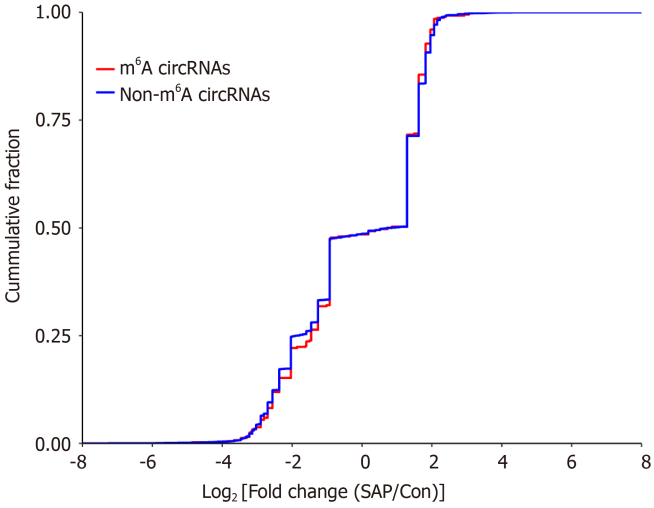


Figure 6 Relationship between N^6-methyladenosine level and expression of circRNAs in severe acute pancreatitis. Cumulative distribution of circRNAs expression between control and severe acute pancreatitis (SAP) groups for N^6-methyladenosine (m^{6}A) circRNAs (red) and non-m^{6}A circRNAs (blue).

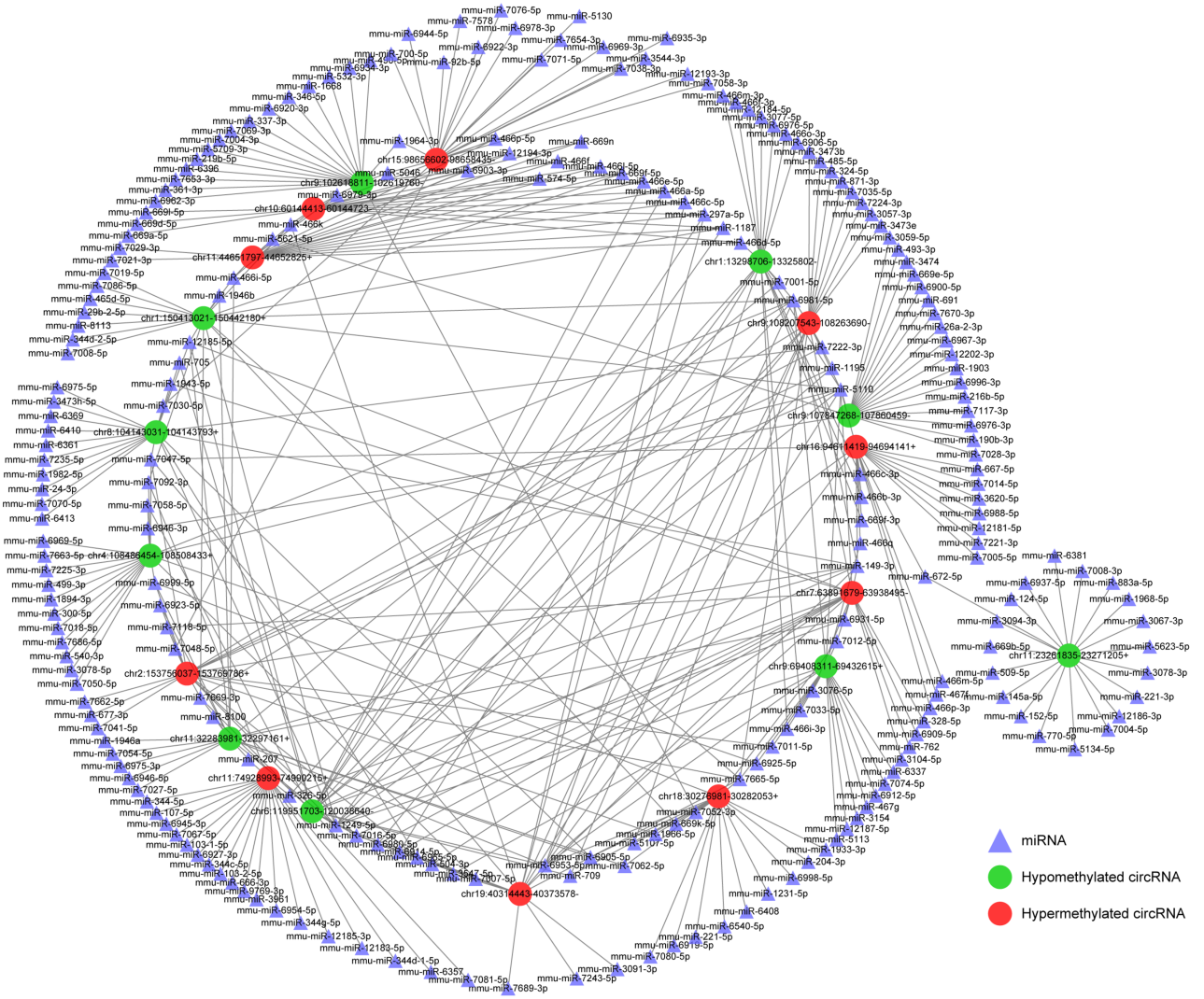


Figure 7 Construction of N^6-methyladenosine circRNA-miRNA networks in severe acute pancreatitis. A map showing the interaction networks of the top 10 upregulated and top 10 downregulated circRNAs according to the level of N6-methyladenosine, and their around 20 target miRNAs with the most stable binding in SAP. Green circles represent hypomethylated circRNAs, red circles represent hypermethylated circRNAs and triangles represent miRNAs, compared with control group.

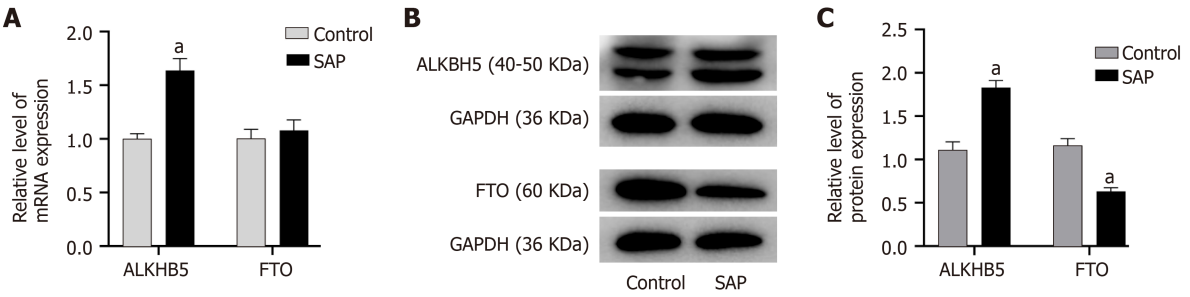


Figure 8 Expression of demethyltransferase in severe acute pancreatitis. A: Relative mRNA levels of alkylation repair homolog 5 (ALKBH5) and fat mass and obesity related protein (FTO) (normalized by the quantity of GAPDH) in each group; B: Representative images of western blot detected with alkylation repair homolog 5 (ALKBH5), FTO, and GAPDH antibodies in control and severe acute pancreatitis (SAP) groups; C: Relative protein levels of ALKBH5 and FTO (measured as the ratio of ALKBH5, FTO to GAPDH by band density) in each group. Data are representative of at least three independent experiments. $^aP < 0.05$ *vs* control group.

ARTICLE HIGHLIGHTS

Research background

Severe acute pancreatitis (SAP) is a lethal inflammatory disease with mortality up to 30%. But the genetic pathological mechanism of SAP remains unclear and SAP is still lack of effective therapeutic options. N^6-methyladenosine (m^{6}A) modification of circular (circ)RNAs plays a key role in many diseases and physiological processes through regulating the metabolism and function of circRNAs. However, the role of m^{6}A circRNA in SAP has been unexplored yet.

Research motivation

The pathophysiology of SAP at the level of gene regulation is complex and remains unclear. circRNAs are found to participate in many physiological processes and play key roles in pathological processes during SAP. m^{6}A modification can affect the "fate" of m^{6}A modified circRNAs, thereby participating in the regulation of diseases. Therefore, we want to explore whether the m^{6}A modification of circRNAs is related to the pathophysiological mechanism of SAP, and determine their biological significance and potential mechanisms.

Research objectives

The present study aims to determine the transcriptome-wide map of m^{6}A circRNAs and explore their biological significance and its possible mechanisms in SAP.

Research methods

The SAP C57BL/6 mice model was induced by retrograde injection of 4% sodium taurocholate salt. m^{6}A-modified RNA immunoprecipitation sequencing was used to determine the transcriptome-wide map of m^{6}A circRNAs. The biological significance of circRNAs with differentially expressed m^{6}A peaks was identified by GO and KEGG analysis. m^{6}A circRNA-microRNA networks was constructed to explore the underlying mechanism of m^{6}A circRNAs in SAP. The expression of demethylases was measured by western blot and qPCR. H&E staining and measurement of serum lipase and amylase were performed to assess the establishment of SAP mice model.

Research results

In the identified transcriptome-wide map of m^{6}A circRNAs, there were 57 circRNAs with differentially expressed m^{6}A peaks; among which, 32 were upregulated and 25 downregulated. Important pathways in the pathogenetic process during SAP were found by functional analysis of these m^{6}A circRNAs, such as protein digestion and regulation of autophagy. m^{6}A circRNA–miRNA networks showed that several important miRNAs in pathogenesis of SAP were bind to these m^{6}A circRNAs, such as miR-24-3p, miR-26a, miR-92b, miR-216b, miR-324-5p and miR-762. To be note, the total m^{6}A level of circRNAs was reduced in SAP, accompanied by the upregulated demethylase ALKBH5.

Research conclusions

The transcriptome-wide profiling of m^{6}A circRNAs in SAP was identified, and the

biological significance and possible potential mechanisms of m^{6}A circRNAs in SAP were predicted, providing new insights into exploring the possible pathophysiological mechanism of SAP and new potential therapeutic targets.

Research perspectives

This present study for the first time identified transcriptome-wide map of m^{6}A circRNAs and determined their biological significance and potential mechanisms. However, the m^{6}A circRNA-mediated precise regulatory mechanisms are need to be explore further *in vivo* and vitro experiments. What's more, further studies are needed to reveal the precise mechanism of ALKBH5 in m^{6}A circRNAs during SAP. In the future, we will explore them and investigate these m^{6}A circRNAs in SAP patients.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 21; 27(43): 7546-7562

DOI: 10.3748/wjg.v27.i43.7546

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

ORIGINAL ARTICLE

Basic Study

Survivin-positive circulating tumor cells as a marker for metastasis of hepatocellular carcinoma

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Author contributions: Yu J and Wang Z contributed equally to this work; Yu J and Wang Z had access to all the clinical data generated by the study and take responsibility for data integrity and accuracy of the data analysis; Yu J conceptualized and designed the study; Zhang H and Wang L contributed to acquisition, analysis, or interpretation of the data; Yu J and Wang Z contributed to manuscript preparation; Li DQ supervised the study.

Institutional review board statement: The study was approved by the ethics committee of Hubei Cancer Hospital (Ethical approval number: LLHBCH2019LW-002).

Conflict-of-interest statement: The authors declare no conflicts of interest for this study.

Data sharing statement: No additional data are available.

Supported by the National Natural Science Foundation of China, No. 81772839; and the Health and

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Abstract

BACKGROUND

Circulating tumor cells (CTCs) and survivin are indicators for tumor stage and metastasis, as well as epitheliomesenchymal transition, in various cancers, including hepatocellular cancer (HCC).

AIM

To explore the potential of survivin-positive CTCs, specifically, as a marker for tumor progression in HCC patients.

METHODS

We examined the survivin expression pattern in CTCs obtained from 179 HCC patients, and investigated the *in vitro* effects of survivin silencing and overexpression on the proliferation and invasion of HCC cells. CTC count and survivin expression in patient samples were examined using RNA *in situ* hybridization.

RESULTS

All 179 patients were positive for CTC markers, and 94.41% of the CTCs were positive for survivin. The CTC and survivin-positive CTC counts were significantly higher in the HCC patients than in the normal controls, and were significantly associated with tumor stage and degree of differentiation. Further, survivin overexpression was found to induce HepG2 cell proliferation, reduce apoptosis, and improve invasive ability.

CONCLUSION

Family Planning Commission Foundation of Hubei Province, No. WJ2019H194.

Country/Territory of origin: China

Specialty type: Oncology

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

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

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Received: May 23, 2021
Peer-review started: May 23, 2021
First decision: June 12, 2021
Revised: July 19, 2021
Accepted: October 27, 2021
Article in press: October 27, 2021
Published online: November 21, 2021

P-Reviewer: Suzuki R
S-Editor: Liu M
L-Editor: Wang TQ
P-Editor: Liu M

Survivin shows upregulated expression (indicative of anti-apoptotic effects) in HCC. Thus, survivin-positive CTCs are promising as a predictor of HCC prognosis and metastasis, and their accurate measurement may be useful for the management of this cancer.

Key Words: Survivin; Circulating tumor cells; Hepatocellular carcinoma; Prognosis; Metastasis

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Core Tip: This study first analyzed surviving-positive circulating tumor cells (CTCs) in patients with hepatocellular carcinoma (HCC). The levels of survivin expression in different CTCs were significantly different. The rates of moderate and high expression in the mesenchymal and hybrid CTCs were significantly higher than those in the epithelial CTCs. Survivin overexpression induced HCC cell proliferation, promoted their invasive ability, and reduced apoptosis. The expression of survivin in mesenchymal CTCs (mCTCs) in liver cancer was associated with metastasis and detection of survivin positivity in mCTCs may have potential value in early detection of tumor metastasis and prognostic evaluation.

Citation: Yu J, Wang Z, Zhang H, Wang Y, Li DQ. Survivin-positive circulating tumor cells as a marker for metastasis of hepatocellular carcinoma. *World J Gastroenterol* 2021; 27(43): 7546-7562
URL: https://www.wjgnet.com/1007-9327/full/v27/i43/7546.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i43.7546

INTRODUCTION

According to 2020 statistics, hepatic cancer ranked sixth among malignant cancers in terms of incidence, and it ranked fourth globally as the main reason for cancer-related death[1]. Further, the 5-year overall survival of this cancer is quite abysmal, as it is less than 12%[1]. The prognosis of this disease continues to remain poor, even though there have been advances in the diagnostic and therapeutic strategies[2]. Hence, understanding the mechanisms underlying the metastasis of this cancer and assessing the status of disease progression are necessary.

The number of circulating tumor cells (CTCs) is an effective marker for solid tumors associated with metastasis[2]. CTC analysis is considered as real-time "liquid biopsy" for cancer patients, as it provides real-time monitoring of tumor progression and recurrence[3]. As a non-invasive biomarker, the CTC level can be used for comprehensive surveillance of cancer progression, in the case of both hepatocellular carcinoma (HCC) and cholangiocarcinoma[4]; in particular, it has been shown to be a good prognostic marker for HCC[5-7]. Currently, the most commonly used technique for CTC isolation is the cell search system, which is the only FDA-approved detection method; this method is based on positive immunoselection of epithelial cell adhesion molecule (EpCAM) and negative immunoselection of leukocytes (for which the general target is CD45). By using this technique, CTCs are divided into epithelial CTCs, mesenchymal CTCs (mCTCs), and hybrid CTCs[8]. According to this classification, CTCs have been reported to be useful as a marker for epithelial-mesenchymal transition (EMT) in HCC[9], and EMT is a known marker for the diagnosis and prognosis of cancer progression. Accordingly, previous studies have revealed that CTCs undergoing EMT are useful as indicators for diagnosing HCC and for predicting its prognosis[10]. The prognostic value of CTCs has also been demonstrated after surgery or during chemotherapy and recurrence[11,12].

Survivin is an anti-apoptotic protein (molecular weight, 16.5 kDa)[13] that plays an important role in inhibiting apoptosis in multicellular organisms and is overexpressed in many tumors, including HCC[14]. Studies have also shown that overexpression of survivin is associated with protection against apoptosis and propensity for metastasis in tumor cells, but this effect was not observed in normal cells[15]. Wurmbach *et al*[16] and Roessler *et al*[17] showed gene overexpression of survivin in HCC samples

compared to normal liver samples, and the difference between the two groups was significant, as shown in Figure 1. Additionally, recent research has shown that insulin-like growth factor-1 induced EMT *via* activation of survivin in HCC cells[18]. Accordingly, overexpression of survivin mRNA and protein has been shown to stimulate EMT in HCC cells; this has been shown to increase their ability for invasion and migration and their cell proliferation rate, and decrease their apoptosis rate[15]. Thus, there is evidence for the potential of survivin as a marker for cancer cell proliferation and metastasis.

As discussed above, the research so far has indicated that both CTCs and survivin are markers for EMT and, therefore, cancer progression in HCC. However, the serum survivin levels of HCC patients are not different from those of healthy controls and patients with nonmalignant chronic liver diseases[19]. And no study so far has analyzed the prognostic value of survivin-expressing CTCs in HCC. Therefore, in the present study, we explored whether survivin-positive CTC levels are associated with cancer stage and clinicopathological characteristics in HCC, and whether survivin expression in CTCs has potential as a predictor of metastasis in HCC patients.

MATERIALS AND METHODS

Genome data

Cancer genome atlas data were retrieved from GEO database (http://www.ncbi.nlm.nih.gov/geo) and TCGA database (http://www.ualcan.path.uab.edu). The GSE6467 and GSE14520 datasets were downloaded for this study. Data was analyzed using software packages including ClusterProfiler, GSEquery, and pheatmap.

Patient cohort

A study cohort (n = 179) included HCC patients who were enrolled at Hubei Cancer Hospital, China between May 2018 and December 2018. Only patients for whom HCC was confirmed based on pathological evidence, according to the World Health Organization criteria, and patients who had not undergone prior anticancer treatment were included. The tumors were staged as 0-A or B-C according to the Barcelona Clinic Liver Cancer (BCLC) staging system. Further, based on TNM classification, the tumors were staged as I, II, III, or IV. A control group comprised 70 healthy persons and 54 patients who tested positive for hepatitis B/C virus. The study was approved by the ethics committee of Hubei Cancer Hospital. (Ethical approval number: LLHBCH2019LW-002)

Collection of blood samples and isolation of CTCs

Samples of peripheral blood (a volume of 5 mL mixed with EDTA as an anticoagulant) were obtained *via* venipuncture. The CanPatrol CTC enrichment technique (SurExam, Guangzhou, China) was used to isolate CTCs from the blood samples and classify them. The samples were filtered through a membrane (pore diameter, 8 μm; Millipore, Billerica, United States), a vacuum plate that provided various valve settings (SurExam, Guangzhou, China), an E-Z 96 vacuum that also had various settings (Omega, Norcross, United States), and a vacuum pump (Auto Science, Tianjin, China). A cell lysis buffer that comprised 154 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, and 0.1 mmol/L EDTA (all the reagents were from Sigma, St. Louis, United States) was used to induce lysis of erythrocytes. The cell suspension was then resuspended for 5 min in phosphate-buffered saline (Sigma, St. Louis, United States) that contained 4% formaldehyde (Sigma, St. Louis, United States), and transferred to the specialized filtration system described earlier. The vacuum pump was turned on and set at a pressure of 0.08 MPa. The CTCs were separated using the membrane as mentioned above.

Classification of CTCs by RNA in situ hybridization

CTC phenotypes were detected by RNA *in situ* hybridization (ISH) with CD45 (a leukocyte marker), EpCAM and CK8/18/19 (epithelial cell markers), and vimentin and Twist (mesenchymal cell markers) as markers (Figure 1). A protease (Qiagen, Hilden, Germany) was used to pretreat the blood cells on the membrane. Following this, the cells were hybridized with a capture probe that targeted the genes for the markers mentioned above. Next, the cells were stained by 5-min incubation with 4,6-diamidino-2-phenylindole (Sigma, St. Louis, United States), and viewed under an automated imaging fluorescence microscope (Keyence, United States). Red fluorescence signals were considered to indicate epithelial cell markers, while green

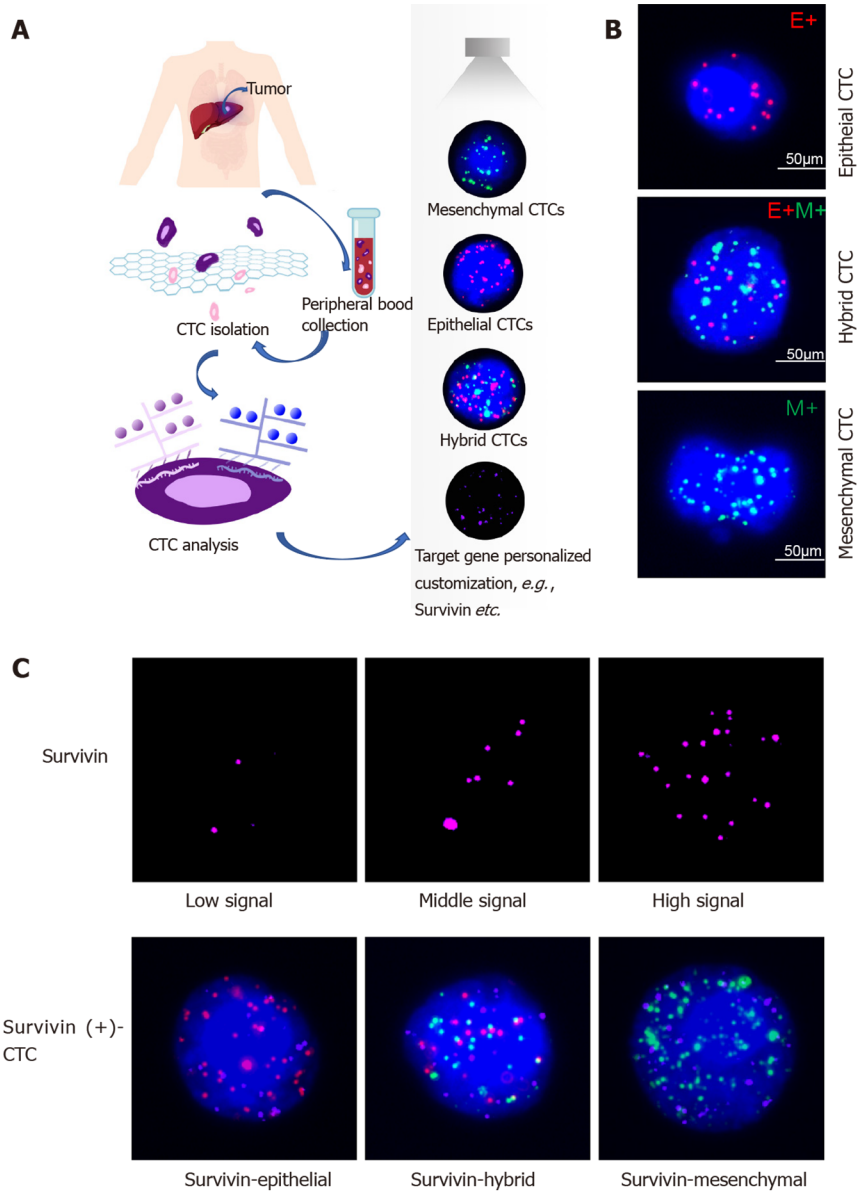


Figure 1 Circulating tumor cell classification and survivin expression in circulating tumor cells by RNA-in situ hybridization. A: Circulating tumor cell (CTC) isolation and RNA *in situ* hybridization analysis of blood samples from patients and healthy controls; B: Detection and classification of CTCs using epithelial-mesenchymal transition markers. CD45 was used as a leukocyte marker, and it is indicated by white fluorescence. Epithelial biomarkers (EpCAM and CK8/18/19) were used for epithelial CTCs, which are represented by red fluorescence, and mesenchymal biomarkers (vimentin and Twist) were used for mCTCs, which are represented by green fluorescence; C: Survivin expression in CTCs is indicated by purple fluorescence (Alexa Fluor 647), with the intensity scored as low, moderate, and high (left to right images). CTC: Circulating tumor cell. CTC: Circulating tumor cell.

fluorescence signals were considered to indicate mesenchymal cell markers. Further, bright white fluorescence signals were considered to indicate CD45 expression.

Survivin expression in CTCs

Survivin expression in CTCs was assessed using the RNA-ISH method (Figure 1C). Survivin expression is indicated by purple fluorescence emitted by the CTCs (Alexa Fluor 647). According to the signal intensity score, survivin expression was classified as absent (0), low (1-2), moderate (3-9), and high (signal > 9), as shown in Figure 1C.

In vitro silencing and overexpression of survivin

The HCC cell line HepG2 was obtained from the cell bank of the Chinese Academy of Science and cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a 5% CO_2 incubator.

Short hairpin RNAs against the survivin sequence (5'-CTTACCAGGTGAGAA-GTGAGGT-3') were designed to knockdown survivin in HepG2 cells, by transfec-tion with the vector pGPU6 harboring siRNAs against survivin. The lentivirus vector

pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro was used to induce overexpression of survivin in HepG2 cells. The vector and siRNA were obtained from Hualian Biotechnology Company (Wuhan, China). HepG2 cells were stably transfected with the survivin siRNA and lentivirus-siRNA plasmid using Lipofectamine2000 (Invitrogen). The transfected cells were incubated in selection medium containing 2 mg/mL puromycin for 2 wk (Bioswamp), and Western blot analysis was used to assess survivin expression.

Cell proliferation and apoptosis analysis

The CCK-8 kit (Bioswamp Company, China) was used to analyze cell proliferation according to the manufacturer's protocol. The staining reagent used was CCK-8 (at a volume of 10 mL); the cells were incubated with the reagent for 2 h at 37 °C for 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h after transfection (with the survivin siRNA or lentivirus). Following this, the absorbance of the cells was detected at 450 nm. Flow cytometry (Beckman, United States) was used to analyze cell apoptosis with the Annexin V-PE and 7AAD assay kit (Becton, Dickinson Company, United States). Apoptotic cells were defined as PE-positive and 7AAD-negative, while necrotic or dead cells were defined as 7AAD-positive and PE-negative.

Cell invasion analysis

For the invasion experiment, cells were seeded at a density of 5×10^4 cells/well in Transwell inserts (pore diameter, 8 μm) coated with Matrigel and containing cold serum-free medium. After an incubation period of 48 h, non-invasive cells that were present in the upper compartment were removed with a cotton swab, and the cells that had successfully adhered to the lower compartment were subjected to fixation with 10% paraformaldehyde and staining with 0.1% hexamethylpararosaniline for 30 min. The number of cells was counted under a microscope (Olympus BX53) in five selected fields. The average number of adherent cells was calculated.

Western blot and immunohistochemistry analysis of survivin protein expression in HCC tissue

Formalin-fixed paraffin-embedded specimens of HCC tissues from Hubei Cancer Hospital were prepared for immunohistochemical staining. The tissue slides were washed in phosphate-buffered saline (Bioswamp, China) after dewaxing and dehydration. The slides were placed in plastic tubes and incubated with boiling citric acid for antigen retrieval. In the next step, endogenous peroxidase activity was inhibited *via* 10-min incubation in methanol containing 3% H_2O_2. Next, the slides were blocked by 15-min incubation in 1% goat serum at 37 °C. Following this, they underwent incubation with anti-survivin rabbit polyclonal antibody (1:100 dilution; Abcam, United Kingdom) at 4 °C overnight in a moist chamber. After treatment with the secondary antibodies for 30 min, the slides were colored with diaminobenzidine and counterstained with hematoxylin. All the images were captured using an Olympus BX53 microscope. The protocol for Western blot assay of survivin protein expression in the tissue specimens was the same as that described for Western blot analysis of the HepG2 cells.

Statistical analysis

All analyses were conducted using SPSS version 20.0 (IBM Corp., Armonk, NY, United States). The Kolmogorov-Smirnov method was used to determine the normality of the CTC and survivin-positive CTC counts, which were found to be non-normally distributed. The phenotypic CTC counts and survivin expression in different groups were examined by the Fisher exact probability test. Correlations between variables were evaluated with the Spearman rank correlation test. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Analysis of genome data

Based on the GSE6467 and GSE14520 datasets, gene expression was found to differ between tumor tissue and normal tissue, as evident from the survivin gene expression data shown in Figure 2A and 2C. Next, we found that DNA replication and cell cycle pathways were downregulated in HCC samples, based on the Ingenuity pathway analysis (Z-score < 0; Figure 2B).

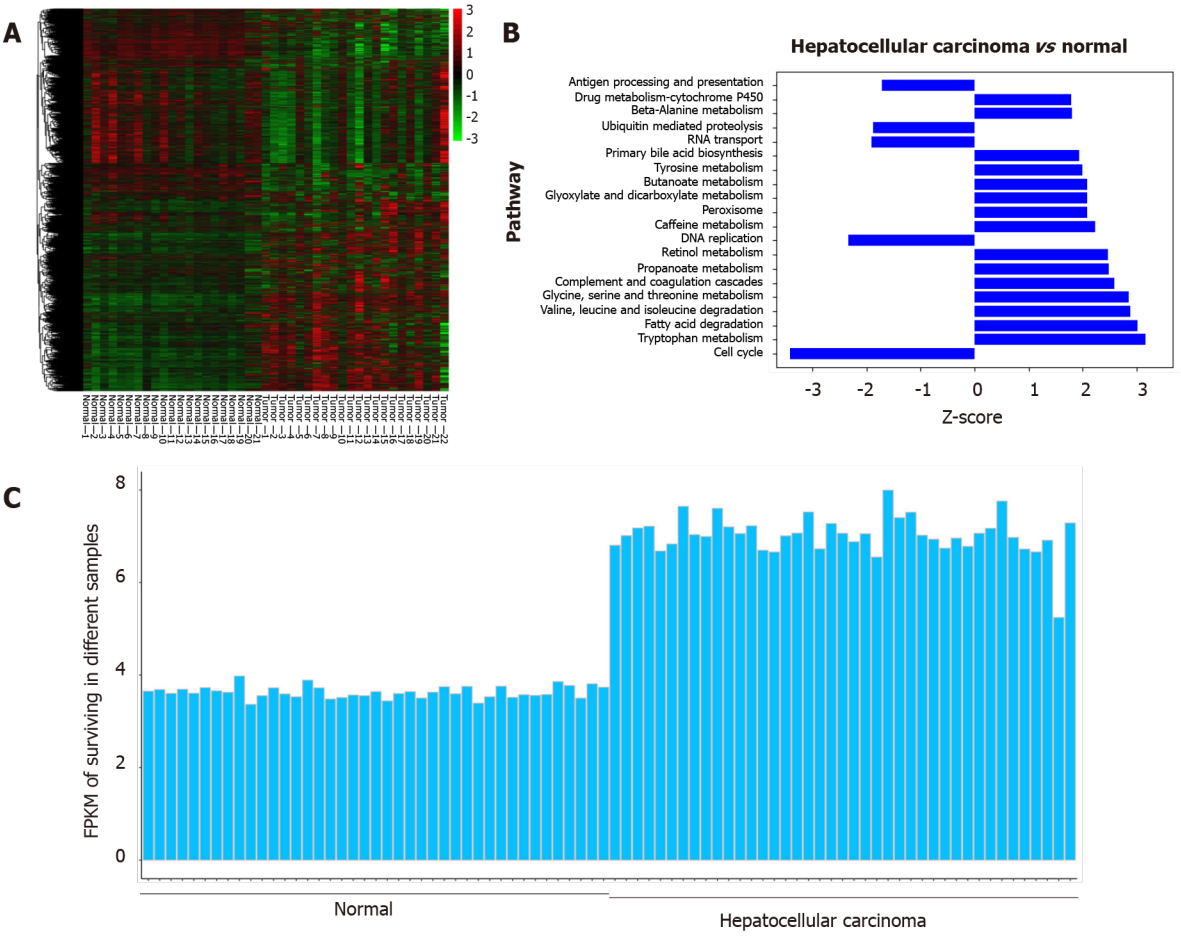


Figure 2 Analysis of survivin gene expression in normal liver tissue and hepatocellular cancer tissue from previously reported genome data. A: The expression patterns in 21 normal tissue samples and 22 hepatocellular carcinoma samples were analyzed by hierarchical clustering; B: Ingenuity pathway analysis was used to identify the upregulated pathways (Z-score > 0) and the downregulated pathways (Z-score < 0) in the normal tissue samples and hepatocellular cancer (HCC) samples; C: The FPKM (fragments per kilobase of transcript per million fragments mapped) of survivin was determined for different samples. Compared with normal liver tissue samples, the expression of the survivin gene was upregulated in HCC samples.

Based on the TCGA data analysis, the expression of survivin in HCC tissue was significantly associated with stage and tumor grade. Survivin expression (fragments per kilobase of transcript per million fragments mapped) in patients with stages III and IV HCC was significantly higher than that in patients with stages I and II HCC ($P < 0.05$). And survivin expression (fragments per kilobase of transcript per million fragments mapped) in patients with grades 3 and 4 HCC was significantly higher than that in patients with grades 1 and 2 HCC ($P < 0.05$) (Supplementary Figure 1).

Survivin expression in HCC specimens and normal hepatic tissues

The survivin expression level in tissue samples from HCC patients ($n = 8$) was significantly higher than that in normal hepatic tissues ($n = 4$), as indicated in the Western blot in Figure 3A and the immunohistochemical staining images in Figure 3B.

Association between survivin expression and cell proliferation, apoptosis, and invasion abilities in HepG2 cells

Silencing of survivin expression and overexpression of survivin were successfully induced by siRNA and lentivirus transfection, respectively (Figure 4A). At 12 h, 24 h, 48 h, 60 h, and 72 h post-transfection, the cell proliferation rate was significantly higher in the survivin-overexpression cells than in the control cells and survivin-silenced cells, while it was significantly lower in the survivin-silenced cells than in the control cells and survivin-overexpressing cells (Figure 5A). Accordingly, the apoptosis rate of survivin-silenced HepG2 cells was significantly higher than that of survivin-overexpressing and normal control cells (Figure 4B). Finally, the results for cell invasion rates obtained from the Transwell migration assays indicated that the survivin-overexpressing HepG2 cells had significantly higher invasive ability than the survivin-

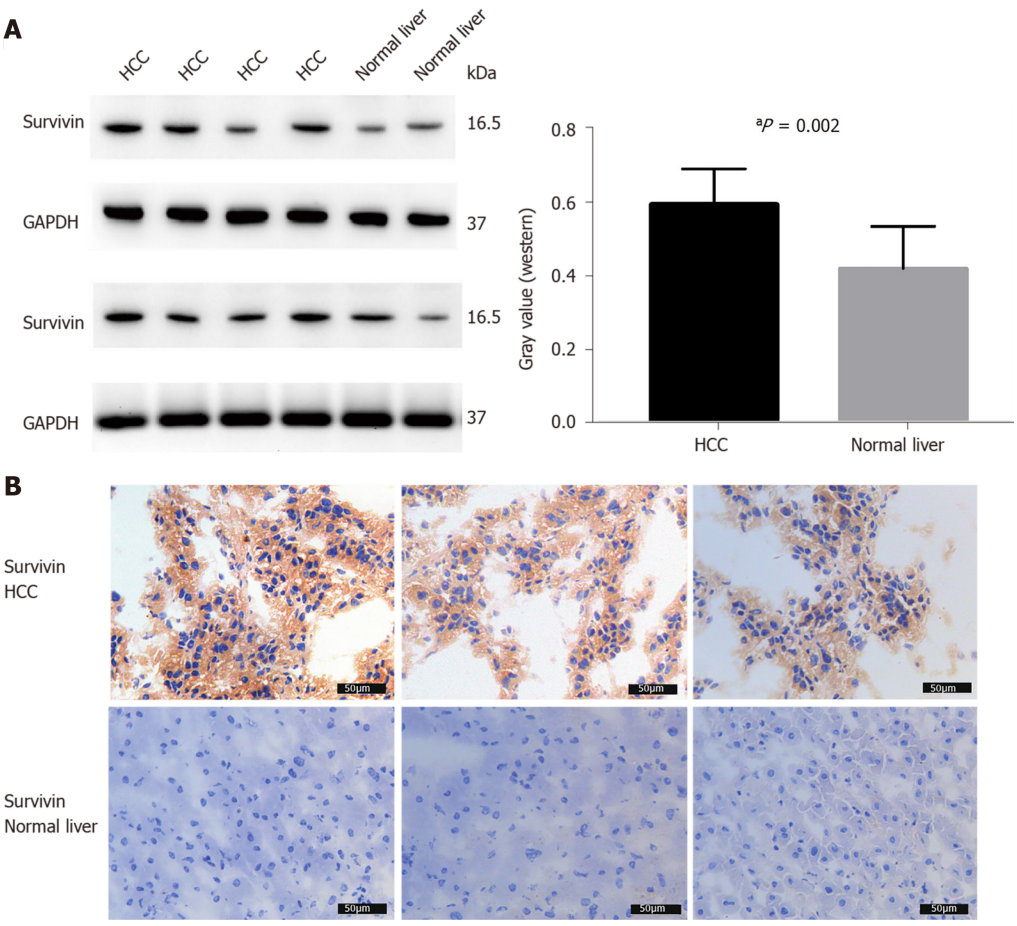


Figure 3 Survivin expression in hepatocellular cancer and normal adjacent tissue samples. A: Western blot analysis showing the protein expression of survivin in hepatocellular cancer (HCC) (n = 8) and normal liver tissue (n = 4); B: Immunohistochemical staining for survivin in HCC (n = 8) and normal liver tissue (n = 4). Survivin expression was higher in HCC than in normal liver tissue. $^aP < 0.05$. HCC: Hepatocellular cancer.

silenced and normal control cells (Figure 5B).

Isolation and classification of CTCs

The observations of RNA-ISH analysis of CTCs based on EMT markers are shown in Figure 1B. The expression of survivin in these subtypes of CTCs is also indicated. The proportion of mCTCs is shown in Figure 6C. The number of mCTCs seemed to be higher in advanced-stage HCC (stage B-C) than in metastatic HCC.

CTC count and its association with clinicopathological characteristics of HCC patients

Table 1 shows the clinicopathological characteristics of the study cohort. A CTC count of > 5/5 mL was detected in 168 out of 179 blood samples (93.85%), and the median CTC count was 16/5 mL (range, 1-193 CTCs/5 mL). Further, survivin-positive CTCs were detected in 94.41% (169 of 179) of the patients. As shown in Figure 6, the median CTC count (Figure 6A) and survivin-positive CTC count (Figure 6B) in HCC patients were significantly higher than those in the HBV/HCV patients and healthy controls (P < 0.05). Patients with stage B/C HCC (or advanced HCC) had a significantly greater median CTC count than those with stage 0/A HCC (or early-stage HCC) (P = 0.024) (Table 1), and the median CTC count in patients with poorly differentiated tumors was significantly higher than that in patients with well-differentiated tumors (P = 0.002) (Table 1).

Correlation between mCTC counts and clinicopathological features

Out of the 179 patients, 138 had an mCTC count of > 2/5 mL. The mCTC count was not significantly associated with AFP level, tumor stage, or degree of differentiation (Table 1). However, the proportion and count of mCTC were associated with BCLC stage and metastasis (Figure 6C).

Table 1 Clinical characteristics of 179 hepatocellular cancer patients

Clinical factor	*n*	[1]CTC count/5 mL Median, SE	[2]mCTC count/5 mL Median, SE	[3]Survivin (+)CTC count/5 mL Median, SE	*P* value
Sex					
Male	119	17, 3.57	6, 1.11	9, 3.05	0.251[1], 0.819[2], 0.909[3]
Female	60	12, 9.61	6, 3.19	9.5, 7.88	
Age					
< 55 yeara	89	19, 4.28	9, 1.50	11, 3.54	0.700[1], 0.141[2], 0.467[3]
≥ 55 years	90	14, 5.75	4, 1.71	8, 4.88	
HBV/HCV					
Positive	130	17, 3.57	7, 1.16	10, 3.03	0.402[1], 0.539[2], 0.436[3]
Negative	49	13, 9.90	4, 3.12	6.5, 8.21	
AFP levels					
< 7.0	80	14, 5.85	5, 1.85	7, 4.66	0.285[1], 0.81[2], 0.254[3]
≥ 7.0	99	22.5, 4.42	7.5, 1.45	11, 3.92	
Cirrhosis					
Yes	119	16, 3.43	6, 1.19	9.0, 2.78	0.498[1], 0.671[2], 0.829[3]
No	60	19.5, 10.03	7.5, 2.94	10, 8.67	
TNM stage					
I-II	79	14, 2.93	6, 1.04	6, 1.49	0.111[1], 0.433[2], 0.002[3]
III-IV	100	24, 6.29	8.50, 2.00	18, 5.95	
BCLC stage					
0-A	58	12.5, 3.04	4, 0.75	4, 1.25	0.024[1], 0.000[2], 0.000[3]
B-C	121	25, 4.94	9, 1.56	20, 4.64	
Differentiation					
Well	29	13, 2.04	5, 2.46	5, 1.70	0.002[1] (well *vs* poor)
Moderate	92	14.5, 5.35	6, 1.77	8.5, 5.15	0.970[2] (well *vs* poor)
Poor	58	37, 6.18	7.5, 1.55	24, 5.44	0.005[3] (well *vs* poor)

[1]Presents circulating tumor cell counts analysis.
[2]Presents mesenchymal circulating tumor cell counts analysis.
[3]Presents survivin positive circulating tumor cell counts analysis.
CTC: Circulating tumor cell; mCTC: Mesenchymal circulating tumor cell; AFP: α-fetoprotein.

ROC curve analysis of CTCs, survivin-positive CTCs, and mCTC proportion

The area under the ROC curve was 0.84 [95% confidence interval (CI) = 0.77-0.91] for CTCs, 0.82 (95%CI = 0.75-0.89) for survivin-positive CTCs, and 0.82 (95%CI = 0.75-0.90) for the proportion of mCTCs (Figure 6D). The rational cut-off value for diagnosis was 12 for the CTC count (sensitivity = 60.89%, specificity = 91.13%), 4 for the survivin-positive CTC count (sensitivity = 81.01%, specificity = 78.23%), and 1.85% for the proportion of mCTCs (sensitivity = 84.92%, specificity = 72.58%).

Correlation between survivin expression in CTCs and clinicopathological factors of HCC patients

The survivin-positive CTC counts were not significantly associated with AFP levels. However, the median survivin-positive CTC count in patients with stages III and IV HCC was significantly higher than that in patients with stages I and II HCC (Table 1). Further, patients with stage B/C HCC (or advanced HCC) had a significantly greater median survivin-positive CTC count than those with stage 0/A HCC (or early-stage HCC) ($P < 0.001$) (Table 1). Finally, patients with poorly differentiated HCC had a

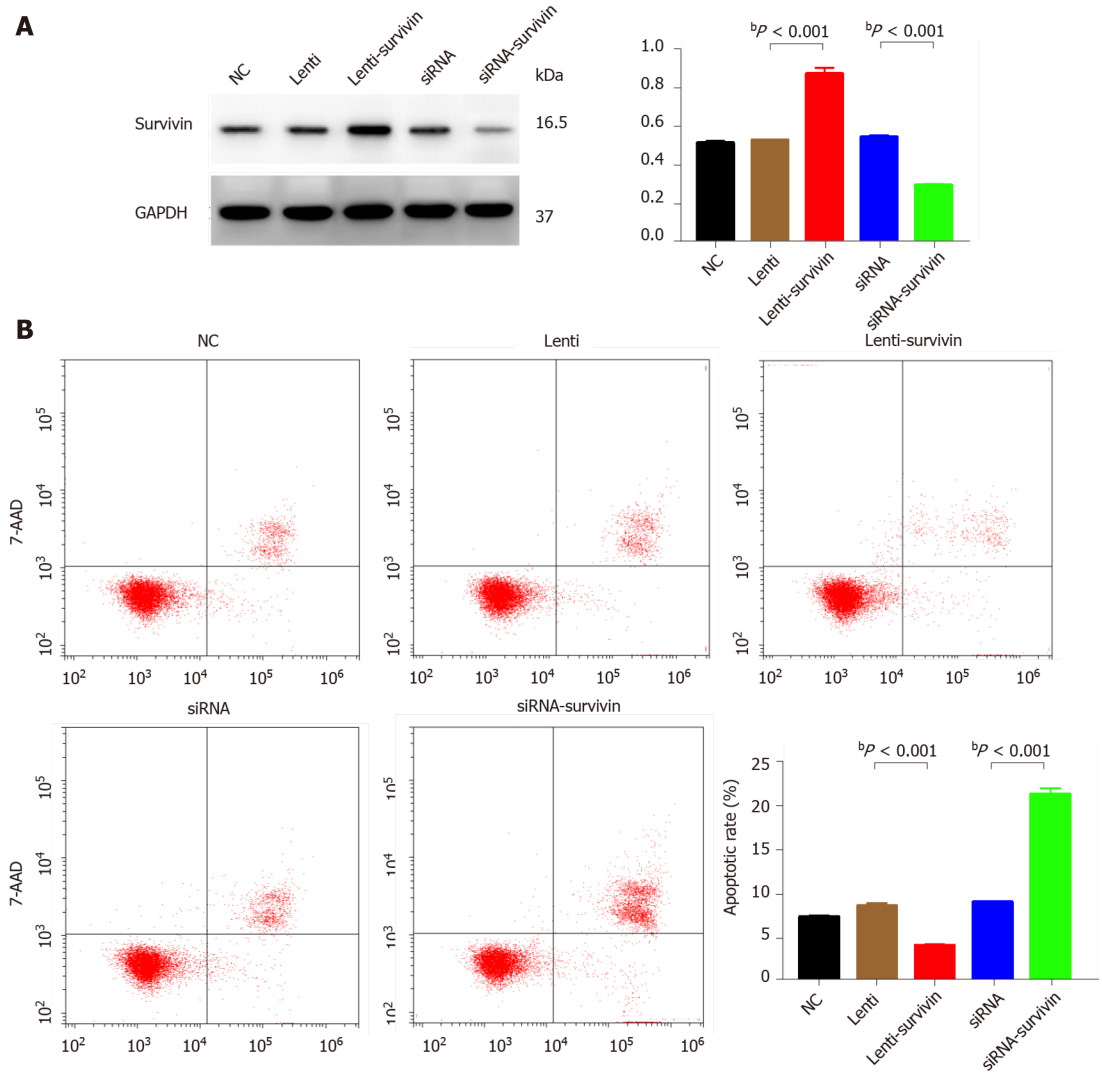


Figure 4 Effect of survivin silencing and overexpression on apoptosis ability of HepG2 cells. A: Western blot analysis confirming survivin protein knockdown and upregulation in the selected clones (siRNA, siRNA-survivin, Lenti, and Lenti-survivin) and controls; B: Cell apoptosis was detected by flow cytometric analysis (n = 3). The apoptosis rate was significantly lower in the survivin-overexpressing cells ($^bP < 0.01$).

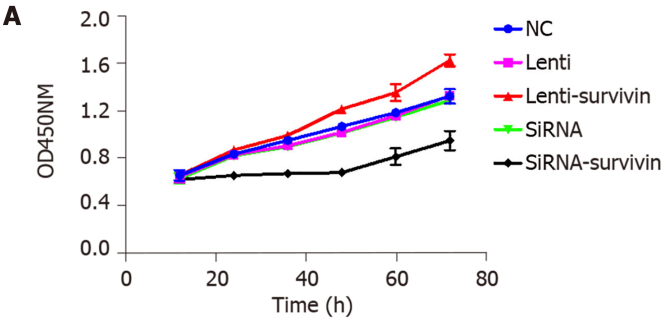
significantly greater median survivin-positive CTC count than those with well-differentiated tumors (P = 0.005) (Table 1).

Survivin expression in CTC subtypes

Survivin expression was detected in all CTC-positive patients (Figure 7), but the level of expression (based on the intensity of the purple fluorescent signal) differed between epithelial, mesenchymal, and hybrid CTCs, although not significantly: Survivin expression was observed in 62.7% of mCTCs, 74.61% of hybrid CTCs, and 73.53% of epithelial CTCs. However, the signal intensities were significantly different: Moderate- and high-intensity signals were observed in 48.53% and 31.91% of mCTCs, respectively, and in 47.65% and 16.5% of hybrid CTCs, respectively. The proportion of mesenchymal and hybrid CTCs with moderate- and high-intensity signals was significantly higher than that of epithelial CTCs (P < 0.001) (Figure 7A and B). In contrast, a significantly greater proportion of epithelial CTCs tended to have low signal intensity for survivin expression: 72.47% of epithelial CTCs *vs* 33.85% and 35.83% of hybrid and mCTCs, respectively (Figure 7A and B).

Dynamic changes in survivin-positive CTC count following surgery and its prognostic significance

The survivin-positive CTC counts were detected in 22 patients at 1 wk following surgical resection. The total CTC count exhibited a considerable decrease after resection, as did the survivin-positive CTC and mCTC counts (Figure 8A-C). During the postoperative period, in 10 out of 22 HCC patients, the total CTC, mCTC, and



P-value	Lenti *vs* lenti-sur	SiRNA *vs* siRNA-sur
60 h	$P < 0.001$	$P < 0.001$
72 h	$P < 0.001$	$P < 0.001$

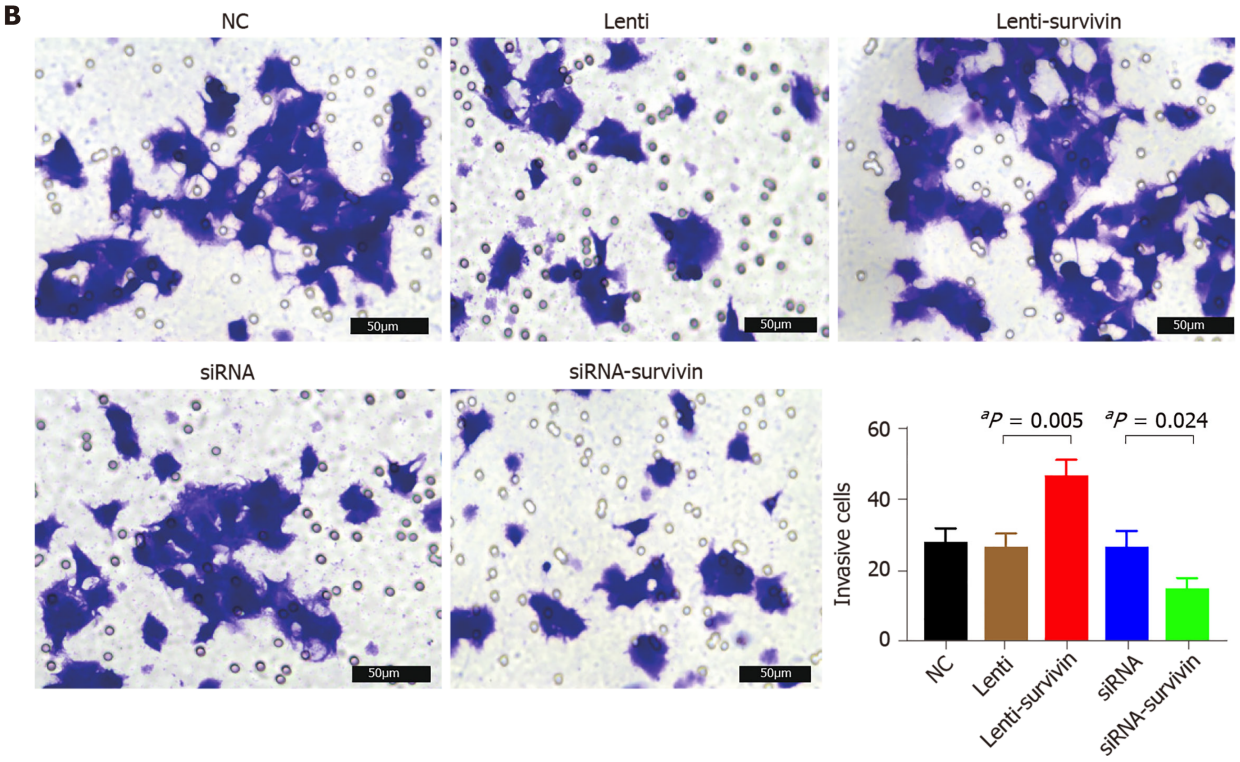


Figure 5 Effect of survivin silencing and overexpression on proliferation and invasion abilities of HepG2 cells. A: Cell proliferation was evaluated at 12 h, 24 h, 48 h, 60 h, and 72 h after transfection with the siRNA or lentivirus using the CCK8 assay ($n = 5$), and it was significantly higher in the survivin-overexpressing cells ($^aP < 0.05$); B: Invasion ability of cells was examined by transwell assay ($n = 3$). The invasive cell count was significantly higher in the survivin-overexpressing cells ($^aP < 0.05$).

survivin-positive CTC counts had increased over the thresholds (12 for CTCs, 4 for mCTCs and sur-CTCs) at 86 d before imaging recurrence or metastatic lesions were detected, while no increase was noted in the other 12 patients for more than 1 year (Figure 8D). Additionally, log-rank test revealed that the recurrence free survival rate was significantly associated with survivin-positive CTC count (Figure 9A). In all 10 cases, over 4 survivin-positive CTCs were detected before recurrence (Figure 9B).

In two HCC patients, initial overexpression of survivin was observed in CTCs (counts/5 mL), while recurrence or metastasis (Figure 10A and B) was observed 4-5 mo after therapy in patients who were in the same BCLC stage before treatment. And increased surviving-positive CTC count is showed in Figure 10C.

DISCUSSION

In the present study, we analyzed the correlation between survivin-positive CTC expression patterns and the prognosis of HCC, in order to explore the potential of survivin-positive CTCs as markers for HCC stage and predictors of HCC metastasis. The prognostic value of survivin-positive CTCs in patients after surgical resection was also demonstrated.

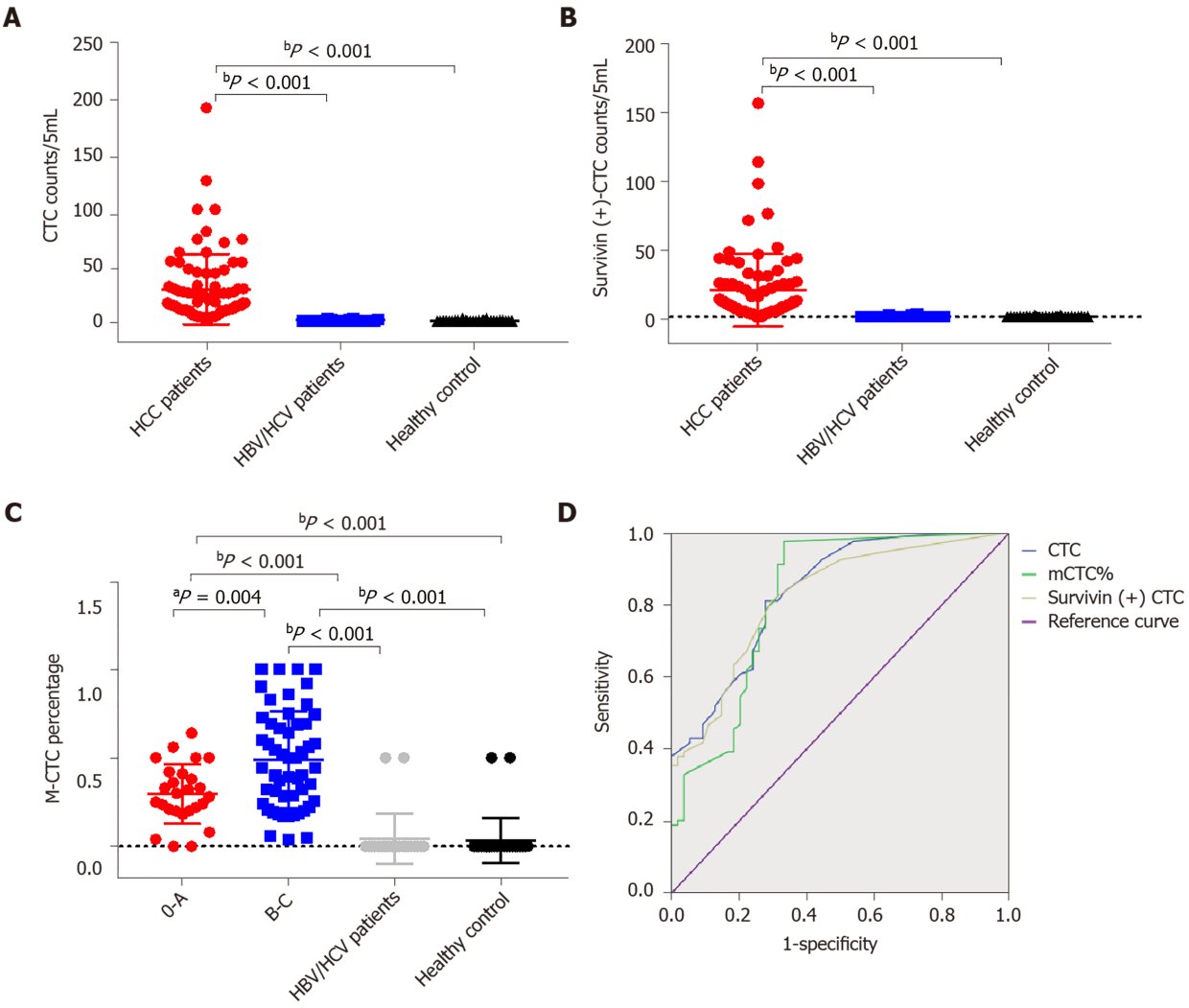


Figure 6 Total circulating tumor cell count and survivin-positive circulating tumor cell count in the study cohort. A and B: Both the median circulating tumor cell (CTC) count (A) and survivin-positive CTC count (B) in hepatocellular cancer (HCC) patients were significantly higher than those in the HBV/HCV patients and healthy controls; C: The proportion of mesenchymal CTCs (mCTCs) in HCC at the advanced BCLC stage (B-C) was significantly higher than that in HCC at the early stage (0-A); D: ROC curves for survivin-positive CTC count, total CTC count, and mCTC proportion. The rational cut-off for diagnosis was 12 for the CTC count, 4 for the survivin-positive CTC count, and 1.85% for the mCTC proportion. $^{a}P < 0.05$; $^{b}P < 0.01$. CTC: Circulating tumor cell.

Our *in vitro* experiments with HepG2 cells showed that siRNA-induced silencing of survivin expression resulted in a significant decrease in the proliferation and invasive ability of these cells, and a significant increase in their apoptosis rate; opposite results were obtained with overexpression of survivin. In agreement with this finding, previous studies have confirmed that survivin deficiency induces apoptosis in HepG2 cells[20,21]. These findings together indicate that survivin promotes the metastasis of HCC, and they are in agreement with a previous study which showed that knockdown of survivin expression suppressed HCC metastasis[22].

In our study, 94.41% of CTCs obtained from the HCC patients were positive for survivin expression. Further, we examined the survivin expression pattern in different phenotypes of CTCs, including mCTCs, hybrid CTCs, and epithelial CTCs. The findings showed that survivin expression was much higher in mesenchymal and hybrid CTCs than in epithelial CTCs, and a major proportion (62.7%) of mCTCs were positive for survivin expression. These findings indicate that survivin plays an important role in promoting EMT in HCC cells. During the process of carcinogenesis, tumor cells gain invasive ability by undergoing EMT, which occurs *via* downregulation of the epithelial cell marker E-cadherin (among others) and upregulation of the mesenchymal cell markers vimentin and N-cadherin (among others)[23]. Accordingly, it has also been reported that survivin mediates EMT in HCC cells *via* modulation of EMT marker expression[24]. Thus, based on these findings, it appears that survivin promotes metastasis of HCC through mediation of EMT events.

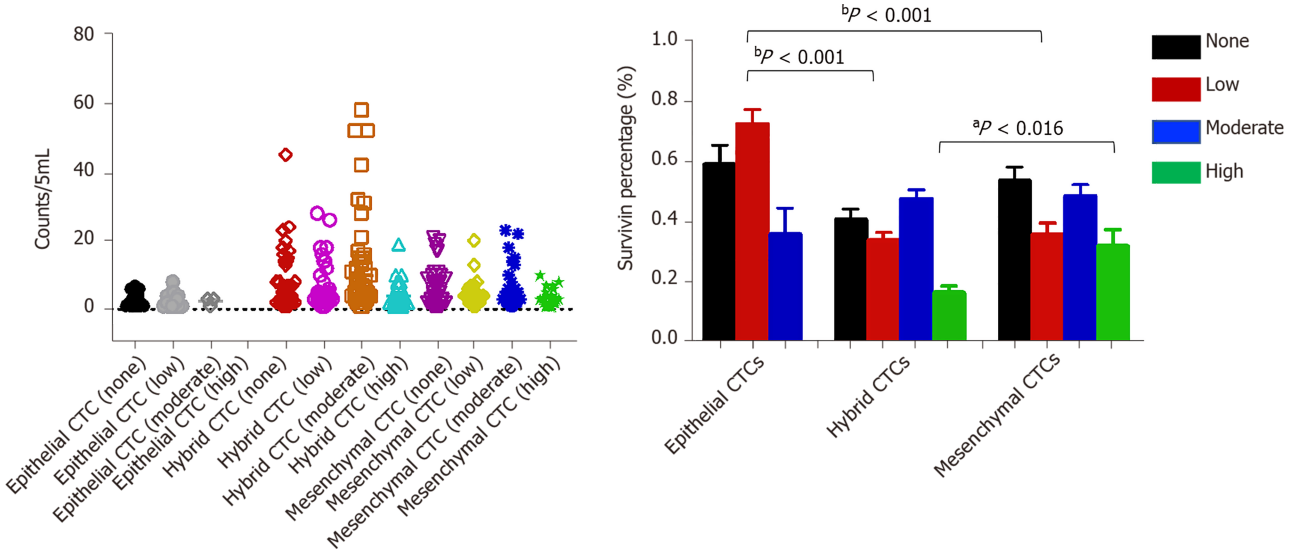


Figure 7 Analysis of survivin gene expression patterns in epithelial, mixed, and mesenchymal circulating tumor cells. A significantly lower percentage of epithelial circulating tumor cells (CTCs) showed moderate staining intensity than mixed and mesenchymal CTCs (mCTCs), while a significantly higher percentage of epithelial CTCs showed low staining intensity than hybrid and mCTCs. $^{a}P < 0.05$; $^{b}P < 0.01$. CTC: Circulating tumor cell.

Our findings showed that survivin was expressed at significantly greater levels in HCC tissue than in normal liver tissue, and the expression levels were significantly associated with the degree of tumor differentiation, that is, poorly differentiated tumors had significantly higher survivin expression. We also found that survivin expression level was significantly greater in HCC tissue than in normal tissue. The high frequency of cytoplasmic survivin expression in HCC observed in our study is in agreement with that reported in previous studies[24,25]. Many studies have demonstrated that survivin plays a crucial role in the early and late stage of carcinogenesis[26]. In agreement with our findings, other *in vitro* studies have reported increased expression of survivin in tumor cells[27,28]. Based on these findings, we assumed that survivin plays an anti-apoptotic role and aids in the progression of HCC. Hence, HCC patients who are positive for survivin should be monitored carefully, and enhanced adjuvant therapy should be considered to help improve their prognosis[29,30]. It is not easy to obtain HCC tissue samples at every follow-up appointment; therefore, detection of survivin in CTCs may be a useful supplementary method.

CONCLUSION

Previous studies have mostly investigated the expression of survivin in cancer cells or tissue specimens from patients[25,31,32], but the strength of our study lies in its investigation of survivin expression in different subtypes of CTCs. This is the first study to have pursued this line of investigation. We found that the survivin-positive CTC counts are significantly associated with the TNM tumor stage, BCLC stage, and degree of differentiation. Thus, the survivin-positive CTC count may particularly be important as an indicator of tumor stage and cancer progression. Additionally, the proportion of mCTCs also increases with tumor progression, but the mCTC counts are not significantly different between the different tumor grades and AFP. This variable needs to be explored further in future studies for its potential as a tumor stage marker.

One of the limitations of this study is the small sample size. Future investigations on survivin-positive CTCs should use a larger sample. Additionally, because of the limited follow-up time, the relationship between survivin-positive CTC counts and overall survival could not be explored. Therefore, an analysis with a longer follow-up duration would be highly useful.

Thus, the present findings indicate that the survivin-positive CTC count might have potential as an indicator for disease stage and a predictor of recurrence in HCC patients. Thus, an efficient and reliable technique to measure survivin expression in CTCs from liver cancer patients could prove useful for predicting the prognosis and recurrence of this cancer, and this could help clinicians select an optimal and customized management strategy for the treatment of this malignancy.

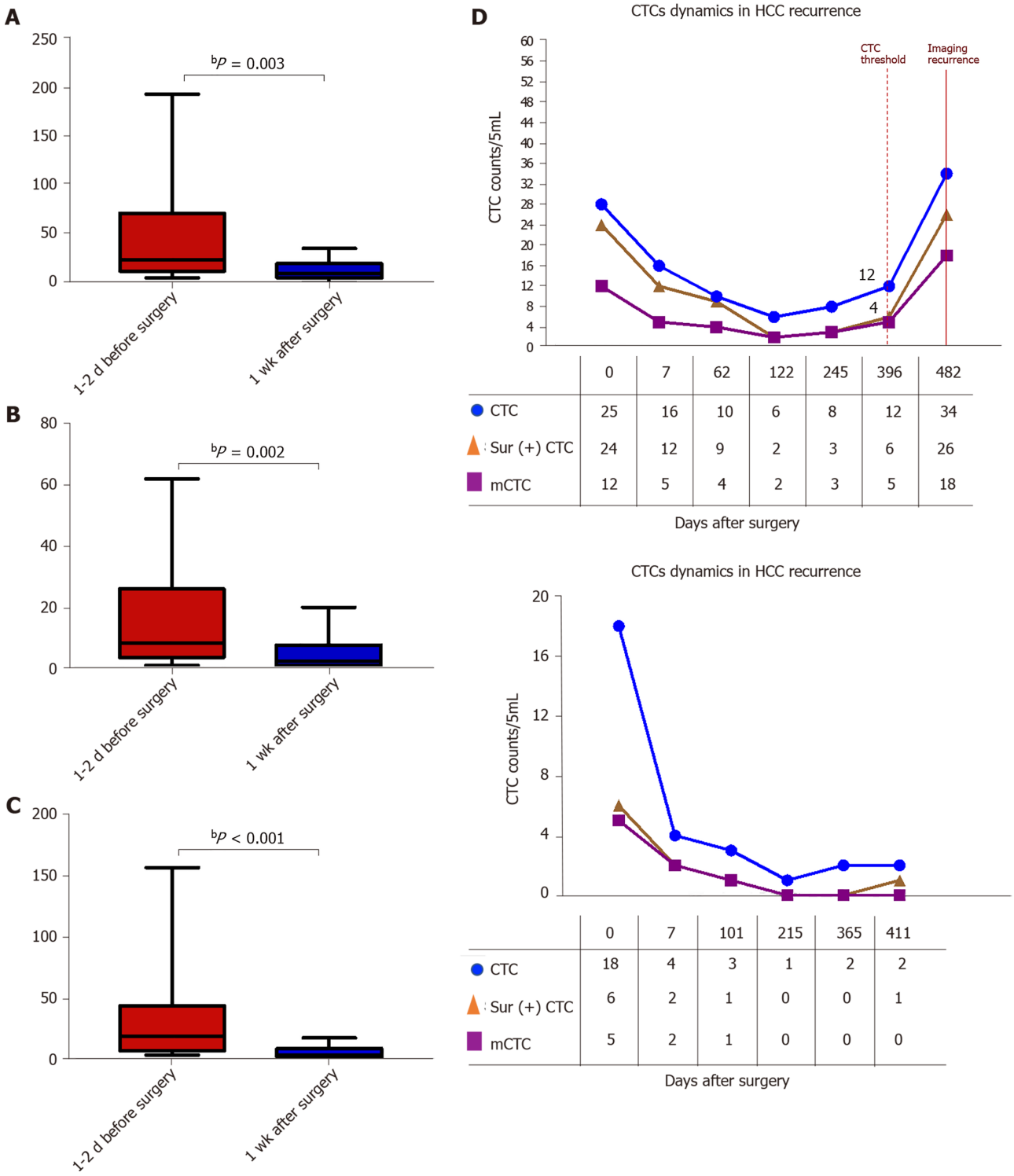


Figure 8 Dynamic changes in circulating tumor cell, mesenchymal circulating tumor cell, and survivin-positive circulating tumor cell counts following surgery. A-C: The total circulating tumor cell (CTC) count, mesenchymal CTC proportion, and survivin-positive CTC count decreased dramatically 1 wk after resection. D: Increased survivin-positive CTC count in postoperative hepatocellular cancer patients (n = 10) at 1-12 mo before detectable recurrence or appearance of metastatic lesions, and decreased surviving-positive CTC count in the last 12 hepatocellular cancer patients with no recurrence. $^{b}P <$ 0.01. CTC: Circulating tumor cell.

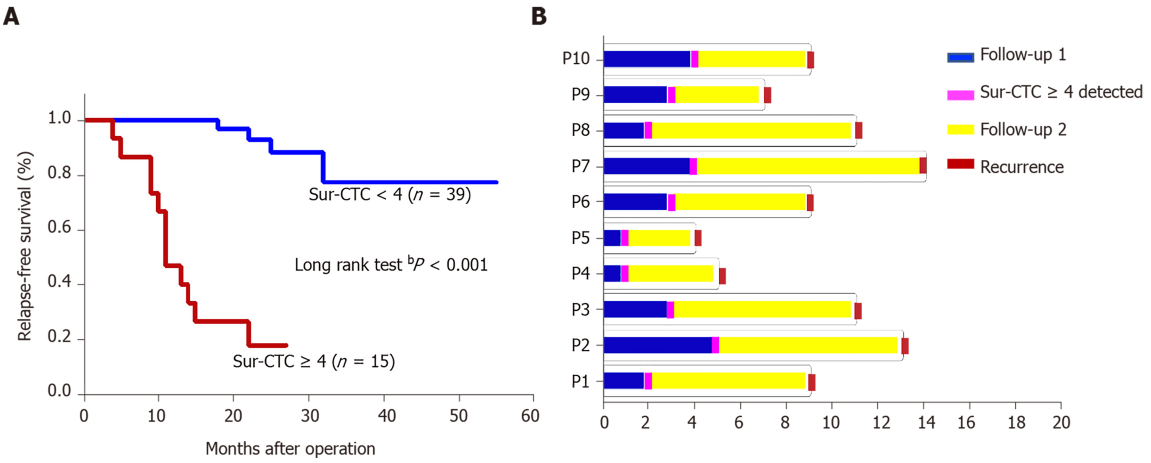


Figure 9 Prognosis of hepatocellular cancer patients with different survivin-positive circulating tumor cell counts. A: Recurrence free survival time after surgery by change in survivin-positive circulating tumor cell (CTC) count; B: The survivin-positive CTCs detected during follow-up of 10 hepatocellular cancer patients after operation predict tumor recurrence. $^{b}P < 0.01$. CTC: Circulating tumor cell.

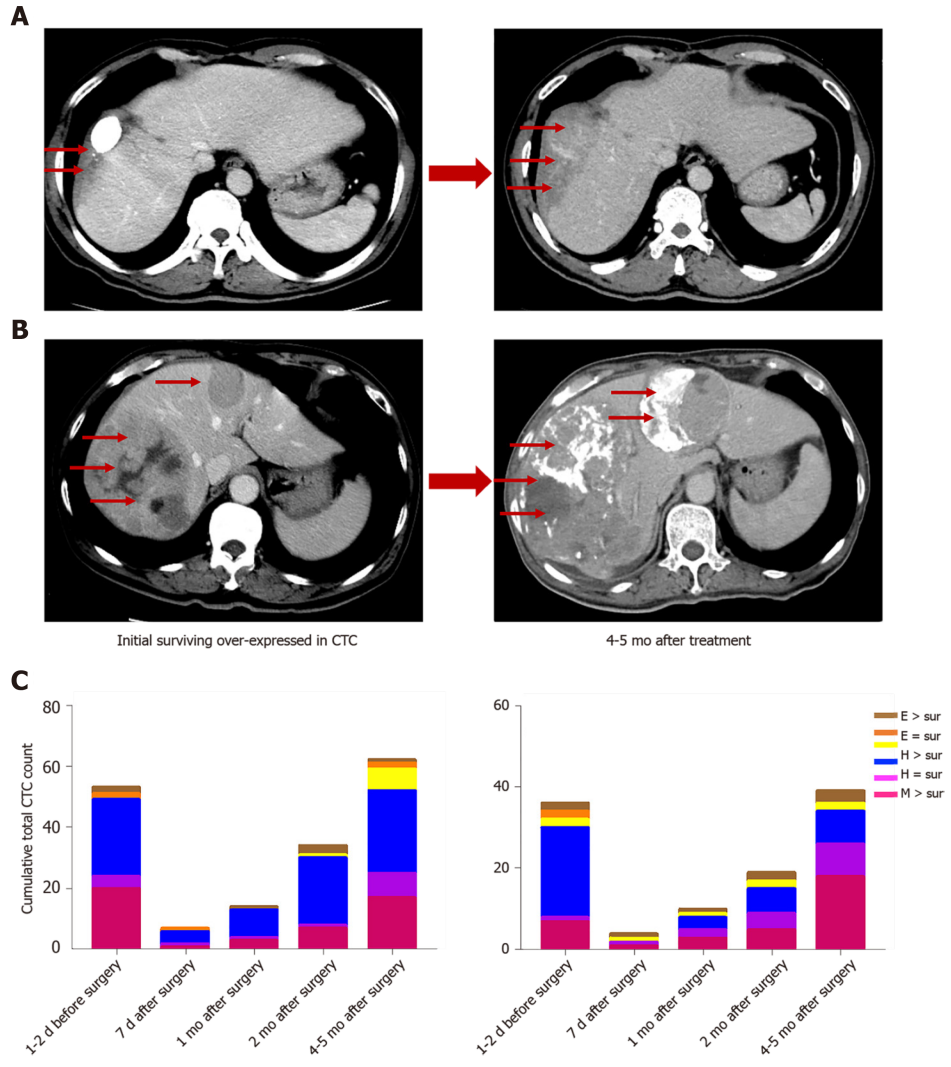


Figure 10 Survivin-positive circulating tumor cells detected during follow-up predict recurrence (m). A and B: Images of two hepatocellular cancer (HCC) patients at the same BCLC stage who expressed survivin in circulating tumor cells (CTCs). At 4 and 5 mo after treatment, the HCC tumors showed more rapid progression in patients with higher survivin expression in CTCs at baseline; C: Cumulative total CTC count by three cell types and survivin-positive CTC count in the two patients before and after operation. The patients' CTC and survivin-positive CTC count increased at the 2-mo follow-up. Letter E represents epithelial CTCs; Letter H represents hybrid CTCs; Letter M represents mesenchymal CTCs. CTC: Circulating tumor cell.

ARTICLE HIGHLIGHTS

Research background

Circulating tumor cells (CTCs), which are also known as liquid biopsy, are mainly used as a predictor to monitor the recurrence and metastasis of tumors including hepatocellular carcinoma (HCC). Survivin is an anti-apoptotic protein that plays an important role in inhibiting apoptosis and is overexpressed in many tumors, including HCC. No study has analyzed the value of survivin-expressing CTCs in HCC.

Research motivation

We aimed to study the expression of survivin in CTCs of HCC and indicate the role of survivin-expressing CTCs as a marker for EMT and cancer progression in HCC.

Research objectives

To explore the prognostic value of survivin-expressing CTCs in HCC, and estimate survivin-positive CTCs as a potential predictor of metastasis in HCC patients.

Research methods

We examined the survivin expression patterns in CTCs of HCC patients, and investigated the *in vitro* effects of survivin silencing and overexpression on the proliferation and invasion of HCC cells. We also analysed the survivin protein expression in HCC tissue. And we observed the dynamic changes in survivin-positive CTC count following surgery and its prognostic significance.

Research results

The CTC and survivin-positive CTC counts were significantly higher in the HCC patients than in the normal controls. Further, survivin overexpression was found to induce HepG2 cell proliferation, reduce apoptosis, and improve invasive ability. Additionally, log-rank test revealed that the recurrence free survival rate was significantly associated with survivin-positive CTC count.

Research conclusions

Survivin-positive CTCs are promising as a predictor of HCC prognosis and metastasis.

Research perspectives

The dynamic monitoring of survivin-positive CTC count would help clinical therapy and improve the prognosis of HCC patients.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 21; 27(43): 7563-7571

DOI: 10.3748/wjg.v27.i43.7563

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

ORIGINAL ARTICLE

Prospective Study

Immunoglobulin G in non-alcoholic steatohepatitis predicts clinical outcome: A prospective multi-centre cohort study

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Author contributions: De Roza MA contributed to study design, data collection and analysis, manuscript writing, critical Review and submission of manuscript; Lamda M contributed to study design, data collection and analysis, manuscript writing and review of manuscript; Goh GBB contributed to study design and critical review of manuscript; Cheah MCC and Lum JHM contributed to data collection; Ngu JHJ contributed to study design, writing and critical review of manuscript; and all authors read and approved the final manuscript.

Institutional review board statement: This study conforms to ethical guidelines and was approved by our institutional review board with waiver of patient consent.

Clinical trial registration statement: This study does not include any intervention and is not a

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Abstract

BACKGROUND

Autoimmune markers including plasma cells (PC), anti-smooth-muscle antibody (ASMA), anti-nuclear antibody (ANA), and raised immunoglobulin G (IgG) are commonly observed in non-alcoholic steatohepatitis (NASH), however their clinical significance is unknown.

AIM

To determine if autoimmune markers in NASH patients are independently associated with poorer clinical outcomes.

METHODS

Consecutive patients with biopsy proven NASH from Christchurch Hospital, New Zealand and Singapore General Hospital (SGH) were included between 2005 to 2016 in a prospective multi-centre cohort study. Patients with other causes of chronic liver disease were excluded. IgG > 14 g/L or globulin fraction > 50%, ANA ≥ 1:40, SMA ≥ 1:40 were considered positive. Multivariate analysis was performed to assess which markers were independently associated with mortality and hepatic decompensation.

RESULTS

randomized controlled trial.

Informed consent statement: Consent was not obtained as data was anonymized and protected with little to no risk of identification.

Conflict-of-interest statement: All authors declare there are no conflicts of interest. None of the authors received financial support or grants for this study.

Data sharing statement: Statistical code, and dataset is available from the corresponding author at jeffrey.ngu@cdhb.health.nz.

CONSORT 2010 statement: The manuscript was checked according to the CONSORT 2010.

Country/Territory of origin: New Zealand

Specialty type: Gastroenterology and Hepatology

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

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

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Received: April 19, 2021
Peer-review started: April 19, 2021
First decision: June 23, 2021
Revised: July 20, 2021

Total 261 patients were included of which 201 were from SGH. The median age was 53 and 51.9% were male. Advanced fibrosis was present in 31.4% at diagnosis. PC, ASMA, ANA and raised IgG were observed in 13.1%, 4.9%, 27.8% and 30.1% of patients respectively. After multivariate analysis, elevated IgG [Hazard Ratio (HR) 6.79, 95%CI: 2.93-17.15] and fibrosis stage (HR 1.37, 95%CI: 1.03-1.87) were found to be independently associated with increased risk of liver decompensation. Age (HR 1.06, 95%CI: 1.02-1.10) and elevated IgG (HR 3.79, 95%CI: 1.90-7.68) were independent factors associated with higher mortality risk.

CONCLUSION

Elevated IgG, rather than ANA, ASMA or plasma cells, is independently associated with increased risk of hepatic decompensation and mortality in NASH. It could hence be important for prognostication.

Key Words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Immunoglobulin G; Autoantibodies; Mortality; Hepatic decompensation

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Core Tip: Autoantibodies such as anti-nuclear antibody (ANA) and anti-smooth-muscle antibody (ASMA) can be present in up to 20%-30% of patients with non-alcoholic steatohepatitis (NASH). However, clinical significance is not well studied and there is no published data on the impact of immunoglobulin G (IgG) and plasma cells on hepatic decompensation and mortality outcomes. Our study found that elevated IgG but not ANA, ASMA or plasma cells is associated with higher risk of mortality, including liver related death, as well as increased risk of hepatic decompensation events. Patients with IgG positive NASH should hence be identified early and monitored closely as they are at higher risk of poorer clinical outcomes.

Citation: De Roza MA, Lamba M, Goh GBB, Lum JHM, Cheah MCC, Ngu JHJ. Immunoglobulin G in non-alcoholic steatohepatitis predicts clinical outcome: A prospective multi-centre cohort study. *World J Gastroenterol* 2021; 27(43): 7563-7571
URL: https://www.wjgnet.com/1007-9327/full/v27/i43/7563.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i43.7563

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a growing phenomenon with an estimated global prevalence of 25%. Non-alcoholic steatohepatitis (NASH) in particular is a progressive form of NAFLD and is associated with poorer clinical outcomes and higher liver related mortality[1]. Independent predictors for poor outcomes include fibrosis[2], obesity and metabolic syndrome, diabetes mellitus (DM)[3], as well as genetic polymorphisms such as PNPLA3[4]. NASH is characterized histologically by hepatic steatosis, inflammation, hepatocellular injury and varying degrees of fibrosis[5]. The inflammatory process in NASH is a complex and heterogeneous "multi-hit" pathway in which the innate immune system plays a critical role, driving the progression of liver fibrosis and leading to cirrhosis, liver failure, the need for liver transplantation and death[6-8]. Less is known, however, about the role of the adaptive immune system and autoantibodies. Autoantibodies are produced by humoral immune responses against self-cellular proteins and nucleic acids and can be physiological or pathological[9]. When used in tandem with clinical findings, they are serological hallmarks for inflammatory autoimmune liver diseases. However, their significance in NAFLD is not well studied despite autoantibodies being present in 25%-35% of patients with NAFLD[10,11].

Adams *et al*[10] reported a higher grade of inflammation in NAFLD patients with positive antinuclear antibodies (ANA) and/or anti smooth muscle antibodies (ASMA) but the difference was slight (1.0 *vs* 1.2, P = 0.02) and there was no correlation to clinical significance or outcomes. More recent data from McPherson *et al*[12] looking specifically at serum immunoglobulins in NAFLD, showed that elevated serum

Accepted: November 2, 2021
Article in press: November 2, 2021
Published online: November 21, 2021

P-Reviewer: Khiatah B
S-Editor: Wang JL
L-Editor: A
P-Editor: Wang JL

immunoglobulin A was significantly associated with advanced fibrosis. Similarly, there was no correlation to immunoglobulins being a predictor for mortality or hepatic events independently from fibrosis and other factors. Despite evidence showing association of autoantibodies and immunoglobulins with higher histological grades of inflammation or fibrosis[10,12,13], other studies dispute these findings[14,15], and correlation to clinical outcomes is still not established in NASH. Ravi *et al*[16] reported no significant difference on liver disease outcomes in steatohepatitis patients with positive ANA or ASMA , however, the major limitation of the study was that overall follow-up was short (median 3 years) and alcoholic and non-alcoholic hepatitis were grouped together.

Overall, autoimmune markers such as ANA, ASMA, plasma cells and immunoglobulins with immunoglobulin G (IgG) in particular, are not well studied in NASH and their clinical significance is unknown in this population. Hence, the aim of this study is to determine if autoimmune markers in patients with biopsy proven NASH are independently associated with poorer outcomes. The outcomes measured being all cause mortality and liver decompensation events.

MATERIALS AND METHODS

Patient selection

Consecutive patients who underwent liver biopsy at Christchurch Hospital, New Zealand (CH) and Singapore General Hospital, Singapore (SGH) were assessed for inclusion in the study. CH cohort consisted of patients with liver biopsy performed from 2008 to 2016 and the SGH cohort from 2005 to 2015. Patients with chronic liver diseases of other aetiologies such as viral hepatitis, alcohol, toxins or drugs, autoimmune including IgG4 related disease, vascular, metabolic and hereditary causes were excluded. All patients, as per local hospital protocol underwent non-invasive liver testing including serology analysis and imaging to exclude other causes of chronic liver disease. Patients were included in the study if the final histological diagnosis was NASH with NAFLD activity score (NAS) ≥ 3 on biopsy with scores for steatosis, lobular inflammation and hepatocyte ballooning[17].

ANA and ASMA were considered positive if titres were observed to be ≥ 1:40. IgG was considered elevated if > 14g/L. Additionally, if quantitative values were not available, globulin fraction (GF) was calculated by the following equation: total protein/(total protein - albumin). Since IgG is the commonest globulin type[18], individuals with GF > 50% were defined as elevated IgG. To assess presence of plasma cells, histology reports were reviewed. Plasma cells were scored as positive if any plasma cells were identified on histology specimens by the pathologist. This study conforms to ethical guidelines and was approved by our respective institutional review boards.

Follow-up

Patients from CH and SGH were followed up for clinical events of liver decompensation and all-cause mortality. Liver decompensation event was defined as the development of any of the following: ascites, gastrointestinal haemorrhage secondary to portal hypertension, hepatic encephalopathy, hepatorenal syndrome. Patients were followed up till 31st December 2017. Follow-up was censored at development of first liver decompensation event, death, liver transplantation, or last clinical contact in case of patients lost to follow-up.

Statistical analysis

Continuous variables were expressed as mean ± SD or median (interquartile range, IQR) and were compared using unpaired *t*-test. Categorical variables were compared using χ^2 test. The associations of putative risk factors and outcomes were analyzed using Cox proportional hazards regression and are summarized as hazard ratios (HR) with 95%CI. The time to event outcomes were also summarized using Kaplan-Meier curves. A two-tailed *P* value of < 0.05 was taken to indicate a statistical significance. All analyses were undertaken using statistical software SPSS version 20.

RESULTS

A total of 261 patients met the study criteria. Of these, 201 patients were recruited from

SGH and 60 patients from CH. Baseline characteristics of patients are listed in Table 1. Majority of patients from CH were of European origin (91.7%) while 97.5% patients from SGH were of Asian origin, reflecting the local population demographic. Median follow-up per patient was 5.1 years (IQR 3.5-7.5). Median age at inclusion in the study was 53 years (± 12.9) and 51.9% were male. The median NAS score at diagnosis was 4 (IQR 3-5) and the mean Metavir fibrosis score was 1.7 (± 1.4). 77% of patients had data available for body mass index (BMI), and comorbidities including presence of DM, of which the mean BMI was 30.61 and DM was present in 45.02% of patients. There were no significant differences in baseline characteristics between patients from SGH and CH (Table 1).

Prevalence of autoimmune markers

ANA was the most common positive autoimmune marker present in 27.8% patients followed by elevated IgG observed in 23.4%. Plasma cells were found on histological examination in 13% of patients with NASH. ASMA was the least common autoimmune marker and was positive in only 4.9% of NASH patients.

Clinical end-points: Liver decompensation and all-cause mortality

During a cumulative follow-up of 1464 person years, 25 patients developed liver decompensation. There was no significant difference between risk of liver decompensation or all-cause mortality between patients from CH and SGH. The 5-year risk of developing liver decompensation after diagnosis of NASH was 8.1% (95%CI: 6.1-11.8) and the 5-year mortality risk was 11.7% (95%CI: 4.4-22.9) (Figure 1). Ten patients developed hepatocellular carcinoma of which 7 were male. Median age at diagnosis of HCC was 65.7 years. Advanced fibrosis or cirrhosis was present in 6 of 10 patients at diagnosis of HCC. During the follow-up period, 36 patients died. Data on cause of death were available for 30 (83.3%) patients. Liver related causes of death were observed in 12 cases (40%), followed by malignancy (30%), septicaemia (17%) and cardiovascular causes of death (13%). Overall, 5-year risk of all-cause mortality was 11.7% (95%CI: 4.4-22.9) (Figure 1).

Predictors of Liver decompensation and all-cause mortality

Liver decompensation: In univariate analysis (Table 2), factors associated with increased risk of liver decompensation were increasing age (HR 1.04, 95%CI: 1.00-1.08), stage of fibrosis (HR 1.67, 95%CI: 1.25-2.26) and elevated IgG (HR 8.20, 95%CI: 3.61-20.30). Other autoimmune markers (ANA, ASMA or Plasma cells) were not found to be associated with risk of liver decompensation (Table 2; Figure 1). Multivariate model was constructed with variables found to be significant in univariate analysis. In multivariate analysis, elevated IgG (HR 6.79, 95%CI: 2.93-17.15) and stage of fibrosis (HR 1.37, 95%CI: 1.03-1.87) were found to be independently associated with increased risk of liver decompensation during follow-up (Table 2).

In a sub-group analysis where only patients with quantitative IgG values (> 14 g/L) were included (n = 43), a trend of association was observed between elevated IgG and increased risk of liver-decompensation during follow-up. (HR 3.1, 95%CI: 0.92-10.8, P = 0.054) (Figure 2).

Mortality: In univariate analysis, predictors of all-cause mortality included: increasing age (HR 1.06, 95%CI: 1.03-1.10), stage of fibrosis (HR 1.27, 95%CI: 1.00-1.61) and elevated IgG (HR 4.5, 95%CI: 2.29-9.00) (Table 2). In multivariate analysis, age (HR 1.06, 95%CI: 1.02-1.10) and elevated IgG (HR 3.79, 95%CI: 1.90-7.68) were found to be independent factors associated with increased risk of mortality (Table 2; Figure 2). Median survival in patients with elevated IgG at baseline was 9.4 years.

DISCUSSION

In this multicentre cohort study, we examined the association between the presence of autoimmune markers such as ANA, ASMA, elevated IgG and plasma cells on histology with clinical outcomes in patients with NASH. The most pertinent finding of our study is that elevated IgG at diagnosis of NASH was associated with increased risk of liver decompensation and reduced overall survival.

Autoimmune markers are commonly encountered in patients with NASH, however their clinical significance is not well defined. In a study of 225 patients with histologically confirmed NAFLD, 20% and 3% respectively were found to have the presence of ANA and ASMA[10]. Similarly, in another cohort study of NASH patients, the

Table 1 Baseline characteristics, *n* (%)

	Overall	CH	SGH	*P* value
Patients	261	60	201	-
Regions				0.07
European	60 (23.0)	55 (91.7)	5 (2.5)	
Asian	197 (75.5)	2 (3.3)	195 (97.5)	
Others	4 (1.5)	3 (5.5)	1 (0.5)	
Age, mean ± SD	53 ± 12.9	52.9 ± 16.9	53 ± 11.7	0.30
Male	51.9%	53.3%	51.7%	0.83
ALT, mean ± SD	112.3 ± 373.9	80.2 ± 66.6	121.9 ± 425.5	0.45
AST, mean ± SD	80.3 ± 216.7	60.8 ± 42.3	86.1 ± 246.1	0.43
NAS, Median (IQR)	4 (3-5)	4 (3-5)	4 (3-5)	0.54
Metavir Fibrosis score, mean ± SD	1.7 ± 1.4	1.6 ± 1.4	1.7 ± 1.3	0.58
F0	53 (20.3)	13 (21.7)	40 (19.9)	
F1	95 (36.3)	25 (41.7)	70 (34.8)	
F2	31 (11.9)	5 (8.3)	26 (12.9)	
F3	48 (18.4)	7 (11.7)	41 (20.4)	
F4	34 (13.1)	10 (16.6)	24 (11.9)	

CH: Christchurch Hospital, New Zealand; SGH: Singapore General Hospital, Singapore; IQR: interquartile range; NAS: Non-alcoholic fatty liver disease activity score.

Table 2 Univariate and multivariate analysis of predictors of clinical outcomes

Predictors	Liver decompensation				All-cause mortality			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age[1] (per 1 yr)	1.04 (1.00-1.08)	0.04	1.01 (0.98-1.05)	0.46	1.06 (1.03-1.10)	< 0.0001	1.06 (1.02-1.10)	0.001
Sex	0.99 (0.44-2.19)	0.98	-	-	1.16 (0.60-2.27)	0.65	-	-
Fibrosis[2]	1.67 (1.25-2.26)	< 0.001	1.37 (1.03-1.87)	0.03	1.27 (1.00-1.61)	0.05	1.01 (0.80-1.29)	0.91
NAS[3]	0.96 (0.67-1.32)	0.79	-	-	0.88 (0.66-1.16)	0.37	-	-
IgG	8.20 (3.61-20.30)	< 0.0001	6.79 (2.93-17.15)	< 0.0001	4.50 (2.29-9.00)	< 0.0001	3.79 (1.90-7.68)	0.0001
ANA	1.15 (0.41-2.86)	0.78	-	-	1.14 (0.47-2.51)	0.76	-	-
ASMA	0		-	-	0.94 (0.05-4.53)	0.95	-	-
Plasma cells	0.97 (0.23-2.81)	0.96	-	-	1.20 (0.41-2.83)	0.71	-	-

[1]Age as continuous variable for every one year increase in age.
[2]Increase in Metavir fibrosis stage.
[3]Increase in non-alcoholic fatty liver disease activity score by 1.
IgG: Immunoglobulin G; ANA: Anti-nuclear antibody; ASMA: Anti smooth muscle antibody; HR: Hazard ratio.

presence of ANA and ASMA was observed in 34% and 6% of all patients respectively [15]. The findings of our study are consistent with the reported prevalence estimates. While inflammation involving plasma cells is typically observed in AIH, the prevalence of plasma cell infiltration in NASH is not known. In the present study, plasma cell infiltration was observed in 13% of patients with histological diagnosis of NASH.

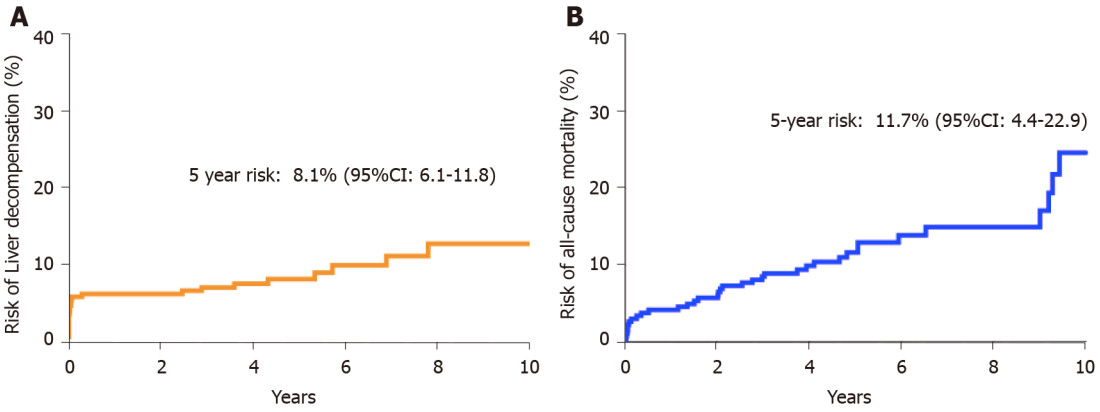


Figure 1 Overall risk of liver decompensation and all cause mortality. A: Overall risk of liver decompensation; B: Overall risk of all cause mortality.

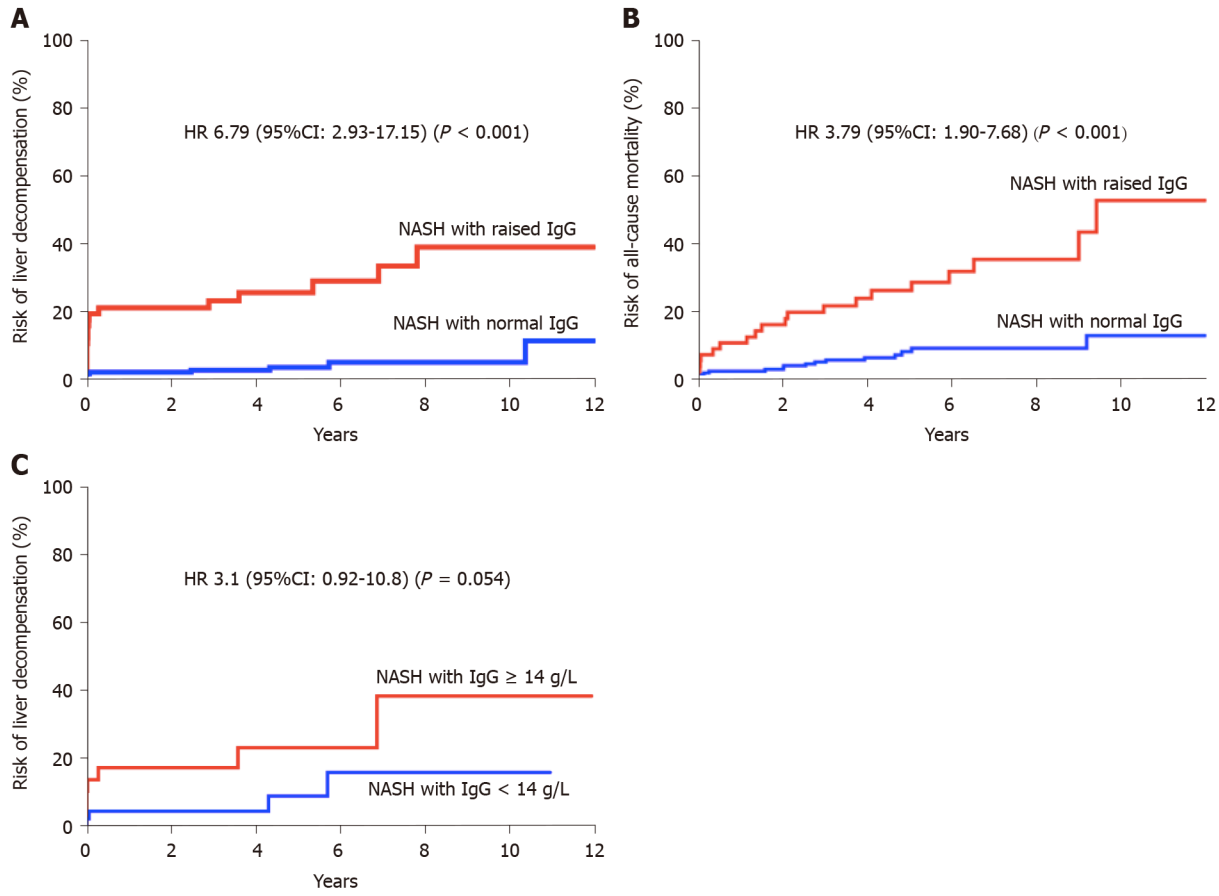


Figure 2 Raised immunoglobulin G and risk of liver decompensation and mortality on multivariate analysis. A: Raised immunoglobulin G (IgG) and risk of liver decompensation; B: Raised IgG and risk of all-cause mortality; C: Raised absolute IgG > 14 and risk of liver decompensation. IgG: Immunoglobulin G.

Association of ANA and/or ASMA with histological severity of NASH has been examined previously in multiple cohort studies and yielded conflicting results[10,12-15,19]. None of the studies, to our knowledge, have assessed association of autoimmune markers with long-term clinical outcomes. We found that the risk of liver decompensation or all-cause mortality were not associated with the presence of either ANA, ASMA or plasma cells, thereby suggesting that these are non-specific markers of inflammation and unlikely to be pathogenically relevant.

On the contrary, elevated IgG at diagnosis of NASH was independently associated with increased risk of liver decompensation (HR 6.79, 95%CI: 2.93-17.15, $P < 0.0001$) and all-cause mortality (HR 3.79, 95%CI: 1.9-7.68, $P = 0.0001$) during follow-up. Majority of the excess mortality in the elevated IgG cohort were liver-related. It needs to be highlighted that diagnosis of probable or definite AIH was conclusively ruled out

in all patients based on the international standardised criteria and no patients were treated with immunosuppression except in cases of organ transplantation.

We do not have a concrete biological explanation for the observed pathogenic role of IgG in NASH, however several mechanisms are plausible. Firstly, oxidative stress in NASH may induce production of IgG by deployment of adaptive humoral response[5,20]. Animal and human based studies have shown that IgG directed against products of lipid peroxidation such as Malondialdehyde and 4-hydroxynonenal appears to be elevated in NASH and correlates with disease severity[20]. Secondly, anti-endotoxins IgG levels were observed to be higher in patients with NASH compared to controls (48 vs 10 GMU/mL), and IgG levels corelated with severity of NASH[20,21]. Endotoxins are generally derived from the gut microbiota[21] and are potential triggers for inflammation and insulin resistance, driving oxidative stress in NASH. Therefore, elevated IgG may be representative of high endotoxemic burden leading to rapid progression of NASH. Lastly, it is possible that elevated IgG in patients with NASH may represent an overlap with a mild degree of autoimmune hepatitis. However, currently no diagnostic criteria exist to define NASH-AIH overlap syndrome.

Our study has several limitations which ought to be acknowledged. Quantitative immunoglobulin values were not available for all patients and globulin fraction was used as a surrogate marker of elevated IgG. While globulin fraction has previously been utilised as an effective surrogate marker of hyper/hypogammaglobulinemia[22] it is possible that we may have under or overestimated the IgG effect on clinical outcomes. However, upon restricting analysis to those patients with quantitative IgG values, a similar effect was observed (although statistically non-significant), suggesting that the observed association between IgG and poor clinical outcomes is true rather than a type-1 error. Data for pre-existing medical comorbidities and current medications were only available for 77% of patients, which may have confounded overall results. However, all patients were on follow up with a specialist and would have received standard of care for hepatic decompensation, regardless of compliance to treatment. Lastly, the diagnosis of NASH was based on unblinded histology interpretation by the local pathology team, consequently an element of inter-observer bias cannot be ruled out. Our study design involved two different population cohorts from two large tertiary centres. Despite having different ethnic compositions, there were no significant differences in baseline-characteristics at inclusion in the study between the two centres suggesting that our results are generalizable. Importantly, both centres have national electronic records available making complete data ascertainment of clinical events possible.

CONCLUSION

In conclusion, we report that ANA, ASMA and plasma cells are commonly present in patients with NASH but carry no prognostic significance. On the contrary, elevated IgG is an independent predictor of increased risk of liver decompensation and reduced overall survival in patients with NASH. Presence of elevated IgG therefore represents a more aggressive NASH phenotype. Identification and close monitoring of these patients is prudent to improve overall clinical outcomes.

ARTICLE HIGHLIGHTS

Research background

Autoimmune markers such as immunoglobulin G (IgG), anti-nuclear antibody (ANA), anti-smooth-muscle antibody (ASMA) can be present in patients with Non-alcoholic steatohepatitis (NASH) but their clinical significance is not well studied.

Research motivation

Knowing the clinical significance of autoimmune markers in patients with biopsy proven NASH can pave the way for future research to better understand why certain sub-groups of patients with NASH deteriorate more rapidly.

Research objectives

This study aimed to determine if any of the autoimmune markers were independently associated with worse outcomes such as mortality and hepatic decompensation. This is important as such patients can be identified for closer monitoring.

Research methods

This is a prospective, multi-center study. Patients with biopsy proven NASH were included and multivariate analysis was performed to determine if any of the autoimmune markers (IgG, ANA, ASMA) were independent risk factors for mortality and hepatic decompensation

Research results

Elevated IgG was an independent risk factor for both mortality and liver decompensation after multivariate analysis with adjustment for age and fibrosis stage. The exact pathophysiology is unknown but IgG levels could possibly correlate to disease severity due to anti-endotoxins IgG and oxidative stress.

Research conclusions

Elevated IgG is an independent predictor of increased risk of liver decompensation and reduced survival in patients with NASH. It could represent a more aggressive NASH phenotype.

Research perspectives

Further research is needed to validate and reproduce this finding and to also establish the pathophysiology and underlying biochemical mechanisms for this observation.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 21; 27(43): 7572-7581

DOI: 10.3748/wjg.v27.i43.7572

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

SYSTEMATIC REVIEWS

Minimum sample size estimates for trials in inflammatory bowel disease: A systematic review of a support resource

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Author contributions: Gordon M conceived the study, contributed to design, analysis and writing; Lakunina S led completion, analysis and write up; Sinopoulou V and Akobeng A contributed to analysis, reviewed and edited the write up.

Conflict-of-interest statement: None to declare.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Country/Territory of origin: United Kingdom

Specialty type: Gastroenterology and hepatology

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

Peer-review report's scientific quality classification

Grade A (Excellent): 0

Grade B (Very good): B, B

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Abstract

BACKGROUND

Of 25% of randomised controlled trials (RCTs) on interventions for inflammatory bowel disease (IBD) have no power calculation.

AIM

To systematically review RCTs reporting interventions for the management of IBD and to produce data for minimum sample sizes that would achieve appropriate power using the actual clinical data.

METHODS

We included RCTs retrieved from Cochrane IBD specialised Trial register and CENTRAL investigating any form of therapy for either induction or maintenance of remission against control, placebo, or no intervention of IBD in patients of any age. The relevant data was extracted, and the studies were grouped according to the intervention used. We recalculated sample size and the achieved difference, as well as minimum sample sizes needed in the future.

RESULTS

A total of 105 trials were included. There was a large discrepancy between the estimated figure for the minimal clinically important difference used for the calculations (15% group differences observed *vs* 30% used for calculation) explaining substantial actual sample size deficits. The minimum sample sizes indicated for future trials based on the 25 years of trial data were calculated and grouped by the intervention.

CONCLUSION

A third of intervention studies in IBD within the last 25 years are underpowered, with large variations in the calculation of sample sizes. The authors present a sample size estimate resource constructed on the published evidence base for

Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

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Received: March 4, 2021
Peer-review started: March 4, 2021
First decision: June 3, 2021
Revised: June 30, 2021
Accepted: October 31, 2021
Article in press: October 31, 2021
Published online: November 21, 2021

P-Reviewer: Liefferinckx C, Vasudevan A, Yuksel I
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

future researchers and key stakeholders within the IBD trial field.

Key Words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Gastroenterology; Statistics; Sample size

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Core Tip: This work has identified a large variation in the estimated minimal clinically important difference (MCID) between study groups in inflammatory bowel disease trials in the literature, with no standard to support study designers or reviewers. We have provided a resource to support sample size estimation based on observed MICD in the literature over the last 25 years.

Citation: Gordon M, Lakunina S, Sinopoulou V, Akobeng A. Minimum sample size estimates for trials in inflammatory bowel disease: A systematic review of a support resource. *World J Gastroenterol* 2021; 27(43): 7572-7581
URL: https://www.wjgnet.com/1007-9327/full/v27/i43/7572.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i43.7572

INTRODUCTION

Sample size estimation (SSE) is an extremely important calculation for designing a clinical trial. Failure to produce an appropriate calculation may lead to imprecise results[1]. If a sample size is too large, statistically significant outcomes may be theoretically detected that may not be clinically relevant (type 1 error). This, however, is rarely a concern as studies are rarely overpowered to balance the study power with the cost. On the other hand, if a sample size is too small then a clinically significant outcome may not be detected statistically (type 2 error)[2,3]. The reporting of SSE in randomised controlled trials (RCTs) is a standard requirement according to the consolidated standards of reporting trials (CONSORT) statement which was introduced as a guide to conducting RCTs in 1996[4].

In a previous systematic review[5], we showed that 25% of RCTs on interventions for inflammatory bowel disease (IBD) have no power calculation (PC). A third of those who report PC do not achieve their target sample size. Based on those results, we decided to conduct a further systematic review.

We set out to systematically review RCTs on interventions for the IBD management, extract the vital parameters needed for sample size calculations, and synthesise the data to demonstrate whether trials across the field are adequately powered. We also set out to use the actual clinical data across these comparisons to synthesise data for minimum sample sizes that would achieve appropriate power to support future researchers designing trials and performing SSEs.

MATERIALS AND METHODS

This review was performed in alignment with Cochrane guidelines[6] in April 2020 and reported in line with the Preferred Reporting Items for Systematic Reviews and Meta-analyses statement[7].

Eligibility criteria

We followed the sampling methodology described within our systematic review protocol (uploaded within our institutional repository)[8] used for our previous review of the reporting of sample size calculations[5].

In brief, we included RCTs investigating either induction or maintenance therapy with biologics, immunomodulators, and microbiome against control, placebo, or no intervention. We conducted a comprehensive search of the Cochrane IBD Specialized Trials Register, CENTRAL, Cochrane library of IBD reviews for primary RCTs. The search terms are presented in Supplementary material.

We included RCTs published since 1996 (after the publication of the CONSORT statement). We excluded reports lacking clear information on the number of participants; cluster RCTs; pilot or feasibility studies; studies with mixed population of people with and without IBD; studies on secondary analyses of follow-up data collection after discontinuation of treatment. We excluded abstracts as these rarely allow space for such information to be presented. As we wanted to assess the established evidence for a PC of treatment for the IBD, we excluded RCTs describing all interventions where work may be at phase 3 (pharmacological: *e.g.* ustekinumab, golimumab, tofacitinib) or not under the three core headings (biologic, immunomodulators or anti-inflammatories).

Complying to the above search strategy, two authors (SL and MG) identified RCTs titles that appeared to be applicable. These were independently screened and in cases of disagreement, a third review author (VS) was involved to reach consensus. Two review authors independently extracted and recorded data on a predefined checklist. When disagreements occurred, a third review author was involved, and the consensus was reached.

We created an excel document to extract data regarding the trials. Firstly, we separated the studies into 8 categories [Crohn's disease (CD)–clinical relapse, clinical remission, endoscopic relapse, endoscopic remission; ulcerative colitis (UC)–clinical relapse, clinical remission, endoscopic relapse, endoscopic remission]. Secondly, we grouped the studies according to the intervention used. One author extracted the data, and in case of any problems, the data was checked by the second author.

The extracted data although is not available publicly can be obtained *via* direct contact with authors. The references of the included stuidies can be found in Supplementary material.

Extracted data included

(1) Number of events and participants originally assigned to each group; (2) Characteristics of participants; (3) The proportion that we calculated according to the number of events and participants (x = n/N), in which n is a number of events and N is a number of participants); (4) The difference achieved that we calculated according to the proportions of two groups (proportion 1–proportion 2); (5) Intervention and control details; (6) Presence of SSE and calculation details [minimal clinically important difference (MCID) used for PC, power, significance level, target sample size]; and (7) Outcomes (the number of patients recruited and completing study; the number of treatment success/failures; and the difference achieved).

We used the studies in which intervention was compared to the control or placebo. We grouped those studies according to the interventions, type of treatment (induction, maintenance), and outcomes (relapse, remission) and calculated mean difference and mean MCID where it was possible.

After resolving all the inconsistencies with data extraction regarding the use of sample size calculations for the studies with achieved difference of less than 10%, we produced two tables (Tables 1 and 2). We recalculated sample size for those groups using the power of 80%, probability of type 1 error 0.05, and the achieved difference. We used those parameters as they were the most commonly used amongst the studies. The parameters we used were two independent groups, dichotomous outcomes. In group 1 we have put the rate reported by the study of the intervention drug, and in group 2 we have put the rate of the placebo.

The small lest MCID that was reported by the studies was 10%, thereby, we decided to not reproduce PC for those studies with the achieved difference of less than 10%. We also calculated the mean sample deficit in percentage based on the target sample size and achieved sample size reported by the studies.

After receiving the sample size of participants, we made a decision whether the study is underpowered, and if yes, then by how many people.

Data synthesis

We produced descriptive statistics regarding the sample sizes for the studies grouped according to the interventions (Tables 1 and 2).

Ethical statement

As all data included already existed within the published scholarly output, no ethical approval was sought.

Table 1 Overall summary of power calculations and sample size deficits

	Total studies	Studies with power calculation	Studies with difference of 10% and less[1]	How many studies didn't achieve target sample size	Mean sample size underpowered (range)	Mean sample size needed	How many studies are underpowered[2]
CD induction	39	26	12	6	28 (2-70)	231	11
CD maintenance	25	19	9	3	52 (7-79)	300	10
UC induction	27	19	8	3	22 (1-55)	219	4
UC maintenance	16	10	0	1 + 1 didn't report	21	196	7

[1]Those studies were not included in analysis.
[2]Either didn't achieve their target sample size, or their achieved sample size is less than mean sample size needed.
UC: Ulcerative colitis; CD: Crohn's disease.

RESULTS

A total of 7451 potential citations were screened and 308 full texts assessed for eligibility. There were 209 texts excluded, 106 because they were published prior to the release of the CONSORT statement and 103 because they did not match our inclusion outcome. This left a total of 99 trials included, with 60 pertaining to CD and 39 to UC. The full details are shown in Figure 1.

The mean proportion of patients achieving clinical remission reported within the placebo groups of induction studies was 34.34% in CD trials and 26.79% for UC. For endoscopic remission, 0% in CD and 29.6% for UC. The mean proportion of patients achieving clinical relapse for maintenance studies were 55% for CD and 46.79% for UC. For endoscopic relapse, 78.85% in CD, and 28.7% in UC.

Within CD induction studies, 26 out of 41 (63.4%) reported a PC and 19 of 26 (73.1%) in maintenance studies. Within UC induction studies, 22 out of 31 (71%) reported a PC and 10 of 17 (58.8%) in maintenance studies.

When considering the MCID that those studies reporting a PC employed for this calculation, within CD induction studies the mean difference was 33% (range 20%-50%) and 27% difference for maintenance studies (15%-40%). Within UC induction studies the mean was 26% (range 19%-40%) and 27% for maintenance studies (18%-40%). The MCIDs these studies reported rarely matched the actual differences achieved by these studies. In fact, the discrepancy between this estimated figure for the MCID used for the PC and the actual differences seen were a mean of 22.8% higher in CD induction studies, 13.8% higher in maintenance studies, 15.7% higher in UC induction studies, and 10.2% higher in maintenance studies.

These discrepancies are proportionally large and in the context of PCs are clearly substantial and led to large numbers of studies being underpowered. These are summarised in Table 1. Study specific data with further details is available upon request.

Table 2 gives the results of our sample size calculations at the intervention specific level that employed the actual achieved clinical differences from previous studies, using the power of 80% and the probability of type 1 error 0.05. This shows the minimum sample sizes that would be indicated for RCTs compared with placebo to use. Within comparisons where the mean difference was less than 10%, no calculation has been given as this would be a very high indicative figure.

DISCUSSION

Within this review, it has been demonstrated that there is no clear basis or accepted standard for current practice for MCID estimation when producing a PC for a primary RCT within IBD. This has led to huge variations in suggested figures for recruitment. These trials present practical and logistical challenges to organisers, with potential inconvenience to patients, as well as the cost to those funding such research. Having an accurate figure for calculations is important to ensure this investment of resource is used most efficiently and effectively. It is key to note that we are not commenting at

Table 2 Proposals for minimum clinically important difference and associated power calculations for future studies

Ulcerative colitis-comparison	Ulcerative colitis-difference achieved (Group 1–Placebo)	Ulcerative colitis-Minimum sample size needed based on data	Crohn's disease-comparison	Crohn's disease-difference achieved (Group 1–Placebo)	Crohn's disease-Minimum sample size needed based on data
Induction studies					
Outcome–clinical remission			Outcome–clinical remission		
Vedolizumab *vs* Placebo	14.8%	190	Glutamine-enriched diet *vs* Placebo	-11.1	634
			Azathioprine *vs* Placebo	-3.6%	NA
			6-MP *vs* Placebo	5%	NA
Fecal Transplant *vs* Control	20.3%	150	6-MP *vs* Placebo	5%	NA
Budesonide *vs* Placebo	6.5%	NA	Interventional diet *vs* Control diet	20.9%	160
Type 1 IFNs *vs* Placebo	5.9%	NA	Elemental diet *vs* Non elemental diet	1.6%	NA
Etrolizumab *vs* Placebo	13.4%	140	N6/N9 rich feeds *vs* non N6/N9 rich food	-1.1%	NA
			Low dose naltrexone *vs* Placebo	9%	NA
5-ASA *vs* Placebo	11.8%	422	GM-CSF *vs* Placebo	7.8%	NA
Outcome–endoscopic remission			Brakinumab *vs* Placebo	8.5%	NA
Vedolizumab *vs* Placebo	37.7%	182	Ustekinumab *vs* Placebo	8.6%	NA
			Natalizumab *vs* Placebo	14.8%	310
Fecal Transplant *vs* Control	26.4%	160	Methotrexate *vs* Placebo	-14.8%	350
Budesonide *vs* Placebo	13.9%	NA	Antibiotics *vs* Placebo	10%	780
Methotrexate *vs* Placebo	46.7%	NA	Outcome–endoscopic remission		
Etrolizumab *vs* Placebo	7.7%	NA	Low dose naltrexone *vs* Placebo	22.2%	60
5-ASA *vs* Placebo	53.7%	306			
Maintenance studies					
Outcome–clinical relapse			Outcome–clinical relapse		
5-ASA *vs* Placebo	-16.4%	290	5-ASA *vs* Placebo, medically induced	3.1%	NA
Vedolizumab *vs* Placebo	-27.4	84	5-ASA *vs* Placebo, surgically induced	-5.4%	NA
Interventional diet *vs* Control diet	-3.6%	NA	Anti-TB *vs* Placebo	-23%	130
Probiotics *vs* Control	-16.7	154	Azathioprine *vs* Placebo, medically induced	-9.9%	NA
Azathioprine *vs* Placebo	-22.4	154	Azathioprine *vs* Placebo, surgically induced	-17.3%	254
Methotrexate *vs* Placebo	19.9%	194	6-MP *vs* Placebo, surgically induced	-10.9%	646
Rectal 5-ASA *vs* Placebo	-29%	90	Omega -3 fatty acids diet *vs* Control diet	-8.5%	NA
Curcumin *vs* Placebo	-9.6%	NA	Elemental diet *vs* No supplemets	-29.4%	88

Outcome–endoscopic relapse			Interventional diet *vs* Control diet	-2.5%	NA
Vedolizumab *vs* Placebo	-34	60	Antibiotics *vs* Placebo	-14.6%	360
			Methotrexate *vs* Placebo	-24.2%	128
5-ASA *vs* Placebo	-16.4%	290	Methotrexate *vs* Placebo	-24.2%	128
			Outcome–endoscopic relapse		
			5-ASA *vs* Placebo	2.7%	NA
			Azathioprine *vs* Placebo	-23%	130
			6-MP *vs* Placebo	-3.8%	NA
			Antibiotics *vs* Placebo	6.6%	NA
Induction studies					
Outcome–clinical remission			Outcome–clinical remission		
Vedolizumab *vs* Placebo	14.8%	190	Glutamine-enriched diet *vs* Placebo	-11.1	634
			Azathioprine *vs* Placebo	-3.6%	NA
			6-MP *vs* Placebo	5%	NA
Fecal Transplant *vs* Control	20.3%	150	6-MP *vs* Placebo	5%	NA
Budesonide *vs* Placebo	6.5%	NA	Interventional diet *vs* Control diet	20.9%	160
Type 1 IFNs *vs* Placebo	5.9%	NA	Elemental diet *vs* Non elemental diet	1.6%	NA
Etrolizumab *vs* Placebo	13.4%	140	N6/N9 rich feeds *vs* non N6/N9 rich food	-1.1%	NA
			Low dose naltrexone *vs* Placebo	9%	NA
5-ASA *vs* Placebo	11.8%	422	GM-CSF *vs* Placebo	7.8%	NA
Outcome–endoscopic remission			Brakinumab *vs* Placebo	8.5%	NA
Vedolizumab *vs* Placebo	37.7%	182	Ustekinumab *vs* Placebo	8.6%	NA
			Natalizumab *vs* Placebo	14.8%	310
Fecal Transplant *vs* Control	26.4%	160	Methotrexate *vs* Placebo	-14.8%	350
Budesonide *vs* Placebo	13.9%	NA	Antibiotics *vs* Placebo	10%	780
Methotrexate *vs* Placebo	46.7%	NA	Outcome–endoscopic remission		
Etrolizumab *vs* Placebo	7.7%	NA	Low dose naltrexone *vs* Placebo	22.2%	60
5-ASA *vs* Placebo	53.7%	306			
Maintenance studies					
Outcome–clinical relapse			Outcome–clinical relapse		
5-ASA *vs* Placebo	-16.4%	290	5-ASA *vs* Placebo, medically induced	3.1%	NA
Vedolizumab *vs* Placebo	-27.4	84	5-ASA *vs* Placebo, surgically induced	-5.4%	NA
Interventional diet *vs* Control diet	-3.6%	NA	Anti-TB *vs* Placebo	-23%	130
Probiotics *vs* Control	-16.7	154	Azathioprine *vs* Placebo, medically induced	-9.9%	NA

Azathioprine *vs* Placebo	-22.4	154	Azathioprine *vs* Placebo, surgically induced	-17.3%	254
Methotrexate *vs* Placebo	19.9%	194	6-MP *vs* Placebo, surgically induced	-10.9%	646
Rectal 5-ASA *vs* Placebo	-29%	90	Omega -3 fatty acids diet *vs* Control diet	-8.5%	NA
Curcumin *vs* Placebo	-9.6%	NA	Elemental diet *vs* No supplemets	-29.4%	88
Outcome–endoscopic relapse			Interventional diet *vs* Control diet	-2.5%	NA
Vedolizumab *vs* Placebo	-34	60	Antibiotics *vs* Placebo	-14.6%	360
			Methotrexate *vs* Placebo	-24.2%	128
5-ASA *vs* Placebo	-16.4%	290	Methotrexate *vs* Placebo	-24.2%	128
			Outcome–endoscopic relapse		
			5-ASA *vs* Placebo	2.7%	NA
			Azathioprine *vs* Placebo	-23%	130
			6-MP *vs* Placebo	-3.8%	NA
			Antibiotics *vs* Placebo	6.6%	NA

NA is put when the difference achieved is less than 10% (which is the least Minimal Clinically Important Difference used by the studies).

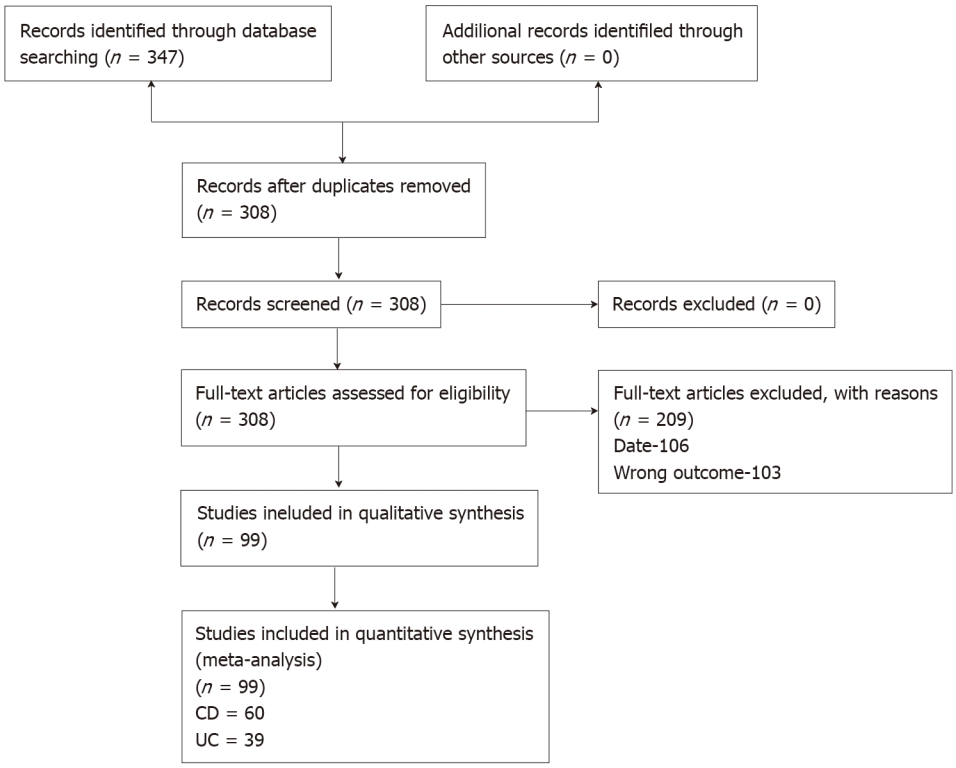


Figure 1 Study flow diagram. UC: Ulcerative colitis; CD: Crohn's disease.

the individual study level. It is inappropriate to look at the projected MCID and PC for a project, if calculated on a reasonable basis, to then retrospectively suggest that the findings of a lesser MCID mean it is underpowered. This not just statistically inappropriate, but methodologically flawed. However, these findings propose that the basis for such MCID estimations is at worst unclear and often can be seen as flawed.

There are further ethical issues these problems raise, such as being forced to give treatments to people without having a statistically proved effect or a high certainty result within the Grading of Recommendations Assessment, Development and

Evaluation analysis (due to reasons of imprecision from statistical sampling issues). The power of a study, therefore, has huge implications on the precision of estimates in the future analysis of data and in turn clinical practice guidelines. Within this review, 30% of studies appeared to be underpowered based on actual achieved clinical differences within the wider comparable evidence base, with mean sample size deficits up to 79 patients per trial. This does impact the overall certainty of the global evidence base within IBD, with precision a key limitation downgrading many outcomes within key guidelines across dozens of interventions.

Within this review, we present a resource for SSE not just for future study authors, but for study peer reviewers and most importantly professionals and the patients. This table gives an estimated PC result for a minimum sample size based on all existing studies within this period. Rather than being based on just single studies or clinical judgement, these represent estimates based on actual achieved clinical data and to our knowledge are the first time such a resource has ever been provided for researchers in the field or indeed for readers of future research. Additionally, for those wishing to calculate key statistics and measures of outcome from their primary studies, this paper provides a systematic and objective resource for baseline risk. This could be used for calculating numbers needed to treat or harm, for example.

This resource can be used by study designers to prevent PCs based on studies that offer a high MCID and as such a lower minimum sample size than is actually warranted. Conversely, it prevents unnecessary over recruitment. Funders can use this to appropriately budget and ensure viability of studies. Ethics boards and other governance groups will be able to consult this resource to support their consideration of research proposals.

There were a number of comparisons where the difference in practice was below 10% and it was deemed inappropriate to make a calculation in such cases, as no previous study has ever indicated an MCID below 10% as clinically significant to patients or practice. In these cases, consideration should be given to the overall figures presented in Table 2 or minimum sample size and MCID in practice in a similar context.

We would also recommend that in practice, patients and key stakeholders should be involved in deciding on an MCID for a given intervention prior to a new study. They may indicate that in spite of any existing MCID evidence that such a difference is not significant enough to matter to those who are most impacted by the findings and such views must be reflected in the process of SSE. It is also worth noting that there will always be settings and contexts when deviation may be warranted, thereby, a resource is not prescriptive but rather presented as evidence-based guidance. We would, however, propose that such deviations can and should be justified to support transparency for the readings these trials report.

There are weaknesses and exceptions to these approaches. The search methods used limited the parameters of the search for pragmatic reasons. However, this does not represent any systematic bias, hence we do not believe it invalidates the findings, and in the future this resource can be updated prospectively. When the achieved difference was less than 10%, rather than reporting extremely large sample size calculations, no such calculation was made. Additionally, in studies comparing active agents, accurate estimates are needed based on the contexts as the hypothesis may not be of the inferiority or superiority but of no difference, which requires a different approach to calculations.

There were some limitations to this review. There are obvious issues of heterogeneity limiting the appropriateness of pooling the data, however, the only way to obtain the previously used MCID was through looking at the past studies. These are mainly related to missing or unclear information in primary studies regarding SSE and as authors were not contacted, assumptions were made for the basis of these calculations which could confer some inaccuracy in our estimations. We also limited our studies to those from after the CONSORT statement release as we felt this was a fair time from which to expect SSE to occur, but earlier studies could potentially have offered more insight. Finally, we have focussed on studies comparing treatment with placebo or no intervention. This was a pragmatic decision as many studies of agents choose to make this comparison, although often these do not reflect current standard clinical practice. In the cases of such comparisons, SSE may not have to be based on a MCID but instead assume clinical equivalency and therefore be informed differently. In essence, this guidance may not be relevant for these scenarios, although may inform statistical considerations within similar contexts. Finally, such a resource of course is likely to become inaccurate rapidly, with the need for updates, but as often no such resource is employed, we believe this is still an improvement on current practices.

Future researcher is needed to potentially validate the calculations with clinical and patient input to ensure the SSE and MCID that the data informs has clinical, as well as statistical relevance. This could lead to a more triangulated resource that is statistically and evidentially sound, but also clinically sound and patient informed. This could conceivably lead to increases or decreases in minimally important differences to reflect complexity in specific clinical scenarios and interventional contexts.

CONCLUSION

In conclusion, a third of intervention IBD studies within the last 25 years are underpowered, with large variations in the approaches to calculating sample sizes and the minimum clinically important differences. The authors present a sample size estimate resource based on the published evidence base for future researchers and other key stakeholders within the IBD trial field.

ARTICLE HIGHLIGHTS

Research background

A third of randomised controlled trials (RCTs) on interventions for inflammatory bowel disease (IBD) have no adequate power calculation (PC).

Research motivation

A key element of PCs is an estimation of a minimally important clinical difference. The basis of these is capricious within the literature, with many not based on any existing or prior studies and as such can lead to massive shifts in PCs for similar studies, with concerns as to the underlying power.

Research objectives

We systematically reviewed RCTs reporting interventions for the management of IBD and to producted a resource for minimum clinically important difference using clinical data for the future researchers to use as a starting point.

Research methods

We included RCTs retrieved from Cochrane IBD trial register and CENTRAL investigating anti-inflammatory, immunomodulator and biologic therapies for either induction or maintenance of remission against control, placebo, or no intervention of IBD in patients of any age. The data was extracted and synthesized. We recalculated sample size and the achieved difference, as well as minimum sample sizes and presented in a tabular format.

Research results

Of 105 trials were included. A large discrepancy between the estimated figure for the minimal clinically important difference used for the calculations (15% differences observed *vs* 30% used for calculation) was observed explaining substantial actual sample size deficits. The minimum sample sizes indicated for future trials based on the 25 years of trial data were calculated and grouped by the intervention.

Research conclusions

There are large variations in the sample size calculatins in the studies of interventions for IBD with a third of all studies being underpowered. The authors present a sample size estimate resource constructed on the published evidence base for future researchers and key stakeholders within the IBD trial field.

Research perspectives

The use of this resource will support research staff, ethics committees and journal editors in ensuring adequate sample sizing and powering of studies across the field.

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7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
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E-mail: bpgoffice@wjgnet.com
Help Desk: https://www.f6publishing.com/helpdesk
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ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2021 November 28; 27(44): 7582-7738



Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

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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, Subrata Ghosh

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

PUBLICATION DATE
November 28, 2021

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E-mail: bpgoffice@wjgnet.com https://www.wjgnet.com

World Journal of Gastroenterology

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World J Gastroenterol 2021 November 28; 27(44): 7582-7596

DOI: 10.3748/wjg.v27.i44.7582

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

FRONTIER

Orexins: A promising target to digestive cancers, inflammation, obesity and metabolism dysfunctions

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Author contributions: Couvineau A, Voisin T, Blais A have equally contributed in writing of the manuscript; Couvineau A, Voisin T, Nicole P, Gratio V and Blais A have read and approved the final version of manuscript.

Conflict-of-interest statement: Authors don't have any conflict of interest.

Supported by INSERM U1149/The Inflammation Research Center, Inserm Transfert, The Institut National du Cancer, No. 2013-213; Ligue Nationale Contre le Cancer, No. R16020HH, GB/MA/CD/EP-12062; and AgroParisTech (INRAE and Université Paris-Saclay).

Country/Territory of origin: France

Specialty type: Biology

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

Peer-review report's scientific quality classification

Grade A (Excellent): 0

Grade B (Very good): 0

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Abstract

Hypothalamic neuropeptides named hypocretin/orexins which were identified in 1998 regulate critical functions such as wakefulness in the central nervous system. These past 20 years had revealed that orexins/receptors system was also present in the peripheral nervous system where they participated to the regulation of multiple functions including blood pressure regulation, intestinal motility, hormone secretion, lipolyze and reproduction functions. Associated to these peripheral functions, it was found that orexins and their receptors were involved in various diseases such as acute/chronic inflammation, metabolic syndrome and cancers. The present review suggests that orexins or the orexin neural circuitry represent potential therapeutic targets for the treatment of multiple pathologies related to inflammation including intestinal bowel disease, multiple sclerosis and septic shock, obesity and digestive cancers.

Key Words: Orexin; Neuropeptide; G-protein coupled receptor superfamily; Inflammation; Metabolic syndrome; Cancer

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Core Tip: Twenty years ago, hypothalamic orexin peptides hypocretin-1/orexin-A and hypocretin-2/orexin-B) and their receptors were identified. They belong to the G-protein coupled receptor superfamily. Orexins and their receptors were involved in the regulation of many functions in the central nervous system: the regulation of wakefulness, drug addictions, food consumption, energy homeostasis and stress.

Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

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Received: April 20, 2021
Peer-review started: April 20, 2021
First decision: June 13, 2021
Revised: June 22, 2021
Accepted: November 15, 2021
Article in press: November 15, 2021
Published online: November 28, 2021

P-Reviewer: Li K
S-Editor: Wang JL
L-Editor: A
P-Editor: Wang JL

However, various biological effects have been also identified in the peripheral nervous system including endocrine and cardiovascular functions. Orexins/orexin receptors have been shown to play a major role in various peripheral diseases encompassing chronic inflammation and cancers. The present review focuses on the impact of orexin exogenous administration, in various pathophysiological conditions including digestive cancers, intestinal bowel disease, septic shock, multiple sclerosis and metabolic syndrome.

Citation: Couvineau A, Voisin T, Nicole P, Gratio V, Blais A. Orexins: A promising target to digestive cancers, inflammation, obesity and metabolism dysfunctions. *World J Gastroenterol* 2021; 27(44): 7582-7596
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7582.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7582

INTRODUCTION

Toward the end of the 1990s, two independent groups managed respectively by J.G. Sutcliffes and M. Yanagisawa have discovered two new hypothalamic neuropeptides which are produced by the same precursor encoded by a single gene[1,2]. One of these two seminal publications co-led by Luis de Lecea and Thomas Kilduff's was based on subtractive cDNA cloning strategy allowing the identification of the hypocretin-1 and hypocretin-2, a contraction between "hypothalamus" corresponding to the location of orexins production and "secretin", one hormone having a slight amino acid homology with hypocretins[1]. At the same time, the Takeshi Sakurai's publication based on the identification of orphan G protein-coupled receptors (GPCRs) by screening with purified unknown peptides from brain extracts has allowed the identification of both hypothalamic orexin-A (OxA) and orexin-B (OxB) referring to the Greek term meaning "appetite" because these two neuropeptides induced feeding after intraventricular brain injection[2]. These two neuropeptides identified by Sutcliffes's group and Yanagisawa's group turned out to be identical. It should be noted that the actual current use assigns the term "hypocretin" for the gene species and "orexins" for the protein species. From this discovery, the two orphans GPCRs have been identified as hypocretin (Hcrt)/orexin receptor 1 (OX1R) and Hcrt/orexin receptor 2 (OX2R) which belong to the large class A rhodopsin-like subfamily of GPCRs[3]. To elucidate the role of orexins in feeding, the Yanagisawa's group has developed a knock out mice invalidated for the hypocretin gene. This model has revealed that the regulation of feeding and appetite were not the major physiologic role of orexins[4]. However, the absence of orexins secretion in this model, led to cataplectic attack symptoms[4]. Moreover, the invalidation of OX2R induces sleep attack whereas the OX1R invalidation led to the sleep disturbance characterized by narcolepsy[5].

The role of the orexins/OXR system was firstly widely studied in the central nervous system (CNS) (Figure 1). Many publications have demonstrated and confirmed that the orexin main role in the CNS was to maintain wakefulness[6]. The absence of orexins production, associated to the loss of orexin neurons, in human CNS induce narcolepsy with cataplexy (Narcolepsy type I). The impact of orexin on sleep regulation had led to the development, by the pharmaceutical industries and the academic laboratories, of orexin receptor-targeting molecules, mainly antagonists, able to regulate the wake-sleep cycle for insomnia treatment[7]. A growing number of antagonists have been developed and classified into two categories: the single orexin-receptor antagonists (SORAs) including selective OX1R antagonist (SORA1s) and OX2R antagonist (SORA2s) and dual orexin-receptor antagonists (DORAs). Recently, two of these antagonists named suvorexant and lately lemborexant were approved by the U.S. Food and Drug Administration in insomnia treatment[8,9]. Moreover, the central action of orexins regulates food intake, energy homeostasis, reward seeking, stress, motivation and drug addictions[10,11] (Figure 1) which included the addiction to cocaine, opioids, amphetamines, cannabis, alcohol and nicotine[12]. Despite the major role of orexins in CNS, these two neuropeptides were also studied, to a lesser extent, in the peripheral nervous system (PNS). In various peripheral organs including the adrenal glands, kidney, cardiovascular system, reproductive tract, adipose tissue and digestive tract, orexins also acted as regulators (Figure 1)[13]. The relatively low

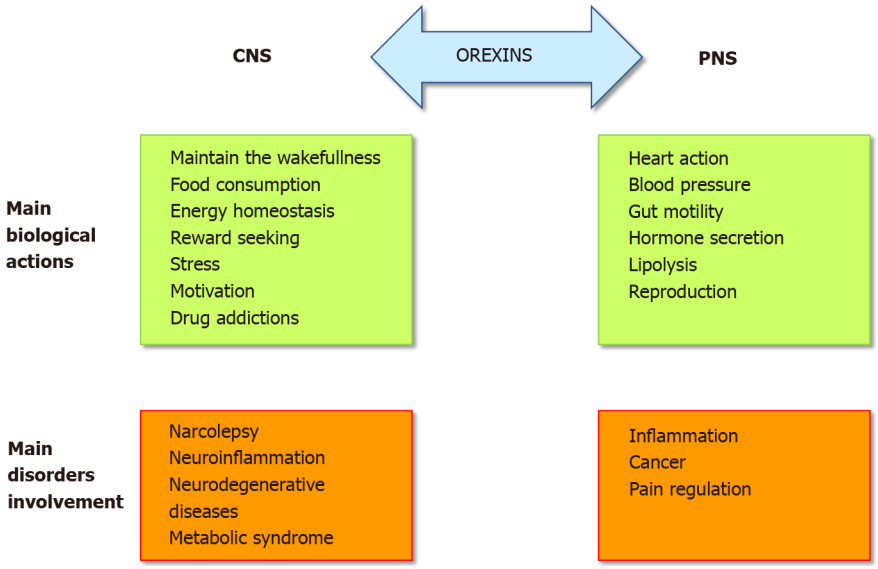


Figure 1 Pathophysiological roles of orexins/orexins receptors system. CNS: Central nervous system; PNS: Peripheral nervous system.

extensive studies of the roles of orexins in the PNS support that their actions were not fully elucidated and controversial[10]. In the digestive tract, orexin has been detected in neurons of the enteric nervous system (ENS) and in the enteroendocrine gut cells [14]. The presence of orexin in enteroendocrine cells supported the idea that this neuropeptide is involved in nutrition and energy homeostasis. Moreover, the use of different antibodies directed against OxA, OxB and prepro-orexin revealed an orexin-immunoreactivity in ENS[15]. Orexins modulate motility and orexin level was regulated by fasting[16]. In the pancreas, OxA was present in pancreatic islets, immunoreactivity being detected in pancreatic beta cells and potentially in alpha cells [14]. In humans, OX1R but not OX2R was also detected in pancreatic islets[17]. Although some studies support the role of OxA in the control of insulin secretion, this role remains conjectural[14]. However, the majority of these studies seemed to show that OxA directly or indirectly *via* the inhibition of glucagon release, regulated the insulin secretion[14]. It should be noted that OxB was also detectable in beta cells[17] but nothing is known about its role in the pancreas. If the presence of orexins in CNS, PNS and ENS was well established, the circulating level of these neuropeptides in healthy human blood was very low (about 2 to 50 pM) which is not enough to activate orexin receptors[18,19].

These biological effects were mediated through two orexin receptor subtypes, OX1R and OX2R which were coupled to Gq protein[10]. The interaction of orexins with its receptors led to the intracellular calcium release involving the phospholipase C (PLC) (Figure 2). Some reports have revealed that orexins were also able to activate the cAMP, PI3K/Akt, JNK and MAPK/Erk1/2 signaling pathways (Figure 2)[13]. The crystallographic structure of OX1R and OX2R associated to the suvorexant antagonist was reported[20,21]. Recently, the structure of OX2R complexed with OxB has been reported, suggesting that the molecular mechanisms which govern the activation or inactivation of receptors were located in the OX2R orthosteric site[22].

In pathological conditions, an abnormal expression of OX1R was observed in human peripheral organs. The presence of an ectopic expression of OX1R in intestinal bowel disease (IBD) including Crohn's disease and ulcerative colitis, in pancreatitis and digestive cancers as colon, pancreas and liver cancers, has been demonstrated[3]. The role of the orexin system in various human pathologies such as narcolepsy[23], neurodegenerative diseases (Alzheimer's disease)[24], ischemia[25], oxidative stress [26], chronic inflammation including IBD[10], multiple sclerosis[10] and metabolic syndrome[27] but also cancers[3], highlighted its potential therapeutic importance (Figure 1). In this context, the present review summarizes the impact of orexins and their receptors in chronic inflammation (*i.e.*, ulcerative colitis, multiple sclerosis, septic shock and metabolic syndrome) and cancers.

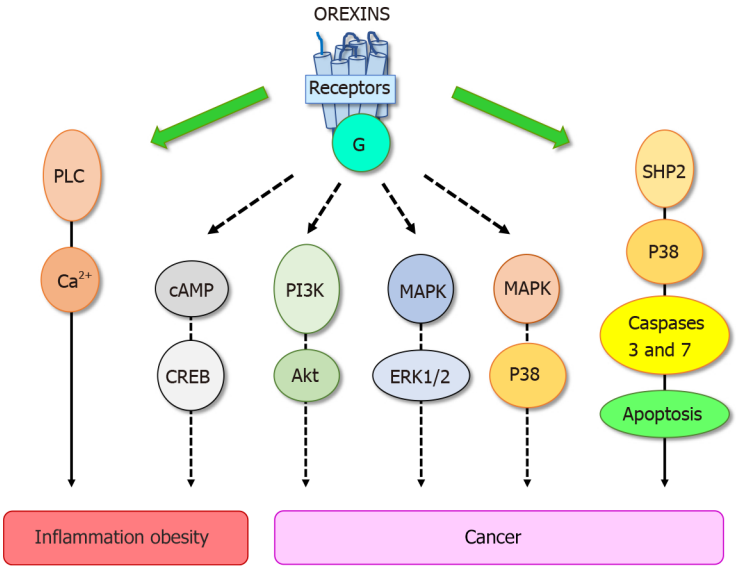


Figure 2 Main signaling pathways activated by orexins/orexins receptors system involved in peripheral diseases. PLC: Phospholipase C; cAMP: Cyclic adenosine monophosphate; CREB: C-AMP response element-binding protein; PI3K: Phosphoinositide 3-kinase; Akt: Protein kinase B; MAPK: Mitogen-activated protein kinase; ERK1/2: Extracellular signal-regulated kinase 1 and 2; P38: Mitogen-activated protein kinase; SHP2: Src homology 2 domains of Src homology 2-containing phosphatase 2.

OREXINS AND DIGESTIVE CANCERS

Despite the constant progress of the therapeutic arsenal, cancer is still the second causes of death worldwide[28]. To date, the treatment range options include surgery, chemotherapy, radiotherapy, hormonotherapy, antibody therapy, gene therapy, immunotherapy which integrate recent treatments based on anti-PDL-1 and CAR-T cells[29,30]. Digestive cancers, including colorectal cancer (CRC), pancreas cancers (PC), liver cancer (HCC), gastric cancer and esophageal cancer represent the second cause of cancer worldwide behind lung cancer[29]. In addition, biliary tract cancers as cholangiocarcinoma (CCH) also belonging to digestive cancers is less frequent. However, it should be noted that the incidence of PC which is mostly depicted (94%) by the pancreatic ductal adenocarcinoma (PDAC), is constantly increasing[31]. The factors increasing risk of digestive cancer include not only tobacco smoke, alcohol use, low physical activity, and diet, but another high-risk factor has also been identified, chronic inflammation such as IBD, pancreatitis, liver fibrosis and metabolic syndrome [32]. As mentioned above, OxA displays anti-inflammatory properties in IBD and other inflammatory diseases. These data indicate that orexin could play a role in triggering cancer. In 2004, our group tested the impact of 26 peptides including neuropeptides, hormones and orexins, on the cell growth of cancer cell line HT-29 derived from colorectal cancer[33]. The vast majority of these peptides had no significant effect on cell growth, only OxA and OxB inhibited the tumoral cell growth [33]. Analysis of this effect showed that orexins had no effect on cell cycle and cell proliferation but were able to induce a mitochondrial apoptosis in cancer cells[34,35]. Apoptosis was mediated by OX1R and OX2R however, only OX1R was ectopically expressed in human digestive cancers including CRC, PDAC, CHC and CCH[3,36]. Moreover, we observed that OX1R was also expressed in hepatic and lung metastasis from CRC[35]. It should be noted that OX1R and OX2R were not expressed in normal colon epithelium and in normal exocrine pancreas and liver[35,36]. However, as mentioned in the introduction, the main signaling pathway activated by orexin receptors involved the intracellular calcium release. Moreover, the inhibition of PLC enzyme which was activated by receptors in the presence of orexins *via* the Gq protein [37] was unable to inhibit the apoptosis process induced by orexins in cancer cells[33]. These observations indicated that the orexins/OX1R system triggered a new signaling pathway in cancer cells responsible of the pro-apoptotic peptide effect. Assessment of the new mechanism of action of orexins/OXR revealed that the interaction between orexins and their receptors induced phosphorylation of two immunoreceptor tyrosine-based inhibitory motifs (ITIM), present in the receptor sequences, induced by Src kinases[34,38]. Receptors phosphorylation led to the recruitment and the activation of tyrosine phosphatase SHP2 (Figure 2) followed by the activation of p38 mitogen-stress

protein kinase, translocation of Bax protein into the mitochondria, release of cytochrome c which participates to apoptosome formation and then activation of caspase 3 and 7 inducing DNA condensation and fragmentation causing the cancer cell death[34,38]. In preclinical mice models, we reported that when cancerous cell lines, such as HT-29 or LoVo or cells obtained from human colon tumors, were subcutaneously xenografted to mice administration of OxA and OxB were able to drastically reduced the tumor volume by apoptosis[35]. Although, preproorexin and OxA were immuno-detectable in total colon, no detection of preproorexin was obtained in normal and dysplastic epithelium[35] suggesting that endogenous OxA has no impact on tumoral growth. The colon cancer treatment was based mainly on surgery and chemotherapy but the primary cause of chemotherapy failure was associated to drug resistance[39]. Global studies indicated that more than 90% of patient cancer mortality was related to chemoresistance. The "gold-standard" treatment used in CRC was the 5-fluorouracil (5-FU). The development of HT-29 colon cancer cell line resistant to 5-FU demonstrated that OX1R was always expressed and orexins were able to induced pro-apoptotic effect in these cells suggesting that orexins response was conserved in drug-resistant cancer cells[35].

In PDAC, which represents the tenth most common cancer and the fifth in term of mortality[40,41], OX1R expression was detected in 96% of adenocarcinomas[36]. This expression was independent of the gender, patient age and tumor size[36]. OxA induced SHP2-dependent apoptosis in AsPC-1 cells derived from human PDAC as well as in PDAC slices from tumor patients maintained in culture[36]. The study using preclinical mouse models xenografted with AsPC-1 cells or cells isolated from patient's tumor indicated that OxA reduced the tumor development by induction of apoptosis [36]. OX1R was expressed at the early stage of development of digestive cancers including PDAC precancerous lesions named pancreatic intraepithelial neoplasia and dysplastic polyps in colon[3,36]. As mentioned above, various OxA antagonists such as almorexant or suvorexant have been developed for the treatment of insomnia. Surprisingly, AsPC-1 cells treatment with those antagonists showed that these two compounds inhibited PDAC cell growth by apoptosis induction[36]. Similarly, the intraperitoneal injection of almorexant in preclinical models led to inhibition of the tumor development indicating that this antagonist acted as OxA which was a full OX1R agonist[36]. These observations indicated that almorexant which blocked the intracellular calcium release induced by orexins was fully able to activate the pro-apoptotic signaling pathway in cancer cells. This type of molecule, able to discriminate various signaling pathways activated by one type of receptor, was termed biased ligand[42]. A very recent study on cryo-electron microscopy structure of OX2R active state revealed that one residue presents on the binding site play a central role in the receptor transition from the inactive to the active state[22]. This report could suggest that one or more residues in the receptor binding site drive the activation/inactivation of various signaling pathways. Moreover, this study confirmed the important role of Lys^{11} and Lys^{15} residues present in OxB, for the peptide interactions with its receptors [43]. OX1R was also expressed in HCC[44] and many studies reported that OxA had pro-apoptotic properties in gastric cancer[45], cholangiocarcinoma[3], esophagus cancer[35] but also in non-digestive cancer including prostate cancer[46] and neuroblastoma[35] in which apoptosis induced in these cancers by OxA was SHP2-dependent. Some report revealed that orexin receptors were also expressed in cortical adenomas[47], pheochromocytomas[48] and in endometrial carcinoma[49]. Observations indicated that OX1R was expressed in early stages of colon and pancreas cancer development[3,36] legitimately asking the following question: is the OxA/OX1R system is involved in chronic inflammation which may represent an important risk factor in tumorigenesis?

OREXINS AND IBD

The two major phenotypes of IBD were represented by ulcerative colitis (UC) and Crohn's disease (CD). These two disorders were characterized by a chronic inflammation of the intestine mucosa mediated by the immune system[50]. CD may affect any part of gastrointestinal tract from mouth to anus but often it concerns the part between the small intestine and the colon which can involve the deeper organ layer [51]. Whereas, UC affects mainly the colon and the rectum with a distal to the proximal gradient, characterized by superficial lesions having relapsing-remitting cycles[52]. These inflammations were characterized by persistent diarrhea, abdominal pain, bloody stools, great fatigue, immune cell infiltration and weight loss[52]. To date,

the etiology of these disorders remains unknown. IBD was associated to an increased risk (2-6 times) to develop CRC as compared to the general population[32,53]. Identification of UC pathogenic factors revealed that this disease involved numerous factors: genetic predisposition, epigenetic modifications, environmental factors including diet, geography, modern lifestyle, smoking, pollution, infections, exercise…, gut microbial factors (dysbiosis), inflammasome signaling pathways, endoplasmic reticulum stress and a disruption of immune response[54,55]. The treatment of UC was based on the severity and extension of the disease, involving the use of anti-inflammatory compounds such as 5-aminosalicylates, corticosteroids and immunosuppressive drugs such as azathioprine, 6-mercaptopurine, methotrexate, cyclosporine A, *etc*.[56]. The understanding of the role of various cytokines [Interleukin (IL)-6, IL-1β, tumor necrosis factor alpha (TNFα)] and other soluble mediators in UC, led to the development of therapies based on anti-TNFα, anti-IL-12/23, anti-integrin α4β7 and Janus kinase inhibitors[57]. The use of these drugs used alone or in combination has undoubtedly been a substantial advance in UC treatment in the last few decades. Unfortunately, important progress remains to be made to obtain curative treatment of IBD patients. The discovery of new targets is a main challenge for the therapeutic treatment of UC. In that respect, GPCR family represents a potential and innovative source of new targets. Several encouraging examples of GPCRs could play this therapeutic role in IBD through interaction with cannabinoid receptors[58], neuropeptide receptors[59], histamine receptors[60] and chemokine receptors[61]. Taking into account that OX1R was expressed in colon cancer and in precancerous lesions[10], the question is "Is OX1R expressed in IBD which represents a high risk to develop cancer?". Indeed, OX1R has been detected in inflamed mucosa from patient suffering of UC and CD[62]. It should be noted that OX1R was not expressed in normal human intestinal epithelium[35]. An epidemiologic study revealed that narcoleptic patients presented a higher prevalence to develop IBD[63]. To determine the role of OxA/OX1R system in ulcerative colitis, the classical DSS-induced colitis mouse model which reproduced the acute phase of colitis, was used. In this model, OX1R was ectopically expressed in inflamed mucosa[62]. Daily intraperitoneal injections of OxA revealed on this model, an anti-inflammatory effect of the peptide on the mucosa integrity and intestinal barrier[62]. OxA inhibited the secretion of various cytokines including IL-6, TNFα, IL-8, IL-1β, IL-1α, IL-17 and MCP-1 cytokines in intestinal mucosa and in immune cells extracted from colon[62]. In contrast, these anti-inflammatory effects were reverted by the SORA1 antagonist, SB-408124 demonstrating the specific effect of OxA which was fully mediated by OX1R *via* PLC signaling pathways (Figure 2)[62]. A recent report also showed that OxA was able to prevent the intestinal barrier disruption caused by lipopolysaccharide (LPS)[64]. To study the role of OxA/OX1R system on chronic inflammatory phases of UC, a genetically engineered mouse model invalidated for the IL-10 cytokine and NADPH Oxidase 1 was used. OxA peripheral injections induced the same anti-inflammatory effect compared to the DSS-induced colitis mouse model. This anti-inflammatory impact was mainly mediated by the activation of PLC that led to intracellular calcium release and inhibit Nuclear factor-κB (NF-κB) activation which plays a key role in pro-inflammatory cytokine secretion[65]. Even if only few studies have been devoted to the peripheral role of OxA in IBD, it seems clear that OxA/OX1R system displays anti-inflammatory properties in UC and may represent a promising new target in the treatment of this disease.

OREXINS AND SEPTIC SHOCK

Septic shock is a dramatic medical condition that represents a major health problem in response to a complex disorder arising from the dysregulation of an inflammatory response to infection that leads to low blood pressure and cellular metabolism abnormalities. Sepsis is caused not only by bacteria, but also by fungi, viruses or parasites. It could be located most frequently in the brain, lungs, urinary tract, skin or abdominal organs. It can lead to multiple organ dysfunction syndromes and death [66]. The pathophysiology of septic shock is not completely understood but an immune and coagulation response to the infection is the key role in the development of severe sepsis involving pro-inflammatory and anti-inflammatory responses. Septic shock was characterized by a widespread inflammatory response which produced a hypermetabolic effect. This effect was manifested by an increase of protein catabolism, cellular respiration and metabolic acidosis which was compensated by respiratory alkalosis[66]. LPS or endotoxins are major cell wall components of Gram-negative

bacteria, which induce systemic inflammatory response responsible of sepsis[67]. LPS-induced endotoxemia mouse model is one of the several well-studied animal models of septic shock[68,69]. Sepsis is characterized by an inflammatory cytokine secretion of TNFα, IL-6, IL-1β, and MCP-1[70]. NF-κB represents a therapeutic target since it induces pro-inflammatory gene transcription implicated in the septic shock[71]. In fact, in LPS-induced septic shock murine models, NF-κB inhibitors such as parthenolide and pyrrolidine dithiocarbamate[71], or an antagonist of toll-like receptor 4, the FP7[72] reverse sepsis effects on organ failure and hypotension. G protein-coupled receptors (GPCRs) may be potential targets for pharmacotherapy in sepsis. They could be involved in re-establishment of vascular endothelial barrier and alleviation of sepsis-induced organ dysfunctions. Several GPCRs and their associated ligands have been shown to play a role in septic shock but also in its treatment, including chemokine receptors (*i.e.*, ACKR2, CCR2, CCR5, CX3CR1, and CXCR1), neuropeptides (*i.e.*, VIP, neuropeptide Y, ghrelin, urotensin II, ocytocin, vasopressin, neurotensin, orexins, substance P, and apelin), proteases [*i.e.*, thrombin (PAR1 and PAR2)], lipid derivatives (*i.e.*, N-arachidonylglycine) and amines (*i.e.*, catecholamines, dopamine histamine, melatonin)[73]. Some reports indicate that intracerebral administration of orexin regulated body temperature and heart rate and increased the adrenocorticotropic hormone level in a mouse sepsis model induced by a caecal ligation associated to perforation[74]. The central administration of OxA to mice with endotoxin shock was shown to increase survival[75]. This report indicates that the exogenous administration of OxA was able to cross the blood barrier in systemic inflammation condition and induced an inhibition of IL-17, IFNγ, IL-6 and TNFα secretion[75]. Moreover, OxA restored body temperature and cardiovascular function in LPS-induced mice[75]. These authors hypothesized that OxA which was able to improve the survival of mice under septic shock condition, acts on the neuroendocrine and autonomic nervous system *via* the CNS, demonstrating a putative interest in treatment of septic shock.

OREXINS AND MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is an immune dysregulation of the blood-brain barrier that affects the CNS through the migration of activated inflammatory cells. In the world, 2.3 million people are diagnosed with MS. The major causes listed for MS are inflammation, demyelination, oligodendrocyte loss, axonal loss and neurodegeneration. The neurodegeneration is a consequence of the inflammation induced by the demyelination which is related to the immune system activation[76]. T-cell mediated destruction of myelin and the autoimmune responses induced are still conjectural. However, this chronic inflammatory process involved a Th1/Th17 autoimmune response in the spinal cord and brain[77]. More recent data define MS as a primary degenerative disorder, which begins in oligodendrocytes that leads to neuroinflammation and to demyelination[78]. These neurodegenerative processes are present in different brain regions, including the hypothalamus and the orexinergic neurons that projected to various brain region encompassing hippocampus, cortical areas, striatum, and spinal cord[79]. As mentioned above, orexin neurons which represent about 70,000 neurons in the human brain, were involved in the regulation of sleep, pain, cognition, anxiety, alertness and motor function[80] and few studies proposed that orexin dysfunction could be connected to fatigue in MS[81]. Moreover, orexins have anti-inflammatory and neuroprotective properties by improving experimental autoimmune encephalomyelitis pathology[82]. In MS the T helper cell 1 cytokines are produced in large concentration by myelin activated T cells to activate microglial cells and macrophages. Then, they induce pro-inflammatory cytokines and chemokines, reactive oxygen, and reactive nitrogen species productions which are associated to demyelination and neurodegeneration. OxA inhibited reactive oxygen species production and also interleukin IL-1β, IL-6, and IL-8 expression. Similarly, to the inhibition of NF-κB activation by OxA in IBD[62], OxA was also able to reduce, in MS, the activation of NF-κB signaling pathways which leads to the expression of matrix metalloproteinase-3 (MMP3) and also to a MMP13 enzyme reduction in the tissue inflammation site[83]. Furthermore, OxA administration was shown to be able to cross the blood-brain barrier and reach the CNS in LPS-induced septic shock murine models [75].

Cerebral ischemia, and neurodegeneration induced by severe oxidative stress models, have been shown to be reduced by OxA. Moreover, OxA can active glucocorticoid secretion and the sympathetic nervous system through catecholamines releases

which had anti-inflammatory properties that reduces immune response[85]. In Parkinson's neurodegeneration disease, OxA had a neuroprotective impact[10] and in Alzheimer's disease, OXR activation also displayed a neuroprotective action[85]. Recently, the use of experimental autoimmune encephalomyelitis (EAE) model mimicking multiple sclerosis shown that orexins were able to induce anti-inflammatory effects. The intraventricular injections of OxA reversed clinical symptoms of EAE including partial or total paralysis of the two hind legs and death[82]. The authors also reported that treatment of the EAE mice model with OxA induced a large reduction in demyelination, microglial activation, and astrogliosis. OxA was shown to reduce nitric oxide synthase gene expression, an oxidative stress target which controls EAE pathology in the CNS. MMP9 (an inflammation enhancer) and IL-12 (a pro-inflammatory cytokine) expressions were also downregulated. Otherwise, OxA treatment limited $CD4^+$ T lymphocytes infiltration and Th1 and Th17 cytokines production induced by myelin oligodendrocyte glycoprotein (MOG35–55). OxA treatment also inhibited chemokine production as MCP-1/CCL2 and IP-10/CXCL10. Moreover, OxA reduced the cytokine production including IFN-γ, IL-17, TNF-α, IL-10, and TGFβ in the CNS[82]. Some of the common symptoms associated with MS are fatigue and sleep disturbances suggesting that MS and narcolepsy/catalepsy can share common genetic aspects[86]. Indeed, the physiological and psychological effects of MS are more severe in patients with sleep disorders[87]. To conclude, orexins which displayed immuno-modulating and neuroprotective properties reinforced by the orexinergic system involvement in the pathological development of multiple sclerosis become an interesting target as anti-inflammatory molecules for MS.

OREXINS AND METABOLIC SYNDROME

Control of Energy balance and metabolism is complex, they are monitored by the nervous and humoral systems. This section will consider the regulation of these functions by OxA and OxB or through their specific receptors. Orexins that are expressed in the lateral hypothalamus were first identified as factors that enhance feeding behavior. However, Hara *et al*[88,89] shown that orexin deficiency or postnatal ablation of orexin neurons induced mice obesity supporting that orexins are negative regulators of energy metabolism. Moreover, in human narcolepsy, related to orexin deficiency, is associated to a greater body mass index and to an increased risk of metabolic syndrome[90]. In contrast orexin overexpression protects rodents from diet-induced obesity and improves glucose control[91].

Orexin functions have been mainly described in the central nervous system but orexins and their receptors are also detected in various organs including the intestine, pancreas, adrenal glands, kidney, adipose tissue and reproductive tract. In peripheral tissues, orexins could affect insulin release, intestinal mobility, hormone secretion and blood pressure regulation[92]. Body weight and energy homeostasis are precisely controlled by many metabolic and hormonal factors including OxA. Orexins and their receptors have been located in the endocrine pancreas where they were co-located with insulin and beta cells[93] suggesting the role of orexin receptors in the glucose homeostasis. Some specificity of both isoforms of orexin and of each receptor in the control of energy balance have been reported but their roles remain unclear[92,94]. Recently plasma OxA have been negatively associated with insulin resistance and positively with insulin sensitivity in type 2 diabetes suggesting a functional role of orexin in the development of correlated obesity diseases[95]. These data support that orexin can modulate appetite, energy expenditure and glucose and lipid metabolism.

Orexin and food intake

Intra-cerebroventricular injections of OxA was shown to increase food intake in rats, while OxB was less effective[96]. However, orexin infusion stimulates feeding during the light period but not at the dark phase and induces no significant increase of the total food intake over 24 h[97]. In a previous study we reported that chronic treatment of mice under standard diet with daily intraperitoneal (i.p.) injections of OxA did not have any important effects on energy intake and energy expenditure, even if the i.p. OxA injections were sensed by the hypothalamus and affected the expression of several receptors and neurotransmitters in the hypothalamus[98]. Moreover, it has been proposed that orexin-induced feeding not simply follow the arousal state but increase the signal of low glucose and hunger[99].

Orexin and obesity

OxA deficiency is associated with narcolepsy and to higher risk of obesity suggesting that OxA deficiency can contribute to glucose homeostasis and insulin sensitivity. Transgenic mice in which orexin-containing neurons are ablated develop narcolepsy and obesity[88]. However, overexpression of OX2R in rats protects them from diet-induced obesity and improves glucose control and leptin sensitivity suggesting that triggering OX1R or OX2R did not regulate the same pathway (Figure 3)[91]. Moreover, lack of the orexins decreased energy expenditure and increase adiposity, principally through a reduction of physical activity. At the opposite exogenous OxA attenuates adiposity in rats and mice ingesting high fat diet. We also reported that i.p. injection of OxA to mice ingesting standard diet induced a small but significant reduction of visceral fat mass and adiposity but we did not observe any decrease of the subcutaneous fat, suggesting a lesser sensitivity of these fat pads to OxA[98]. These results support the potential anti-obesity effects of orexins. However, conflicting results have reported by different studies. *In vitro* studies using 3T3-L1 showed that OxA stimulates preadipocytes proliferation whereas OxB suppresses. Both OxA and OxB can stimulate pig adipocytes but no convincing evidence have been reported in humans[17,100]. It has also been shown that OxA contributes to changes of the white fat morphology *via* stimulation of preadipocytes proliferation and inhibition of apoptosis but OxA has no effect on lipolysis in fat tissue derived from human explants [101]. Moreover, OxA can raise corticosterone levels and glucocorticoids that can stimulate lipolysis in adipocytes[102]. In addition, *in vivo* studies reported that OxA potentiates physical activity and energy expenditure which reduce lipid accumulation.

Orexin and brown adipose tissue

Brown fat cell functions are different they generate heat. Many data support that OxA is required for adipogenesis of brown adipose tissue (BAT) in rodents. Activation of brown adipose tissue is controlled by environmental and hormonal factors as well as sympathetic neurons[103]. Ida *et al*[104] reported that cold stress stimulates the expression of preproorexin mRNA expression suggesting that orexin may be involved in body temperature control. Moreover, ablation of hypothalamic orexin neurons reduced BAT thermogenesis[105] and at the opposite central administration promotes thermogenesis[106]. A recent report, contrary to previous studies[107,108], did not show a direct effect of orexin on BAT development but a regulation of BAT by orexin signaling through the sympathetic system[109]. Moreover, *in vivo* OxA fail to stimulate the differentiation of human brown preadipocytes as well as the expression of the genes regulating thermogenesis.

Orexin and regulation of glucose homeostasis

Orexin deficiency has been associated with obesity, glucose intolerance and insulin resistance in rodents and humans[110]. OxA i.p. injections have been shown to increase GLUT4 expression in the liver suggesting that orexin can improve glucose uptake in hepatocytes, adipocytes and skeletal muscle[111]. Moreover, several studies show that orexin stimulates insulin secretion[112-114]. In rat model of type 2 diabetes mellitus (T2DM), an obesity treatment with OxA decreases fasting glucose and plasma levels of TNFα improve glucose control by increasing insulin sensitivity, increases plasma concentration of adiponectin and protects β-cells from apoptosis[114]. More studies are needed to better elucidate the mechanisms by which OxA modulates adipokines levels and other metabolic parameters such as the ability to reduce glucose. This effect can be a potential therapeutic approach for the treatment of DM and of its complications.

To summarize, a variety of data are presented in the literature. This disparity depends if the studies present *in vitro* or *in vivo* data and if the orexin administration was central (intraventricular) or peripheral. More studies will be needed to better define the mechanism by which the orexins regulate food intake, energy expenditure and glucose metabolism.

CONCLUSION

Since the identification of orexin peptides in hypothalamus demonstrating their crucial roles in sleep/wake regulation, the importance of their peripheral effects revealed their potential interests as therapeutic molecules in a wide range of human pathologies including also digestive diseases such as acute/chronic inflammation (IBD, septic shock, MS), metabolic syndrome and cancers.

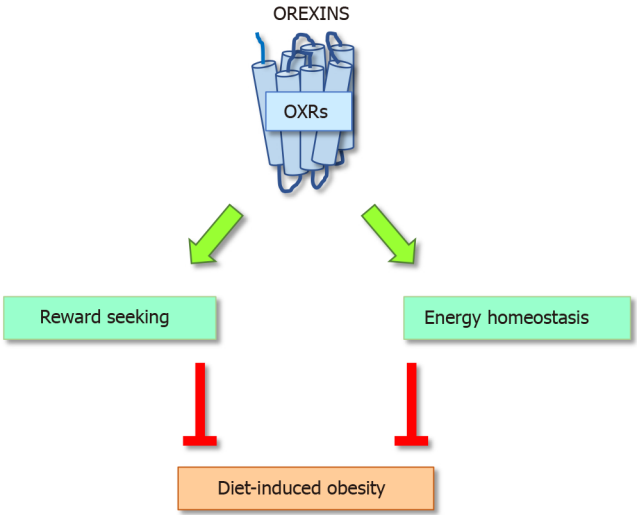


Figure 3 Main actions of orexins and their receptors on diet-induced obesity.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 28; 27(44): 7597-7611

DOI: 10.3748/wjg.v27.i44.7597

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

OPINION REVIEW

Endoscopic management of difficult common bile duct stones: Where are we now? A comprehensive review

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Author contributions: Anderloni A conceived the idea for the manuscript; Tringali A and Costa D reviewed the literature and drafted the manuscript; Fugazza A, Colombo M, Khalaf K and Repici A reviewed the manuscript.

Conflict-of-interest statement: Alessandro Fugazza is a consultant of Boston Scientific and Olympus; Alessandro Repici is a consultant of Boston Scientific, Fujifilm and ERBE; Andrea Anderloni is a consultant of Boston Scientific, Olympus and Medtronic; No others conflict of interest for the remaining authors.

Country/Territory of origin: Italy

Specialty type: Gastroenterology and hepatology

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

Peer-review report's scientific quality classification

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Abstract

Endoscopic management for difficult common bile duct (CBD) stones still presents a challenge for several reasons, including anatomic anomalies, patients' individual conditions and stone features. In recent years, variable methods have emerged that have attributed to higher stone removal success rates, reduced cost and lower adverse events. In this review, we outline a stepwise approach in CBD stone management. As first line therapy, endoscopic sphincterotomy and large balloon dilation are recommended, due to a 30%-50% reduction of the use of mechanical lithotripsy. On the other hand, cholangioscopy-assisted lithotripsy has been increasingly reported as an effective and safe alternative technique to mechanical lithotripsy but remains to be reserved in special settings due to limited large-scale evidence. As discussed, findings suggest that management needs to be tailored to the patient's characteristics and anatomical conditions. Furthermore, we evaluate the management of CBD stones in various surgical altered anatomy (Billroth II, Roux-en-Y and Roux-en-Y gastric bypass). Moreover, we could conclude that cholangioscopy-assisted lithotripsy needs to be evaluated for primary use, rather than following a failed management option. In addition, we discuss the importance of dissecting other techniques, such as the primary use of interventional endoscopic ultrasound for the management of CBD stones when other techniques have failed. In conclusion, we recognize that endoscopic sphincterotomy and large balloon dilation, mechanical lithotripsy and intraductal lithotripsy substantiate an indication to the management of difficult CBD stones, but emerging techniques are in rapid evolution with encouraging results.

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

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Received: April 27, 2021
Peer-review started: April 27, 2021
First decision: June 13, 2021
Revised: July 23, 2021
Accepted: November 18, 2021
Article in press: November 18, 2021
Published online: November 28, 2021

P-Reviewer: Ozkan OF, Pelaez-Luna M
S-Editor: Wu YXJ
L-Editor: Filipodia
P-Editor: Wu YXJ

Key Words: Common bile duct stones; Balloon dilation; Endoscopic retrograde cholangiopancreatography; Endoscopic ultrasonography; Anastomoses, Roux en y; Double balloon enteroscopy; Mechanical lithotripsy; Cholangioscopy

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Core Tip: The endoscopic management of difficult common bile (CBD) stones remains a challenge, whilst emerging techniques such as endoscopic sphincterotomy and large balloon dilation, mechanical lithotripsy and intraductal lithotripsy provide a procedural indication that align with the patient's condition, comorbidities, feature of the CBD stone and the patient's anatomical features. This review focuses on comprehensively outlining a stepwise approach for the management of difficult CBD stones and comparatively discusses indications depending on surgical altered anatomy and future indications in the management of difficult CBD stones.

Citation: Tringali A, Costa D, Fugazza A, Colombo M, Khalaf K, Repici A, Anderloni A. Endoscopic management of difficult common bile duct stones: Where are we now? A comprehensive review. *World J Gastroenterol* 2021; 27(44): 7597-7611
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7597.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7597

INTRODUCTION

About 10%-15% of biliary stone extraction procedures are demanding and require additional endoscopic techniques in order to allow stone clearance[1]. Factors that influence the technical difficulty of common bile duct (CBD) endoscopic clearance can be attributed to the patient's clinical condition, the stone's characteristics and anatomical factors (Table 1). Furthermore, the concomitant presence of Mirizzi syndrome and/or primary sclerosing cholangitis are also agreed upon by experts to make stone extraction a challenging procedure[2,3].

In accordance with the European Society of Gastrointestinal Endoscopy (ESGE) guidelines on endoscopic management of common bile duct stones, endoscopic sphincterotomy combined with endoscopic papillary large balloon dilation is considered the first line approach, reserving mechanical lithotripsy in case of failure [4]. Cholangioscopy-assisted lithotripsy has been increasingly reported as an effective and safe alternative technique to treat difficult CBD stones, even though its availability is still limited to referral centers[4,5]. Moreover, endoscopic management of CBD stones in patients with surgically altered anatomy (SAA) is technically demanding with a reduced rate of technical success[6]. Balloon assisted enteroscopy (BAE) has revealed to be effective in this setting, although its rate of failure has been reported to be up to 35%[7]. The use of interventional endoscopic ultrasonography (I-EUS) has been reserved to cases of BAE failure, due to its higher rate of adverse events when compared with BAE in previous reports[8-10]. Nevertheless, recent studies showed that I-EUS is efficacious with a low risk of adverse events, so it should be considered as first line treatment in expert hands and in referral centers[11]. The future of I-EUS requires the development of dedicated devices, making the procedure easier and safer with expanded indications[6,12]. Further studies will help to assess the role of I-EUS as the first approach for the management of CBD stones in patients with SAA. Finally, percutaneous cholangioscopy is also a novel alternative technique that should allow to treat difficult CBD stones in patients with SAA[13].

STEPWISE APPROACH FOR THE MANAGEMENT OF DIFFICULT COMMON BILE DUCT STONES

The first step is gaining access to the biliary tree, in order to remove the stone, which can be achieved by three different techniques: Endoscopic sphincterotomy (EST), endoscopic papillary large balloon dilation (EPLBD) and a combination of EST and

Table 1 Causes of difficult stone extraction

Category	Risk factors
Patient's clinical condition	Age > 65 yr; Bleeding tendency; Very poor medical condition
Stone characteristics	Stone size > 15 mm; Barrel or square shaped; Multiple stones > 3; Hard stone consistency; Intrahepatic/cystic duct location
Anatomical factors	Anatomical CBD factors: Narrowing of the bile duct distal to the stone, sigmoid-shape CBD, distal CBD angulation > 135°, short distal CBD < 36 mm; Periampullary diverticulum; Duodenal stricture; Surgically altered anatomy (Roux-en-Y gastric bypass or Billroth II with long afferent limb)

EPLBD [endoscopic sphincterotomy and large balloon dilation (ESLBD)]. Although the optimal choice remains debatable among endoscopists, the recently published ESGE guidelines[4] recommend ESLBD as the first-line approach to difficult CBD stones (in particular large stones), due to a 30%-50% reduction of the use of mechanical lithotripsy (ML) and a similar rate of technical success when compared to EST alone [14-20] (Figure 1).

However, balloon dilation is contraindicated in cases with distal biliary strictures, due to the increased risk of perforation[21], whereas EST increases the risk of bleeding in patients on antithrombotic agents[22]. A recently published systematic review and metanalysis including 13 randomized controlled trials conducted on 1990 patients[23] focused on the treatment of large CBD stones using the three techniques. Analyzing the surface under the cumulative ranking curve index, Lyu *et al*[23], concluded that EPLBD had the highest overall and initial success rates and the lowest probability of bleeding. ESLBD also had the lowest probabilities for the need for ML, risk of perforation, morbidity rates and risk of mortality. On the other hand, EST was associated with the lowest rates of post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis (PEP) and cholangitis. However, when each outcome was analyzed on pooled network analysis, no significant differences among the three groups emerged. There are many limitations in this study: The definition of the success rate and post-procedure complications varied in the included studies, the details of the different endoscopic procedures, such as the size of the dilation balloon, differed in the studies and the patient's characteristics changed among studies, such as bleeding predisposing factors and anatomical factors (*e.g.*, the presence of a peripapillary diverticulum and the size of the distal bile duct). To conclude, the technique for removing difficult CBD stones is still debated among endoscopists and needs to be tailored to patient's characteristics and anatomical conditions[24]. Higher numbers of high-quality trials are required.

When the size of CBD stones, even after balloon dilation, exceeds the diameter of the distal CBD, ML should be performed (Figure 2). The success of ML has been reported to range between 79% and 96%[25-29], with a low mortality rate and an overall adverse events (AEs) rate of 3.5% in a multicenter study[30], including trapped/broken basket, wire fracture and broken handle perforation/duct injury. Even though previous studies have focused on the importance of stone size as the factor that hampers stone clearance[29], Garg *et al*[28] demonstrated that the only predictor of unsuccessful ML was the stone impaction into the CBD. This determined the inability to push the basket proximal to the stone or the incapability to open fully the basket to grasp the stone.

ML can require multiple sessions to achieve stone clearance, undergo AEs and still be ineffective for difficult CBD stones[31]. In such cases, cholangioscopy-assisted intraluminal lithotripsy with electrohydraulic (EHL) or laser lithotripsy (LL) is the technique of choice to fragment large stones under direct visualization. There are three cholangioscopy techniques available: The oldest "dual-operator" mother-baby approach, the "single-operator" mother-baby approach (Spyglass, Boston Scientific, Natick, MA, United States) and the "direct" technique using currently available ultrathin gastroscopes[32].

Each of the three cholangioscopy technique allows EHL or LL under direct visualization (Figure 3). Nevertheless, in regards to which specific type of cholangioscopy and lithotripsy to use, it depends on local expertise and availability, as mentioned in ESGE guidelines[4]. There are currently no studies that compare cholangioscopy techniques. Concerning the type of lithotripsy used, Veld *et al*[33] recently published a systematic review comparing LL, EHL and extracorporeal shock wave lithotripsy (ESWL) in the treatment of difficult CBD stones after a previously failed ERCP. In their study, LL had a significantly higher complete ductal clearance rate (95.1%) compared

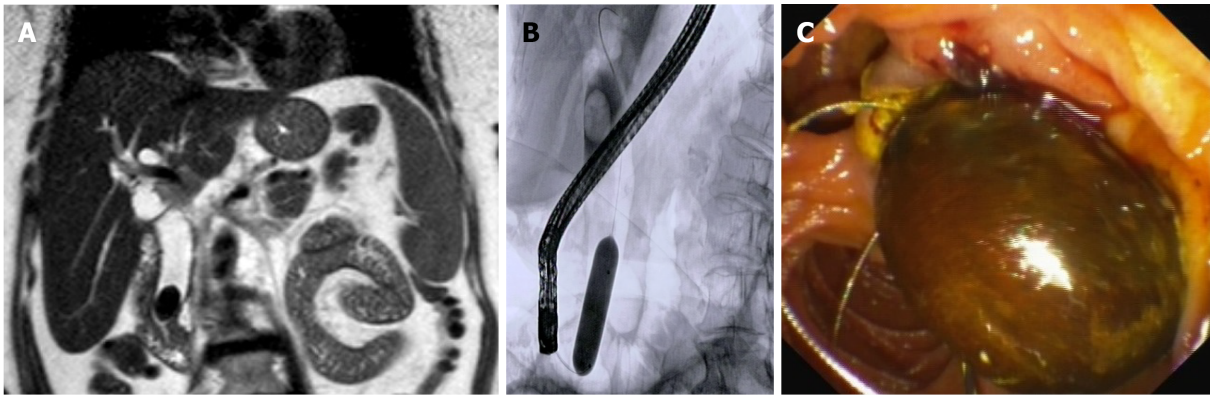


Figure 1 Management of difficult common bile duct stone by endoscopic sphincterotomy and large balloon dilation. A: Magnetic resonance imaging showing a large stone in the distal common bile duct; B: Fluoroscopic appearance of endoscopic papillary large balloon dilation with a pneumatic balloon filled with contrast medium; C: Final endoscopic view of the stone extracted by a Dormia basket.

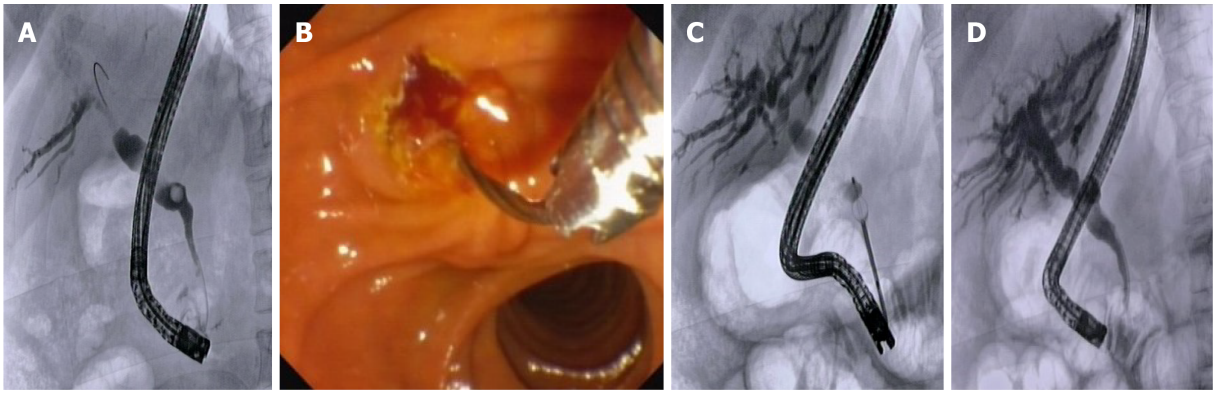


Figure 2 Management of common bile duct stones with distal biliary stricture by mechanical lithotripsy. A: Cholangiogram showing distal common bile duct (CBD) stricture with stone in the medium CBD; B: Introduction of a mechanical lithotripter over the Dormia basket; C: Mechanical lithotripsy under fluoroscopic control; D: Final cholangiogram showing complete CBD clearance.

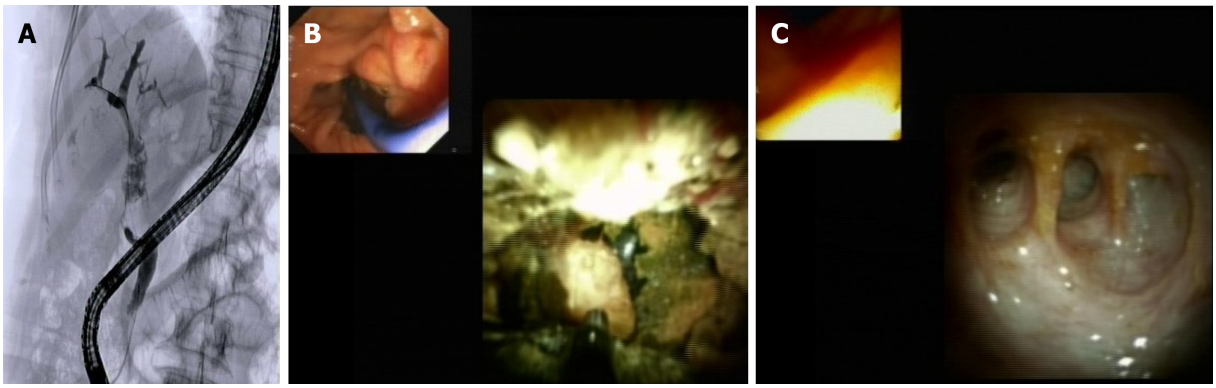


Figure 3 Management of impacted common bile duct stones with distal biliary stricture by cholangioscopy assisted lithotripsy. A: Cholangiogram showing distal common bile duct (CBD) stricture with large impacted stone in the medium CBD and multiple stones above; B: Cholangioscopy assisted lithotripsy by electrohydraulic of the impacted stone; C: Final cholangioscopy showing complete CBD clearance with biliary confluence appearance.

with EHL (88.4%) and ESWL (84.5%), while EHL had a higher post-procedural AEs rate (13.8%, including cholangitis, hemobilia and pancreatitis) compared with ESWL (8.4%) or LL (9.6%). In opposition to these results, a more recent meta-analysis[34] reported a superiority of EHL *vs* LL (mean successful endoscopic clearance rate 91.4% and 88.6%, respectively), explaining a more selective study inclusion than that made by Veld *et al*[33]. The last published meta-analysis comparing cholangioscopy-guided lithotripsy *vs* conventional therapy for complex bile duct stones[34] showed no

significant difference between ERCP and cholangioscopy in terms of therapeutic success, AEs rate and mean fluoroscopy time, but a shorter mean procedure time for conventional ERCP methods were found. In detail, cholangioscopy-guided lithotripsy showed a successful endoscopic clearance rate of 88.29% [95% confidence interval (CI): 86.9%-90.7%], first session successful endoscopic clearance rate of 72.7% (95%CI: 69.9%-75.3%), mean procedure time of 47.50 ± 6 min for session, number of sessions to clear bile duct of 1.5 ± 0.18 and adverse event rate of 8.7% (95%CI: 7%-10.9%). The majority of patients in the considered studies had a history of failure to remove stones on prior ERCP attempt. Therefore, Galetti *et al*[34] concluded that cholangioscopy-guided lithotripsy should be reserved to cases where the conventional techniques failed to achieve initially stone clearance. The majority of data published by Galetti *et al*[34] are in line with an older meta-analysis by Korrapati *et al*[35]. Regarding AEs, cholangitis is the most frequently reported, while PEP and perforation rarely occur [35].

Cholangioscopy-assisted lithotripsy should be reserved to selected cases and in the setting of tertiary care centers due to its costs, complexity and AEs rate. However, an increasingly number of authors claim that cholangioscopy-assisted lithotripsy may be considered first-line therapy for patients with difficult CBD stones in order to avoid serial procedures[36-38] and decrease costs[39]. Moreover, this procedure must be performed at tertiary centers by expert endoscopists.

When conventional ML fails and intraluminal lithotripsy is not available, the ESGE guidelines suggest to perform ESWL[4]. However, this procedure often requires multiple sessions, the placement of a naso-biliary drainage and subsequent ERCP to extract stone fragments. Ductal clearance rate appeared lower compared with LL (53%-73% *vs* 83%-97%, respectively)[40,41] but similar to EHL (78.5% *vs* 74%, respectively)[42]. ESWL-related AEs, including mostly cholangitis and pancreatitis, range from 9%-35.7%[40,42].

When biliary stones remain irretrievable but patients still need biliary drainage due to the risk of cholangitis, the placement of a temporary plastic stent is highly recommended before a second attempt at stone extraction can be made[4]. Horiuchi *et al*[43] documented that stent placement for 2 mo is associated with large and/or multiple CBD stones becoming smaller and/or disappearing without any complications, with a successful stone removal of 93% on a second ERCP[43]. Likewise, the disintegration of stones depends on the continuous friction between the plastic stent and the stones, which produces stress forces on the stone[43]. In a recent retrospective study, Jang *et al*[44] compared the use of different stents (7-Fr rather than 10-Fr plastic stents) in this setting, showing that the mean stone size reduction did not differ between the stents (5.7 mm in the 7-Fr stent group and 5.5 mm in the 10-Fr stent group; $P = 0.91$). However, when performing multivariate analyses, 7-Fr double pigtail stents significantly improved the complete clearance rate. On the other hand, the reduction in stone size was greater in the double-stenting group than in the single-stenting group, but the use of a double stent did not alter the complete stone clearance rate[44]. Covered self-expandable metal stent have been also used as an alternative to plastic stents to drain CBD after unsuccessful difficult stone removal. However, their high cost has not been certainly related to improved benefits[45-48].

DIFFERENT APPROACHES FOR PATIENTS WITH ALTERED ANATOMY

Endoscopic management in patients with SAA is still challenging for the endoscopists. Before performing ERCP, it is paramount to understand the anatomy and length of the afferent limb in order to select the appropriate approach, through the revision of the surgical report. The success of interventional endoscopy will depend on the correct choice of the endoscope and devices that should be tailored to the patient's anatomy [49].

Billroth II reconstruction

According to the ESGE guideline, a duodenoscope should be the first choice, reserving a forward-viewing endoscope (gastroscope, pediatric colonoscope, device assisted enteroscope) in case of failure[50]. Endoscopic sphincterotomy, where an inverted sphincterotome rather than precut following biliary stent placement in case of dedicated sphincterotome unavailability is used, is the standard of care in this setting. Furthermore, EPLBD could be used as an alternative method to sphincterotomy for CBD stones extraction, especially for stones larger than 10 mm[51,52]. Usually, the length of the afferent limb is short (less than 50 cm), but in cases of antecolic gastroje-

junostomy, it could be too long to be reachable by a duodenoscope. In this case, a forward viewing endoscope, allowing better visualization and easier intubation of the afferent limb compared with the lateral viewing endoscope, should be used. However, duodenoscope makes biliary cannulation easier using the elevator, even though it emerged to be associated with a higher perforation rate when compared with the forward viewing endoscope. This is due to limited visualization, difficult control of the scope, and the need to apply more pressure to overcome looping[53,54]. However, according to a recently published review by Krutsri[53], in patients with Billroth II gastrectomy, the duodenoscope has an afferent limb intubation success rate ranging from 62.5%-100%, cannulation success rate 88.2%-100% and complication rate 0%-12.5%. On the other hand, gastroscope with or without cap is reported to have similar results with an afferent loop intubation success rate of 76.8%-100%, cannulation success rate 81.4%-100% and complication rate 0%-10%. A subsequent retrospective study comparing sideviewing duodenoscope and forwardviewing endoscope to perform ERCP in patients with Billroth II gastrectomy reported afferent loop intubation rates of 95.1% for the side-viewing duodenoscope and 100% for the forward-viewing endoscope (P = 0.49). The rates of reaching the papilla were 70.7% and 91.1%, respectively (P = 0.06). Cannulation success rate after reaching the papilla was 100% in the side-viewing duodenoscope group and 90.3% in the forward-viewing endoscope group[55].

In 2015, Bove *et al*[56] reported a 30-year experience, showing that, in tertiary referral centers, patients with Billroth II (BII) that underwent ERCP had similar rates of morbidity and mortality when compared with patients with normal anatomy. In a recent systematic review and meta-analysis, Park *et al*[57] compared the efficacy and safety of forward viewing *vs* lateral viewing endoscopes and demonstrated that there was no statistically significant difference between the two endoscopes.

In referral centers, device-assisted enteroscopies (DAE) could be the first option because of higher technical success rate and lower adverse events, when compared with duodenoscopes and forward viewing endoscopes[53]. The majority of CBD stones in patients with Billroth II anatomy can still be removed by standard techniques such as EST and EPBD.

In a recent retrospective study, Duo *et al*[58] analyzed the risk factors for technical ERCP failure in Billroth II anatomy, demonstrating that in two or more CBD stones where the largest CBD stone measures to 12 mm or larger in size, stone characteristics for failed stone removal were included. Moreover, after the first ERCP attempt, Braun anastomosis and the use of no cap-assisted gastroscope were risk factors for technical failure of ERCP in this patients' cluster[58]. The initial stone removal rates of EPLBD have been reported to range from 66.7%-92.5%, while the overall stone clearance rates were from 96%-100%[51,59,60]. EPLBD, with or without EST, showed a high rate of first session stone clearance, reducing the need of endoscopic ML[51]. EPLBD has resulted to be associated with higher risk of post-ERCP pancreatitis in some studies [61-63], unlike most of the recent papers which showed the efficacy of EPLBD without increasing adverse events, including PEP[59,64]. On the other hand, EST is associated with a higher risk of bleeding[65]. However, sometimes lithotripsy is necessary to achieve stone clearance, especially when stones are too large to extract even after EPLBD, or when EPLBD is too risky in cases of distal bile duct narrowing or stricture [66].

There are three treatment options for lithotripsy: Endoscopic ML, cholangioscopic guided lithotripsy (LL or EHL) and ESWL. ML is a first treatment option, although it failed in cases where bile duct stones were larger than 2-3 cm, due to the difficulty to catch with a mechanical lithotriptor. In those cases, intraductal lithotripsy could be used, although cholangioscopy is difficult to carry out in patients with SAA. In these setting of patients, ESWL should be considered as a second option. However, endoscopic naso-biliary drainage is necessary before performing ESWL, which has a lower efficacy compared to LL or EHL[40].

Finally, EUS-guided intraductal lithotripsy or percutaneous transhepatic biliary drainage should be considered if ESWL is ineffective. In the percutaneous transhepatic biliary drainage approach, CBD stones are extracted in the antegrade fashion after balloon dilation of the papilla. In cases with large CBD stones, percutaneous transhepatic cholangioscopy with intraductal lithotripsy or ESWL could be attempted to facilitate stone removal. EUS-guided approach has gained popularity in the management of bile duct stones in patients with BII anatomy. In a retrospective study [9], EUS-guided antegrade (EUS-AG) treatment had a technical success rate of 72%, due to the technical difficulty of the antegrade stone extraction. Moreover, mechanical or intraductal lithotripsy, through an enterobiliary fistula after fistula maturation, could be made[67].

Roux-en-Y reconstruction

Roux en Y reconstruction can be divided in Roux en Y without gastric bypass (*e.g.*, Roux en Y gastrojejunostomy, Roux en Y hepaticojejunostomy, pancreatico-duodenectomy) and with gastric bypass (RYGB). Roux-en-Y reconstruction, compared to BII surgery, results in a longer and tortuous limb, increasing the difficulty to reach the papilla. In this setting, patients should be managed in referral centers where DAEs are the first option[68], with a reported cannulation rate ranging from 58%-95.6%, and an AEs rate between 7% and 10%, with a perforation rate of 0%-3.2%[69,70].

A systematic review and meta-analysis showed that BAE has a high diagnostic and procedural success rate in patients with Roux-en-Y reconstruction (69.4% and 61.7%, respectively), with an overall AEs rate of 6.5%[71]. Different studies have shown that there is no superiority among different DAE methods [single balloon enteroscopy (SBE), double balloon enteroscopy (DBE) and spiral enteroscopy][72]. Nevertheless, three systematic review and meta-analyses demonstrated better results for DBE compared to SBE (the success rate of reaching the papilla and treatment were 89.7% and 63.5% for DBE and 80.9% and 61.7% for SBE, respectively)[71,73,74].

The success rate of forward viewing endoscopes could be increased by using an underwater cap assisted technique, which combines the use of a cap applied to the tip of a pediatric colonoscope, with the injection of water as an alternative medium to carbon dioxide or air to distend the bowel lumen. It appeared that on one side the underwater technique reduces loop formation and bowel distension, while on the other side, the use of a cap improves the visualization of the papilla and helps in maintaining a stable position[75].

Even after cannulation, extraction of CBD stones can be difficult in patient with Roux-en-Y reconstruction, although different techniques have been described in this cluster of patients. EPLBD has been reported to achieve complete stone removal on a single-session in 66.7%-100%, while overall complete stone removal was obtained in 96.7%-100% of cases[49]. ML can be used, even though it is often technically challenging during enteroscopy-assisted ERCP and may fail.

Therefore, direct peroral cholangioscopy, through direct insertion of an ultra-slim endoscope or an enteroscope into the bile duct, allowing to perform lithotripsy, has been described in some case reports[76-80]. Some authors have also described the placement of an overtube through the scope, which allows the insertion of a cholangioscope (Spyglass, Boston Scientific) and direct lithotripsy[81,82].

EUS-AG stone treatment has also gained popularity. Biliary access is achieved from the stomach or jejunum under EUS-guidance, using a guidewire that is passed through the ampulla into the duodenum. Then the ampulla is dilated using a balloon, and finally, CBD stones are pushed into the duodenum using a stone extraction balloon.

The reason for technical failure is the failed puncture of the intrahepatic bile duct, guidewire passage and difficulty to stone extraction due to large stones size. However, these reasons could be overcome using large balloon dilation. Nevertheless, the maximum balloon size is limited to the size of the distal CBD, therefore intraductal lithotripsy is needed in cases with stones larger than the size of the distal CBD, increasing the risk of bile leak. However, a two-step approach has been proposed to prevent bile leaks and allowing safe usage of ML and cholangioscopy in EUS-AG stone treatment[67].

Mechanical lithotriptor can be introduced over the guidewire and into the bile duct, through the fistula. A fistula dilation up to 10-F using a plastic stent, a fully covered self-expandable metal stent after endoscopic ultrasound-guided hepaticogastrostomy or endoscopic ultrasound-guided hepaticojejunostomy, should be made in order to prevent bile leak and to allow easy access of the cholangioscope into the biliary system [83].

For the management of complex CBD stones, with the use of DAEs in patients with SAA has been studied in larger cohort of patients, EUS-guided therapy in this setting has been increasingly reported in case reports[8-11,84-86]. There are advantages and disadvantages in enteroscope-assisted stone management and EUS-guided interventions in patients with Roux-en-Y anatomy.

Enteroscopy-assisted ERCP uses the physiological biliary access and has a lower risk of bile duct leakage, although scope insertion can be challenging. On the other hand, EUS-guided approach involving the puncture of the left intrahepatic bile duct has a lower risk of bile leak but can be challenging when the intrahepatic bile duct is minimally dilated. Enteroscopy-assisted ERCP should be the first approach, reserving EUS-guided approach in case of failure as a salvage technique. We should keep in mind that enteroscopy-assisted ERCP and EUS-AG stone treatment need expertise as well as dedicated devices, therefore these procedures should be performed by skilled endoscopists in high volume referral centers.

Roux-en-Y gastric bypass

The treatment of CBD stones in patients who underwent weight loss surgery, especially RYGB, is challenging due to difficult access to the CBD. In fact, in Roux en-Y reconstruction, the afferent limb length can be more than 200 cm, with sharp angulation of the jejunojejunal anastomosis, severe adhesion and looping of the scope. Moreover, the incidence of symptomatic gallbladder disease is around 15% after significant weight loss, therefore prophylactic cholecystectomy has been suggested [87]. The percentage of therapeutic success of ERCP using a pediatric colonoscope or with DAEs has been reported around 60%[88]. New techniques to perform ERCP in this setting are EUS-directed transgastric ERCP (EDGE) and laparoscopic-assisted transgastric ERCP (LA-ERCP), which reach a success rates of 80%-100%[89-91]. However, both procedures have some limits. EDGE requires expertise in interventional EUS and ERCP, has higher costs and can be associated with stent migration and subsequent perforation[92,93] and permanent gastro-gastric fistula with weight regain [94]. On the other hand, LA-ERCP needs coordination between surgeon and endoscopist with a gastrostomy tube left *in situ*, if multiple ERCP procedures are required to obtain stones clearance[95].

EDGE is a two-step procedure; procedures can be performed in the same session or in two separate sessions (Figure 4). Single session EDGE is associated with a higher risk of perforation due to lumen apposing metal stent dislodgement, while dual session has a lower perforation risk, but it requires 10-14 d interval to allow fistula maturation. A shortened interval dual session (2-4 d) has been proposed to overcome this limitation, decreasing the risk of intraprocedural 20 mm lumen apposing metal stent dislodgement and allowing a timely transgastric ERCP[96].

An international, multicenter trial comparing EUS-guided gastro-gastrostomy-assisted ERCP *vs* enteroscopy-assisted ERCP (e-ERCP) in patients with RYGB has shown that EUS-guided gastro-gastrostomy-assisted ERCP may be superior to e-ERCP in terms of higher technical success and shorter procedural time, with similar safety profile[97]. LA-ERCP provides the opportunity to perform cholecystectomy concomitantly with CBD stones clearance, in case the gallbladder is still in place[98].

Post-liver transplantation

CBD stones after liver transplantation have an incidence between 4% and 10% of cases [99]. Biliary strictures are the major predisposing risk factor for biliary CBD stones, occurring in up to 90% of liver transplant patients with biliary stones[100]. Other possible agents for stone formation are cold ischemia, hyperlipidemia, hypercholesterolemia, infections and cyclosporine assumption[100,101]. Biliary stones in post-liver transplantation patients may cause severe complications such as pancreatitis, biliary infections and biliary cirrhosis, which can drastically worsen the graft's course. The endoscopic management of CBD stones in this setting of patients is successful in over 90% of cases, although the presence of anastomotic strictures results in a challenging and demanding procedure and increases the difficulty of stones removal[99]. In this context the strictures have to be treated simultaneously with stone extraction, using balloon dilation and mechanical lithotripsy and reserving the use of cholangioscopy-assisted lithotripsy in case of failure[102]. CBD stones may be mistaken with biliary casts, which occur in 2.5%-18% of post-liver transplant patients due to hepatic ischemic injury and are associated with poorer graft survival[103]. The differential diagnosis is mainly based on cholangiography features in addition to the endoscopist experience and can be confirmed by cholangioscopy direct visualization. This is fundamental to direct the treatment approach.

FUTURE RESEARCH DIRECTIONS IN THE MANAGEMENT OF DIFFICULT CBDS

The use of cholangioscopy-assisted lithotripsy in patients with difficult CBD stones as a first step rather than after failed traditional treatment, need to be assessed in randomized controlled trials. Many endoscopic techniques have been used in patients with difficult CBD stones and altered anatomy, with variable results. Percutaneous transhepatic cholangioscopy, which has been recently reported as a novel and alternative approach for patients with SAA, allows access to the biliary tree and stones fragmentation under direct visualization[13,104,105]. However, efficacy and safety of its use need to be further investigated.

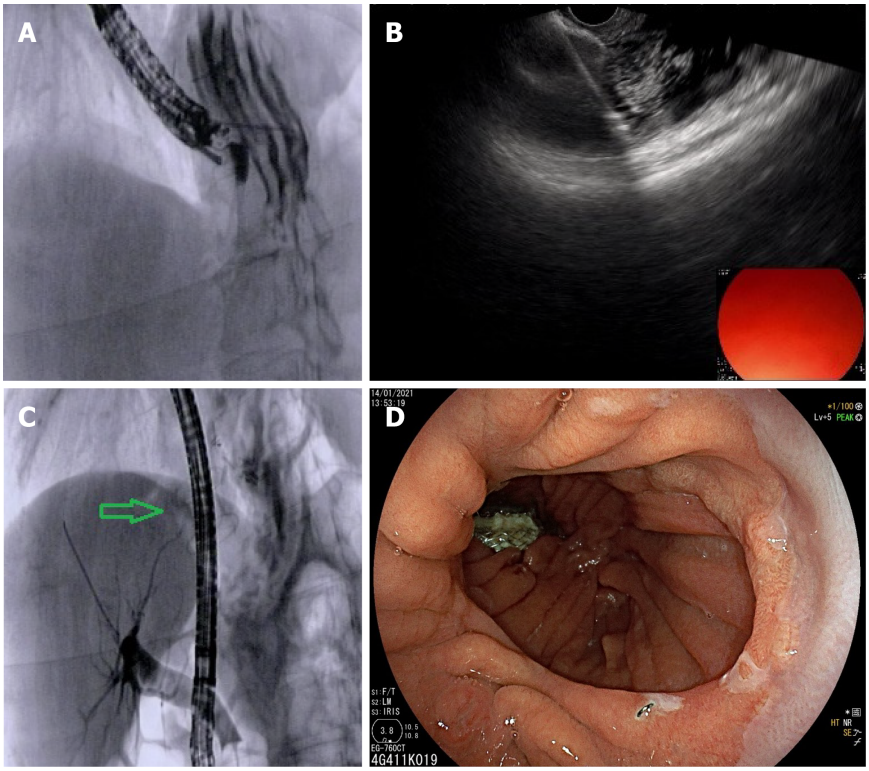


Figure 4 Endoscopic ultrasonography-directed transgastric endoscopic retrograde cholangiopancreatography for management of common bile duct stone in patient with previous Roux-en-Y gastric bypass for bariatric surgery. A: Endoscopic ultrasonography (EUS)-guided puncture of the excluded stomach with a 19G EUS needle with injection of contrast medium and sterile saline for gastric distension under fluoroscopic control; B: EUS guided first flange deployment of 20 mm lumen apposing metal stent (LAMS) into the gastric remnant; C: Endoscopic retrograde cholangiopancreatography for stone removal was performed after advancing the duodenoscope through the LAMS (green arrow); D: Endoscopic image confirming placement of the LAMS within the gastric pouch.

EUS guided interventions for biliary drainage after failed ERCP are in rapid evolution[106-107], including management of CBD stones in patients with normal anatomy when other techniques have failed[108]. Two retrospective studies and a recent review have compared EUS-rendezvous with precut papillotomy technique, showing that treatment success was significantly higher for EUS-rendezvous than for those with precut papillotomy, without significant differences in terms of complication rate[109-111]. Therefore, in tertiary referral centers, EUS-rendezvous could be used instead of precut papillotomy. The role of EUS-guided biliary treatment in patients with SAA should be investigated as primary technique in well-designed studies comparing safety and efficacy of EUS-guided interventions with enteroscopy-assisted ERCP. Finally, regarding patients with RYGB, more studies are needed in order to assess the role of the three different methods used in this setting (EDGE, LA-ERCP, e-ERCP), allowing endoscopists to tailor the technique to the patient.

CONCLUSION

The step-up approach involving ESLBD, ML and intraductal lithotripsy for patients with difficult CBD stones has been quite well validated. On the other hand, many different techniques involving the use of EUS in this setting are in rapid evolution with encouraging results. In patients with SAA, the endoscopic management of CBD stones is still challenging and should be managed in referral centers. The role of EDGE compared with LA-ERCP, e-ERCP as well as the efficacy and safety of percutaneous transhepatic cholangioscopy need to be evaluated in further well-designed studies. Finally, the definition of difficult CBD stones includes many different clinical scenarios with distinctive outcomes depending on the treatment choice. The correct pre-operative evaluation of the patient could help in choosing the best treatment strategy, in order to avoid unnecessary, ineffective ERCP session/attempt and can offer the best therapeutical approach to our patients.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 28; 27(44): 7612-7624

DOI: 10.3748/wjg.v27.i44.7612

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

EVIDENCE REVIEW

Role of early transjugular intrahepatic portosystemic stent-shunt in acute variceal bleeding: An update of the evidence and future directions

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Author contributions: Khan F performed research and wrote the manuscript; Tripathi D contributed to critical revision of the manuscript for important intellectual content.

Conflict-of-interest statement: Khan F has no conflict of interest. Tripathi D receives Speaker fees and consultancy for Gore Medical, and Research grant award for early TIPSS trial (NIHR, UK).

Country/Territory of origin: United Kingdom

Specialty type: Gastroenterology and Hepatology

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

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 open-access article that was

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Abstract

Variceal bleeding is a serious complication of cirrhosis and portal hypertension. Despite the improvement in management of acute variceal bleed (AVB), it still carries significant mortality. Portal pressure is the main driver of variceal bleeding and also a main predictor of decompensation. Reduction in portal pressure has been the mainstay of management of variceal bleeding. Transjugular intrahepatic porto-systemic stent shunt (TIPSS) is a very effective modality in reducing the portal hypertension and thereby, controlling portal hypertensive bleeding. However, its use in refractory bleeding (rescue/salvage TIPSS) is still associated with high mortality. "Early" use of TIPSS as a "pre-emptive strategy" in patients with AVB at high risk of failure of treatment has shown to be superior to standard treatment in several studies. While patients with Child C cirrhosis (up to 13 points) clearly benefit from early-TIPSS strategy, it's role in less severe liver disease (Child B) and more severe disease (Child C > 13 points) remains less clear. Moreover, standard of care has improved in the last decade leading to improved 1-year survival in high-risk patients with AVB as compared to earlier "early" TIPSS studies. Lastly in the real world, only a minority of patients with AVB fulfil the stringent criteria for early TIPSS. Therefore, there is unmet need to explore role of early TIPSS in management of AVB in well-designed prospective studies.

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Received: April 19, 2021
Peer-review started: April 19, 2021
First decision: May 12, 2021
Revised: May 27, 2021
Accepted: October 29, 2021
Article in press: October 29, 2021
Published online: November 28, 2021

P-Reviewer: Debnath P
S-Editor: Wang JL
L-Editor: A
P-Editor: Wang JL

In this review, we have appraised the role of early TIPSS, patient selection and discussed future directions in the management of patients with AVB.

Key Words: Transjugular intrahepatic portosystemic stent-shunt; Early transjugular intrahepatic portosystemic stent-shunt; Salvage transjugular intrahepatic portosystemic stent-shunt; Portal hypertension; Acute variceal bleed; Hepatic encephalopathy

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Core Tip: Outcome of high-risk patients following episode of acute variceal bleeding (AVB) is poor and insertion of transjugular intrahepatic portosystemic stent-shunt (TIPSS) within 72 h of index endoscopy (early or pre-emptive TIPSS) is associated with remarkable outcomes in a selection of patients (Child C up to 13 points). However, it's efficacy in Child B patients is debatable and criteria for high-risk patients needs to be refined. Moreover, management of variceal bleeding has improved in last decade and provision of early TIPSS (within 72 h) is challenging in most healthcare facilities. In this paper we have discussed the role of early TIPSS, patient selection and future directions in management of AVB.

Citation: Khan F, Tripathi D. Role of early transjugular intrahepatic portosystemic stent-shunt in acute variceal bleeding: An update of the evidence and future directions. *World J Gastroenterol* 2021; 27(44): 7612-7624
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7612.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7612

INTRODUCTION

Acute variceal bleeding (AVB) is a severe complication of portal hypertension and occurs at a rate of around 10%-15% per year in patients with cirrhosis. The risk of variceal bleeding depends on the severity of liver disease, size of varices, and presence of red wale marks[1]. Six-week mortality following an episode of AVB (the endpoint identified as the key outcome in variceal bleeding) is reported to be between 15% and 25%[2-4]. Early mortality was reported to be 50% in the early eighties[5]. The presence of clinically significant portal hypertension is the main factor determining the risk of development of varices and other liver-related decompensations. Transjugular intrahepatic portosystemic stent-shunt (TIPSS) was initially used for management of refractory variceal bleeding only (salvage or rescue TIPSS), followed by prevention of rebleeding or as secondary prophylaxis. There has been recent interest in early or pre-emptive TIPSS (e-TIPSS) in selected patients at high risk of treatment failure and mortality. There remains considerable controversy in the utility of early TIPSS, and we aim to provide a summary of the current evidence and discuss unresolved issues and future directions.

IDENTIFYING PATIENTS AT RISK OF A POOR OUTCOME FOLLOWING AVB

Although the prognosis of AVB has significantly improved over the last decades due to better management of haemorrhage and its associated complications, mortality is still as high as 15%-20%[2].

Patients presenting with AVB do not benefit equally from standard treatment as not all patients have the same risk profile of treatment failure, re-bleeding and mortality. The risk of rebleeding (and subsequently death) is greatest in the first 48-72 h after the initial episode and over 50% of rebleeding episodes occur within the first 10 d[6-8]. Therefore, it is important to identify those patients who are at high risk of treatment failure and death in whom a more aggressive approach, like implantation of early or pre-emptive TIPSS (within 72 h of index bleeding) can be utilised.

Measurement of the hepatic venous pressure gradient (HVPG) is the gold standard method for evaluating portal hypertension[9]. Portal hypertension is defined as an increase of HVPG > 5 mmHg; and HVPG ≥ 10 mmHg is defined as clinically significant portal hypertension as above this threshold, varices usually appear and risk of developing overt clinical decompensation (variceal bleeding, ascites and hepatic encephalopathy) increases[9,10]. If varices remain untreated, rebleeding and death occur in approximately 60% and 30% of patients respectively, one to two years after the index bleeding[1].

HVPG measured within 24 h of the bleeding episode is shown to be a prognostic indicator for outcome following AVB. HVPG > 20 mmHg has been associated with up to 5-fold increased risk of failure to control bleeding and one-year mortality[11,12]. Decrease in portal pressure of ≥ 20% from the baseline or to HVPG ≤ 12 mmHg is associated with significant reduction in risk of decompensation and with improved survival[13].

Recent data also show that decreasing HVPG by > 10% from baseline, or to absolute values < 10 mmHg, reduces the risk of development of varices and AVB regardless of the presence of varices[14]. Therefore, lowering HVPG has been one of the treatment strategies in management of AVB.

Portal hypertension correlates strongly with severity of liver disease measured by Child-Pugh score[13]. The severity of liver disease remains the main determinant of prognosis in patients with AVB[15,16]; There is a strong relationship between the presence of HVPG > 20 mmHg and Child–Pugh class[11,17]. Therefore, Child-Pugh Class C is associated with poor outcome following AVB. Moreover, presence of ascites and bacterial infections are also associated with poor outcome[18].

Severity of bleeding (active bleeding on endoscopy and haematocrit level) as well as presence of portal vein thrombosis are also among the significant predictors of early treatment failure following AVB[19].

Recalibrated MELD score (−5.312 + 0.207 × MELD) has been developed to predict early mortality after an episode of AVB. MELD score of 19 or higher is associated with a higher mortality risk of 20%[2]. The utility of recalibrated MELD in predicting outcome has recently been validated in 2 observational studies[20,21]. Similarly, Child-Pugh Class C is associated with higher mortality risk than in Child–Pugh class A and B cirrhosis, regardless of the presence of active bleeding[21].

In a recently published study acute-on-chronic liver failure (ACLF) at baseline is also found to be an independent risk factor for rebleeding and mortality in patients presenting with AVB. Presence of ACLF almost doubled the risk of rebleeding[22].

SALVAGE TIPSS

In the 1980s, the prognosis in patients with refractory or uncontrolled variceal bleeding was poor with mortality of over 90% in Child-Pugh B and C patients[23]. Though rescue surgical treatments (oesophageal transection or surgical porto-systemic shunting) were effective in decreasing portal hypertension, these procedures were associated with high mortality, ranging from 50% to 90% in this situation[24,25]. Moreover, subsequent liver transplantation may become technically more difficult to perform following porto-systemic shunt surgery[25].

The concept of percutaneous transjugular porto-systemic shunt in context of oesophageal variceal bleeding in humans was first introduced by Colapinto *et al*[26] in 1982 (in which intrahepatic portosystemic shunt was created by dilating the track with an angioplasty balloon). First (prospective) study evaluating the role of salvage TIPSS in patients with variceal haemorrhage refractory to (then) standard medical and endoscopic treatment was published in 1994[27]. In that study though salvage TIPSS (with bare stent) was associated with immediate control of bleeding in all 20 patients, 40-d mortality was very high at 60% mainly due to liver failure and sepsis[27].

Several (retrospective) studies were published afterwards, evaluating the role of salvage (rescue) TIPSS (using uncovered stents) in setting of refractory variceal bleeding[28-30]. Salvage TIPSS was effective in controlling the variceal bleeding but early mortality rate remained high in these patients, approaching 48% at 45-d. Majority of the patients died due to multi-organ failure and sepsis. Child-Pugh (CP > 11), APACHE II and MELD scores (> 20) were associated with increased mortality[29,30]. These studies were uncontrolled, mainly involved uncovered stents and sclerotherapy was the choice of endoscopic treatment.

Standard treatment of AVB has improved considerably in the recent decade and covered TIPSS has lower risk of stent dysfunction as compared to bare metal stents[31,

32]. In subsequently reported retrospective studies of salvage TIPSS using both covered and uncovered stents, the use of covered stent did not culminate in survival advantage at both 6 wk and 1-year[33,34]. However, use of bare metal stent was associated with increased rate of re-bleeding due to stent dysfunction and salvage TIPSS appeared to be futile in patients with Child-Pugh score of > 13[34].

A recently published Chinese retrospective study of 58 patients, in which 55 patients had covered stents, showed better 6-weeks and 1-year transplant free survivals (87.7% and 81.8%, respectively) following salvage TIPSS[35]. Treatment failure at 6 wk was associated with bare stents and white cell count. It is important to note that 62% patients had Child B disease and over 60% had hepatitis B related disease. Only 30% of patients had Child C disease. Median MELD score was 10 and mean Child score was 8.7, indicating that majority of patients had less severe disease (but with high portal pressure)[35]. Moreover, 82% of patients had variceal embolization[35], an effective tool to prevent re-bleeding[36,37].

EARLY TIPSS

Randomised control trials in e-TIPSS

It is important to clarify the concept of e-TIPSS. e-TIPSS strategy refers to a pre-emptive placement of TIPSS in those at a high-risk of treatment failure before treatment failure or re-bleeding occurs. In this setting, TIPSS is usually placed within 24–72 h of successful therapeutic endoscopy (with patients already on pharmacological therapy with vasoactive drugs and antibiotics). The rationale of this strategy is that reducing portal pressure early on, will prevent rebleeding, the associated liver failure and development of multi-organ failure with a lot worse outcome. This is in contrast to salvage TIPSS, where TIPSS is placed in patients with refractory variceal bleeding, not controlled with standard treatment; and this group of patients has very high mortality (as described above).

As stated earlier, reduction in portal hypertension is one of the mainstays of management of AVB. Utilising this evidence, Jalan *et al*[38] introduced the concept of preventive insertion of TIPSS (pre-emptive or early TIPSS placement, within 72 h) to lower portal pressure in cirrhotic patients with AVB in 1990s. They published a randomised control trial (RCT) in 1997 including 58 patients and compared endoscopic band ligation (EBL) with e-TIPSS (with bare-metal stent) randomised within 24 h after controlling of first episode of AVB. Mean time to TIPSS in that study was 2.2 d. e-TIPSS placement was superior to EBL in preventing rebleeding and was cost-effective in this setting. However, no survival difference was seen, although ITU requirement was significantly less with TIPSS. The Child-Pugh score of 9 was similar in the two groups, although there were some Child's A patients included[38]. Patient selection was not as strict as for subsequent studies. This could explain the lack of difference of survival.

Since then, the role of e-TIPSS in the management of acute variceal bleeding in patients with cirrhosis has been evaluated in several studies. The safety and efficacy of e-TIPSS in high-risk cirrhotic patients has been evaluated in a few RCTs (Table 1).

Monescillo *et al*[12] performed the first study applying high-risk selection criteria by using measurements of HVPG. Fifty two patients with HVPG ≥ 20 mmHg measured with 24 h of bleeding episodes were randomised to either TIPSS group or standard therapy. Their study showed that "early" TIPSS placement was associated with a significantly lower rate of treatment failure (50% *vs* 12%) and lower 6-wk mortality (38% *vs* 19%). 46% of study population had Child C disease[12]. However, bare-metal stents were used in TIPSS patients and standard of care (SOC) in the non-TIPSS group did not reflect current practice (sclerotherapy rather than combination of endoscopic band ligation and non-selective beta-blocker therapy). Patients in non-TIPSS arm received only non-selective beta-blockers (NSBBs) to prevent rebleeding and EBL was used in whom NSBBs were not tolerated or were contraindicated.

Measurement of early HVPG for risk stratification and treatment assignment in AVB is not easily applicable in clinical practice nor readily available. Therefore, it is important to identify non-invasive predictors of treatment failure and early mortality in patients with AVB. In this regard, Abraldes *et al*[11] not only showed a strong relationship between the presence of HVPG > 20 mmHg and Child-Pugh class C but also showed that 6-wk mortality is more strongly determined by the severity of underlying liver disease (assessed by Child- Pugh classification) than by HVPG > 20 mmHg. Therefore, subsequent studies used clinical criteria to define high-risk patients and used only covered stents. A schema of the study design of these trials is illustrated

Table 1 Early transjugular intrahepatic portosystemic stent-shunt in acute variceal bleeding: Key studies

Ref.	Main inclusion criteria	Primary and secondary outcomes	Results	Comments
Randomised controlled trials				
Monescillo *et al* [12], 2004 (Italy)	HVPG > 20 mmHg within 24 h of admission.	(1) Primary: Sensitivity and specificity of HVPG cut-off value (20 mmHg) in predicting transplant-free survival (TFS), and assessment of TFS as well as short- and long-term survival; and (2) Secondary: Transfusional needs, ICU stay, complications during the first week of treatment, and causes of death.	6-wk mortality = 17% in e-TIPSS *vs* 38% in control ($P \leq 0.05$). 1-yr mortality = 31% in e-TIPSS *vs* 65% in control ($P \leq 0.05$). Treatment failure = 12% in e-TIPSS *vs* 50% in control ($P = 0.001$).	46% of study population had Child C and 40% had Child B cirrhosis. mean Child score = 9.2. SOC does not reflect current management and only bare metal stents were used.
García-Pagán *et al*[39], 2010 (Europe)	Child- B with active bleeding or Child C < 14 points.	(1) Composite Primary: Failure to control bleeding and failure to prevent clinically significant VB within 1 yr; and (2) Secondary: Mortality at 6 wk and at 1 yr, failure to control acute bleeding, early rebleeding, rate of rebleeding between 6 wk and 1 yr, other complications of PHTN, number of days in ICU, days spent in the hospital, use of alternative treatments.	6-wk survival = 97% in e-TIPSS *vs* 67% in control (NNT = 3.3). 1-yr survival = 86% in e-TIPSS *vs* 61% in control ($P < 0.001$). 1-yr re-bleeding = 3% in e-TIPSS *vs* 50% in control ($P < 0.001$, NNT = 2.1).	mean Child score = 9.4. mean MELD score = 16.2. About 50% of study participants had Child C cirrhosis. Majority had ALD. NSBB (propranolol or nadolol) was administered with EBL in 25 patients.
Lv *et al*[44], 2019 (China)	Child B and C < 14 points, regardless of active bleeding.	(1) Primary: Transplant-free survival; and (2) Secondary: Failure to control bleeding or rebleeding, new or worsening ascites, overt HE, and other complications of portal hypertension and adverse events.	6-wk TFS = 99% in e-TIPSS *vs* 84% in SOC ($P = 0.02$). 1-yr TFS = 86% in e-TIPSS *vs* 73% in SOC ($P = 0.046$; NNT = 8). 1-yr re-bleeding/uncontrolled bleeding = 11% in e-TIPSS *vs* 34% in SOC ($P < 0.0001$).	mean Child Score = 8.0. mean MELD score = 13.8. More than 55% patients had Child-Pugh B without active bleeding. 75% of patients had Hepatitis B and had Child B cirrhosis. No significant difference in the incidence of HE was observed between two groups.
Dunne *et al* [46], 2020 (United Kingdom)	Child B and C, 8-13 points (regardless of active bleeding at the endoscopy).	(1) Primary: 1-yr survival; and (2) Secondary: Survival at 6 wk, early rebleeding (within 6 wk) and late rebleeding (between 6 wk and 1 yr), and the development of HE.	1-yr survival = 79.3% in e-TIPSS *vs* 75.9% in SOC ($P = 0.79$). e-TIPSS group showed a trend to reduced variceal re-bleeding ($P = 0.09$).	Median Child score = 9.8. Median MELD score = 17. More than 90% of participants had ALD. More than 55% had Child-C disease. 23/29 received TIPSS, 13 within 72 h. 18/29 (62%) in SOC group had carvedilol, 3 had cardio-selective beta- blocker and 2 had rescue-TIPSS for early re-bleeding. Incidence of HE was higher in e-TIPSS group ($P < 0.05$).
Observational studies				
Garcia-Pagán *et al*[49], 2013 (Europe)	Child-B with active bleeding or Child-C < 14 points.	(1) Composite primary: Failure to control acute bleeding or to prevent clinically significant variceal rebleeding; and (2) Secondary: mortality, development of other complications related to portal hypertension and the percentage of follow-up days spent in hospital.	1-yr survival = 86 % in e-TIPSS *vs* 70% in SOC ($P = 0.056$); e-TIPSS had lower incidence of failure to control bleeding or rebleeding than patients receiving SOC (3 *vs* 15, P <0.001).	mean Child score = 10. mean MELD score= 17. No significant difference in incidence of HE. Incidence of development of new or worsening ascites was low in e-TIPSS group ($P < 0.01$).
Rudler *et al* [52], 2014 (France)	Child-C 10–13 cirrhosis or Child-B with active bleeding	(1) Primary: prevention of rebleeding at 1 yr; and (2) Secondary: 3 and 6-mo survival, liver transplantation, control of bleeding, rate of rebleeding at 6 wk, between 6 wk and 1 yr, and the occurrence of adverse events (HE, acute cardiac failure, sepsis).	1-yr survival = 71% in e-TIPSS *vs* 74% in control ($P = 0.77$). 1-yr free of rebleeding = 97% in e-TIPSS *vs* 51% in control ($P < 0.001$).	mean Child score = 11.2. mean MELD score = 21.5. 77% had ALD and 77% had Child-C cirrhosis. Patients with previous history of variceal bleeding or with PVT were also included.
Thabut *et al* [50], 2017 (France)	Child-C (< 14) or Child-B with active bleeding	Survival at 5-d, 6-wk and 1-yr.	1-yr survival = 85% in e-TIPSS *vs* 59% in control ($P = 0.04$).	67% had ALD. 52% undergoing TIPSS had Child C cirrhosis. 35% were eligible for e-TIPSS. Severity of liver disease was the only parameter that influenced survival.
Hernández-Gea *et al*[51], 2018 (Europe and Canada)	Child-C score (< 14 points) or Child-B plus active bleeding	(1) Primary: Survival at 6 weeks and 1 year; and (2) Secondary: (a) The composite end-point of failure to control acute bleeding (up to day 5), early rebleeding (from day 5 to day 42), and late rebleeding (from day 42);	6-wk survival = 92% in p-TIPSS *vs* 77% in control. Overall, 1-yr survival = 78% in p-TIPPS *vs* 62% in control ($P = 0.014$). 1-yr survival in Child C patients = 78% in e-TIPSS *vs* 53% in control ($P = 0.002$). 1-yr survival in Child-B + AB = 77% in	Median MELD score= 15.5. Median Child Score= 10. More than 75% of patients had ALD. Development of de novo or worsening of previous ascites was significantly less in p-TIPSS group ($P < 0.001$). No

		(b) onset or worsening of ascites; and (c) development of HE.	p-TIPSS *vs* 75% in control (P = 0.935).	difference in incidence of HE was observed in two groups.
Lv *et al*[45], 2018 (China)	Any grade of cirrhosis (with Child score < 14) and AVB.	(1) Primary: All-cause mortality; and (2) Secondary: Failure to control acute bleeding or rebleeding, new or worsening ascites and development of overt HE.	Overall 6-wk mortality = 3.6% in e-TIPSS *vs* 10.6 % in SOC (P = 0.002). Overall 1-yr mortality = 14.1% in e-TIPSS *vs* 17.3% in SOC (P = 0.218). e-TIPSS group had significantly lower mortality in MELD ≥ 19 category.	Patients with Child A cirrhosis were also included. Only small number (< 20%) had Child C cirrhosis. Survival benefit was not seen in Child B patients without active bleeding. Incidence of HE was not significantly different between two groups.
Trebicka *et al* [22], 2020 (Multicentre)	Child-C, Child-B with active bleeding.	(1) Primary: All-cause mortality or liver transplantation at 6 wk and 1 yr; and (2) Secondary: Rebleeding.	6-wk mortality = 13.6 % in e-TIPSS *vs* 51% in SOC group of patients with ACLF (P = 0.002). 1-yr mortality = 22.7% in e-TIPSS *vs* 56.5% in SOC group with ACLF (P = 0.002).	Patients with ACLF had a higher rate of rebleeding compared to patients without ACLF (42-d: P < 0.001; 1-yr: 22.9% *vs* 17.7%, P = 0.024).

e-TIPSS: Early transjugular intrahepatic portosystemic stent-shunt; RCT: Randomised controlled trial; HVPG: Hepatic venous pressure gradient; HCC: Hepatocellular carcinoma; PHTN: Portal hypertension; PVT: Portal vein thrombosis; TFS: Transplant-free survival; HIV: Human immunodeficiency virus; ICU: Intensive care unit; NSBB: Non-selective beta-blockers; EBL: Endoscopic band ligation; IGV: Isolated gastric varices; MELD: Model for end-stage liver disease; ALD: Alcohol-related liver disease; NNT: Number needed to treat; HE: Hepatic encephalopathy; SOC: Standard of care; ACLF: Acute on chronic liver failure.

in Figure 1 and Figure 2.

In García-Pagán *et al*[39] landmark RCT published in 2010, patients with Child-Pugh C < 14 or Child-Pugh B with active bleeding at index endoscopy were considered high-risk patients. While there is clear justification of including patients with Child C disease in this category[11], the selection of Child B patients (with active bleeding on endoscopy) was not very clear. The composite primary end point in their study was of failure to control acute bleeding or to prevent clinically significant variceal rebleeding within 1 year. Their trial of 63 patients showed that early covered TIPSS (placed within 72 h of index bleeding) not only reduced re-bleeding at 1 year (3% *vs* 50%, P < 0.001) but also improved 6-wk [97% *vs* 67%; absolute risk reduction = 30%; number needed to treat (NNT) = 3.3]; and 1-year survival rates (86 *vs* 61%, P < 0.001; NNT = 4.0) in high-risk patients with cirrhosis when compared to standard of care (NSBB plus EBL). It is important to note that rates of treatment failure and death were higher in Child C patients than in those with Child-B disease, and mortality rates in Child B category did not appear to be significantly different statistically between SOC and TIPSS arms (2/16 *vs* 1/16). However, the trial was not powered enough to conduct appropriate subgroup analyses[39]. Patients with prognostic factors unlikely to benefit from TIPSS placement were excluded from this trial and the subsequent studies (Table 1).

In the light of emerging evidence, subsequent guidelines incorporated the use of pre-emptive or e- TIPSS, as a treatment option in patients with AVB at high risk of treatment failure[40-43] (Table 2).

An RCT from China included 132 cirrhotic patients who were randomly assigned (2:1) to receive pre-emptive TIPSS or standard of care (NSBB + EBL)[44]. This RCT showed better 1-year transplantation-free survival (primary outcome) in e-TIPSS group than in the control group; with greatest benefit for those with a MELD score between 12 and 19 (P = 0.04, NNT = 8)[44]. However, all patients with Child B and C (< 14 points) cirrhosis were included regardless of active bleeding at the index endoscopy. Secondly, the patient demographics were significantly different from other studies and most patients were Child-Pugh B without active bleeding (57%). Over 75% of patients had Hepatitis B related cirrhosis. Only 43% of patients were high risk according to the previously described criteria[39], and considered to benefit from e-TIPSS intervention[45]. Therefore, absolute risk difference of 13% for 1-year (transplant-free) survival in e-TIPSS group appeared to be lower than in the previous RCTs (34% and 25%)[39]. There was no significant difference in incidence of hepatic encephalopathy between the two groups.

A recently published RCT from the UK included 58 patients with a Child-Pugh score of 8-13, without previous treatment for portal hypertension related bleeding, regardless of active bleeding on endoscopy[46]. Patients were randomised to receive e-TIPSS or standard of care (carvedilol + EBL). There was no difference in 1-year survival rate (primary outcome) between the SOC and e-TIPSS groups (75.9% *vs* 79.3% respectively, P = 0.79). More than 90% of participants had alcohol related liver disease and majority (over 80%) were actively consuming alcohol at inclusion reflecting real Western world population. Over 55% had Child-C disease with median MELD score of 17, comparable with Garcia-Pagan study[39]. In the e-TIPSS group, 23/29 patients

Table 2 Summary of current Guidelines regarding early transjugular intrahepatic portosystemic stent-shunt

Ref.	Guidelines	e-TIPSS recommendations
[40]	Baveno VI Consensus Workshop (2015)	An early TIPSS (p-TIPSS) with PTFE-covered stents within 72 h (ideally < 24 h) must be considered in patients bleeding from EV, GOV1 and GOV2 at high risk of treatment failure [*e.g.*, Child-Pugh class C < 14 points or Child-Pugh class B with active bleeding) after initial pharmacological and endoscopic therapy (1b; A)]. Criteria for high-risk patients should be refined.
[41]	American Association for the Study of Liver Diseases (2017)	In patients at high risk of failure or rebleeding (CTP class C cirrhosis or CTP class B with active bleeding on endoscopy) who have no contraindications for TIPSS, an "early" (pre-emptive) TIPSS within 72 h from EGD/EVL may benefit selected patients.
[42]	The European Association for the Study of the Liver (2018)	Early pre-emptive covered TIPSS (placed within 24–72 h) can be suggested in selected high-risk patients, such as those with Child class C with score < 14 (I; 2). However, the criteria for high-risk patients, particularly Child B with active bleeding, remains debatable and needs further study. Up to 10%–15% of patients have persistent bleeding or early rebleeding despite treatment with vasoactive drugs plus variceal ligation, and prophylactic antibiotics. TIPSS should be used as the rescue therapy of choice in such cases (I; 1).
[43]	British Society of Gastroenterology (2020)	In patients who have Child's C disease (C10-13) or MELD ≥ 19, and bleeding from oesophageal varices or GOV1 and GOV2 gastric varices and are hemodynamically stable, early or pre-emptive TIPSS can be considered within 72 h of a variceal bleed where local resources allow (weak recommendation, moderate quality of evidence). However, large multi-centre randomised controlled trials are necessary to determine whether patients with Child's B disease and active bleeding or with MELD 12-18 benefit from early pre-emptive TIPSS.

PTFE: Polytetrafluoroethylene; p-TIPSS: Pre-emptive transjugular intrahepatic portosystemic shunt; EV: Oesophageal varices, GOV: Gastro-oesophageal varices; CTP: Child-Turcotte-Pugh; EGD: Oesophago-gastro-duodenoscopy; EVL: Endoscopic variceal ligation; MELD: Model for end-stage liver disease.

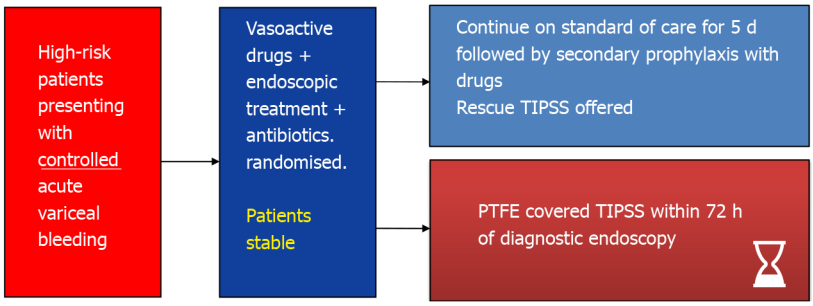


Figure 1 Early transjugular intrahepatic portosystemic stent-shunt – study design. High risk criteria: Child's C or Child's B + active bleeding, Child-Pugh score 8-13, Child's B + C; Maximum threshold: CPS > 13; TIPSS: Transjugular intrahepatic portosystemic stent-shunt; PTFE: Polytetrafluoroethylene.

(79%) actually underwent TIPSS and only 13 within 72 h, but all within 5 days. There was no difference in worsening or new ascites, with more encephalopathy (46.1% *vs* 20.7%, $P < 0.05$) and a trend towards lower variceal rebleeding in the e-TIPSS group ($P = 0.09$). Notably, previous RCT[39] and recent individual data metanalysis[47] did not show significant difference in development of hepatic encephalopathy between the two groups. Though the study was not powered enough to reach valid conclusions, it demonstrated better survival in the SOC arm than the previous European RCT[39] (76% *vs* 61%), although SOC survival rate is comparable to the Chinese RCT[44].

Better survival in SOC group could be explained by improved initial management of AVB (vasoactive drugs, antibiotics and endoscopic band ligation), with better access to intensive care. Furthermore, carvedilol was used to a greater extent. The improved SOC could be major factor in the lack of difference in survival between the two groups. In the SOC arm, 18/29 patients received carvedilol (at a median dose of 6.25 mg a day). Carvedilol with its additional alpha-1 antagonism profile, seems to have greater effect on reducing HVPG than other traditional NSBB (propranolol and nadolol) and may have a beneficial effect in SOC group but this needs further validation. This study has led to much debate in relation to patient selection[48].

Observational studies

The benefits of e-TIPSS have been shown by several (but not all) observational studies. Most of these studies used similar clinical high-risk and exclusion criteria as the study by Garcia-Pagan[49] (Table 1). It is important to note that in a French national audit and in large multicentre study, only a minority of patients eligible for e-TIPSS (according to defined criteria) actually received e-TIPSS (6.7% and 9.8% respectively) [50,51]. Survival benefit of e-TIPSS was only seen in those with Childs-Pugh C disease

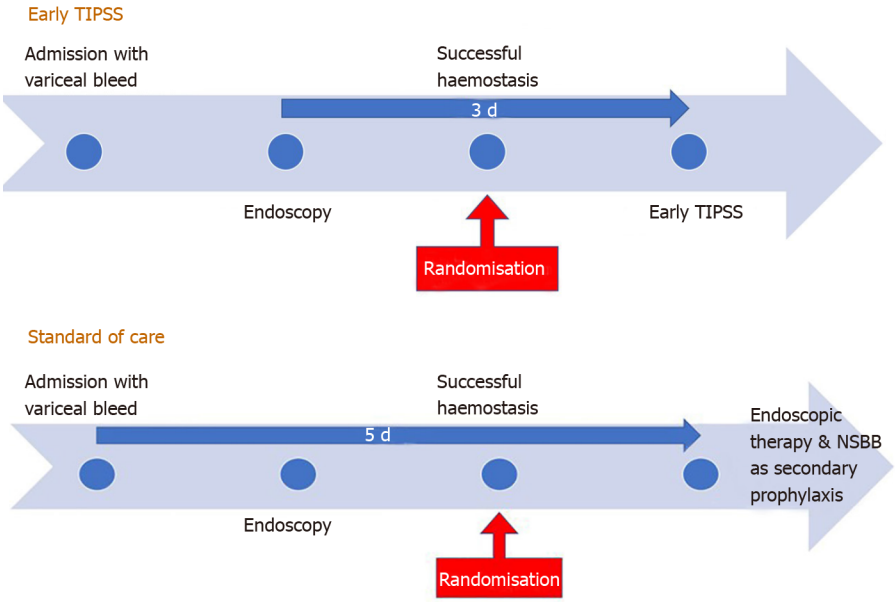


Figure 2 Design of early transjugular intrahepatic portosystemic stent-shunt and standard of care. TIPSS: Transjugular intrahepatic portosystemic stent-shunt.

in a large multicentre study including 671 patients[51]. These large observational multicentre studies underscore the lack of adherence of physicians to concept of e-TIPSS and difficulty in arranging e-TIPSS (within limited timeframe) in a real-life practice. Most physicians did not believe in the role of e-TIPSS. Two European studies did not find a statistically significant increase in survival in e-TIPSS group[49,52]. One of these studies included patients with Child-Pugh score up to 15 points i-e, patients with significantly advanced liver disease[52].

A recent observational study from China included 1425 patients with cirrhosis and variceal bleed[45]. Most of the patients had cirrhosis due to viral hepatitis and e-TIPSS was also offered to Child-Pugh A patients and Child-Pugh B patients without active bleeding. Survival benefit was observed in patients fulling the high-risk criteria used in Garcia Pagan RCT[39] and with MELD score ≥ 19 but not in patients with Child-Pugh A or Child-Pugh B without active bleeding.

In a recently published retrospective study, e-TIPSS has also shown improved 6-weeks and 1-year survival ($P < 0.05$) in patients with ACLF[22]. 671 patients were eligible for e-TIPSS and only 66 received e-TIPSS. 22 out of 66 e-TIPSS patients had ACLF. However, the findings need to be interpreted with caution due to the small sample size and require validation in larger prospective studies.

Systemic reviews and meta-analyses

A few metanalyses of studies looking at the role of early TIPSS in patients with AVB have been published in recent years. A well-designed meta-analysis of two earlier RCTs[39] and two observational studies[49,52] comparing e-TIPSS with standard of care showed that e-TIPSS is associated with reduced overall mortality (odds ratio = 0.38, 95%CI: 0.17-0.83, $P = 0.02$)[53] (Table 3). It is important to note that sensitivity analysis looking separately at Child B patients with active bleeding and those with Child C (< 14 score) showed that survival benefit was only observed in Child C (< 14 score) patients but not so in Child B patients. There was also significant reduction in rebleeding with e-TIPSS without significant difference in incidence of hepatic encephalopathy. Moderate heterogenicity was observed among the studies and the recent RCTs by Lv *et al*[44] and Dunne *et al*[46] were not included in this metanalysis. The authors concluded that further study was required to identify factors associated with poor outcome after e-TIPSS.

A recently published individual patient data meta-analysis assessed the efficacy of e-TIPSS in high-risk patients[47]. They included 7 studies: 3 randomized controlled trials[12,39,44] and 4 observational studies[45,49,51,52] comprising 1327 patients. As discussed previously, one of the RCTs[44] and one of the observational studies[45] included patients in all Child-Pugh categories, therefore only individual data of those patients fulfilling the current high-risk criteria (Child-Pugh B with active bleeding and Child- Pugh C up to 13 points) were included in this individual meta-analysis. This

Table 3 Early transjugular intrahepatic portosystemic stent-shunt in acute variceal bleeding: Key meta-analyses

Ref.	Design	Results	Comments
Deltenre *et al*[43]	4 studies (2 RCTs[12,39] and 2 Observational[49,52]) included.	e-TIPSS was associated with fewer deaths [odds ratio (OR) = 0.38, P = 0.02], and with lower rates of bleeding (OR = 0.08, 95%CI: 0.04–0.17, P < 0.001) within 1 year when compared to SOC, without increase in incidence of encephalopathy (OR = 0.84, 95% CI: 0.50–1.42, P = 0.5).	There was moderate heterogeneity between studies. No significant difference in mortality was observed between Child–Pugh B and C patients. This could be explained by inclusion of sicker patients (C-P score < 14) in Rudler *et al* [52] study.
Nicoară-Farcău *et al*[47]	Individual data meta-analysis from 7 studies (3 RCTs[12,39,44] and 4 observational studies[45, 49,51,52]), comprising 1327 patients (310 received e-TIPSS, 1017 received SOC (drugs + endoscopic treatment).	Overall, e- TIPSS significantly increased 1- year survival compared with SOC [hazard ratio (HR) 0.443; P < 0.001]. e-TIPSS significantly reduced the risk of failure to control bleeding/preventing variceal rebleeding (HR = 0.338; P < 0.001) and ascites without increasing risk of HE, compared with SOC.	Only individual data of those patients fulfilling the high-risk criteria (Child-Pugh B with active bleeding and Child-Pugh C < 14 points) from included studies were included. On multivariate analysis patients with Child-Pugh score > 7 points had a significantly worse survival than those with Child-Pugh score ≤ 7. Both prospective and observational studies were included and latest UK RCT[46] and the multicentre French audit[50] were not included.
Tripathi *et al*[54]	3 RCTs[39,44,46] comparing e-TIPSS (with covered stent) with SOC, comprising 152 patients.	e-TIPSS significantly reduced incidence of re-bleeding (RR = 0.20; P ≤ 0.001). Improvement in overall survival at 1 and 2 yr was not statistically significant between two groups (RR = 0.62; P = 0.16 and RR = 0.62; P = 0.19 respectively).	There was no significant difference in incidence of HE. RCTs are underpowered to reach firm conclusion about the survival benefit of e-TIPSS.

e-TIPSS: Early transjugular intrahepatic portosystemic stent-shunt; RCT: Randomised controlled trial; SOC: Standard of care; HE: Hepatic encephalopathy.

meta-analysis showed overall survival benefit of e-TIPSS over standard of care. Six-week and 1-year survival were significantly higher in the e-TIPSS group than in the SOC group (93% *vs* 76.8% and 79% *vs* 62%, respectively P < 0.001). Moreover, benefit of e-TIPSS was observed in both CP- B patients with active bleeding (P = 0.008;) and in CP-C patients (P < 0.001). Number of patients needed to treat to save one life was 4.23 (95%CI: 3.57–6.94). Multivariate analysis showed that patients with a CP score > 7 points had a significantly worse survival than those with CP score of 7 points or less. e-TIPSS significantly reduced the risk of failure to control bleeding/preventing variceal rebleeding (P < 0.001) in all patients. Moreover, risk of developing new or worsening ascites was significantly reduced by the e-TIPSS in the overall population (P < 0.001). This meta-analysis showed no significant differences in the risk of developing hepatic encephalopathy in the overall population (P = 0.553). However, a limitation of this meta-analysis is the inclusion of both prospective and observational studies, and the authors concluded that further prospective studies are necessary. The latest UK RCT[46] and the multicentre French audit[50] were not included in this meta-analysis, thus somewhat limiting its utility.

A recent meta-analysis of 152 patients in three prospective RCTs, including the latest UK RCT[39,44,46], concluded that e-TIPSS is more effective in preventing variceal rebleeding than standard of care (EBL and medical management) without increase in adverse events[54]. e-TIPSS with covered stents significantly reduced incidence of bleeding (RR = 0.20, 95%CI: 0.09–0.42, P < 0.001). This was associated an improvement in overall survival, but it did not quite reach statistical significance, at 1 and 2 years (RR = 0.62, 95%CI: 0.33–1.19 and RR = 0.62, P = 0.16 95%CI: 0.31–1.26, respectively). Incidence of hepatic encephalopathy was similar across the studies[54].

FUTURE DIRECTIONS

Patients with advanced liver disease i-e with Child-Pugh C score (up to 13 points) and MELD score ≥ 19 benefit from the e-TIPSS intervention in the described studies. However, benefit of this intervention in patients with less severe disease i-e with Child-Pugh B or MELD < 19 is not very robust and there is further need to define the high-risk category.

Though patients with child score > 13 points are considered too sick for early TIPSS intervention with high mortality, it is not very clear from the literature if there is a maximal threshold of severity of liver disease beyond which there is no benefit from e-TIPSS intervention. Indeed certain patients with ACLF may benefit from e-TIPSS following AVB. This concept needs further revalidation in a multi-centre trial

collecting large numbers of patients.

Outcomes after an episode of variceal bleed have improved in the last decade with improved 1-year survival in patients receiving standard care[44,46] as compared to the earlier landmark RCT[39], causing reluctancy to adopt e-TIPSS approach among the practicing physicians. Moreover, providing e-TIPSS (within 72 h of admission) is challenging in most healthcare systems, even in centres providing 24/7 TIPSS service, and is a significant barrier to adoption of e-TIPSS. Indeed, recruitment in trials[39,46] was very slow and careful reading of the manuscripts suggests that the included patients may not be truly representative of the entire population of patients with severe cirrhosis and variceal bleeding. With such stringent inclusion criteria, the applicability of this therapeutic approach is questionable in a larger cohort of cirrhotic patients.

Even if TIPSS was performed outside the 72 h window, so called "late e-TIPSS", it may not have a significant impact on the outcomes given the time frame for acute bleeding is 5 d as defined by the Baveno consensus[40]. Indeed, benefits of e-TIPSS placement following oesophageal variceal bleeding have been observed for up to 28 d after index endoscopy[55,56]. Therefore, a more pragmatic approach to the time window for e-TIPSS is an important consideration when designing future trials.

CONCLUSION

The role of e-TIPSS in acute variceal bleeding requires further prospective study with adequately powered trials. Studies should focus on careful patient selection, investigate optimal timing of TIPSS, and explore quality of life and health economics.

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World J Gastroenterol 2021 November 28; 27(44): 7625-7648

DOI: 10.3748/wjg.v27.i44.7625

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

REVIEW

COVID-19: Effect on gastroenterology and hepatology service provision and training: Lessons learnt and planning for the future

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Author contributions: Anjum MR led with literature search and submission of the article; Anjum MR and Chalmers J drafted the article; Chalmers J, Hamid R, and Rajoriya N contributed to editing of the article; Hamid R and Rajoriya N did critical appraisal of the article; Rajoriya N is the guarantor of the article.

Conflict-of-interest statement: All authors have no conflict of interests to declare.

Country/Territory of origin: United Kingdom

Specialty type: Gastroenterology and hepatology

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

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

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Abstract

In late 2019, reports arose of a new respiratory disease in China, identified as a novel coronavirus, severe acute respiratory syndrome coronavirus 2. The World Health Organisation named the disease caused by the virus 'coronavirus disease 2019 (COVID-19)'. It was declared a pandemic in early 2020, after the disease rapidly spread across the world. COVID-19 has not only resulted in substantial morbidity and mortality but also significantly impacted healthcare service provision and training across all medical specialties with gastroenterology and Hepatology services being no exception. Internationally, most, if not all 'non-urgent' services have been placed on hold during surges of infections. As a result there have been delayed diagnoses, procedures, and surgeries which will undoubtedly result in increased morbidity and mortality. Outpatient services have been converted to remote consultations where possible in many countries. Trainees have been redeployed to help care for COVID-19 patients in other settings, resulting in disruption to their training - particularly endoscopy and outpatient clinics. This has led to significant anxiety amongst trainees, and risks prolongation of training. It is of the utmost importance to develop strategies that continue to support COVID-19-related service provision, whilst also supporting existing and future gastroenterology and Hepatology services and training.

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Received: March 27, 2021
Peer-review started: March 27, 2021
First decision: June 14, 2021
Revised: June 28, 2021
Accepted: November 15, 2021
Article in press: November 15, 2021
Published online: November 28, 2021

P-Reviewer: Zelai NT
S-Editor: Liu M
L-Editor: A
P-Editor: Liu M

Changes to healthcare provision during the pandemic have generated new and improved frameworks of service and training delivery, which can be adopted in the post-COVID-19 world, leading to enhanced patient care.

Key Words: COVID-19; Gastroenterology; Hepatology; Training; Service provision

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Core Tip: The coronavirus disease 2019 (COVID-19) pandemic has led to adverse effects on many aspects of life. Healthcare professionals have faced unique challenges with COVID-19 including surges in cases with easing of lockdown measures, the emergence of new variants, and the roll-out of mass vaccination. The pandemic has had a largely negative impact on gastroenterology and hepatology service provision and training across the world. These difficulties have affected job-roles and training across the medical profession. We review the available evidence on the COVID-19 disruption to gastroenterology and hepatology service provision and training, discussing recommendations to minimise the interference going forward.

Citation: Anjum MR, Chalmers J, Hamid R, Rajoriya N. COVID-19: Effect on gastroenterology and hepatology service provision and training: Lessons learnt and planning for the future. *World J Gastroenterol* 2021; 27(44): 7625-7648
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7625.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7625

INTRODUCTION

In December 2019, China's regional World Health Organisation (WHO) office was informed of a new respiratory disease of unknown cause[1], detected in Wuhan, Hubei Province, China. It was characterised as a novel coronavirus strain, severe acute respiratory syndrome coronavirus 2; the WHO subsequently designated the disease caused by the virus as 'coronavirus disease 2019 (COVID-19)'. The virus spread rapidly and a Public Health Emergency of International Concern was declared at the end of January 2020, followed by pronouncement of a global pandemic in March 2020. The WHO asked for global preparedness to detect and manage COVID-19[1], urging countries to ensure they had adequate hospital resources and functional test-and-trace systems. The epicentre of the pandemic shifted to Europe in March 2020 and cases began to rise exponentially in the United Kingdom. The first United Kingdom case of COVID-19 was in a 75-year-old lady on February 21, 2020, identified through a retrospective analysis of sputum samples by the University of Nottingham[2]. At the time of writing, COVID-19 has claimed over 128000 Lives in the United Kingdom[3] and over 3.9 million worldwide[4]. These figures from United Kingdom pertain to individuals that died within 28 d of a positive test; they do not consider the likely substantial number of indirect deaths. With the global spread of COVID-19, there has been an unprecedented impact on healthcare services. Wards and intensive care units (ICUs) have been inundated with COVID-19 patients, and healthcare staff have been redeployed from their base specialities to help care for COVID-19 patients. Resource scarcity, in addition to the risk of virus transmission with face-to-face interaction means that speciality services have been decimated. In the field of gastroenterology and hepatology, many different aspects of service provision have been affected such as a significant reduction in diagnostic and therapeutic endoscopy. Out-patient clinics have been cancelled or moved to remote consultations; liver transplantation programmes have been temporarily halted or limited to only super-urgent transplantation at times. These changes, combined with the redeployment of trainees to COVID-19 wards and ICUs, have resulted in restricted gastroenterology and hepatology training, and an associated impact on trainees' morale. This article will review the available evidence regarding gastroenterology and hepatology service provision and training during the pandemic. We will provide an overview of the disruption caused by the COVID-19, and also review recommendations to minimise the impact of this disruption. The United Kingdom will be used as a reference, as it is

familiar to the authors; other countries will be compared and contrasted throughout. At time of writing, the United Kingdom healthcare system is dealing with a further peak of infections (January/February 2021).

THE EFFECT OF COVID-19 ON GASTROENTEROLOGY AND HEPATOLOGY SERVICES

An overview of the disruption

Worldwide, most countries imposed national lockdowns to tackle rising COVID-19 cases. In the United Kingdom, this was first implemented on March 23, 2020. In the NHS most elective medical and surgical work was halted in an attempt to increase availability of bed spaces for COVID-19 patients requiring hospital admission. Staff were redeployed to departments with increasing pressures - primarily ICUs, Acute Medicine and Respiratory wards with doctors often required to fill other staffing vacancies such as ICU nursing. In gastroenterology and hepatology, the effects from COVID-19 have not been discriminatory; all ranks of staff, from junior doctors to consultants, have been affected[5-8]. In the United Kingdom, the British Society of Gastroenterology (BSG) issued consensus guidelines on endoscopy service provision, and the management of specific disease patient cohorts, including: Inflammatory bowel disease (IBD), chronic liver disease, immunosuppressed patients, coeliac disease, and on transjugular intrahepatic portosystemic shunt service provision[9]. Similarly, gastroenterology and hepatology associations and experts worldwide have issued consensus opinions and guidelines regarding the management of patients during the pandemic[10-15]; which will be expanded upon throughout this article.

Endoscopy

During the peak of the pandemic, a number of major gastroenterology societies worldwide advised to postpone non-urgent examinations[16] including: The European Society of Gastrointestinal Endoscopy[17], the World Endoscopy Organisation[18], the American College of Gastroenterology, the American Society for Gastrointestinal Endoscopy[19], the Canadian Association of Gastroenterology[20], joint statements from Indian Gastroenterology societies[21], and the Gastroenterological Society of Australia[22]. Recommendations from major organisations have been summarised in Table 1.

In conjunction with other position statements and expert opinions[16,17,23,24], the BSG issued a statement during the first wave of the pandemic stating that 'all non emergency Gastrointestinal (GI) endoscopic procedures should stop immediately'[25], for an initial period of six weeks. The recommendation included the Bowel Cancer Screening Programme (BCSP), symptomatic two-week-wait (2WW) and Urgent Suspected Cancer (USC) referrals. The statement concluded that very few patients were likely to come to harm from the pause, highlighting that the lack of ICU beds and potential risk of higher surgical mortality during the initial COVID-19 peak may in fact cause harm if endoscopy was continued as before. The BSG accepted that a small number of patients may have a delay in their diagnosis[26]; and advised that 2WW and USC referrals should be individually triaged and risk-assessed by gastroenterology consultants. An 'Urgent Deferred Waiting List' was created, so as to prioritise follow-up and investigations when services resumed. Of note, urgent 2WW referrals by primary care physicians, primarily for patients with suspected cancer, decreased by up to 80% during the first peak of the infections[27]. Endoscopy service disruption occurred worldwide. Alboraie *et al*[7] obtained data from 163 participating centres, across 48 countries and 6 continents. The majority (93.9%) of the centres were hospital-based endoscopy units, affiliated with teaching hospitals. The centres reported a significant reduction in their procedural numbers during the pandemic: 85% reported that procedure volume decreased by > 50%, with only emergency services being continued, and four endoscopy units (2.45%) completely suspended procedures. The top three indications for endoscopic procedures included upper GI bleeding (89.6%), lower GI bleeding (65.6%), and cholangitis (62.6%)[7]. A study from Melbourne, Australia also demonstrated that significantly fewer procedures were performed during the peak of COVID-19, as compared to the pre-pandemic era[28]. A survey of 123 North American gastroenterology practices, comprising of 1379 Gastroenterologists in 32 United States states and 4 Canadian provinces observed a 90% decrease in endoscopy volume during the COVID-19 pandemic[29]. A further survey of 252 centres from 55 countries, reported a consistent reduction in endoscopic activity across all continents[30]. The United Kingdom's BSG issued guidance on the resumption of

Table 1 Summary of advice on Endoscopy service provision during the peak of COVID-19 pandemic from major societies around the world (March-April, 2020)

World Endoscopy Organization[18]
Postpone routine and elective procedures.
Take patient temperature at presentation, and screen for travel to high risk area, contact with COVID patient and occupational exposure.
Upper GI procedures (OGD, EUS, ERCP) carry highest risk of aerosolization.
Colonoscopy and flexible sigmoidoscopy carries some risk of aerosols generation.
In a positive patient or those at high risk of COVID, only perform highly urgent/emergent procedures.
Use enhanced PPE during Upper GI procedures, and standard PPE with surgical mask during lower GI procedure but use enhanced PPE if available or if high risk patient.
Perform GI endoscopy in negative pressure room if available.
If, endo-tracheal intubation required, perform in negative pressure room and minimize staff in the room during intubation.
Standard endoscope reprocessing is sufficient to kill COVID virus.
Essential person only in the room to conserve PPE.
Consider pre-procedure COVID testing for risk stratification.
European Society of Gastrointestinal Endoscopy[17]
Postpone all elective and surveillance endoscopy.
Case by case triage for non-urgent/emergent procedures.
Appropriate training of staff on the infection prevention strategies for COVID.
Health Care Professionals in endoscopy units should be triaged daily for sign symptoms of COVID and tested if needed.
COVID can effectively be inactivated by commonly used disinfectants having virucidal activity, so, reprocessing of flexible endoscopes and endoscopic accessories should be performed according to published guidelines.
Cleaning the endoscopy unit with virucidal agents is recommended as infection by contact is possible.
If feasible, online care should be provided (e.g. telemedicine) for pre-procedure clinics and assessment.
Before procedure, both patient and health care professional to use surgical face mask and face shield/visor if available. Temperature check all patients.
Relatives and caregivers should not have access to the GI endoscopy unit.
For patients who are considered at high risk for COVID, separate pre- and post-GI endoscopy recovery areas(or timeslots) should be arranged.
Same enhanced personal protection measures are recommended for all procedures, both upper or lower GI endoscopies for simplification.
Use negative pressure procedure rooms if available for high risk or positive COVID patients.
Post-procedure, consider tracing and contacting patients at 7 d and 14 d to inquire about any new COVID diagnosis, or development of COVID symptom.
American Society for Gastrointestinal Endoscopy[19]
Postpone on urgent procedures.
On arrival patients have their temperature checked and screened for COVID symptoms, contact or travel history.
Guidance on use of PPE.
Use negative pressure rooms if available.
Reprocessing of endoscopes as per standard guidelines.
Contact patient 14 d after the procedure to inquire about any COVID symptoms.
British Society of Gastroenterology[25,31]
All non-emergency GI endoscopic procedures should stop immediately, including Bowel Cancer Screening and fast-track referrals.
All emergency upper GI endoscopic procedures are classified as AGPs, irrespective of the COVID status of the patient, because the virus can be shed before any symptoms are present.
All staff in the room should wear PPE.
Case by case triage of cancer suspicious and other referrals.
Maintain a separate Urgent Deferred Waiting List to prioritise their proactive follow-up and investigation when services resume.

Subsequent guidance recommended to consider pre-procedural symptom screen and COVID testing with separation of high risk COVID sites from COVID minimised sites for low risk patients.
Indian Society of Gastroenterology[21]
Postponed routine non-urgent procedures
Screen patients pre-procedures with symptoms screen, travel and contact history.
Take temperature of all patients pre-procedure.
Minimum number of staff in the procedure room.
Use appropriate PPE based on risk assessment and stratification.
Standard disinfection processes are effective against COVID.
Surgical masks for patients' use too, if they have respiratory symptoms.

AGP: Aerosol-generating procedure; ERCP: Endoscopic retrograde cholangiopancreatography; EUS: Endoscopic ultrasound; GI: Gastro-intestinal; OGD: Oesophago-Gastro-Duodenoscopy; PPE: personal protective equipment.

GI endoscopic services in the United Kingdom[31] during the recovery phase after the pandemic's first peak in 2020. In addition to restoring specialist staff, it was advised that there needed to be increased infection control to minimise peri-procedural infection spread[32], including additional time and space for procedures, secure supplies of personal protective equipment (PPE), and the need for COVID-19-minimised facilities, where COVID-19 positive patients, are separated from those that are unlikely to have the infection. To accurately separate patients, individuals were screened for symptoms and tested for COVID-19 prior to endoscopy, with COVID-19 patients often deferred to the end of an endoscopy list, or indeed their procedure undertaken in a different hospital area. Similar guidelines were issued by gastroenterology organisations in other parts of the world, *e.g.*, America[33], Europe[17] and Asia[34]. A multi-centre prospective study of COVID-19 transmission in 6208 patients having outpatient endoscopy, found low prevalence of transmission peri-endoscopy if performed in a 'COVID-19-minimised pathway', which consisted of symptom screening and/or a COVID-19 reverse transcriptase polymerase chain reaction (RT-PCR) swab of patients prior to procedure. In total, 2611 patients in the study had a COVID-19 swab pre-procedure, and only 3 tested positive, all of whom were asymptomatic. None of the patients developed symptoms of COVID-19 within two weeks after the procedure based on telephone follow-up[35]. It should be noted that data for this study was collected during the recovery phase of the first peak in United Kingdom (Summer 2020), when community prevalence of COVID-19 was low. In contrast, Alboraie *et al*[7] found that 25.8% of participating centres (from 48 countries) reported positive cases of COVID-19 amongst patients within two weeks of their procedure date, although the percentage of positive cases within each individual centre was not reported. The data, however, included patients requiring urgent endoscopy and was not limited to COVID-19-minimised sites, which may have accounted for the higher infection rates. GI endoscopic procedures generate significant aerosol[36]. Therefore, the importance of air flow was imperative, as it was recognised that post-procedure turnover time or 'down-time' was required to let aerosolised particles settle and therefore reduce potential cross-infection. The down-time is dependent on air flow cycles within the procedure room to reduce the particulate burden of air, the procedure type and the Covid status of patient[37]. Infection control reviews of some units revealed suboptimal air exchange in endoscopy rooms and a lack of infection prevention training in some units. A survey of 83 institutes by Hungarian Society of Gastroenterology revealed 33.33% participants had infection prevention training, less than 1% said that they have negative pressure procedure rooms, and only 20% reported that they have some form of mechanical ventilation or air purification system[38]. It was not easy to implement all the recommendations quickly with resultant delays in resumption of endoscopic services. These operational issues and others, such as: redeployment of endoscopy staff, reconfiguration of endoscopy units/ service pathways, pre-procedure triage of patients and COVID-19 testing, and enhanced PPE significantly added to the workload of staff.

Colorectal cancer services

The COVID-19 pandemic has had a major disruptive impact on colorectal cancer service provision. Deviation from the national guidelines was observed at every point

in the patient care pathway including referrals, endoscopy/colonoscopy, surgery provision, surveillance and patient follow-up[39]. The degree of disruption varied between units both in the United Kingdom[40] and internationally[39,41]. Following cessation of non-emergency endoscopy and BCSP during the first wave of COVID-19, Rutter *et al*[42] analysed the United Kingdom's 'National Endoscopy Database'[42], and demonstrated that endoscopic activity reduced significantly compared to pre-COVID-19 Levels by an average of 12%, but declining to 5% at the lowest point (Figure 1). The authors reported an increase in the per-procedure cancer detection rate, owing to labour-intensive triage of the existing endoscopy waiting lists and of new referrals by senior clinicians. Despite this, the weekly number of cancers detected decreased by an average of 58% compared to pre-COVID, suggesting 72% of colorectal, 37% of oesophageal, 52% of gastric, and 19% of pancreato-biliary cancers may have been missed. This was a substantial and concerning reduction in cancer detection due to decreased endoscopic activity. Reduced procedure rates were confirmed by other studies, with reports of decreased adherence to colorectal screening programmes[39], and an 81% drop in colonoscopic activity, during the pandemic[7]. Maringe *et al*[43], modelled the impact of diagnostic delays on patient survival in the United Kingdom, and estimated that there would be 15.3%-16.6% additional deaths in colorectal cancer patients, and an increase of 5.8%-6.0% in deaths by oesophageal cancers. This data highlighted the severe consequences of reduced endoscopy services during the COVID- 19 pandemic, with substantial foreseeable increases in the number of avoidable cancer deaths, as a result of these diagnostic delays. A cross-sectional study from the United States assessed the weekly number of newly identified breast, colorectal, lung, pancreatic, gastric and oesophageal cancers during the COVID-19 pandemic. The authors found that detection fell 46.4% (from 4310 to 2310) for the 6 cancers combined[44].

Management of liver diseases and liver transplantation

COVID-19 affected liver transplant services throughout the world. In the United Kingdom, 7 adult and 3 paediatric centres performed 8740 Liver transplants performed in the United Kingdom in the last ten-year period[45]. National Health Service Blood and Transplant and the United Kingdom Liver Advisory Group updated and reviewed guidance throughout the pandemic. Liver transplantation centres were advised to continue procedures on a case-by-case basis - largely for patients in a clinically urgent category [based on high 'United Kingdom Model for End-Stage Liver Disease' scores or hepatocellular carcinoma (HCC) on verge of going out of transplant criteria]. Where possible, activity was continued for all super-urgent liver transplantation. Age for donation after brainstem death was restricted to 60 years and donation after circulatory death restricted to 50 years on March 23, 2020, with a projected transplant reduction of 51%[46]. After further appraisals, these donor criteria were relaxed later in the 2020. During the peaks of COVID-19, some centres temporarily suspended all liver transplant activity (except for extremely super-urgent cases) due to a surges in hospital admissions, shortages of ICU beds, and organ procurement restrictions implemented by the organ donation authority[47,48]. The number of liver transplantations in the United Kingdom fell by 84% (3 nationally per week) during the first COVID-19 peak[46]. On average, the number of patients on the United Kingdom liver transplant waiting list at the end of each month between January 2019 to March 2020, was above 400. This number fell rapidly to 109 during the first peak, by May 2020, with only clinically urgent patients being left on the waiting list and the remaining patients temporarily suspended[46]. At the time of writing, the United Kingdom again has severe restrictions in place for liver transplantation activity, due to a further surge of COVID-19 infections (February 2021). Analyses from the National Organ Procurement Agency in France, and the United Network for Organ Sharing in the United States, in April 2020, demonstrated a reduction in solid-organ donor transplantations by 90.6% and 51.1% respectively[49]; this was predominantly due to fewer kidney transplants, but there was also a substantial decrease in liver, lung and heart transplants. The American Association for the Study of Liver Diseases (AASLD) recommended postponing liver transplantation, advising each programme to consider its capability regarding ICU beds, ventilator availability and blood donation[50]. The Saudi Association for the Study of Liver Diseases and Transplantation published a position statement, advising that efforts should be made to persevere with normal transplant activity, but with the adoption and flexibility of individual transplant pathways, including virtual tele-medicine consultation to avoid patient contact and stricter preventive measures[51]. The European Association for the Study of the Liver (EASL) advised that centres should prioritise patients who have a poor short-term prognosis without liver transplant, including those with acute liver

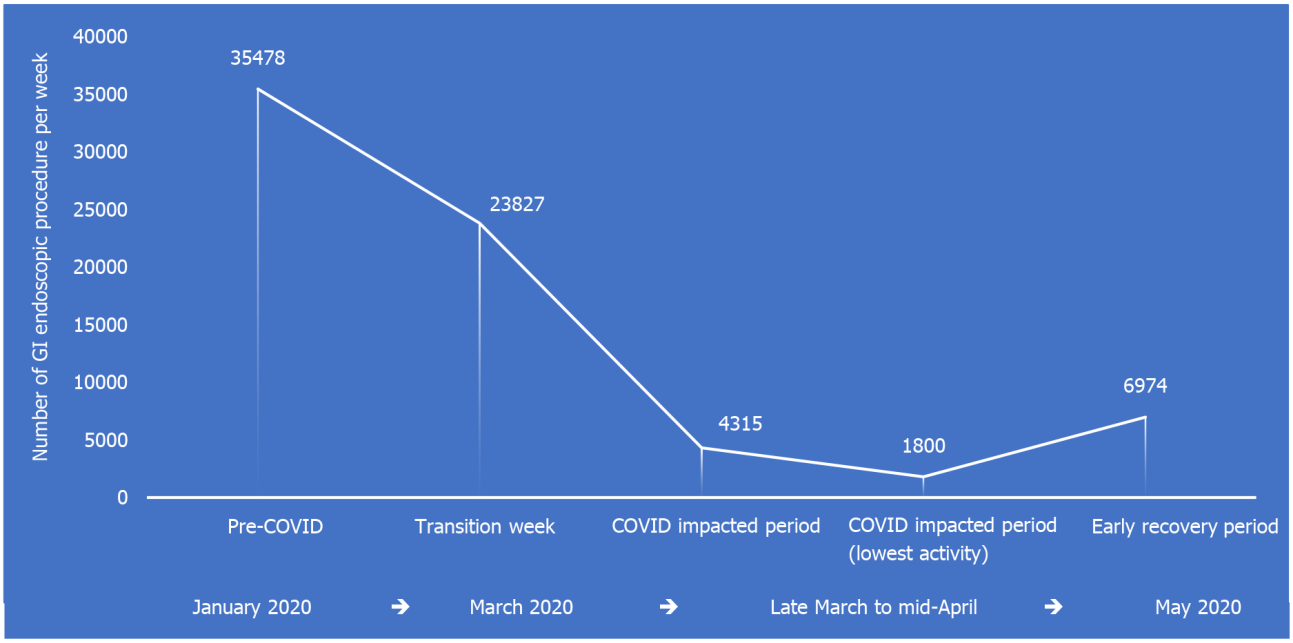


Figure 1 Reduction in United Kingdom wide endoscopic activity (cumulative number of procedures/week) during 1st peak of COVID-19 infections. Source: United Kingdom's National Endoscopy Database analysis, Rutter *et al*[42].

failure, high MELD score and HCC[52]. The liver transplant society of India recommended a moratorium on all non-urgent transplants during the initial COVID-19 peak[53]; similarly, the Pan-Arab Association of Gastroenterology recommended that non-urgent transplants should be postponed[54]. Hepatology societies across the world provided guidance for the management of patients with liver diseases in both inpatient and outpatient settings, and on liver transplant (Figure 2). The AASLD[55], EASL[56] and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID)[13] all advised: To separate COVID-19 positive and negative in-patient cohorts with liver disease, minimise contacts by reducing staff levels during ward rounds, limit the number of investigations performed and have restrictions on visitors. Similarly, for outpatients, the societies recommended that units offer remote consultation for appointments and only perform essential investigations. Bollipo *et al*[10] summarised these recommendations including advice on the management of liver transplant, endoscopy, liver cancer, and both inpatient and outpatient care. A multicentre, retrospective, cross-sectional study by Amaddeo *et al*[57] found that fewer patients with HCC presented to the multidisciplinary meetings, and had a treatment delay that was longer in the COVID-19 period than in 2019.

IBD services

Outpatient IBD services were severely impacted during the pandemic with cancellations of clinic appointments and subsequent conversion to remote consultations[58]. IBD surveillance endoscopy services were temporarily halted as a non-urgent endoscopic procedure. This has affected the follow-up of known IBD patients, as well as new IBD cases. With regards to new patients, cases were triaged to assess urgency and consideration made to delay diagnostic endoscopy and imaging in those with mild symptoms and moderate biomarkers[59]. It was recommended that initiation of therapy be on a case-by-case basis. Known IBD patients were advised to continue their current medications given the increased risk of infection associated with active disease and hospitalisation. Similar guidelines were issued by other IBD societies around the world. The European Crohn's and Colitis Organisation advised that centres adopt virtual consultations, use a home faecal calprotectin test for monitoring, and limit endoscopic evaluations to those patients in whom it was felt to be absolutely necessary [60]. Recommendations to minimise contact and reduce endoscopic intervention were echoed in guidance from the International Organization for the study of Inflammatory Bowel Disease and Crohn's and Colitis Foundation of America[61]. Healthcare providers adopted and modified their IBD unit protocols to minimise both patient-patient and patient-health care worker contact. Centres also screened patients for COVID-19 symptoms before they attended infusion units[62]. A significant proportion of new IBD patients were diagnosed and treated without having endoscopic or

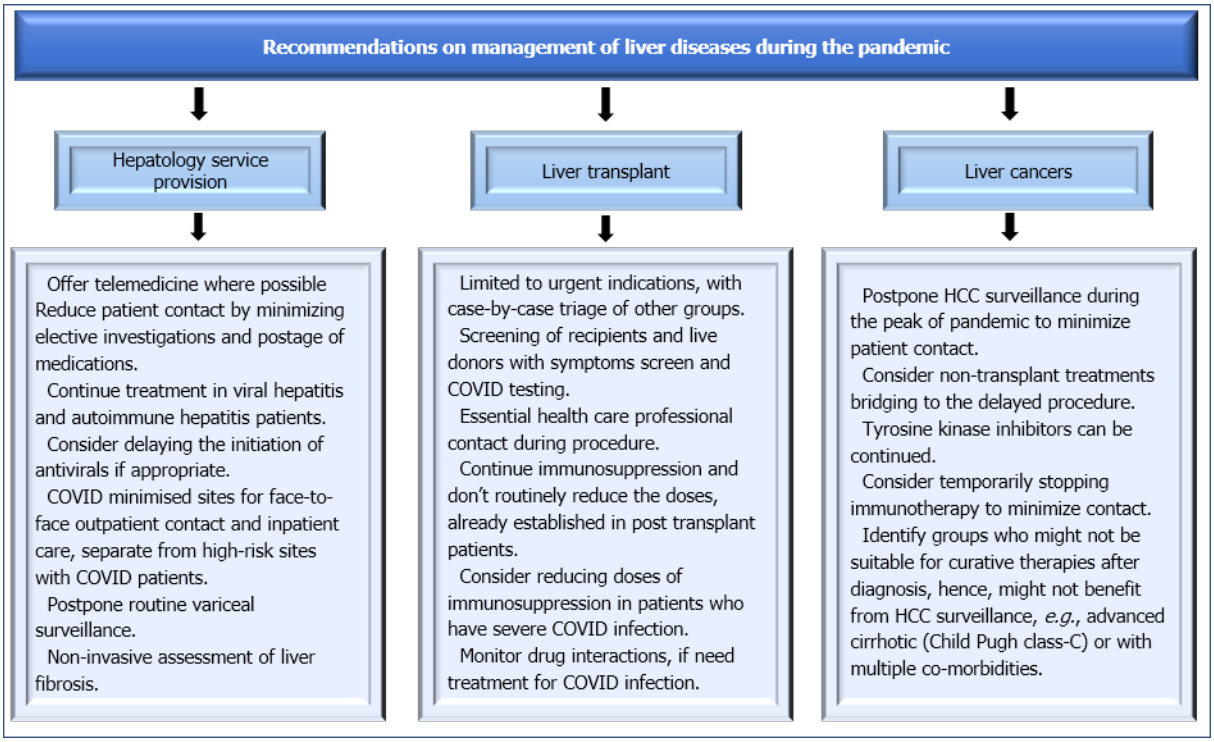


Figure 2 Summary of recommendations from major Hepatology societies across the world on management of liver diseases during the pandemic. Sources: American Association for the Study of Liver Diseases[55]; British Society of Gastroenterology[9]; European Association for the Study of the Liver[56]; Indian Gastroenterology Society[21]; International Liver Transplantation Society[10]; Pan-Arab Association of Gastroenterology[54]. HCC: Hepatocellular carcinoma.

histological evaluation[63]. Based on early observations from China and Italy, IBD patients were felt to be at higher risk of severe COVID-19, particularly older patients with comorbidities, patients on high doses of systemic corticosteroids, and patients with active disease[64,65]. The United Kingdom's BSG issued guidance to stratify IBD patients into low, medium and high-risk of a poor outcome from COVID-19 infection [59]. High-risk patients included those on: (1) Concomitant therapies (immunomodulator and biologic) who were over 70 and/or had selected co-morbidities; (2) Those who were receiving daily steroids (≥ 20 mg prednisolone or equivalent); (3) Those who had recently commenced biologics with immunomodulators or systemic steroids; and (4) Those who had short bowel syndrome and required nutritional support or were on parenteral nutrition. IBD patients identified as high-risk were advised to shield by the United Kingdom government during the peaks of the pandemic; they were advised to stay home wherever possible and only to leave the house for essential reasons including medical appointments. This risk assessment was consistent with findings from Brenner *et al*[66] of 525 international COVID-19 cases reported to the 'Surveillance Epidemiology of Coronavirus Under Research Exclusion for Inflammatory Bowel Disease' (SECURE-IBD) registry[66]; 50% of IBD patients with a severe COVID-19 outcome (*i.e.*, ICU admission, ventilator use, and/or death) were over 70 years of age, and 50% of those who died had cardiovascular co-morbidities. The SECURE-IBD registry was also analysed to assess COVID-19 outcomes with various IBD medications. The major finding from these studies[66,67], was that thiopurine treatment, as both monotherapy and combination therapy, was associated with an increased risk of severe COVID-19 infection when compared with tumour necrosis factor alpha (TNF-α) antagonist monotherapy. Further findings were that TNF-α antagonist monotherapy may have a protective effect against severe COVID-19 and that no significant differences were observed in outcomes when comparing classes of biologics[67]. This data was consistent with previous findings that thiopurines increase the risk of viral infections compared with TNF-α antagonists[68]. This was reflected in real-world practice; Sharma and Meade[58] assessed prescribing of IBD medications in a United Kingdom tertiary hospital and found increased de-novo biologic therapy compare to pre-pandemic, particularly among thiopurine-naïve patients. The BSG recommended: not to stop current medications (to avoid any flares of IBD), to consider anti-TNF monotherapy if needed and to avoid immunomodulators[59]. A panel of 15 IBD experts convened to review the management of acute severe ulcerative colitis

(UC) in the context of COVID-19, recommending that patients with UC flares should be isolated throughout their hospital stay, and Infliximab along with steroids should be considered as a rescue therapy if needed. It was not deemed appropriate to commence thiopurine therapy for maintenance however steroids and Infliximab continuation was considered more appropriate[69]. Provision for follow-up and investigations to monitor disease response in IBD patients was impacted throughout the pandemic, providing a further rationale for initiating treatment with more efficacious agents earlier. Kennedy *et al*[70] surveyed 125 IBD services during the first peak of COVID-19 infections and found that there was a significant reduction from baseline in whole-time equivalent gastroenterologists and IBD nurses providing elective outpatient care. Of concern, 27% of services reported no access to faecal calprotectin, and a further 32% reported reduced access to faecal calprotectin testing [70], making management more challenging. Curtailment in IBD-specific services, *e.g.*, outpatient services, endoscopic services, regular multidisciplinary meetings to discuss complex patients and re-deployment of specialist nurses was accompanied by an increase in IBD helpline queries from patients[70]; 94% of services reported an increase in IBD helpline activity. In summary, worldwide, organisations made significant changes to ensure safe care provision to the IBD population. These adaptations included: diagnosing IBD clinically in several instances without endoscopic, histological and in most cases radiological investigations, remote prescribing/ Laboratory investigations and medicine infusion arrangements outside of high risk COVID-19 main hospital sites along with provisions of tele-consultations for patients [63,70,71]. Due to rapid changes in IBD patient services, governance structures for development of novel ways of working remains a major focus. Changes to IBD delivery structure should be discussed with patients where possible. In a survey of 685 IBD patients[72], participants reported that the COVID-19 pandemic has had a negative impact upon their psychological well-being and quality of life. The patients reported an increase in perceived stress with 39% of respondents worried about their IBD care, but respondents were happy with delivery of care remotely.

Outpatient gastroenterology and hepatology clinic provision

Outpatient clinics were disrupted by COVID-19, with temporary suspension of many routine outpatient clinics, and with conversion of standard outpatient face-to-face clinic consultations to telephone or virtual appointments - referred to as "telemedicine". Emergency clinic appointments were provided depending on clinical urgency, with some centres adopting temporary email addresses or telephone helplines for patients to contact the hospital. As the first wave subsided, face-to-face appointments were re-introduced in some centres, depending on local COVID-19 policies; however, telemedicine has now been adopted and sustained by many gastroenterology and hepatology outpatient services including viral hepatology and IBD clinics. The advent of telemedicine preceded the pandemic[73], but its use has increased exponentially in the past year, with a study from New York[74] reporting an 8729% increase in the use of video consultations during COVID-19, accounting for 21.9% of outpatient visits. Worldwide, medical organisations issued guidance regarding the use of telemedicine[75-77] outlining recommendations for establishing telemedicine systems, as well as ethical considerations. Broadly, telemedicine is considered appropriate for patients with straightforward complaints, who do not require physical examination, feel comfortable using the required technology, and who can be provided with all necessary information and prescriptions remotely. Key principles regarding patient identification, capacity, consent and safe information storage hold true for telemedicine as they do for in-person consultations. A multi-modal telemedicine network was established in Sichuan Province, Western China, in January 2020, in response to the COVID-19 pandemic[78]. The network synergised a new 5G service, a smartphone application, and an existing telemedicine system; it was funded in the short-term by disaster funds. An expert group was established to provide education to medical staff. By March 23, 2020, the authors reported that 9085 patients had received online consultations or interventions through the application, and 1094 *via* telephone. Four hundred and twenty-four consultations were conducted for severe and critical COVID-19 patients highlighting the substantial potential of telemedicine. In addition, radiologists used the network to successfully perform remote computed tomography (CT) scanning of 152 patients, allowing quality imaging in areas with a severe shortage of qualified technicians. Various other organization have issued guidelines for remote consultations[79-81]. Key principles include: Correct identification of patient at start of teleconsultation and use of available medical records/referral letters in conjunction with patient history to decide whether a safe management plan can be made remotely, or is face to face consultations required. Any

remote examinations requires patient consent, with the United Kingdom's GMC recommending the use of a chaperone during video consultation in a same way as one would do during face-to-face consultation[82]. Medicolegal organizations have also generated their recommendations on tele-consultations on similar basic principles[83]. Similar recommendations to the United Kingdom GMC guidelines were issued for physicians by Ghosh *et al*[84] in India. The American Telemedicine Association has described similar principals and framework of remote consultations even before the pandemic[85]. The impact of telemedicine has been studied by Lee *et al*[86] in the setting of liver transplantation in a randomised prospective trial; 106 patients were randomised to standard of care face-to-face practice, or telemedicine home-based care - utilising tablets, video-calls, and texts. Participation rates, quality of life and 90-d hospital re-admission rates were compared, with lower re-admission rates at 90 d (28% *vs* 58%, $P = 0.004$) and improved quality of life with regards to physical function and general health in the telemedicine group. Munroe *et al*[87] designed a single-arm, crossover study during the COVID-19 pandemic. A telemedicine consult, *via* phone or video call, was offered to patients referred to the practice; patients could accept a virtual consult or request a review in person. The authors reported high levels of patient acceptance of telemedicine, and no discernible changes in outcomes or care-use related to medical decision-making, time to appointment or patient satisfaction. In a retrospective observational study from New York, United States, Ramaswamy *et al*[74] analysed feedback from telemedicine consultations pre- and mid-pandemic; in both cohorts, satisfaction was significantly higher with video consultations vs. in-person visits (94.9% *vs* 92.5%, $P < 0.001$). McKenna *et al*[88] surveyed 212 general neurological patients from Dublin, Ireland who had attended remote consultations and found that 76% of patients felt remote consultations were either "just as good" (67.1%) or "better" (9.0%) than face-to-face consultations. Those who reported remote consultations to be 'not as good' were older (52.3 years *vs* 46.6 years, $P = 0.045$) and had neurological conditions that required clinical examination (66.7%) or an undiagnosed condition awaiting investigation/ review (46.7%). The United Kingdom's Medical Protection Society assessed doctors' opinions on telemedicine[89]; of 1250 respondents 70% agreed that the benefits of telemedicine were 'unquestionable' during the pandemic. However, 80% feared that the doctor-patient relationship could 'break down' or were worried telemedicine might deny some patients' treatment. Almost three-quarters of doctors expressed concern about medically missing something in a remote consultation and 60% were worried about a claim or investigation.

THE EFFECT OF COVID-19 ON GASTROENTEROLOGY AND HEPATOLOGY TRAINING

Worldwide, COVID-19 has had a disruptive effect on the training of gastroenterology and hepatology doctors due to redeployment and cancellation of educational activities. This has been compounded in some settings by medical staff exhaustion and burnout. Most routine and non-emergency specialty services have been suspended, including a drastic reduction in endoscopic activity, and hence training opportunities for gastroenterology and hepatology trainees. Global shortages of PPE led to 'non-essential persons' being excluded from endoscopic lists[90] to conserve the supply in some hospitals. In addition, to minimise risk of infection, centres were advised to consider restricting staffing for procedures, limiting endoscopy to a small number of specialist consultants, and excluding trainees[91]. A survey of Australian gastroenterology trainees demonstrated a 75% reduction in endoscopic activity, with 30% of trainees prohibited from performing emergency endoscopy; again, to limit staff exposure and to conserve PPE[92]. Most training assessments, specialty exams, and continuous professional development (CPD) activities were cancelled during the first peak of COVID-19. In May 2020, the BSG surveyed its members and found that 66% of United Kingdom trainees were not doing any specialty clinics, 29% were unable to continue their formal research commitments, and 53% were unlikely to achieve their Annual Review of Competency Progression targets, which would therefore risk prolongation of training[93]. In a survey of Canadian gastroenterology trainees[94], 94% were concerned about achieving and/or maintaining clinical competence, and 71% were concerned about prolongation of training due to the pandemic. During the pandemic, trainees have experienced an increase in workload, often in new clinical environments, leading to exhaustion and burnout. This, alongside missed training opportunities, has generated anxiety and stress amongst specialty trainees. In an international survey of 770 endoscopy trainees from 63 countries[95], 52.4% of respondents reported anxiety

and 18.8% reported burnout. Cravero *et al*[96] surveyed 1420 Internal Medicine trainees from the United States, China, Saudi Arabia, Taiwan and other countries[96] ,and found that the trainees caring for COVID-19 patients were more likely to have worked additional hours compared to pre-pandemic, and that the incidence of reported burnout was proportionate to the number of COVID-19 patients that trainees had cared for. Fifty-nine percent trainees expressed concerns about their preparedness for independent practice while 20% trainees reported that pandemic has negatively effected the progress towards their career goals. Karampekos *et al*[97] surveyed Greek gastroenterology fellows, and fellowship programme directors regarding the impact of COVID-19 on endoscopy training. The two groups broadly agreed on the factors associated with a negative impact: an unknown timeframe of COVID-19 measures, cancellation of endoscopy, and fewer endoscopies performed by fellows. The fellows were significantly more concerned about their ability to acquire and/or maintain endoscopy competence than their programme directors (83.1% *vs* 27.8%, $P < 0.001$). In addition, proposed strategies to address training post-pandemic varied with fellows predominantly suggesting prolongation of training (49.4%) and programme directors suggesting an increase in daily workload (44.4%). This study highlighted the importance of involving both trainees and programme leaders in planning and decision-making for training during and after the COVID-19 pandemic. Finally, remote working (*e.g.*, telemedicine) has allowed some flexibility in doctors' working patterns - clinics can be performed from offices or from home with remote access to patient data. This was actively encouraged by some hospitals to reduce clinician footfall within the hospital during the COVID-19 pandemic. However, there was a resultant effect on training with fewer opportunities for direct learning and an inability to gain immediate, face-to-face advice from supervisors.

PROPOSALS FOR GASTROENTEROLOGY AND HEPATOLOGY SERVICE-PLANNING AND TRAINING PROVISION DURING THE COVID-19 PANDEMIC AND BEYOND

There has been a significant increase in the caseload of COVID-19 patients in recent months, and it is clear that the pandemic is far from over. In addition to direct COVID-19-related morbidity and mortality, there has been significant collateral damage, due to disruption in routine and urgent health services, such as cancer workload. After the 1st wave, the rate of COVID-19 related admissions slowed and the services began to recover. However with mutation of the virus, surges in COVID-19 cases recurred with most countries thereafter experiencing a 2nd and some 3rd waves. At time of writing, the delta-variant is on the increase in the United Kingdom. With experience gained in planning for surge capacity in hospitals, reconfiguration of services has been easier for many in the 2nd wave and now 3rd wave. Services however are constantly running at high volume, often exacerbated by winter pressures (in Europe) meaning healthcare services remain at constant risk of being overwhelmed. In this section, we discuss recommendations to ensure optimal continuity of patient care and gastroenterology/hepatology training during the pandemic (Figure 3), and indeed new approaches could continue well after the pandemic has ended. It should be reinforced that general principles of adequate PPE, social-distancing measures, robust contact-tracing systems and the roll-out of COVID-19 vaccines remain crucial pillars for controlling COVID-19 on mass population levels.

Protecting the workforce

Adequate PPE and infection-control measures are essential to allow staff members to remain safe whilst caring for COVID-19 patients. A study of 420 healthcare workers deployed to care for COVID-19 patients in Wuhan, China[98], demonstrated that effective infection prevention measures protected the workforce from getting infected - despite working an average of 100-130 h in the ICU, none of the participants reported symptoms of COVID-19 and all remained antibody negative. The study highlighted the importance of the procurement and distribution of PPE, as well as providing adequate training to healthcare professionals in its use. Thomas *et al*[99] appraised global PPE guidance and the available scientific evidence regarding aerosols, virus transmission, and respiratory protection. The authors concluded that there were shortcomings with the Public Health England's PPE guidelines and recommended urgent revision to protect the United Kingdom's NHS workforce during the pandemic. Rising infections and deaths amongst healthcare workers worldwide prompted calls

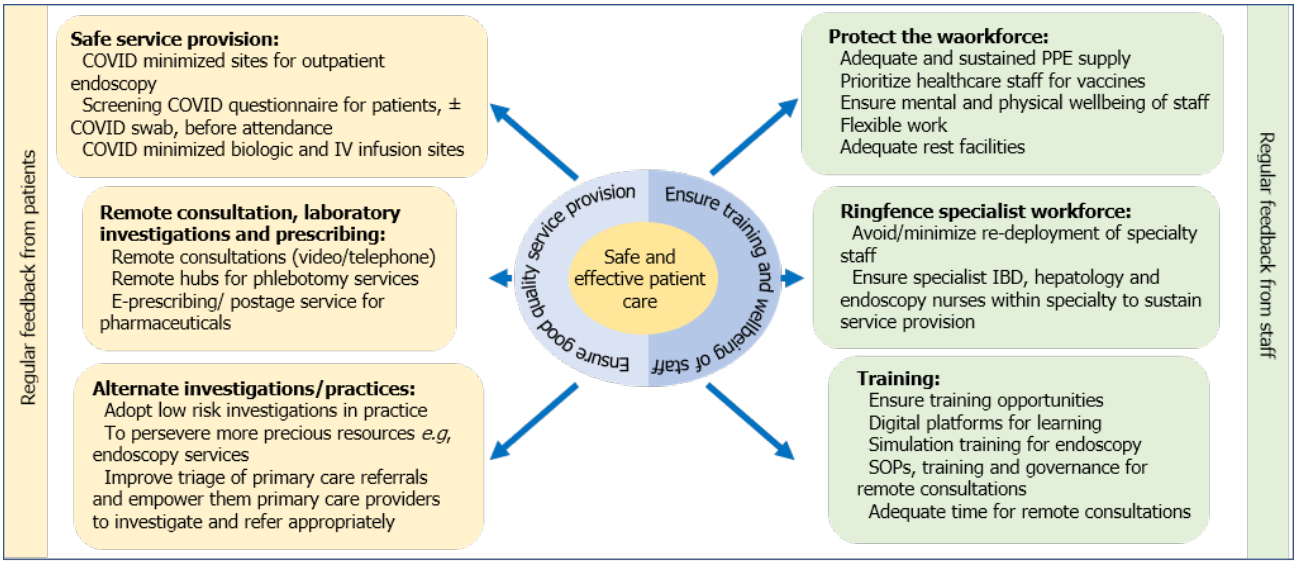


Figure 3 Recommendations for safe and effective care provision and continuation of training during Pandemic. IBD: Inflammatory bowel disease; PPE: Personal protective equipment; SOP: Standard operating procedure.

for urgent action and PPE provision[100-103]. In parallel to PPE provisions, the vaccine rollout remains key in protecting the workforce. In many countries, healthcare staff have been identified at a high-risk group and targeted for vaccination early. This is in conjunction with medically at-risk patients and also the elderly. Mental and physical wellbeing of healthcare workers is required for optimal performance, and health care organisations and medical unions should have resources in place. Hospital organisations should ensure where possible, that staff have adequate breaks on shifts, and there are adequate provision of rest facilities, especially given 'social-distancing' requirements. It is increasingly recognised that the pandemic will have a psychological impact on the majority of healthcare staff, and support must therefore be put in place [104]. Early experiences from China[105], and more recently from Europe[106], suggest that healthcare staff are likely to experience negative mental health outcomes due to the pandemic. In addition, with the increased workload burnout is becoming more common[96]. In the United Kingdom, NHS England recommended support and flexibility for staff working during the pandemic[107]. Tomlin *et al*[106] proposed a phased model of the mental health burden, in which stressors from different phases of the pandemic are considered and coping strategies were suggested for both the individual and the organisation for each stage. For example, in the preparation phase individuals should be aware of anxiety levels and stress triggers, and the organisation is advised to identify those who may experience challenges to their mental wellbeing. This may be particularly relevant to those with existing mental health difficulties, those with caring responsibilities and those who have recently survived a stressful or traumatic experience. By identifying vulnerable staff members and putting resilience and well-being plans in place, hospital organisations can take a proactive approach to supporting staff thereby minimising harm.

Ring-fence the specialist workforce

During surges of COVID-19 infections, much of the specialist workforce was redeployed to care for COVID-19 inpatients. Initially, it was assumed that a short period of disruption in services would cause minimal harm; however, more recent data has highlighted significant missed and delayed diagnoses as a result[42,43]. In subsequent waves of infection, a proportion of the specialist workforce where possible should aim to continue working within the specialty; for gastroenterology and hepatology this would include doctors, specialist nurses and endoscopy staff, all of whom are essential to sustain the components of the service - in-patient caseload, out-patient clinics and endoscopy lists. A balance between ongoing service provision for the COVID-19 pandemic and that of existing and ongoing services for gastroenterology and hepatology must be found.

COVID-19-minimised sites

The objective of 'COVID-19-minimised' sites, also known as 'cold' sites, is to

physically segregate COVID-19 patients from those that are not infected, ideally on separate sites (cold *vs* hot sites). This allows units to provide endoscopic and outpatient services to gastroenterology and hepatology patients while minimising their risk of COVID-19 contacts. The United Kingdom's BSG proposed a telephone screening questionnaire, termed the 'SCOTS criteria'[108]; 3-7 d prior to endoscopy, the patient is asked if they have symptoms of COVID-19 or have come into close contact with a known or suspected case in the preceding 14 d. They interviewer should also consider supplementary factors such as the patient's occupational risk of exposure, recent travel from a known risk area, and if that patient is in a shielded category. For patients reporting any of the SCOTS criteria, clinicians should consider if the procedure can be delayed for 14 d or if an alternative can be offered. If not, level 2 PPE should be used and procedure performed in a 'hot' location. Screening should be combined with a COVID-19 test pre-procedure. Kim *et al*[109] performed a meta-analysis of 19 studies, predominantly from China, to assess the diagnostic performance of RT-PCR, the commonly used COVID-19 screening test; they reported a pooled sensitivity of 89%. The negative predictive value reduced as COVID-19 prevalence increased, ranging from 99.9% with 1% prevalence to 93.4% at 39% prevalence. Despite this variation, the data suggests that combining a screening questionnaire with an RT-PCR swab prior to procedure would allow accurate triage of patients to either COVID-19-minimised or high-risk sites for endoscopy. COVID- 19-minimised sites ensure safe service provision and enhance patient confidence in attending healthcare facilities during the pandemic. With the advent of vaccinations, COVID-19 passports for visits for endoscopic procedures or to hospital may be considered when risk stratifying patients, however remains to be clarified based on long-term immunity data and risk of infection thereafter.

Training and governance for telemedicine

Despite advances in technology, telemedicine has been a novel concept for a substantial proportion of health care workers. There was no formal training or governance structure in place in many hospitals due to the speed of rollout of this modality during the pandemic. It is recommended, especially for junior trainees, that remote consultations are undertaken at a location where a consultant is available for opinion and supervision (*i.e.*, hospital clinic rather than from home). This would provide the structured approach and will enable trainees and supervisors to have close liaison and immediate contact for questions. Another format proposed could be mixture of face-to-face and remote consultations within a same session. This would ensure social distancing for visiting patients at intervals while having teleconsultations in between in appropriate patients. There are pros and cons to remote clinics[110]. The pros include working remotely both for clinician and patient without the need for travel thus minimizing COVID-19 contact risk and preserving PPE used during face to face consultations. Remote clinics also acts as initial triage of patients to identify those who would benefit from face-to-face consultation. Cons include no clinical examination and a lack of visual clues. Issues also may exist contacting patients or conversing with those not speaking native language to the healthcare professional. It is important to consider patients who either can't use or don't have access to the internet or telephone resources. Consultation from all stake holders is required before starting a new remote consultation service, otherwise conflicts may arise. One recent example was that Government of India launched the 'e-sanjeevani', a national teleconsultation service during the pandemic while Indian Medical Association issued an advisory against the use of telemedicine in most situations, creating confusion amongst medical community[111]. Appropriate allocation of time and planning is required for both to face-to-face consultations and remote consultations in terms of time allocations for appointment and job planning[112]. Clinical exam might not be performed for remote consultations but other logistic issues like arranging investigation requests and prescriptions are important part of remote consultations and require allotted time. From the patient perspective, these remote consultations should be booked and organized as timed slots as one would expect with face-to-face consultations, rather than them being called at random time or date.

Exploring alternative investigations and practices

Using evidence-based practices, alternative investigations and modified pathways (Figure 4) may have a role to replace some of the more resource-intensive services and tests.

Primary care referral triage: Advanced triage of primary care referrals is not a new concept. An example of this was The Royal Wolverhampton Trust, United Kingdom,

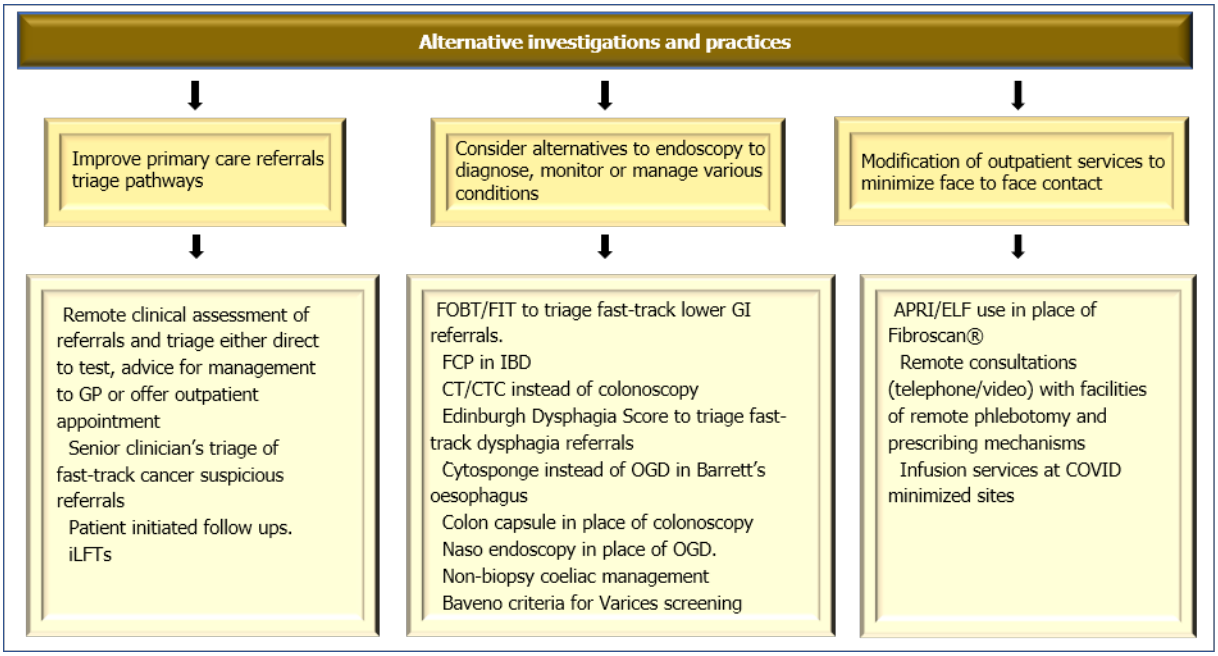


Figure 4 Recommendations for exploring alternative investigations and practices during the pandemic. FOBT: Faecal occult blood test; FIT: Faecal Immunochemical Test; FCP: Faecal Calprotectin; CTC: CT colonography; iLFT: intelligent liver function testing;APRI: aspartate aminotransferase to platelet ratio index; ELF: Enhanced Liver Fibrosis; OGD: Oesophagogastroduodenoscopy.

developing a 'Clinical Assessment Service' (CAS) in 2014-2016[113]. A Gastroenterologist reviewed primary care referrals, arranged investigations if necessary, and either discharged the patient back to primary care with advice, or arranged an outpatient appointment. The authors reported that 32% of triaged CAS patients were managed without the need for an outpatient appointment. In the first three years of using CAS, 3136 fewer outpatient appointments were required, which translated into a 481613 GBP cost saving. A Californian study of Rheumatology referrals reported similar results with 1/4 of e-referrals being resolved without a clinic appointment over a 4-year period[114]. The COVID-19 pandemic has demonstrated a need for widespread development of such services. During the pandemic, triaging has been used for 2WW and USC referrals; there is scope for this to be expanded to include many other primary care gastroenterology and hepatology referrals. Another concept is 'patient-initiated' follow-up or review, which has been pioneered by NHS Scotland (United Kingdom) during the pandemic[115]. For certain patients - those who are stable or maintained on long-term treatment, an alternative approach can be offered, in which the patient requests reviews based on their wants and needs, rather than being allocated routine appointments. Caveats exist for this method: patients require clear guidelines for when to request reviews and they must be able to confidently self-manage their condition. As a result a patient-initiated approach is unlikely to be suitable for certain cohorts *e.g.* alcoholic liver disease patients. In March 2019, Whear *et al*[116] conducted a meta-analysis of 17 randomised trials assessing patient-initiated follow up in patients with chronic health conditions. The authors demonstrated that patient-initiated appointment approach had little or no effect on patient anxiety/depression and patient satisfaction scores, when compared with consultant-led appointment systems. The need to adopt such frameworks in routine practice has become crucial during the pandemic, to minimise contact and to prioritise service provision for those who need it most urgently.

Endoscopy: Endoscopy services have been disrupted and limited during the COVID-19 pandemic, and since services have resumed, there is added cost to each procedure due to associated infection-control procedures and PPE. Therefore, alternative pathways and resources have been trialled, to triage patients and streamline services. Faecal occult blood or Faecal immunochemical tests (FITs) have been used to triage 2WW lower GI suspected cancer referrals[117]. In 2019, six United Kingdom 'FIT pioneer sites' shared data regarding FIT positive and negative cancers, in a combined 9182 patients[118]. The number of FIT negative cancers was 0.01%-0.75%, with a negative predictive value of at least 99.05%. The incidence of FIT-negative cancers was

highest in those with iron deficiency anaemia, suggesting that alternative methods of triage may be required in these patients. A pre-pandemic single centre study from Scotland, United Kingdom of 5422 patients[119], assessed the use of FITs in significant bowel disease (SBD) including colorectal cancer, high risk adenoma and IBD. The findings suggested that use of FIT in conjunction with a full blood count and clinical assessment correctly identified 93.9% of all SBD. There were fewer referrals to secondary care (15.1% reduction) and an increased yield of SBD detection (13.9% to 20.5%), suggesting that patients were appropriately targeted for colonoscopy. Of those not immediately referred, only 0.7% were found to subsequently have SBD. Widespread implementation of FIT testing may therefore provide an effective and safe way to identify patients at risk of SBD. Faecal calprotectin is an effective tool to distinguish between IBD and functional GI disorders[120]. It also correlates well with endoscopic and histological disease activity in known IBD patients[121], and therefore is a suggestion that serial faecal calprotectin measurement could be used to monitor disease activity and to detect relapse early. During the pandemic, CT-colonography (CTC) could be used preferentially to optical colonoscopy to screen patients for colorectal cancer[122]. CTC imaging is acquired by a single healthcare worker, and requires only a limited duration of close proximity with the patient. It is performed with disposable equipment and there is minimal exposure to stool. Such radiological alternatives may therefore reduce the risks associated with COVID-19 and preserve PPE. Staffing requirements would be minimised, but it should be noted that radiology services may also see increased caseloads with surges in COVID-19 infections. Validated clinical questionnaires could replace previous initial evaluation pathways and 'direct to test' endoscopy referrals during and following the pandemic. The Edinburgh Dysphagia Score[123] uses six parameters to stratify patients into high and low risk of cancer: age, sex, weight loss, duration of symptoms, location of dysphagia and acid reflux. In 435 patients, it was found to have a sensitivity of 97.5% in correctly stratifying cancer patients to the high-risk group; 30% of referrals were stratified to the low risk group and could therefore be investigated less urgently. The Eckardt Score could be used for evaluation in achalasia[124]. Cytosponge is a nonendoscopic, ingestible, sampling device and may provide a non-AGP alternative for diagnosis of Barrett's oesophagus[125] during the pandemic. It has also been shown to have potential as a triage tool for endoscopy, in patients with mild to moderate dysphagia with suspected oesophageal cancer[126]. Similarly, colon capsule endoscopy is an ingestible device that allows visualisation of the bowel without attendance at hospital. It is equally effective when compared to colonoscopy for identifying polyps more than 10mm in size and more sensitive than radiological investigations in the detection of colorectal cancers[127]. It may be an effective and safe alternative to colonoscopy during the pandemic[128]. New methods to triage and stratify patients who require endoscopic procedures could significantly reduce the demands on this service. However, the clinical effectiveness of using these new approaches will need to undergo rigorous testing and trials to ensure patients with significant pathology are not experiencing delays to endoscopy, or indeed being missed altogether. The United Kingdom's BSG updated guidelines on polyp surveillance towards end of 2019[129] that could mean reduction in number of surveillance colonoscopy procedures[130]. The units needed to validate their existing waiting lists in view of this updated guidance but pandemic hit in early 2020. It is imperative that validation work continues, especially when there is additional backlog of procedures when the activity was reduced during the pandemic. Non-biopsy protocol for coeliac disease diagnosis could also avoid need for endoscopy in patients who are symptomatic and IgA TTG ≥ 10 × upper normal limit on two occasions or one positive IgA TTG accompanied by positive endomysial antibodies (especially in children). Clinicians should be mindful of proceeding with requesting endoscopy though, if there are alarm symptoms present or if the patients belong to older age groups (≥ 55 years)[131]. It is important to incorporate alternative pathways as much as possible, as a modelling study from United Kingdom suggests that even with mitigation measures, it may take till after 2022 to clear backlog of endoscopic procedures[132].

Hepatology: Routine (non-urgent) Fibroscan® services were deferred in many hospitals during the peak of the COVID-19 pandemic[133]. Tests such as the Enhanced Liver Fibrosis test[134] or AST to Platelet Ratio Index[135] could be used as an alternative to assess fibrosis remotely. For patients with cirrhosis for whom endoscopy was recommended prior to the COVID-19 pandemic, for screening and surveillance of varices, it was suggested to initiate non-selective beta blocker (NSBB) therapy based on clinical judgement, taking into account Child Pugh class and platelet count[136].

Similarly, in patients with advanced fibrosis or cirrhosis, or at high risk of having portal hypertension and varices, the BSG advised to consider starting NSBBs treatment prophylactically during the pandemic[136,137]. The Baveno criteria[138] could be used to identify patients at low risk of having varices, though this would require Fibroscan services to be operational; arguably easier and associated with a reduced risk of COVID-19 transmission *vs* traditional endoscopic services. AASLD advised to postpone HCC surveillance from 6 mo to 8 mo in most cirrhotic patients who do not have key risk factors for HCC development[139]. The BSG[140] and EASL[13] also advocated delaying HCC surveillance during the peak of infections. Mehta *et al*[141] reviewed guidelines from various hepatology societies and provided recommendations on HCC surveillance and monitoring. The authors advised that surveillance should not be performed in patients who are unlikely to benefit, such as those who were not transplant-eligible with Child Pugh Class C cirrhosis or significant, life-limiting, co-morbidities. Similarly, it was advised against surveillance in low-risk groups such as Hepatitis C and non-alcoholic steatohepatitis patients without cirrhosis given the marginal risk-benefit ratio. Clinicians at NHS Tayside, Scotland, United Kingdom developed an automated 'intelligent liver function testing' (iLFT) algorithm in 2018[142]. Abnormal liver function tests (LFTs) were combined with clinical features, diagnostic criteria, investigation ordering and reporting, and a tracked blood sciences system; the algorithm then generated a diagnosis or descriptor of the abnormality, with fibrosis staging. Of 568 abnormal LFTs, two thirds were managed in primary care, reducing the need for secondary care referrals. The iLFT algorithm is currently being assessed in other United Kingdom centres; this and similar systems could be incorporated into healthcare services during and after the pandemic, minimising secondary care burden. COVID-19 infection has worse outcomes in obese patients. Hence it is important to stress the need for enhanced resources to prevent and treat metabolic syndrome and associated conditions including liver disease[143], and the need strengthen the pathways for recognition and management more than ever.

Provision for remote laboratory investigations and prescribing

Conventionally when patients attend outpatient appointments, they have laboratory investigations and collect prescriptions during their hospital visit. With the increasing use of telemedicine, it is vital that there are safe mechanisms in place to arrange prescriptions and investigations if required. In order to avoid attendance to hospital for tests, organizations can establish blood-hubs outside main hospital sites, preferably multiple, so patients can have laboratory tests done closer to their home and without coming in contact with high risk patient areas[13]. These sites may be able to also provide day-case infusion services including biologics and other intravenous infusions (such as iron infusions) away from main hospitals, *i.e.*, 'hot' sites. In the current digital age, healthcare providers may aim to setup a smart phone apps or a website link for patients to book appointments for tests at convenient times to them which may also avoid overcrowding at these hubs. There should also be a more conventional system (*e.g.*, telephone appointment booking system) in place for patients who may have limited access or knowledge of using online systems or smart-phones. Remote prescribing systems are helpful for patients with chronic liver and GI diseases. A process whereby patient can be posted medication scripts is helpful in institutions that have this in place. Monitoring of medication (*e.g.*, blood tests in patients of immunosuppression agents) is imperative to ensure safe practice. Digital prescribing - whereby the prescriber can send digital prescription to hospital or community pharmacy and these can either by collected by patients or can be posted to them, is an important resource where available. Such E-prescribing provides an auditable trail for governance purposes. These however require robust information technology systems in place and will depend on availability of resources across different parts of the world.

Optimising training opportunities

For endoscopy training there is need to ensure adequate PPE supply so this doesn't hinder trainees' attendance to training lists. Simulation endoscopic training can be utilized in the current pandemic, allowing trainees sufficient hands-on time. Various organizations have already adopted provision of CPD activities and have moved to online platforms. With numerous available online GI/Liver teaching resources, there has also been a boom in modalities for education such as Twitter. FitzPatrick *et al*[144] reviewed how gastroenterology training can thrive during COVID-19[144]. The authors discussed the challenges in learning environment during COVID-19 pandemic

in endoscopy, outpatient and educational settings, and provided proposed solutions. These included adequate PPE supply, use of simulation training along with directed access to limited endoscopic activity for hands-on training opportunities (like involvement in GI bleed management), reinstatement of formal specialty training days, supervisor's proactive discussion with trainee regarding the remote consultation undertaken. Keswani *et al*[145] have reviewed the importance of internet-based learning, simulator training, and adoption of new educational models to maximize training during the pandemic[145]. Digital learning has flourished during COVID-19 crisis and has provided trainees the option of distanced learning.

CONCLUSION

It is clear there has been a major impact of gastroenterology and hepatology training but also service provision due to COVID-19. Healthcare teams throughout the world have attempted to continue care for patients with pre-existing and new presentations of GI and liver conditions; however this provision has been extensively modified and impacted by the pandemic. Now, with new treatments for COVID-19 along with the vaccines, healthcare professionals are moving forward with a hopeful reduction in burden of the disease for patients. This in turn will allow some manoeuvrability with regards to the ongoing required non-COVID-19 service provision. A balance must exist in the fight against COVID-19, but also ensuring ongoing high levels of care to patients with non-COVID-19 diseases. The training of the specialists of tomorrow remains vital, allowing trainees where possible a safe environment to hone their skills gaining relevant expertise but also providing high quality care to patients in the current pandemic. Whilst the COVID-19 pandemic has had a significant impact upon services and patients, novel approaches of service reconfigurations along with optimisation of existing pathways/protocols have been implemented worldwide in an attempt to maintain optimal care for gastroenterology and hepatology patients and service providers.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 28; 27(44): 7649-7660

DOI: 10.3748/wjg.v27.i44.7649

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

MINIREVIEWS

Challenges in the diagnosis of intestinal neuronal dysplasia type B: A look beyond the number of ganglion cells

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Author contributions: Terra SA and Gonçalves AC collected and interpretated the data, drafted the initial manuscript and revised the article critically; Lourenção PLTA and Rodrigues MAM analysed and interpreted the data, drafted the initial manuscript and revised critically; all authors read and approved the final version of the manuscript.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

Supported by Supported by São Paulo Research Foundation (FAPESP), No. 2017/26205-9.

Country/Territory of origin: Brazil

Specialty type: Pathology

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

Peer-review report's scientific quality classification

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Abstract

Intestinal neuronal dysplasia type B (IND-B) is a controversial condition among gastrointestinal neuromuscular disorders. Constipation is its most common clinical manifestation in patients. Despite intense scientific research, there are still knowledge gaps regarding the diagnostic criteria for IND-B in the histopathological analysis of rectal biopsies. The guidelines published in the past three decades have directed diagnostic criteria for quantifying the number of ganglion cells in the nervous plexus of the enteric nervous system. However, it is very complex to distinguish numerically what is pathological from what is normal, mainly because of the difficulty in determining a reliable control group composed of healthy children without intestinal symptoms. Thus, a series of immunohistochemical markers have been proposed to assist in the histopathological analysis of the enteric nervous system. Several of these markers facilitate the identification of other structures of the enteric nervous system, in addition to ganglion cells. These structures may be related to the etiopathogenesis of IND-B and represent new possibilities for the histopathological diagnosis of this disease, providing a view beyond the number of ganglion cells. This review critically discusses the aspects related to the disease definitions and diagnostic criteria of this organic cause of constipation.

Key Words: Intestinal neuronal dysplasia type B; Constipation; Diagnosis; Gastrointestinal neuromuscular diseases

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

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Received: February 12, 2021
Peer-review started: February 12, 2021
First decision: April 18, 2021
Revised: April 26, 2021
Accepted: November 11, 2021
Article in press: November 11, 2021
Published online: November 28, 2021

P-Reviewer: Choi YS
S-Editor: Gong ZM
L-Editor: A
P-Editor: Gong ZM



Core Tip: There are knowledge gaps regarding the diagnostic criteria for intestinal neuronal dysplasia type B (IND-B) in the histopathological analysis of rectal biopsies. Several immunohistochemical markers have been proposed to identify other structures of the enteric nervous system, beyond the ganglion cells. These structures may be related to the etiopathogenesis of IND-B and represent new possibilities for the histopathological diagnosis of this disease. This review critically discusses the aspects related to the disease definitions and diagnostic criteria of this organic cause of constipation.

Citation: Terra SA, Gonçalves AC, Lourenção PLTA, Rodrigues MAM. Challenges in the diagnosis of intestinal neuronal dysplasia type B: A look beyond the number of ganglion cells. *World J Gastroenterol* 2021; 27(44): 7649-7660
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7649.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7649

INTRODUCTION

The histopathological diagnosis of colon diseases presenting with severe constipation in childhood has been a challenge in recent decades. Hirschsprung's disease (HD), the best known among the gastrointestinal neuromuscular diseases, is defined by the absence of ganglion cells in the submucosal and myenteric plexuses of the enteric nervous system (ENS). Intestinal neuronal dysplasia type B (IND-B) is characterized by hyperplasia of the submucosal nerve plexuses[1-4] (Table 1).

IND-B was first described in 1970, when Nezelof *et al*[5] reported three cases of megacolon associated with hyperplasia of the myenteric nervous plexus. A year later, Meier-Ruge[6] defined it as a condition usually associated with low intestinal obstruction that could simulate HD but showed distinct histopathological characteristics such as hyperplasia of the nerve plexuses and increased activity of the enzyme acetylcholinesterase (AChE) in the parasympathetic nerve fibers of the lamina propria of the mucosa.

Almost 50 years have passed and, despite the intense scientific research conducted during this period, there are still uncertainties, including IND-B etiopathogenesis, diagnostic criteria, and therapeutic possibilities. Thus, IND-B is currently considered as a controversial condition among the differential diagnoses of organic causes of intestinal constipation[7-10].

Most patients with IND-B are children showing chronic constipation. The main symptoms are very similar to those observed in patients with HD. Delay in the meconium passage, abdominal distention, vomiting, and difficulty in eating can occur during the first days of life[7,9]. A portion of these patients can show symptoms throughout their lives, evolving to severe constipation refractory to different types of clinical treatment[7,9,11,12]. Severe symptoms such as episodes of enterocolitis, acute intestinal obstruction, volvulus, and intussusception, although rare, are possible complications in different age groups[12-14]. Recent studies have highlighted the growing number of cases in adults, some showing symptoms of constipation since childhood, and others with late onset of symptoms[15-19]. The oldest patient reported in the literature with a diagnosis of IND-B was 71 years old. This patient showed symptoms since childhood, with severe constipation for more than 60 years, including several hospitalizations and surgeries during this period[19].

Two therapeutic modalities have been used in patients with IND-B: conservative clinical treatment and surgical treatment[9,20]. Conservative treatment is based on changes in diet, laxatives, and enemas in cases of fecal retention[7,20,21]. Surgical treatment can be performed through sphincterotomy, extensive surgical resection, or temporary colostomy[22-25]. However, the outcomes obtained using different treatment modalities show conflicting results[11,20,21]. Currently, the most accepted trend is that patients diagnosed with IND-B who have no complications must receive conservative treatment[1,3,10,20].

Table 1 Comparison between Hirschsprung's disease and intestinal neuronal dysplasia type B regarding the main clinical and histopathological aspects

	HD	IND-B
Histopathological findings on rectal biopsies	Absence of ganglion cells in the submucosal and myenteric plexuses	Hyperplasia of the submucosal nerve plexuses
Clinical Picture	Neonatal bowel obstruction or severe constipation	Neonatal bowel obstruction or severe constipation
Treatment	Surgical (colorectal pull-through)	Diet, laxatives, enemas or surgical (if complications)

HD: Hirschsprung's disease; IND-B: Intestinal neuronal dysplasia type B.

CONTROVERSIES REGARDING THE EXISTENCE OF IND-B

One of the main controversies in the understanding of IND-B is related to the existence of a cause-effect relationship between the histopathological findings and clinical symptoms. In most cases, a diagnosis of IND-B is based on the histopathological analysis of rectal biopsies in patients with constipation that is usually refractory to clinical treatment. A minority of patients have acute intestinal obstruction or enterocolitis[7,9]. In contrast, histopathological alterations compatible with the diagnosis of IND-B were observed in the colon segments of 36 asymptomatic children[26]. Other studies have failed to demonstrate a direct correlation among histopathological findings, clinical symptoms, and radiological and manometric changes[27,28]. Given these controversies, some authors show skepticism concerning the definition of IND-B as a true clinical condition, preferring to define it as a histopathological alteration of the ENS, which may not present symptoms[7,10,29].

In a recent review on the subject, Kapur and Reyes-Mugica[10] concluded that IND-B remains an undefined histopathological phenotype of uncertain clinical relevance, and that it is imprudent to make clinical decisions based on this histopathological diagnosis, while claiming that this type of histopathological finding may represent deviations from normality. Moreover, these authors criticize the most recent scientific publications that investigated patients with this diagnosis, arguing that they have perpetuated the debate on dubious and controversial concepts. However, in medical practice, we continue to find children with severe constipation or intestinal obstructions who undergo diagnostic investigation for HD and, in the histopathological analysis of rectal biopsies, present ganglion cells hyperplasia in the nervous plexuses of the submucosa. Schmittenbecher *et al*[11] considered that these morphological changes were compatible with a diagnosis of IND-B, which should be considered as a distinct clinical condition, since the symptoms presented by the patients were evident and often persisted for several years, directly influencing their quality of life. The authors argued that patients with IND-B should be treated in a specific manner and highlighted the need to develop specific treatment algorithms. The majority of experts who participated in the Fourth International Symposium on Hirschsprung's disease and related neurocristopathies in Genova, Italy, in 2004 and in a recent survey by the European Association of Pediatric Surgeons, consider IND-B to be a clinicopathological condition, justifying the need for further studies on this disease[30,31].

LACK OF DIAGNOSTIC CRITERIA

The lack of well-established criteria for the histopathological diagnosis of IND-B can be considered as the basis for many of the uncertainties described. To understand any disease, it is mandatory to know how to diagnose it. In the case of IND-B, the morphological criteria for histopathological diagnosis have been modified over the years, making comparisons between studies difficult and increasing the controversies and doubts concerning its existence[7,32,33]. Hyperplasia of the nervous plexuses of the ENS is a morphological finding that defines IND-B (Figure 1), but it is differently characterized by varying criteria[6,9].

Based on the 1990 Frankfurt Consensus discussions, Borchard *et al*[32] established morphological criteria for the histopathological diagnosis of IND-B by using biopsies

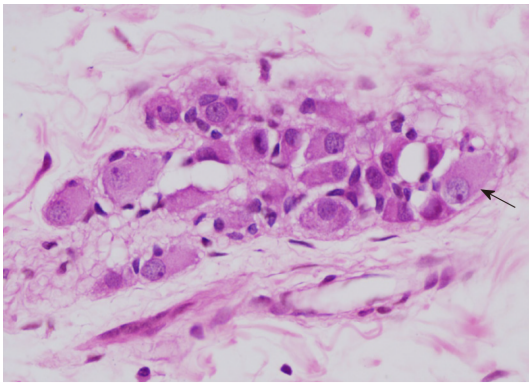


Figure 1 Intestinal neuronal dysplasia type B. Submucosal nerve plexus with hyperganglionosis: giant ganglion. Ganglion cell (arrow) (H&E, 400 ×).

of the rectal wall (Table 2). Since then, these criteria have been widely used, both in clinical practice, follow-up studies and in investigations into the pathophysiology and etiopathogenesis of IND-B[24,27,34].

New diagnostic criteria were proposed in the 1990s, highlighting the need to identify giant ganglia in the submucosa, characterized by the presence of a minimum number of ganglion cells, ranging from six to more than 10 per ganglion[35,36]. In 2004, Meier-Ruge *et al*[33] proposed a quantitative criterion for the histopathological diagnosis of IND-B (Table 2). Since this diagnosis depends on quantitative data, which can be compromised by technical variables, the entire laboratory process must be highly standardized. The biopsy must be adequate to obtain a sufficient quantity of submucosal tissue for analysis, and the frozen sections must be 15-μm thick and analyzed for a specific histochemical panel composed of lactate dehydrogenase, succinyl dehydrogenase, and nitric oxide (NO) synthase[1,33]. Although these criteria have been accepted by the scientific community, they were used in few case reports [37]. The need for fresh material and specific histochemical stains, the availability of which is restricted to certain centers, is a major limitation for diagnosis[9,38,39]. It is also uncertain whether these criteria can be applied to 5-μm thick histological sections from paraffin-embedded material used for conventional histological analysis with hematoxylin and eosin (H&E) and immunohistochemical methods[4,39]. Taguchi *et al* [37] analyzed data from 161 Japanese gastroenterological and pediatric surgery institutions from January 2000 to December 2009 and found 355 patients with HD and allied disorders. Of these, 13 patients were diagnosed with IND-B based on the histopathological criteria commonly used, but only four met the quantitative criteria proposed by Meier-Ruge *et al*[33]. Terra *et al*[39] analyzed surgical specimens from 29 patients diagnosed with IND-B, previously established by the criteria of the Frankfurt Consensus[32], in the histological sections processed for conventional histology by H&E. Only one patient met the numerical diagnostic criteria proposed by Meier-Ruge *et al*[33]. The authors concluded that the recommended quantitative criteria for 15-μm cuts stained by specific histochemical panels have limited applicability when transposed to conventional histological analysis.

Thus, the changes proposed in the last three decades have oriented the diagnostic criteria for quantifying the number of ganglion cells in the nervous plexuses of the ENS[33,35,36]. However, it is very complex to numerically distinguish the pathological from the normal, mainly because of the difficulty in determining a reliable control group composed of healthy children without intestinal symptoms[1,10,40]. Obtaining intestinal samples for use as controls has ethical limitations because of the need for invasive procedures to obtain tissue samples. In some studies, the control group was composed of intestinal samples from patients with congenital intestinal malformations such as anorectal abnormalities, which cannot be considered healthy controls[41]. In addition, the variation in the number of ganglion cells related to age, especially during the first year of life, leads to the need for age-matched controls, making it difficult to develop an appropriate control group[26]. The thickness of the histological section, the staining techniques, and the methods of histopathological analysis directly influence the number of ganglion cells identified in the count[38,39]. For all these reasons, the quantitative criteria seem to have contributed to more uncertainty rather than greater objectivity for the diagnosis of IND-B. There are important differences regarding the minimum number of ganglion cells and nerve ganglia, questions concerning the different methods of histopathological analysis, and the possibility of a minimum age

Table 2 Summary of the histopathological criteria for the diagnosis of intestinal neuronal dysplasia type B

Diagnostic criteria	Definition	
Frankfurt Consensus[32], 1990	Mandatory criteria	Submucosal plexus hyperplasia
		Increased activity of the AChE enzyme in nerve fibers around submucosal blood vessels
	Complementary criteria	Neuronal heterotopy
		Increased AChE activity in the lamina mucosa
Meier-Ruge *et al*[33], 2004	Quantitative criteria	At least 20% of giant nerve ganglia[1] in the submucosa, in 25 submucosal ganglia
	Age criteria	Patients must be older than 1 yr

[1]Giant nerve ganglia: A ganglion with more than eight ganglion cells.
AChE: Acetylcholinesterase.

for diagnosing this disease[39].

UNCERTAINTIES AND RECENT ADVANCES

Pathophysiology and etiopathogenesis are poorly defined aspects of IND-B[7,30]. Some authors consider these aspects as part of the normal development of the ENS[3]. As age advances, there is an increase in the size of ganglion cells and a decrease in their number per plexus[26,36,40]. These findings can be related to a physiological process of maturation, which involves the apoptosis of components of the ENS[1,33]. Schimpl *et al*[21] retrospectively analyzed 105 patients with IND-B: 60.95% of patients were less than 6 mo old, 30.47% were between 6 and 12 mo old, and only 8.57% were more than 1 year old. This percentage distribution, which decreases with age, suggests a maturation process of the ENS or some abnormality in the development of the intestinal nervous system, with spontaneous normalization with advancing age.

Other studies have raised the possibility that IND-B represents an adaptive response of the ENS, secondary to obstructive or inflammatory bowel phenomena that occur in the fetal, perinatal, or postnatal period. There are reports of morphological findings suggestive of IND-B in the intestinal areas immediately above intestinal atresia, rectal mucosa prolapse, imperforate anus, and necrotizing enterocolitis[27,42,43]. These secondary responses to obstructions or inflammations have been studied experimentally, and the results are conflicting[44,45]. Using a model of partial intestinal colon obstruction in rats, Moore *et al*[44] showed a decrease in the number of ganglion cells compared to that in the control group, explained by an increase in colon diameter due to the distention of its wall. Using a model of chronic intestinal obstruction in adult rats, Gálvez *et al*[45] identified histopathological changes compatible with IND-B, with an increase in the number of ganglion cells.

Recent studies have shown that IND-B can originate from genetic alterations, which directly influence the embryological development of tissues derived from the neural crest. Angelini *et al*[41] showed significantly unregulated expression of microRNAs in the submucosal and muscular layers of the colon and peripheral blood of patients with IND-B. The molecular pathways biologically regulated by these microRNAs (axon guidance, nerve growth factor signaling, neural cell adhesion molecule (NCAM) signaling for neurite outgrowth, neuronal system, and apoptosis) show activities related to the ENS and, therefore, can be related to the pathogenesis of IND-B. Liu *et al*[46] showed a decrease in the methylation levels of locus 32 on the *Sox10* promoter gene in the peripheral blood of patients with IND-B. There was a negative correlation between these levels and Sox10 expression in the colons of these patients. These changes can contribute to the regulation of the number of intestinal glial cells and the maturation of neurons in the ENS[46].

In addition, reports on familial occurrence of IND-B and its association with other intestinal and extraintestinal diseases reinforce the theory that IND-B has a primary, genetically determined origin[34,47-49]. Among these associations, HD, incomplete intestinal rotation, and multiple endocrine neoplasia type 2 (NEM 2) are the most important[23,28].

Histopathological findings compatible with IND-B in segments proximal to areas of aganglionosis are not uncommon and have been considered as a possible cause of the persistence of obstructive symptoms in patients undergoing surgical treatment for HD.

This association was first reported in the 1970s[50]. Since then, the association between IND-B and HD has been described with rates ranging from 6% to 75% of HD cases[38,51,52]. In these cases, the presence of colonic segments with IND-B can be explained both by primary embryological alteration of the ENS, which gives rise to neuropathies, and by secondary adaptation to distal intestinal obstruction, caused by the spastic aganglionic intestinal segment[22,53].

IMMUNOHISTOCHEMISTRY AND LOOKING BEYOND THE NUMBER OF GANGLION CELLS

Over the past two decades, a series of immunohistochemical markers have been proposed to assist in histopathological analysis of the ENS[2,4,54-57]. The use of calretinin, well established in HD, is the primary example of how an immunohistochemical method can contribute substantially to the diagnosis of enteric neuropathy. The absence of immunohistochemical expression of calretinin in colorectal aganglionic segments has been routinely used and is part of the main guidelines for HD diagnostic management[58,59]. Terra *et al*[39] identified the expression of calretinin in ganglion cells and mucosal nerve fibers in 29 patients with IND-B. Immunoexpression of this marker was observed in ectopic neurons in the lamina propria and in the muscularis mucosa (Figure 2), which was not previously identified using standard histology (H&E).

Several studies have assessed the role of immunohistochemical methods both in investigating aspects related to etiopathogenesis and in improving the histopathological diagnosis of IND-B. These studies[39,46,54,60-71] are summarized in the table presented in Supplementary Table 1. The pattern of immunoexpression of the main markers described in patients with IND-B is shown in Table 3.

Bosman *et al*[66] identified an increase in the number of ganglion cells, with positive expression of NO synthase in the intestine of patients with IND-B compared to that in the control group. This enzyme is responsible for the synthesis of NO, a neurotransmitter capable of inducing smooth muscle relaxation that may be responsible for the clinical symptoms of intestinal pseudo-obstruction presented by patients.

Yamataka *et al*[62] showed a reduction in c-kit and 171B5 (synaptophysin) expression in the muscle layers, with the exception of the myenteric plexuses, in the intestine of patients with IND-B compared to that in the control group. The c-kit protein is expressed in the interstitial cells of Cajal and is considered to be a regulator of gastrointestinal tract motor activity[72,73]. Synaptophysin is expressed in the protein membrane of synaptic vesicles and is present during the neurotransmission of the central and peripheral nervous systems, including the ENS. The reduction in the expression of these two markers raises the hypothesis of the role of interstitial cells of Cajal in the pathophysiology of IND-B[62].

Kobayashi *et al*[63] reported that the markers NCAM, growth-associated protein 43 (GAP43), and synaptophysin showed absence of expression or markedly decreased expression in the muscular layers of the intestine of patients with IND-B compared to that in the control group. No change was observed in the expression of these markers in the nervous plexus of the ENS. NCAM is a surface glycoprotein that is important for the interaction between neurons and muscle cells during synaptogenesis and is considered as a marker of the neuromuscular junction[74]. GAP43 is a marker associated with neuron development and regeneration during axonal growth and is found in high concentrations in presynaptic areas[63]. These changes in the expression patterns of these markers led the authors to propose the hypothesis that patients with IND-B presented defects in the innervation of neuromuscular junctions, which would explain the changes in colonic motility. However, Nogueira *et al*[65] were unable to reproduce this pattern of NCAM and synaptophysin expression, weakening this hypothesis.

Kobayashi *et al*[64] showed an increase in the number of mast cells, identified using immunohistochemical methods, in the intestinal segments of patients with IND-B and in the aganglionic segments of HD patients compared to that in the normoganglionic segments of HD patients and individuals in the control group. Mast cells were also identified by the marker nerve growth factor (NGF) close to the nerve ganglia in patients with IND-B and close to the hypertrophied nerve trunks in the aganglionic segments of HD. Based on these observations, the authors suggested the possible participation of mast cells in the pathogenesis of IND-B and HD.

Table 3 Immunohistochemical markers for the diagnosis of intestinal neuronal dysplasia type B

Immunohistochemical marker	Immunoexpression pattern
Bcl-2[54]	Positive expression in immature and mature ganglion cells
Calretinin[39,54]	Positive expression in mature ganglion cells in submucosal and myenteric plexuses
	Positive expression in intrinsic nerve fibers and ectopic neurons in the mucosa
c-kit[60-62]	Positive expression in Cajal cells in muscle layers and myenteric nerve plexuses
GAP43[63]	Absence of expression in muscularis mucosae and/or in the circular muscular and/or longitudinal muscular
	Positive expression in nerve plexuses
Hu C/D[38]	Positive expression in mature ganglion cells in submucosal and myenteric plexuses
NGF (mast cells)[64]	Expression of mast cells close to ganglia
NCAM (CD56)[60,61,63,65]	Positive expression in neurons of the muscular layers
	Variable expression in nerve fibers in muscle layers and mucosa
NO synthase[66]	Positive expression in ganglion cells
NSE[61]	Positive expression in nerve fibers in the muscle layers and mucosa
Peripherin[66,67]	Positive expression in ganglion cells
PGP9.5[60,68]	Positive expression in enlarged nerve trunks, hyperplastic nerve ganglia, and heterotopic nerve cells
	Positive expression in neurons of the inner intestinal muscle layer
PTEN[69]	Reduced expression in submucosal and myenteric nerve plexuses
RET[54,66]	Positive expression in mature and immature ganglion cells
S100[60,61,66]	Positive expression in ganglion cells
	Positive expression in nerve fibers in the muscular layers and mucosa
Synaptophysin[60,62,63,65,66,70]	Positive expression in synaptic vesicles in plexuses and nerve fibers
SMA (1a4)[61,71]	Positive expression in muscle fibers from muscular layers and muscularis mucosa
Sox10[46]	Positive expression in glial and ganglion cells in nerve plexuses

Bcl-2: B-cell lymphoma 2; c-kit: Transmembrane tyrosine kinase receptor; GAP43: Growth associated protein 43; Hu/CD: RNA binding protein Hu (HuC and HuD) - also referred to as type-1 anti-neuronal nuclear antibodies (ANNA-1); NGF: Nerve growth factor; NCAM: Neural cell adhesion molecule; NO: Nitric oxide; NSE: Neuron-specific enolase; PGP9.5: Protein gene product 9.5; PTEN: Phosphatase and tensin homologue deleted on chromosome 10; RET: Rearranged during transfection - immunohistochemical detection of RET proto-oncogene; SMA: Smooth muscle antibody; Sox10: SRY-box transcription factor 10.

O'Donnell and Puri[69] evaluated the immunohistochemical expression of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) in patients with IND-B. PTEN is a protein involved in cell proliferation, survival, and migration and has a potential modulatory role in neurogenesis and synaptic plasticity. The authors showed significantly reduced expression of this marker in the myenteric and submucosal plexuses of the intestine of patients with IND-B, absence of PTEN expression in the aganglionic segments of HD patients, and strongly positive expression in ganglionic intestinal segments of HD patients and individuals in the control group. Based on these results, the authors suggested that the giant ganglia with immature cells, present in IND-B, might be associated with a reduction in PTEN level, which might be responsible for the lack of control in neuronal growth and proliferation.

Some markers have demonstrated the potential to assist in neuron counting, which is fundamental for the quantitative diagnosis of IND-B. Geramizadeh *et al*[60] evaluated the immunohistochemical expression of the protein gene product 9.5 (PGP9.5), a specific cytoplasmic marker of the nervous system, S100, a nerve cell nucleus and cytoplasm marker, c-kit, synaptophysin, and NCAM to count nerve cells in the muscle layers and myenteric plexuses of the distal colon of patients with HD,

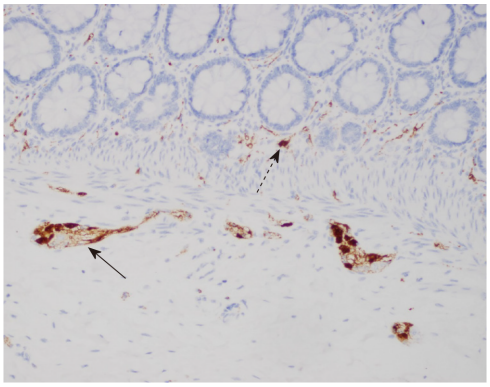


Figure 2 Calretinin immunohistochemistry in intestinal neuronal dysplasia type B. Positive nuclear calretinin staining in neurons from a submucosal nerve plexus (arrow). Heterotopic neuron in the muscularis mucosa (dotted arrow) (200 ×).

IND-B, and the control group. The authors showed a significantly higher number of nerve cells, stained by these immunohistochemical methods, in the muscle layers and/or the myenteric plexuses in patients with IND-B than in HD patients. However, this difference was not significant when patients with IND-B were compared to those in the control group. Kim *et al*[61] showed no significant differences in the counts of interstitial cells of Cajal (c-kit immunopositive) in muscle layers and myenteric plexuses in patients with IND-B compared to those in patients with other types of intestinal pseudo-obstructions.

Swaminathan *et al*[38] evaluated the use of the pan-neuronal immunohistochemical marker Hu C/D in the quantitative analysis of ganglion cells in colonic segments with IND-like submucosal ganglion cell hyperplasia at the proximal margins of HD resections. This marker detects neuronal cell bodies, facilitating the recognition of neurons, and has been used as a neuronal marker for the central and peripheral nervous systems[75]. By counting the nuclei of ganglion cells immunoexpressing Hu, it was possible to develop and validate a quantitative histopathological criterion for the diagnosis of giant ganglia, defined by the presence of at least seven ganglion cells [38].

Wang *et al*[54] determined the number of ganglion cells and the area of myenteric nerve plexuses using immunohistochemical expression of the RET protein, which is encoded by the *RET* gene and is related to cell growth and differentiation, and of B-cell lymphoma 2 (Bcl-2), a protein encoded by the homonymous gene located on chromosome 18q21, which plays a role in the maintenance of cell survival. The authors identified a significantly higher number of ganglion cells and a larger myenteric plexus area in the colon of patients with IND-B than in that of the control group.

CONCLUSION

IND-B remains a controversial clinicopathological condition, with a poorly understood etiopathogenesis and a series of challenges related to its diagnosis. There is no consensus regarding the criteria used by different centers for the histopathological diagnosis of IND-B. Despite these uncertainties, patients continue to show severe constipation or intestinal obstruction, associated with histopathological alterations compatible with this morphological phenotype, which fully justifies all the efforts directed toward new diagnostic strategies. Only the correct diagnosis of these patients can lead to more effective treatment and better prognosis. In this regard, immunohistochemical techniques have identified other ENS structures, in addition to the ganglion cells of the submucosa and muscular nerve plexuses. These structures may be potential candidates for biomarkers in the histopathological diagnosis of IND-B, expanding the horizon beyond counting the number of ganglion cells.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 28; 27(44): 7661-7668

DOI: 10.3748/wjg.v27.i44.7661

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

MINIREVIEWS

Treatment of *Helicobacter pylori* infection in the presence of penicillin allergy

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Author contributions: Dutta AK and Phull PS contributed equally to this work, jointly undertook the literature review; Phull PS had the idea for the review; Dutta AK wrote the first draft of the manuscript, which was revised by Phull PS; all authors have read and approved the final manuscript.

Conflict-of-interest statement: The authors have no conflicts of interest to declare

Country/Territory of origin: United Kingdom

Specialty type: Gastroenterology and hepatology

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

Peer-review report's scientific quality classification

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

Open-Access: This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external

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Abstract

Therapy of *Helicobacter pylori* (*H.pylori*) requires a combination of antibiotics together with an acid suppressing agent; most treatment regimens include Amoxicillin as one of the antibiotics, which is an important constituent as resistance to it is low. However, allergies to the penicillin group of antibiotics are not uncommon, and treating *H.pylori* infection in such individuals can be challenging due to the restricted choice of regimens. The aim of this review is to summarise the evidence for therapeutic options in patients with *H.pylori* infection and penicillin allergy. A literature search was conducted in PubMed for English language publications using the key words 'Helicobacter' and 'treatment' or 'therapy' and 'penicillin' or 'beta-lactam' and 'allergy' or 'anaphylaxis'. Eighteen studies were identified that specifically evaluated *H.pylori* treatment success in penicillin allergic patients. The number of subjects in most of them was low and many were retrospective, uncontrolled, single cohort studies. The most effective option for first-line treatment appears to be Bismuth-based quadruple therapy for 10-14 d. The evidence supports second-line treatment with Levoflaxacin-based triple therapy for 10 d. Patients with persistent *H.pylori* infection after 2 treatment courses should be considered for testing to confirm penicillin allergy. Further treatment should be guided by the results of *H.pylori* culture and sensitivity testing.

Key Words: *Helicobacter pylori*; Infection; Treatment; Penicillin-allergy; Stomach; Duodenum

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Received: April 12, 2021
Peer-review started: April 12, 2021
First decision: June 3, 2021
Revised: June 17, 2021
Accepted: November 10, 2021
Article in press: November 10, 2021
Published online: November 28, 2021

P-Reviewer: Cheng H, El-Bendary M, Ma H
S-Editor: Wang LL
L-Editor: A
P-Editor: Wang LL



Core Tip: Penicillin allergy is a not uncommon occurrence and treating *Helicobacter pylori* infection in such individuals can be challenging. This review highlights the lack of high-quality studies to help guide management strategies. Recommendations have been made based on the limited data, but it would be important to monitor the success of treatment regimens and use what can be demonstrated to be effective locally.

Citation: Dutta AK, Phull PS. Treatment of *Helicobacter pylori* infection in the presence of penicillin allergy. *World J Gastroenterol* 2021; 27(44): 7661-7668
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7661.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7661

INTRODUCTION

Infection with *Helicobacter pylori* (*H.pylori*) is prevalent worldwide with about half of world's population estimated to be affected by this gram negative spiral bacterium[1]. The organism is causally implicated in the pathogenesis of peptic ulcer disease[2] and gastric adenocarcinoma[3]. Guidelines for the management of *H.pylori* infection have been published by a number of national societies and organisations[4-7].

Therapy of *H.pylori* requires a combination of antibiotics together with an acid suppressing agent (proton-pump inhibitor, PPI); most treatment regimens include Amoxicillin as one of the antibiotics, which is a particularly important constituent as resistance to it is low[8]. However, allergies to the penicillin group of antibiotics are reported in 5% to 15% of patients in developed countries[9] and, consequently, the treatment options in individuals allergic to penicillin are significantly restricted.

In this review we summarise the available evidence for therapeutic options in patients with *H.pylori* infection and penicillin allergy.

LITERATURE SEARCH

A literature search was conducted in PubMed using the key words 'Helicobacter' and 'treatment' or 'therapy' and 'penicillin' or 'beta-lactam' and 'allergy' or 'anaphylaxis' for English language publications from database commencement until January 31, 2021. Of the 77 publications identified, 18 studies were included in the review (48 were excluded as not relevant, and 11 were review articles)[10-27].

EVIDENCE FOR TREATMENT OF *H.PYLORI* INFECTION IN THE PRESENCE OF PENICILLIN ALLERGY

Data from studies specifically targeting penicillin allergic patients (Table 1). Considering the large volume of publications on *H.pylori* therapy, there is relatively little data on treatment of this bacterium in penicillin allergic individuals. A summary of data available from the 18 identified studies is shown in the Table. It should be noted that the number of subjects included in most of them is quite low and many are retrospective, uncontrolled, single cohort studies. All results discussed below are presented on an intention-to-treat basis.

First line therapy

Dual therapy: Prach *et al*[10] reported 100% treatment success with a 14 d combination of Omeprazole and Clarithromycin; however, this was only in 3 patients.

Triple therapy: The success rate with the 7 d PPI-Clarithromycin-Metronidazole regimen, has been reported as 50%-83.3% in retrospective studies[20,21] and 54-58% in prospective studies[11,14,18]. A longer 14 d regimen also resulted in a low success rate at 63.6%[23]. The European Registry on *H.pylori* management (Hp-EuReg) has provided the largest experience of treatment in penicillin allergic patients[25]. Although drug dose, frequency and duration details were not provided, the PPI-Clarithromycin-Metronidazole regimen achieved 69% success rate. Two studies from

Table 1 Published studies of *Helicobacter pylori* eradication therapy in patients allergic to penicillin

Ref.	Yr	Country	Study type	Treatment details	*n*	Success rate (PP, %)	Success rate (ITT, %)
Prach *et al*[10]	1998	United Kingdom	Prospective,single cohort	*1st line* O 20 mg b.d., C 500 mg t.d.s, 14 ds	3	100;	100;
Gisbert *et al*[11]	2005	Spain	Prospective, single cohort	*1st line* O 20 mg b.d., C 500 mg b.d., M 400 mg b.d., 7 d; *2nd line* RBC 400 mg b.d., T 500 mg q.d.s., M 250 mg q.d.s., 7 d; *3rd line* O 20 mg b.d., C 500 mg b.d., RIF 150 mg b.d., 10 d; *4th line* O 20 mg b.d., C 500 mg b.d., LF 500 mg b.d.,10 ds	12; 17; 9; 2	64; 53; 17; 100	58; 47; 11; 100
Rodriguez-Torres *et al*[12]	2005	Puerto Rico	Prospective, single cohort	*1st line* E 40 mg q.d.s., T 500 mg q.d.s., M 500 mg q.d.s.,10 d; *2nd line* E 40 mg q.d.s., T 500 mg q.d.s., M 500 mg q.d.s.,10 d	17; 3	NA; NA	85; 100
Matsushima *et al*[13]	2006	Japan	Retrospective, single cohort	*1st line* PPI o.d., T 500 mg b.d., M 250 mg b.d., 7-14 d	5	100	80
Gisbert *et al*[14]	2010	Spain	Prospective, single cohort	*1st line* O 20 mg b.d., C 500 mg b.d., M 400 mg b.d., 7 d; *2nd line* O 20 mg b.d., C 500 mg b.d., LF 500 mg b.d.,10 d	50; 15	55; 73	54; 73
Tay *et al*[15]	2012	Australia	Prospective, single cohort	*2nd line* R 20 mg t.d.s., B 240 mg q.d.s., RIF 150 mg b.d., CF 500 mg b.d., 10 d	69	94.2	94.2
Liang *et al*[16]	2013	China	Prospective, randomised	*2nd line* 109 pen allergic overall but results reported for whole group including non-allergic; L 30 mg b.d., B 220 mg b.d., T 500 mg t.d.s., F 100 mg t.d.s., 14 d; L 30 mg b.d., B 220 mg b.d., T 500 mg q.d.s., M 400 mg q.d.s., 14 d	108; 107	96.1; 93.1	91.7; 87.9
Furuta *et al*[17]	2014	Japan	Retrospective, single cohort	*1st line* PPI b.d., SF 100 mg b.d., M 250 mg b.d., 7 d; *1st line* PPI b.d., SF 100 mg b.d., M 250 mg b.d., 14 d; *2nd Line* PPI b.d., SF 100 mg b.d., M 250 mg b.d., 7 d; *2nd Line* PPI b.d., SF 100 mg b.d., M 250 mg b.d., 14 d; *3rd Line* PPI b.d., SF 100 mg b.d., M 250 mg b.d., 7 d; *3rd Line* PPI b.d., SF 100 mg b.d., M 250 mg b.d., 14 d	7; 4; 9; 3; 3; 2	100; 100; 100; 100; 100; 100	100; 100; 100; 100; 100; 100
Gisbert *et al*[18]	2015	Spain	Prospective, single cohort	*1st line* O 20 mg b.d., C 500 mg b.d., M 400 mg b.d., 7 d; *2nd line* O 20 mg b.d., B 120 mg q.d.s., T 500 mg q.d.s., M 500 mg t.d.s., 10 d; *3rd line* O 20 mg b.d., C 500 mg b.d., LF 500 mg b.d.,10 d; *4th line* O 20 mg b.d., C 500 mg b.d., RIF 150 mg b.d., 10 d; *3rd line* O 20 mg b.d., C 500 mg b.d., RIF 150 mg b.d., 10 d; 4th line O 20 mg b.d., C 500 mg b.d., LF 500 mg b.d.,10 d; *2nd line* O 20 mg b.d., C 500 mg b.d., LF 500 mg b.d.,10 d; *3rd line* O 20 mg b.d., B 120 mg q.d.s., T 500 mg q.d.s., M 500 mg t.d.s., 10 d; *1st line* O 20 mg b.d., B 120 mg q.d.s., T 500 mg q.d.s., M 500 mg t.d.s., 10 d; *2nd line* O 20 mg b.d., C 500 mg b.d., LF 500 mg b.d.,10 d	112; 24; 3; 2; 7; 2; 50; 3; 50; 14	59; 38; 50; 0; 20; 100; 73; 100; 75; 64;	57; 37; 33; 50; 14; 100; 64; 100; 74; 64;
Mori *et al*[19]	2017	Japan	Prospective, single cohort	*1st line* E 20 mg b.d., SF 100 mg b.d., M 250 mg b.d.,10 d; *2nd line* E 20 mg b.d., SF 100 mg b.d., M 250 mg b.d.,10 d; *3rd line* E 20 mg b.d., SF 100 mg b.d., M 250 mg b.d.,10 d	33; 19; 5	100; 84.2; 40	100; 84.2; 40
Ono *et al*[20]	2017	Japan	Retrospective,single cohort	*1st line* PPI b.d., C 200 mg b.d., M 250 mg b.d., 7 d; *1st line* V 20 mg b.d., C 200 mg b.d., M 250 mg b.d., 7 d; *1st line* PPI b.d., SF 100 mg b.d., M 250 mg b.d., 7 d; *1st line* V 20 mg b.d., SF 100 mg b.d., M 250 mg b.d., 7 d; *2nd line* PPI b.d., C 200 mg b.d., M 250 mg b.d., 7 d; *2nd line* V 20 mg b.d., C 200 mg b.d., M 250 mg b.d., 7 d; *2nd line* PPI b.d., SF 100 mg b.d., M 250 mg b.d., 7 d; *2nd line* V 20 mg b.d., SF 100 mg b.d., M 250 mg b.d., 7 d	10; 13; 20; 14; 3; 1; 24; 3	55.6; 92.3; 100; 100; 33.3; 100; 100; 66.7	50; 92.3; 100; 92.9; 33.3; 100; 100; 66.7
Sue *et al*[21]	2017	Japan	Prospective& retrospective, single cohort	*1st line* V 20 mg b.d., C 200 or 400 mg b.d., M 250 mg b.d., 7 d; *1st line* PPI b.d, C 200 or 400 mg b.d, M 750 mg b.d., 7 d	20; 30	100; 86.2	100; 83.3
Osumi *et al*[22]	2017	Japan	Prospective, single cohort	*1st line* R 20 mg b.d, Mi 100 mg b.d, M 250 mg b.d., 7 d	5	100	100
Long *et al*[23]	2018	China	Prospective, randomised	*1st line* E 20 mg b.d., C 500 mg b.d., M 400 mg b.d., 14 d; *1st line* E 20 mg b.d., B 600 mg b.d., C 500 mg b.d., M 400 mg b.d., 14 d	33; 33	70; 96	63.6; 84.8

Song *et al*[24]	2019	China	Prospective, single cohort	*1st line* E 20 mg b.d, B 220 mg b.d., LF 500 mg o.d., Cef 500 mg b.d., 14 d	152	90.1	85.5
Nyssen *et al* [25]	2020	Europe	Retrospective,multi-centre registry	*1st line* PPI, C, M; *1st line* PPI, C, LF; *1st line* PPI, B, T, M; *2nd line* PPI, C, LF; *2nd line* PPI, M, LF; *2nd line* PPI, B, T, M; *3rd line* PPI, B, T, M; *3rd line* PPI, B, C, LF; *3rd line* PPI, C, LF *(NB drug dose, frequency and duration not specified)*	285; 54; 250; 20; 13; 70; 18; 1; 2	69; 82; 92; 73.7; 76.5; 81.8; 77.8; 100; 50	69; 80; 91; 75; 76.5; 78.3; 77.8; 100; 50
Luo *et al*[26]	2020	China	Prospective, single cohort	*1st & 2nd line* E 20 mg b.d., C 500 mg b.d., M 400 mg b.d., 14 d; *1st & 2nd line* E20 mg b.d., B 220 mg b.d., C 500 mg b.d., M 400 mg q.d.s., 14 d; *1st & 2nd line* E 20 mg b.d., LF 500 mg o.d., M 400 mg b.d., 14 d; *1st & 2nd line* E 20 mg b.d., B 220 mg b.d, LF 500 mg o.d., M 400 mg q.d.s., 14 d; *1st & 2nd line* E 20 mg b.d., T 500 mg q.d.s., M 400 mg b.d., 14 d; *1st & 2nd line* E 20 mg b.d., B 220 mg b.d, T 500 mg q.d.s., M 400 mg q.d.s., 14 d	5; 22; 1; 10; 2; 72	100; 94.1; 100; 100; 100; 100	100; 81.8; 100; 80; 100; 97.2
Sue *et al*[27]	2021	Japan	Prospective, single cohort	*2nd line* V 20 mg b.d,, SF 100 mg b.d., M 250 mg b.d., 7 d	17	88.2	88.2

PP: Per protocol analysis; ITT: Intention to treat analysis; B: Bismuth compound; C: Clarithromycin; Cef: Cefuroxime; CF: Ciprofloxacin; E: Esomeprazole F: Furazolidone; LF: Levofloxacin; M: Metronidazole; Mi: minocycline O: Omeprazole; PPI: proton pump inhibitor; R: Rabeprazole; RBC: ranitidine bismuth subcitrate; RIF: Rifabutin; SF: Sitafloxacin; T: Tetracycline; V: Vonoprazan.

Japan, have shown higher success rates (92.3%-100%) for this 7 d triple therapy when combined with Vonoprazan (a potassium-competitive blocker that inhibits gastric H+K+-ATPase) instead of a PPI[20,21].

In a prospective study, Rodriguez-Torres *et al* reported a success rate of 85% with a 10 d triple therapy combining Esomeprazole, Tetracycline and Metronidazole[12]. A small retrospective study from Japan also reported a similar success rate of 80% when this regimen was used for 7-14 ds[13]. Osumi *et al* achieved a 100% success rate using a modified 7 d regimen, substituting Minocycline for Tetracycline in a small study of 5 patients[22].

Levofloxacin in combination with Clarithromycin and PPI has been reported to achieve an 80% success rate[25]. Recent studies from Japan have evaluated treatment regimens utilising the fluoroquinolone, Sitafloxacin, which has a lower minimum inhibitory concentration for *H.pylori* than Levofloxacin and is effective in strains with the gyrA mutation, which denotes resistance to Levofloxacin[19]. Remarkably high success rates of 100% were reported for 7-14 d treatment regimens combining Sitafloxacin with Metronidazole and PPI, in two retrospective[17,20] and one prospective study[19].

Quadruple therapy: Retrospective data has demonstrated a 91% success rate for the PPI-Bismuth-Tetracycline-Metronidazole quadruple therapy[25]. Three prospective studies have reported success rates of 74% with a 10 d PPI-Bismuth-Tetracycline-Metronidazole combination[18], 84.8% with a 14 d PPI-Bismuth-Clarithromycin-Metronidazole regimen[23] and 85.5% with a 14 d PPI-Bismuth-Levofloxacin-Cefuroxime treatment[24].

Second line therapy

In view of the attrition of successfully treated patients with each course of treatment, patient numbers for studies evaluating second line therapies tend to be low, often in single digits[12,17,20].

Triple therapy: Gisbert *et al*[14,18] have treated a relatively large number of patients with a 10 d combination of PPI-Clarithromycin-Levofloxacin, demonstrating success rates of 64%-73%. Levofloxacin based triple therapy using Clarithromycin or Metronidazole appears to achieve similar success rates, of 75% and 76.5%, respectively [25]. Sitafloxacin-based triple therapy has shown success rates of 100% in 2 small retrospective studies evaluating a 7 d regimen[17,20], whilst a prospective study investigating a 10 d treatment course reported a lower success rate of 84.2%[19]. Sue *et al*[27] demonstrated a success rate of 88.2% in a prospective study of a 7 d Sitafloxacin regimen using Vonoprazan instead of a PPI.

Quadruple therapy: An early study from Spain reported a low success rate of 47% using a 7 d regimen of Ranitidine Bismuth citrate-Tetracycline-Metronidazole, which has been considered as quadruple therapy due to an acid-suppressing agent and bismuth being combined into one tablet[11]. The same group of investigators also reported a low success rate of 37% for 10 d PPI-Bismuth-Tetracycline-Metronidazole quadruple therapy[18]. However, the European Registry has demonstrated a success rate of 78.3% for this regimen[25]. In a large prospective study, Liang *et al*[16] included 109 penicillin allergic patients randomised to 2 wk quadruple therapy with either PPI-Bismuth-Tetracycline-Metronidazole or PPI-Bismuth-Tetracycline-Furazolidine; success rates were 87.9% and 91.7%, with no difference between penicillin allergic and non-allergic patients[16].

A relatively large study from Australia reported on 69 patients with penicillin allergy, who had all failed prior therapy with PPI-Clarithromycin-Metronidazole. Treatment with a 10 d regimen of PPI-Bismuth subcitrate-Rifabutin-Ciprofloxacin achieved a success rate of 94.2%[15].

Luo *et al*[26] prospectively evaluated an antibiotic susceptibility approach using a variety of 14 d quadruple therapies, and demonstrated high success rates of 80%-100%. However, the results were not presented separately for first-line and rescue treatments [26].

Salvage (third-line) therapy

The published data for salvage therapy after failure of second-line treatment is very limited with 4 studies reporting on patient numbers in single figures[11,17-19]. Details are provided in the Table but it is difficult to draw any meaningful conclusions from the results.

Evidence from non-penicillin combination regimes in unselected groups of patients: Meta-analyses of trials on the efficacy of non-penicillin regimes in treating *H.pylori* infection are an alternate source of useful information when making treatment decisions about penicillin allergic individuals. These trials generally included unselected group of individuals without considering penicillin allergy status.

The meta-analysis by Gisbert *et al*[28] demonstrated a success rate of 81% with 7 d triple therapy regimen of PPI-Clarithromycin -Nitroimidazole, similar to the success rate with the regimen containing amoxicillin instead of nitroimidazole.

Two meta-analyses of randomised controlled trials on first line therapy of *H.pylori* with quadruple therapy of PPI-Bismuth-Tetracycline-Metronidazole have shown success rates of 77%[29,30]. A longer duration (10-14 d) of quadruple therapy was more effective than the 7 d triple therapy of PPI-Clarithromycin-Amoxicillin[30].

SUMMARY AND RECOMMENDATIONS

The triple therapy regimen of PPI-Clarithromycin-Metronidazole is still frequently used as first line therapy for penicillin allergic subjects[25]. However, whilst it demonstrates an acceptable success rate of approximately 80% in unselected patients [28], it does not perform well in penicillin allergic patients[11,14,18,20,21,23,25]. The reasons for this discrepancy are unclear, but it is possible is that the studies of unselected patients may only have had small numbers of penicillin allergic individuals, or the study design may have excluded individuals with antibiotic allergy. Whilst there is a paucity of recent data for this specific regimen, the efficacy of Clarithromycin-based triple therapy has been shown to be significantly impaired in

the presence of Clarithromycin resistance, which is an increasingly encountered issue [29]. Whilst increasing the duration of PPI-Amoxicillin-Clarithromycin triple therapy has been shown to improve success rates, this has not been demonstrated convincingly for the PPI-Clarithromycin-Metronidazole regimen[31]. If available, Vonoprazan could be considered as a substitute for PPI in clarithromycin-based triple therapy to improve its efficacy[20,21]. Sitafloxacin-based triple therapy is an alternative option, although this antibiotic is not widely available[17,19,20]. Bismuth-based quadruple therapy, lasting 10-14 d, is the most attractive option for first-line treatment of *H.pylori*, with a high success rate in patients with penicillin allergy[18,23,24,25], matching that in unselected patients[29,30]. In order to optimise the success of first line treatment, a detailed history of prior antibiotic use could aid the choice of regimen prescribed.

In the event of treatment failure, the published evidence suggests that second-line therapy should be instituted with the 10 d PPI-Levofloxacin-Clarithromycin regimen [14,18]; a Sitafloxacin-based triple therapy is an alternative option[17,19,20,27] . If Bismuth-based quadruple therapy has not been used as first-line treatment, then this regime could be considered for subsequent treatment, although there is variable evidence for the efficacy of PPI-Bismuth-Tetracycline-Metronidazole quadruple therapy[16,18,25]. Alternative antibiotic combinations may be more successful such as PPI-Bismuth-Tetracycline-Furazolidine[16] or PPI-Bismuth-Rifabutin-Ciprofloxacin [15], although there are concerns about the potential for side-effects with rifabutin, especially myelotoxicity[32].

It is not possible to provide any evidence-based recommendations for salvage therapy after failure of two treatment courses. It is generally recommended that in this situation, further treatment should be guided by the results of *H.pylori* culture and sensitivity testing[5,6,26]. Another approach is to confirm penicillin allergy at this stage, as many patients with this label turn out not to be truly allergic[5,6,9]. A negative penicillin skin test allows the safe use of amoxicillin-containing salvage regimens, as recommended for non-allergic patients.

CONCLUSION

This review of the evidence for treating *H.pylori* in penicillin-allergic individuals has highlighted the lack of high-quality studies to help guide management strategies. Whilst recommendations have been made based on the limited data, it would be important to monitor the success of treatment regimens and use what can be demonstrated to be effective locally[33]. Regional differences in drug availability will influence the choice of regimen, and patterns of antibiotic resistance rates will influence treatment success.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 28; 27(44): 7669-7686

DOI: 10.3748/wjg.v27.i44.7669

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

ORIGINAL ARTICLE

Basic Study

Calycosin attenuates severe acute pancreatitis-associated acute lung injury by curtailing high mobility group box 1 - induced inflammation

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Author contributions: Zhu CJ and Yang WG designed the experiments; Li DJ, Chen SY, Wang QF and Liu YN performed the experiments; Wu ZW, Cui ZC, Zhang Y and Cheng B performed data analysis; Li DJ drafted and revised the manuscript; all authors wrote, read and approved the final manuscript.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University.

Institutional animal care and use committee statement: All animal experiments were conducted in accordance with relevant

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Abstract

BACKGROUND

Acute lung injury (ALI) is a common and life-threatening complication of severe acute pancreatitis (SAP). There are currently limited effective treatment options for SAP and associated ALI. Calycosin (Cal), a bioactive constituent extracted from the medicinal herb Radix Astragali exhibits potent anti-inflammatory properties, but its effect on SAP and associated ALI has yet to be determined.

AIM

To identify the roles of Cal in SAP-ALI and the underlying mechanism.

METHODS

SAP was induced *via* two intraperitoneal injections of L-arg (4 g/kg) and Cal (25 or 50 mg/kg) were injected 1 h prior to the first L-arg challenge. Mice were sacrificed 72 h after the induction of SAP and associated ALI was examined histologically and biochemically. An *in vitro* model of lipopolysaccharide (LPS)-induced ALI was established using A549 cells. Immunofluorescence analysis and western blot were evaluated in cells. Molecular docking analyses were conducted to examine the interaction of Cal with HMGB1.

guidelines and regulations and approved by the Animal Ethics Committee of The National Drug Clinical Trial Institution of The First Affiliated Hospital of Zhengzhou University (Ethic Review Number: 2019-KY-140).

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Supported by the Key Research Projects of Henan Higher Education Institutions, No. 20A320064.

Country/Territory of origin: China

Specialty type: Immunology

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

Peer-review report's scientific quality classification

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

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Received: May 17, 2021
Peer-review started: May 17, 2021

RESULTS

Cal treatment substantially reduced the serum amylase levels and alleviated histopathological injury associated with SAP and ALI. Neutrophil infiltration and lung tissue levels of neutrophil mediator myeloperoxidase were reduced in line with protective effects of Cal against ALI in SAP. Cal treatment also attenuated the serum levels and mRNA expression of pro-inflammatory cytokines tumor necrosis factor-α, interleukin-6, IL-1β, HMGB1 and chemokine (CXC motif) ligand 1 in lung tissue. Immunofluorescence and western blot analyses showed that Cal treatment markedly suppressed the expression of HMGB1 and phosphorylated nuclear factor-kappa B (NF-κB) p65 in lung tissues and an *in vitro* model of LPS-induced ALI in A549 cells suggesting a role for HGMB1 in the pathogenesis of ALI. Furthermore, molecular docking analysis provided evidence for the direct interaction of Cal with HGMB1.

CONCLUSION

Cal protects mice against L-arg-induced SAP and associated ALI by attenuating local and systemic neutrophil infiltration and inflammatory response *via* inhibition of HGMB1 and the NF-κB signaling pathway.

Key Words: Severe acute pancreatitis; Acute lung injury; Calycosin; Mouse model; High-mobility group box 1; Nuclear factor-kappa B

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Core Tip: In this study, we showed that Calycosin protects mice against L-arginine-induced severe acute pancreatitis (SAP) and associated acute lung injury (ALI) by attenuating local and systemic inflammatory response *via* inhibition of high mobility group box 1 (HGMB1) and the nuclear factor-kappa B signaling pathway. Suppression of HMGB1 expression is a potential target for the treatment of SAP-ALI.

Citation: Zhu CJ, Yang WG, Li DJ, Song YD, Chen SY, Wang QF, Liu YN, Zhang Y, Cheng B, Wu ZW, Cui ZC. Calycosin attenuates severe acute pancreatitis-associated acute lung injury by curtailing high mobility group box 1 - induced inflammation. *World J Gastroenterol* 2021; 27(44): 7669-7686
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7669.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7669

INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease with wide clinical variation, resulting in an approximately 35% mortality when progressing to severe AP (SAP)[1]. Acute lung injury (ALI) is the most common cause of death in patients with severe AP (SAP), occurring in 10%-25% of SAP cases and responsible for up to 60% of AP-associated deaths[2]. Inflammation and pro-inflammatory cytokines play a key role in the development of SAP; therefore, inhibition of inflammation and the release of inflammatory factors are thought to be potential approaches for the therapy of SAP-ALI.

High mobility group box 1 (HMGB1), a highly conserved DNA binding nuclear protein, plays a vital role in the pathogenesis of inflammatory diseases such as pancreatitis[3]. Secreted HMGB1 released from necrotic acinar cells has been shown to aggravate the pancreatic inflammatory process[3,4]. Secreted HMGB1 exhibits cytokine-like properties that induces both the local and systemic inflammatory cascade that ultimately leads to multi-organ dysfunction[5,6]. HGMB1 has been shown to activate pro-inflammatory nuclear factor κB (NF-κB) signaling *via* interaction with multiple cell-surface receptors including Toll-like receptor (TLR) 2, TLR4 or TLR9 and receptor for advanced glycation end products (RAGE)[7]. The activation of NF-κB upregulates the gene expression of pro-inflammatory cytokines, chemokines and adhesion molecules which further aggravates the inflammatory response[4]. HMGB1 has also been shown to serve as a chemo-attractant recruiting neutrophils to site of

First decision: June 26, 2021
Revised: July 9, 2021
Accepted: September 10, 2021
Article in press: September 10, 2021
Published online: November 28, 2021

P-Reviewer: Jin CH, Lee SH, Samadder S, Şurlin VM
S-Editor: Wang LL
L-Editor: Webster JR
P-Editor: Wang LYT

inflammation and prevents neutrophil apoptosis which exacerbates tissue damage[8-10]. Blockade of HMGB1 by administration of anti-HMGB1 neutralizing antibodies was shown to inhibit the recruitment and accumulation of neutrophils in the lung[11, 12]. Thus, HMGB1 is a potential target for the treatment of ALI that is commonly found in SAP.

Calycosin (Cal) is one of the bioactive constituents extracted from the Chinese medicinal herb *Radix Astragali*, one of the five herbs of the Wutou Decoction, a classic herbal formula concocted by ancient Chinese medical doctor, Zhongjing Zhang, widely used for the treatment of rheumatoid arthritis[13]. Cal is a phytoestrogen isoflavone that has been shown to exhibit various biological effects including potent anti-inflammatory properties[14], as well as anti-cancer[15], neuroprotective[16], anti-Parkinson activity[17].

However, no studies have assessed the potential use of Cal for the expression of HMGB1 in the treatment of ALI in SAP. Hence, this study aims to address this question by exploring the effects of Cal administration on the expression of HMGB1 both in LPS induced ALI *in vitro* and an L-arginine induced ALI model in mice with SAP.

MATERIALS AND METHODS

Chemicals and reagents

L-arginine (L-arg: purity > 98%, endotoxin-free) and the BCA Protein Assay Kit were purchased from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). Calycosin (Cal: $C_{16}H_{12}O_5$, purity > 98%;) was from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Enzyme-linked immunosorbent assay (ELISA) kits for interleukin (IL)-6, HMGB1, IL-1β and MPO were obtained from Wuhan Cloud-Clone Corp. (Wuhan, China). ELISA kits for tumor necrosis factor (TNF)-α and CXCX-1 were procured from Proteintech Group (Rosemont, IL, United States). The amylase ELISA kit was bought from Shanghai BlueGene Biotech Co., Ltd. (Shanghai, China). Primary antibodies against NF-κB p65 (p65), phosphorylated NF-κB p65 (p-p65), and GAPDH were purchased from Cell Signaling Technology Inc. (Danvers, MA, United States). Primary antibody against lymphocyte antigen 6 complex locus G6D (Ly6G) was obtained from Abcam (Cambridge, United Kingdom). Primary antibody against HMGB1 and Fluorescent secondary antibody were produced by Proteintech Group. Horseradish peroxidase (HRP)-conjugated secondary antibodies and Hypersensitive WB Chemiluminescent Substrate Reagent were from Beyotime Biotechnology (Jiangsu, China).

Animals

Twenty-four male C57BL/6N mice (weight: 18-22 g, age: 8–10 wk) were purchased from Charles River Company (Beijing, China). The mice were housed in a specific pathogen-free facility with a dark/light cycle of 12/12 h in ambient temperature of 22 ± 2 ℃ and humidity of 50% ± 10%. Mice were fed standard rodent chow and clean water *ad libitum*. All animal experiments were conducted in accordance with relevant guidelines and regulations and approved by the Animal Ethics Committee of The National Drug Clinical Trial Institution of The First Affiliated Hospital of Zhengzhou University (Ethic Review Number: 2019-KY-140). All mice received humane care and the study was conducted following the ARRIVE guidelines.

Murine model of L-arginine-induced severe acute pancreatitis

L-arg was dissolved in normal saline and then sterilized by filtration (pH approximately equal to 7.0). Mice were randomly divided into four groups (n = 6 in each group): Control (Saline), L-arg (4 g/kg + Saline), L-arg + Low-dose Cal (L, 25 mg/kg bodyweight), and L-arg + High-dose Cal (H, 50 mg/kg bodyweight). The Cal treatment groups received prophylactic Cal treatment (25 or 50 mg/kg) *via* intraperitoneal injection 1 h before the first injection of L-arg. The Control and L-arg groups received an intraperitoneal injection of normal saline before L-arg injection. After the 1 h prophylactic treatment, the mice received intraperitoneal injections of either normal saline (Control group) or 4 g/kg of L-arg every hour for 2 h to induce severe AP as previously described by Dawra *et al*[18]. Blood samples were drawn from the retro-orbital venous plexus under general anesthesia using sodium pentobarbital. Blood samples were centrifuged at 3000 rpm for 15 min at 4°C and plasma serum stored at -80°C for downstream biochemical analyses of serum amylase and cytokine levels. Mice were then sacrificed, and the pancreas and lung tissues from each mouse were

quickly removed. The pancreas and left lung were dissected in two with one half being fixed in 4% paraformaldehyde (PFA) for histopathological assessment and the other half snap-frozen in liquid nitrogen and stored at -80°C for biochemical analysis.

Measurement of wet-to-dry weight ratio in lung tissue

Lung tissue wet-to-dry weight (W/D) ratio was employed to determine the extent of pulmonary edema following L-arg administration. The right lung was excised and surface water was removed by blotting with filter paper. The lung weight was immediately measured on a standard electronic laboratory scale and recorded as the wet weight (W). The lung was then dried in an oven at 60°C for 48-72 h and reweighed as dry weight. The W/D ratio was calculated based on the following formula: W/D = (wet weight − dry weight)/dry weight.

Histopathological assessments

Fixed pancreatic and lung tissues were embedded in paraffin blocks and 4 μm thin sequential sections were prepared. Tissue sections were stained with hematoxylin and eosin as per our standard laboratory protocol. Stained sections were visualized and imaged under an optical light microscope (CX31, Olympus Optical Co., Ltd., Japan), and histopathological changes were assessed by three experienced pathologists who were blind to the experimental procedure. Pancreatic tissue damage was graded using a modified Schmidt Scoring System[19] as normal to severe (scale of 0-4) based on the degree of inflammatory cell infiltration, vacuolization and acinar cell necrosis. Similarly, lung tissue sections were assessed for alveolar thickening and inflammatory cell infiltration with scoring system ranging from 0-3[20].

Immunohistochemical evaluation of Ly6G expression

For immunohistochemical staining, lung tissue sections were deparaffinized and rehydrated in graded ethanol. Sections were immersed in 3% hydrogen peroxide (in methanol) for 20 min to block endogenous peroxidase activity, and then boiled in 0.1% citrate buffer for antigen retrieval, followed by incubation in 3% BSA serum (in PBS) for 30 min at room temperature to block non-specific immuno-reactivity. Tissue sections were incubated overnight at 4°C with anti-Ly6G antibody (1:500 dilution in 3% BSA-PBS) to stain neutrophils. Following incubation with HRP-conjugated secondary antibody color development was achieved by incubating sections with diaminobenzidine color development reagent and visualized under an optical light microscope. Five non-overlapping high-power fields (×100 magnification) for each section were captured. The integrated optical density (IOD) of positive expression for Ly6G in lung tissue sections were measured from gray-scale images using Image J software (Image J 1.52, National Institute of Health) following calibration of hue (0 – 25), saturation (0-255), and intensity (0-255) levels in the area of interest. The AOD (relative expression) was determined as IOD/positive area.

Serum enzymes and cytokine quantification using ELISA

Blood samples collected were subjected to ELISA analysis to determine serum levels of amylase, TNF-α, IL-6, IL-1β, CXCL-1 and HMGB1 in accordance with the corresponding manufacturer's protocol. Serum amylase level was expressed as U/L and serum TNF-α, IL-6, IL-1β, CXCL-1 and HMGB1 Levels were expressed as pg/mL.

MPO activity in lung tissues

To assess lung MPO activity, 20 mg of frozen lung tissue were homogenized in homogenization buffer (0.5% hexadecyl trimethylammonium bromide, 5 mmol/L EDTA, and 50 mmol/L potassium phosphate buffer; pH 6.2) on ice. The homogenate was then centrifuged at 12000× g for 15 min at 4°C and the resulting supernatant was retained. MPO activity was determined in accordance with the corresponding manufacturer's protocol, MPO levels were expressed as pg/mg.

In vitro model of LPS-induced ALI in A549 cells

The lung adenocarcinoma A549 cell line was purchased from ATCC (VA, United States) and maintained in RPMI-1640 medium containing 10% fetal bovine serum and 100 IU/mL streptomycin and 100 IU/mL streptomycin. An *in vitro* cellular model of ALI was established by treating A549 cells with 1 μg/mL lipopolysaccharide (LPS, Sigma, United States) for 24 h[21]. To test the effects of Cal, cells were pretreated with various concentrations of Cal (1 μM, 5 μM, 10 μM, and 20 μM) for 1 h before LPS stimulation. After 24 h of stimulation, cells were either fixed for immunofluorescence assessment, or harvested for protein extraction for downstream western blot analyses.

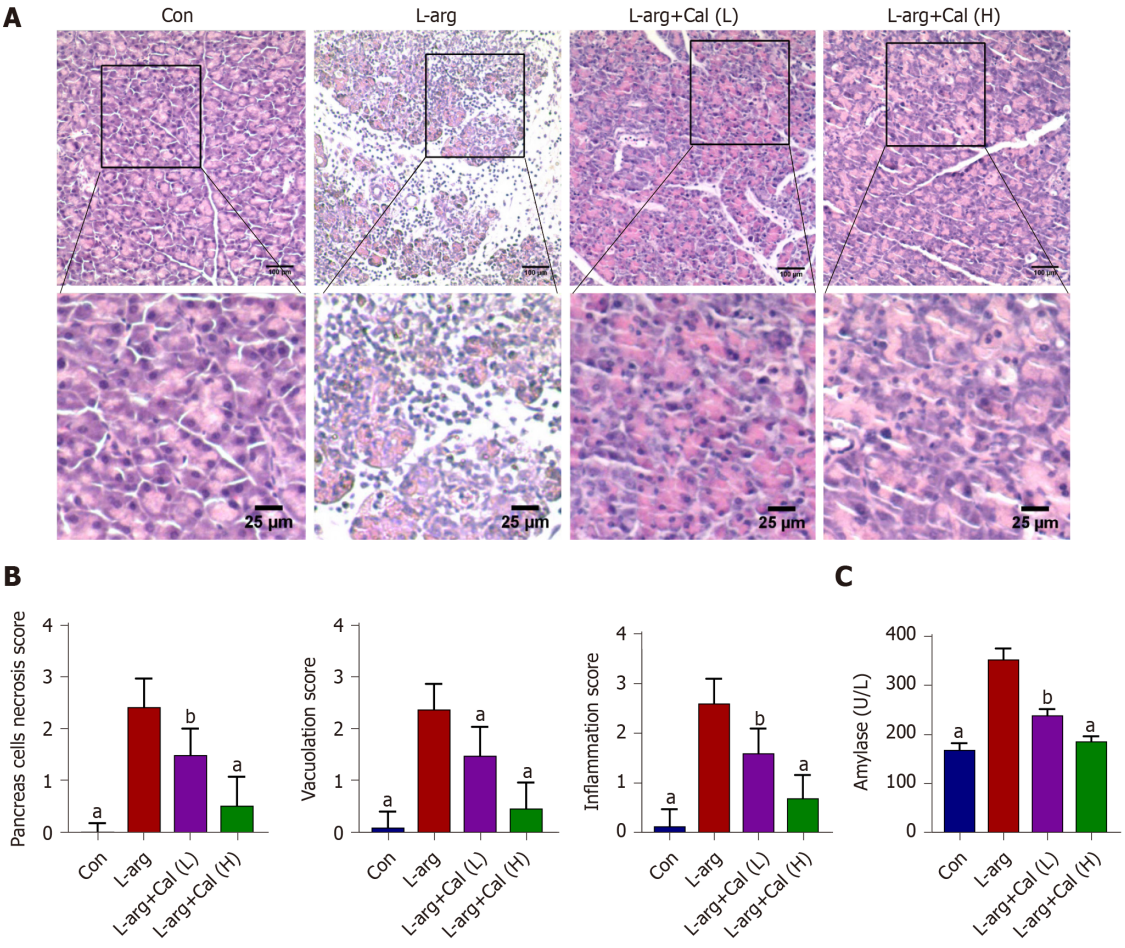


Figure 1 Effects of calycosin on L-arginine induced severe acute pancreatitis. Mice were pretreated with calycosin [calycosin (Cal): 25 and 50 mg/kg] for 1 h, L-arginine (L-arg: 4 g/kg) was intraperitoneally (*i.p.*) injected every hour for two consecutive hours. A: The histological assessment of pancreatic tissue damage in the control (Con), L-arg and Cal group (L: 25 mg/kg; H: 50 mg/kg); B: Pancreatic histological scores; C: Serum amylase and lipase levels. Data represent mean ± SD values. $^{a}P < 0.001$ *vs* L-arg group; $^{b}P < 0.01$ *vs* L-arg group. Con: Control group; Cal: Calycosin group; L-arg: L-arginine group.

CCK-8

The CCK-8 assay was used to assess the effects of Cal on A549 cell viability. Cells were seeded onto 96-well plates at a density of 1×10^{4} cells/well and then treated with various concentrations of Cal (1, 5, 10 and 20 μM) for 24 h. DMSO (0.1%) was added to the 0 μM group. After treatment, the cells were incubated with 10 μl of CCK-8 reagent for 1 h (Bosterbio, United States) and then the absorbance at 450 nm was measured using a Microplate Reader.

RT-qPCR

Total RNA was extracted using TRIzol reagent. Two microgram of total RNA was reversed transcribed into cDNA using Hiscript II Q RT SuperMix for qPCR in accordance with the manufacturer's instructions (Nanjing, China). Real-time quantitative PCR was carried out using the SYBR Green qPCR Master Mix, containing template cDNA and specific primers for TNF-α, IL-6, IL-1β, CXCL-1, or HMGB1. PCR reactions were carried out on an ABI Prism Real-time PCR System (Applied Biosystems, Foster City, CA, United States). GAPDH was used as an internal housekeeping control. The primers used are listed in Supplementary Table 1.

Immunofluorescence staining

The expression of HMGB1 and NF-κB (p-p65) in A549 cells and lung tissue was evaluated by immunofluorescence. Briefly, cells was washed twice with PBS, fixed with 4% paraformaldehyde (PFA) for 30 min, and then permeabilized with 0.5% Triton X-100 in TBS-Tween 20 (TBST) for 5 min. For lung tissues, sections were dewaxed, hydrated, and treated with EDTA-containing antigen retrieval buffer (pH 8.0) in a microwave oven, and then blocked with 5% BSA for 1 h. Samples (cells or tissue sections) were then incubated with the primary antibodies (HMGB1, 1:100; or NF-κB

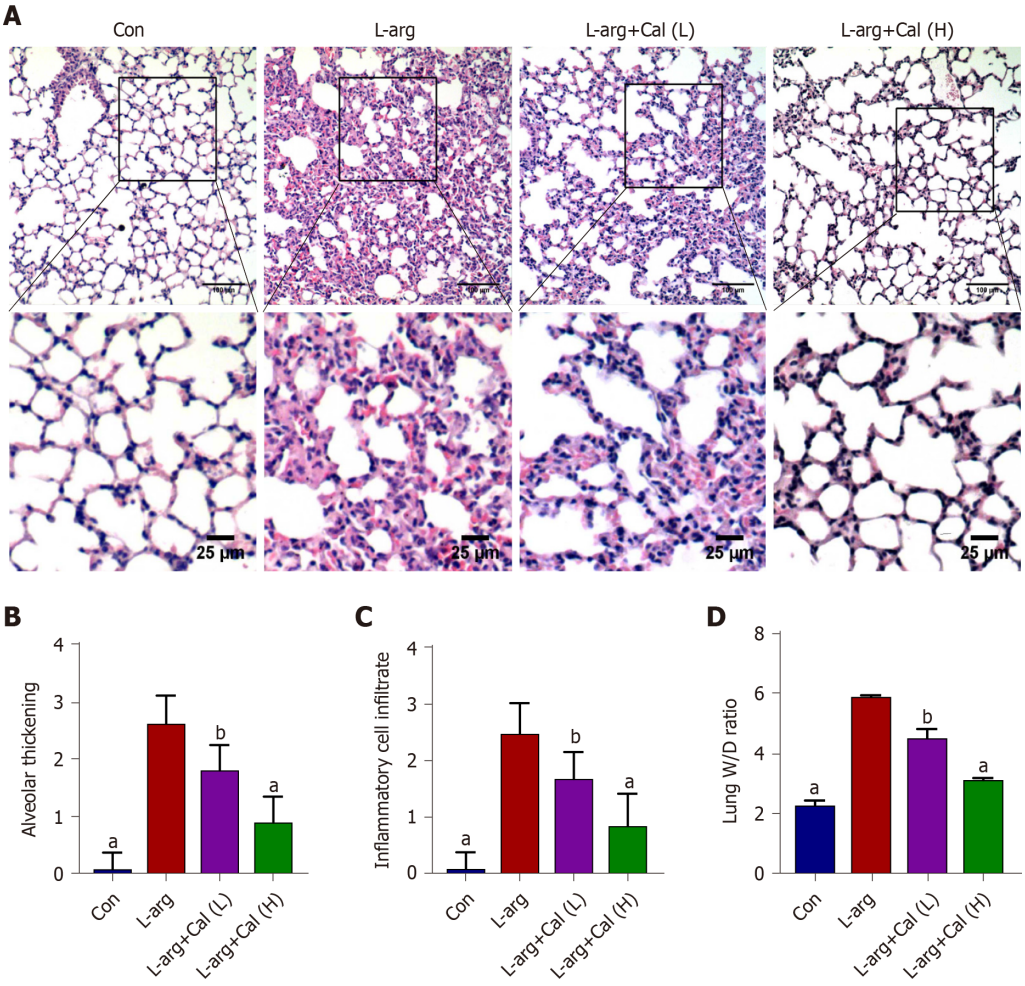


Figure 2 The effects of calycosin on histopathological findings of lung tissue in mice. H&E sections were examined by light microscopy. A: Representative pathological images of the lung tissue; B: Lung injury scores in alveolar thickness; C: Inflammatory infiltrate; D: The lung wet/dry weight ratios are shown. Data represent mean ± SD values. $^{a}P < 0.001$ *vs* L-arg group; $^{b}P < 0.01$ *vs* L-arg group. Con: Control group; Cal: Calycosin group; L-arg: L-arginine group.

p-p65, 1:200) in TBST overnight at 4°C. Samples were washed three times with TBST and then incubated with Cy3–conjugated Affinipure Goat Anti-Rabbit IgG (H + L) antibody (1:100, Proteintech) for 1 h. After washing three times with TBST, the samples were incubated with DAPI to stain the nuclei and then mounted in anti-fade mounting medium for assessment by fluorescence microscopy.

Western blot analysis

Frozen cells or lung tissues were lysed and homogenized in RIPA lysis buffer containing protease inhibitors and 1 mmol/L phenylmethanesulfonylfluoride (PMSF) on ice. Lysates were then mixed with SDS loading buffer and denatured by heating at 100°C for 10 min. Each protein sample was separated by SDS-PAGE electrophoresis (10% gel), and then transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% (w/v) skim milk in TBST for 1 h at room temperature and then incubated with primary antibodies overnight at 4°C. The following primary antibodies and dilutions were used: anti-p65 (1:1000), anti-p-p65 (1:1000), anti-HMGB1 (1:500) and anti-GAPDH (1:1000). Following extensive washing with TBST the membranes were incubated with the HRP-conjugated secondary antibody (1:1000 in 1% (w/v) skim milk in TBST) for 1 h at room temperature. Protein-antibody immunoreactivity was detected by Hypersensitive Chemiluminescent Reagent and imaged on a LI-COR Odyssey Imaging System (LI-COR, Lincoln, NE, United States). Densitometry analysis was performed using the associated software and the band intensity of target proteins was normalized to GAPDH signals.

Molecular docking

Molecular docking analysis of the interaction(s) between Cal and HMGB1 was carried out using the open-source Autodock Vina v1.1.2 software (Scripps Research, CA,

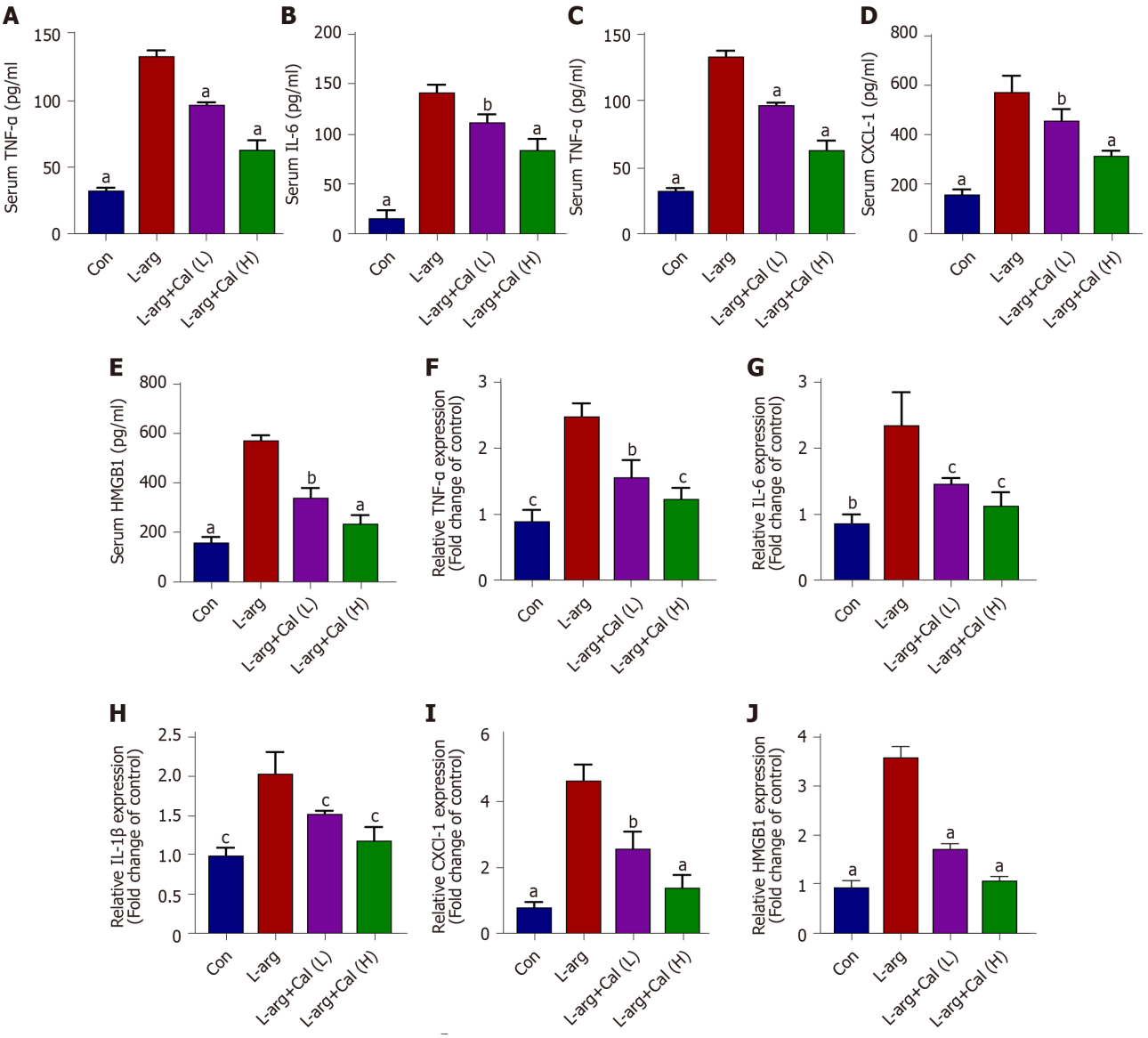


Figure 3 Effect of calycosin on tumor necrosis factor α, interleukin-6, interleukin-1β, chemokine ligand 1 and high mobility group box 1 in serum and mRNA levels. A: TNF-α; B: IL-6; C: IL-1β; D: CXCL-1; E: HMGB1 in serum levels were determined by ELISA kits. F: TNF-α; G: IL-6; H: IL-1β; I: CXCL-1; J: HMGB1 in mRNA levels. Data represent mean ± SD values. $^{a}P < 0.001$ *vs* L-arg group; $^{b}P < 0.01$ *vs* L-arg group; $^{c}P < 0.05$ *vs* L-arg group. TNF-α: Tumor necrosis factor α; IL-6: Interleukin-6; IL-1β: Interleukin-1β; HMGB1: High mobility group box 1; CXCL-1: Chemokine ligand 1; L-arg: L-arginine; Con: Control group; Cal: Calycosin group; L-arg: L-arginine group.

United States). Two-dimensional (2D) coordinates of Cal were retrieved through the PubChem website (https://pubchem.ncbi.nlm.nih.gov). The three-dimensional structure of HMGB1 A-box (PDB ID: 2RTU) was retrieved from the RCSB Protein Data Bank. Optimized binding conformations were generated using criteria such as energy minimization and cluster size. To increase the accuracy of the binding conformations generated, the value of exhaustiveness was set to 1. Finally, the superposition of Cal and HMGB1 was carried out. Receptor proteins and ligand molecules were converted into PDBQT formats.

Statistical analysis

All bar graphs presented in this study are expressed as mean ± SD values of at least three independent experiments. Differences between 4 groups were compared by one-way analysis of variance using GraphPad Prism 8.0.2 (GraphPad Software Inc., San Diego, CA, United States). Statistical significance was set at a P value < 0.05 unless otherwise stated.

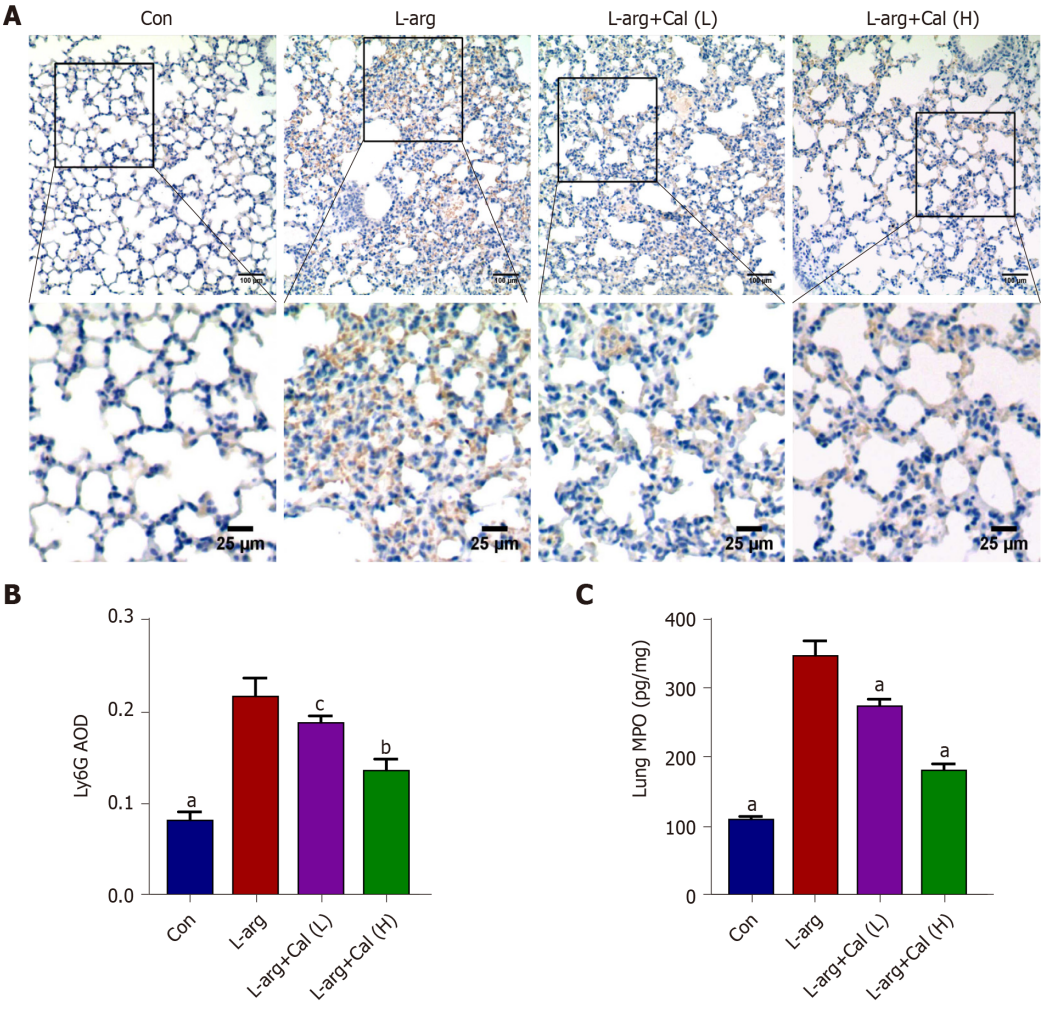


Figure 4 The effect of calycosin on the immunological staining of lung slices for Ly6G. A: Micrographs of lung section stained with immunological staining of lung slices for Ly6G (brown); B: Average optical density for Ly6G was determined; C: Levels of myeloperoxidase in per milligram lung tissue were determined by enzyme-linked immunosorbent assay kits. Data represent mean ± SD values. $^{a}P < 0.001$ *vs* L-arg group; $^{b}P < 0.01$ *vs* L-arg group; $^{c}P < 0.05$ *vs* L-arg group. Con: Control group; Cal: Calycosin group; L-arg: L-arginine group.

RESULTS

Cal treatment protects against ALI in mice with SAP

As shown in Figure 1A and B, the pancreas from mice treated with L-arg exhibited classical histological signs of SAP including significant tissue damage, acinar cell vacuolization and necrosis, as well as abundant inflammatory cell infiltration when compared with control mice ($P < 0.001$). On the other hand, mice that were administered prophylactic Cal throughout the experimental period showed a dose-dependent reduction in pancreatic tissue damage (Figure 1A). Elevated serum amylase is a key indicator of pancreatic acinar cell injury in AP. Amylase activity was markedly elevated (more than 2-fold) at 72 h following the induction of SAP in the L-arg group when compared to the control group (Figure 1C; $P < 0.001$). Consistent with histological findings, Cal administration dose-dependently decreased serum amylase levels when compared to the L-arg SAP group ($P < 0.001$). Taken together, these results indicated that Cal administration can protect mice against L-arg-induced SAP.

SAP is often accompanied by ALI and contributes to the majority of AP-associated death[22,23]. We examined whether secondary ALI was similarly induced in our SAP mouse model following L-arg treatment. As shown in Figure 2A-C, lung tissue from control mice showed normal pulmonary architecture, while the lung tissue in the L-arg SAP group exhibited significant lung edema, alveolar wall thickening, local hemorrhage, and inflammatory cell infiltration ($P < 0.001$). In contrast, the aforementioned histopathological observations in the Cal treatment groups exhibited noticeable improvement particularly when mice were treated with 50 mg/kg Cal (high-dose). Alveolar capillary permeability or pulmonary edema was assessed using the W/D

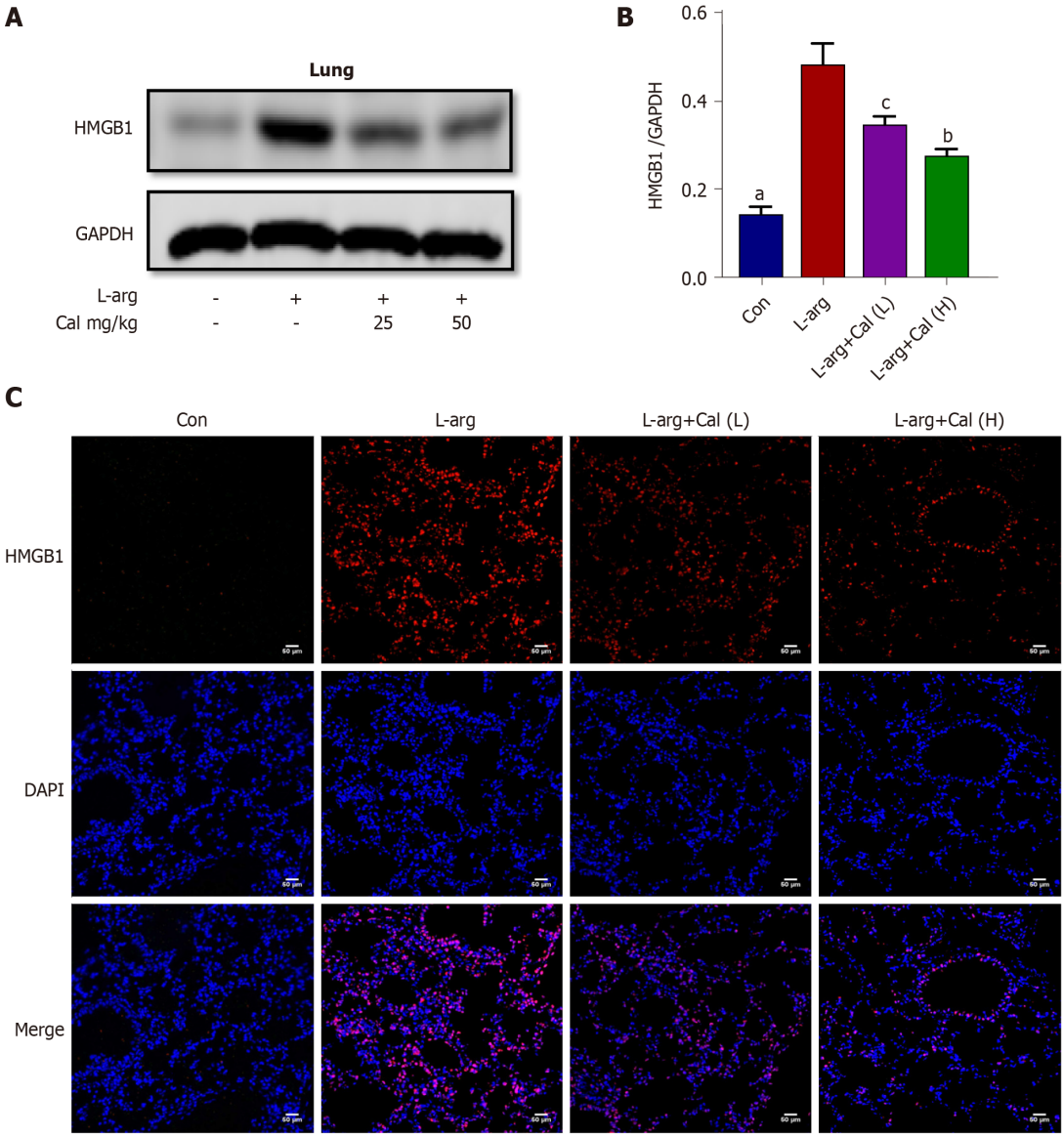


Figure 5 Effects of calycosin on the expression of high mobility group box 1 in lung tissues. A: Western blot detection of high mobility group box 1 (HMGB1) expression in lung tissue; B: Quantitative analysis of HGMB1 in lung tissue; C: Immunofluorescence staining of HMGB1 in lung tissue. Data represent mean ± SD values. $^{a}P < 0.001$ *vs* L-arg group; $^{b}P < 0.01$ *vs* L-arg group; $^{c}P < 0.05$, *vs* L-arg group. L-arg: L-arginine; HMGB1: High mobility group box 1; Con: Control group; Cal: Calycosin group.

ratio. Consistent with secondary ALI, L-arg SAP mice exhibited a markedly elevated W/D ratio compared with controls (Figure 2D; $P < 0.001$), whereas Cal treatment significantly reduced pulmonary edema (lower W/D ratio) in a dose-dependent manner. Together, our results showed that Cal treatment can further alleviate secondary ALI in mice with SAP.

Cal inhibits pro-inflammatory cytokine expression and secretion

The expression and release of pro-inflammatory cytokines and mediators are critical effectors that exacerbate local pancreatic tissue damage and mediate systematic inflammation during SAP[24]. Thus, serum levels of pro-inflammatory cytokines and chemokines such as TNF-α, IL-6, IL-1β, CXCL-1 and HMGB1 were determined using ELISA and their mRNA levels in lung tissue were also determined. Consistent with histological observations, serum levels of TNF-α, IL-6, IL-1β, CXCL-1 and HMGB1 were markedly elevated in the L-arg SAP group when compared with controls (Figure 3A-E, respectively; $P < 0.001$) as well as their mRNA expression levels (Figure 3F-J). In contrast, Cal treatment dose-dependently reduced the secretion and serum concentration of these pro-inflammatory cytokines and chemokine when compared with the L-arg SAP group.

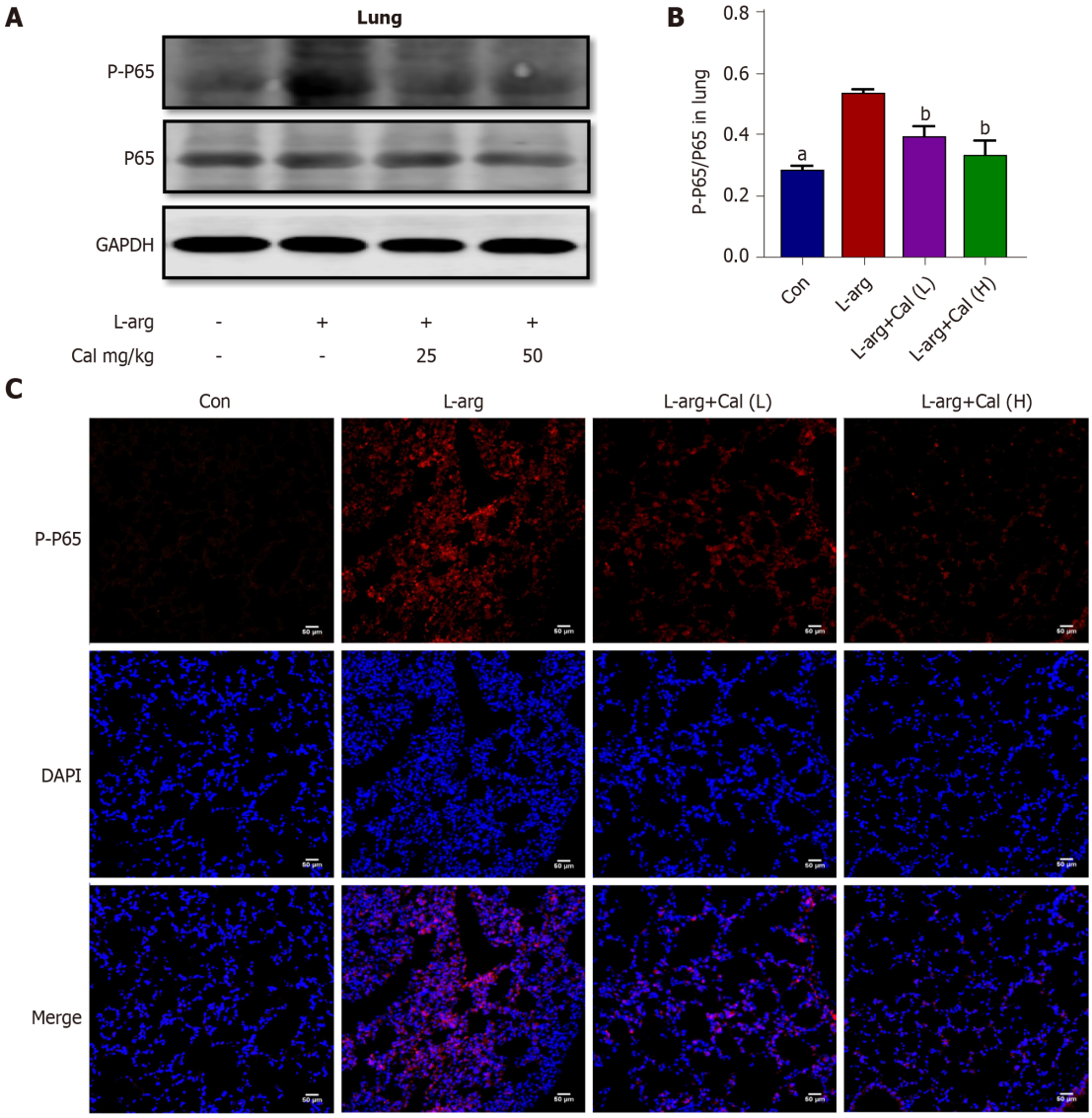


Figure 6 Effects of calycosin on the expression of phosphorylated nuclear factor-kappa B-p65 expression in lung tissues. A: Western blot detection of phosphorylated nuclear factor-kappa B-p65 (NF-κB-p65) expression in lung tissue; B: Quantitative analysis of phosphorylated NF-κB-p65 in lung tissue; C: Immunofluorescence staining of phosphorylated NF-κB-p65 in lung tissue. Data represent mean ± SD values. $^{a}P < 0.001$ *vs* L-arg group; $^{b}P < 0.01$ *vs* L-arg group. p-p65: Phosphorylated nuclear factor-kappa B-p65; Con: Control group; Cal: Calycosin group; L-arg: L-arginine group.

Cal prevented neutrophil infiltration in the lung

Previous studies have shown that the accumulation of a large number of infiltrating neutrophils in the lungs is a pathophysiological feature of ALI[7,8]. We assessed whether this corresponded with reduced neutrophil infiltration in the lungs. To this end, lung tissue sections were stained for Ly6G, a specific marker that separates neutrophils from other inflammatory cells such as leukocytes[25]. As shown in Figure 4A and B ($P < 0.001$), a significantly greater number of Ly6G-positive cells was observed in L-arg SAP mice than in control mice. This elevation in Ly6G positivity in the L-arg SAP group was also correlated with marked increases in lung MPO activity (Figure 4C; $P < 0.001$) further attesting to significant neutrophil infiltration in the lungs following L-arg-induced SAP. On the other hand, Cal treatment dose-dependently reduced neutrophil infiltration as demonstrated by reduced Ly6G positivity in lung tissues (Figure 4A and B; $P < 0.05$ and $P < 0.01$) and diminished lung MPO activity (Figure 4C; $P < 0.001$). These results further strengthened the protective effects of Cal against secondary ALI associated with AP.

Cal suppressed the activation of HMGB1 and the NF-κB signaling pathway in vivo

Previous studies have shown that many of the pro-inflammatory effects of extracellular HMGB1 is driven by activation of the NF-κB signaling pathway[3-4]. To examine whether NF-κB signaling is involved in mediating the inflammatory effects of

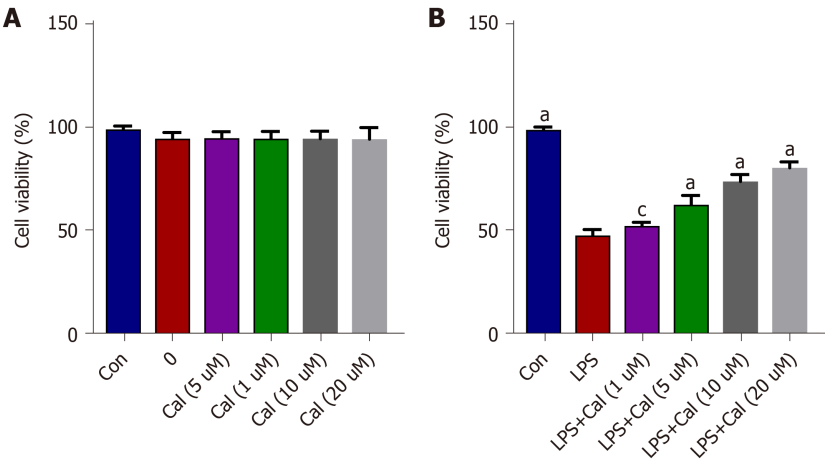


Figure 7 Effects of calycosin on the cell viability of A549 cells. A: Cell vitality was detected by the CCK8 assay after calycosin Cal (1 μM, 5 μM, 10 μM, and 20 μM) treatment; B: Cal pretreatment markedly increased cell vitality induced by lipopolysaccharide (LPS, 1 μg/mL). Data represent mean ± SD values. $^{a}P < 0.001$ *vs* LPS group; $^{c}P < 0.05$ *vs* L-arg group. Con: Control group; Cal: Calycosin group; LPS: Lipopolysaccharide group.

HMGB1 in ALI *in vivo*, we examined HMGB1 and NF-κB expression and signaling activation using immunofluorescence and western blot analyses, respectively. Our results showed that the expression of HMGB1 (Figure 5A-C) and p-p65 (activated form of NF-κB p65 subunit) (Figure 6A-C) was significantly elevated ($P < 0.001$) in lung tissues following L-arg induced SAP. Treatment with Cal resulted in a dose-dependent decrease in HMGB1 and p-p65 expression in the lungs. These results suggest that the inhibition of HMGB1, NF-κB signaling activation and pro-inflammatory cytokine secretion is in part associated with the protective effect of Cal against ALI in mice with SAP.

Cal treatment improved LPS-induced A549 cells viability and inflammatory response

To further define the molecular mechanism of the protective effects of Cal against ALI, an *in vitro* model of ALI was established by stimulating the lung adenocarcinoma cell-line A549 with LPS in the absence or presence of Cal. As shown in Figure 7A, treatment with the indicated concentrations of Cal for 24 h did not exert detrimental effects on A549 cell viability (Figure 7A). On the other hand, treatment with LPS (1 μg/mL) markedly reduced A549 cell viability as compared with untreated controls, while treatment with Cal resulted in a dose-dependent enhancement of cell viability especially at 20 μM (Figure 7B). Based on these data, 20 μM of Cal was used for downstream investigations.

Cal suppressed the activation of HMGB1 and the NF-κB signaling pathway in vitro

The effects of Cal on HMGB1 and NF-κB p65 expression and activation were examined using the LPS-induced ALI cellular model. Immunofluorescence and western blot analyses demonstrated that LPS stimulation markedly induced HMGB1 and p-p65 expression (Figure 8 and Figure 9; $P < 0.001$) and this was markedly attenuated following treatment with Cal.

The theoretical binding mode of the Cal and HMGB1 pathway

The interaction between HMGB1 A-box and Cal was assessed using molecular docking software. The HMGB1 structure consists of the A-box and B-box, two DNA binding domains and a negatively charged acidic C-terminal tail. The B-box fragment has a pro-inflammatory effect, while the A-box fragment is known to be antagonistic to inflammatory responses, when the A-box binds to the TLR4 receptor but is not able to trigger TLR4 dimer formation due to a lack of critical interactions with the TLR4[26]. As shown in Figure 10, amino acid residues Arg73, and Gly14 of HMGB1 interacts with Cal to form hydrogen bonds. Amino acid residues Glu77, Lys11, Lys10, Asp8, Arg8, Arg13, and Pro12 of the HMGB1 A-box form hydrophobic interactions with Cal. These molecular docking results suggest that Cal exhibits distinct binding affinities to the HMGB1 A-box that inhibited its downstream signaling pathways.

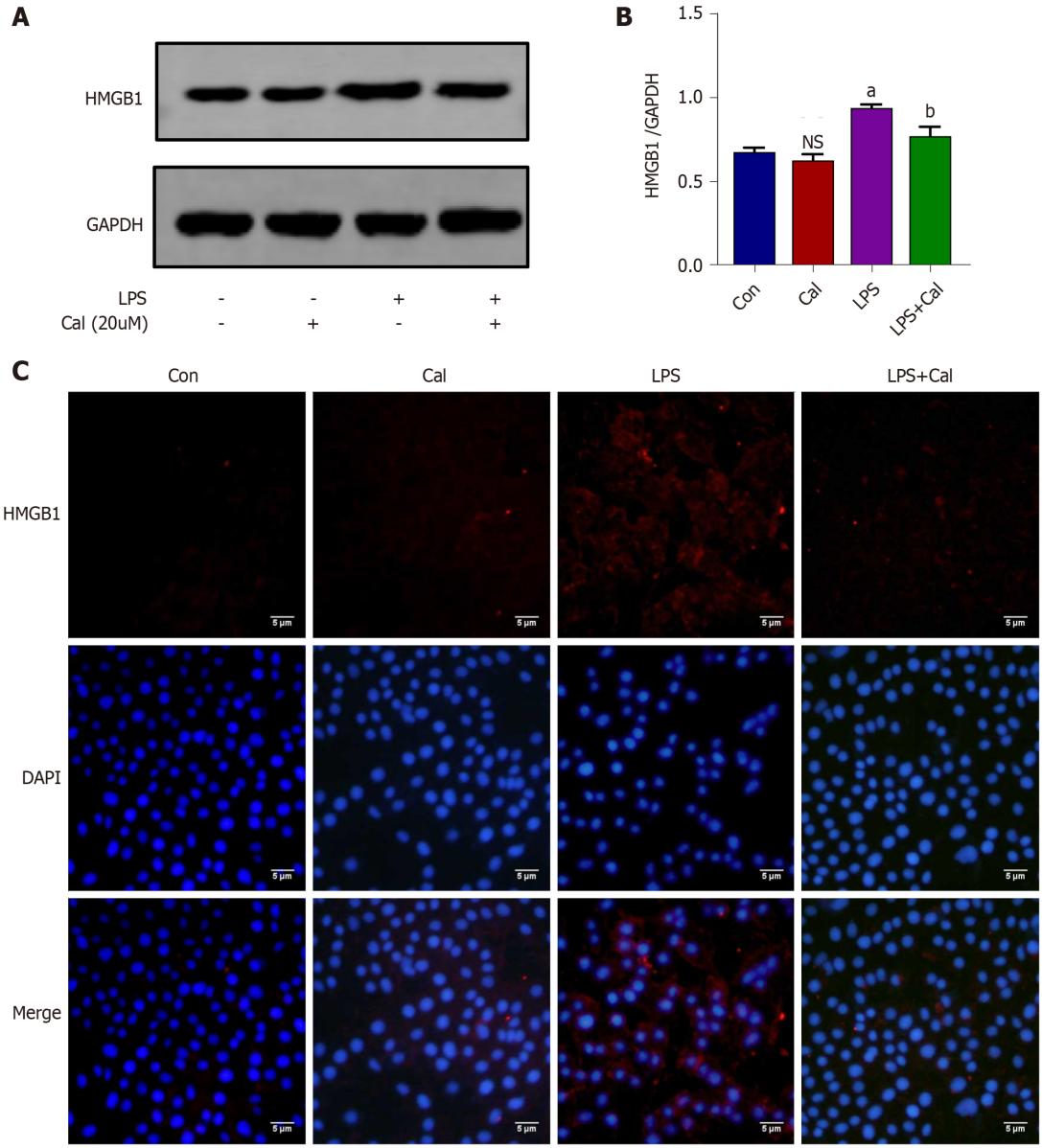


Figure 8 Effects of calycosin on the expression of high mobility group box 1 *in vitro* model of LPS-induced ALI using A549 cells. A: Western blot detection of high mobility group box 1 (HMGB1) expression in A549 cells; B: Quantitative analysis of HMGB1 in A549 cells; C: Immunofluorescence staining of HMGB1 in A549 cells. Control group (untreated), Calycosin group (Cal, 20 μM), LPS group and LPS + Cal group were treated with 1 μg/mL LPS for 24 h in the absence or presence of Cal (20 μM) pretreatment for 1 h. Data represent mean ± SD values. $^{a}P < 0.001$ *vs* control group; $^{b}P < 0.01$ *vs* LPS group. ns: No significance *vs* Con group. HMGB1: High mobility group box 1; Con: Control group; Cal: Calycosin group; LPS: Lipopolysaccharide group.

DISCUSSION

SAP is one of the most common causes of acute abdominal pain and often manifests with many complications, resulting in high mortality[1]. The pathogenesis of AP is multifactorial and involves a multi-step process. In the early stages of the disease, intra-pancreatic activation of pancreatic enzymes such as trypsin, leads to auto-digestion of acinar cells and initiates the production and release of various pro-inflammatory mediators (or cytokine cascade). The elevation in pancreatic pro-inflammatory cytokine levels induces pancreatic oxidative stress and increases vascular permeability, leading to pancreatic edema and acinar cell necrosis, which augments pancreatic inflammation[27,28]. At this stage, inflammatory cell infiltration and macrophage activation results in the further release of systemic cytokines and inflammatory mediators, leading to systemic inflammatory response syndrome. ALI is a common and severe complication associated with SAP[29]. Thus, identifying effective therapies that can effectively treat local and systemic tissue damage remains an unmet medical challenge.

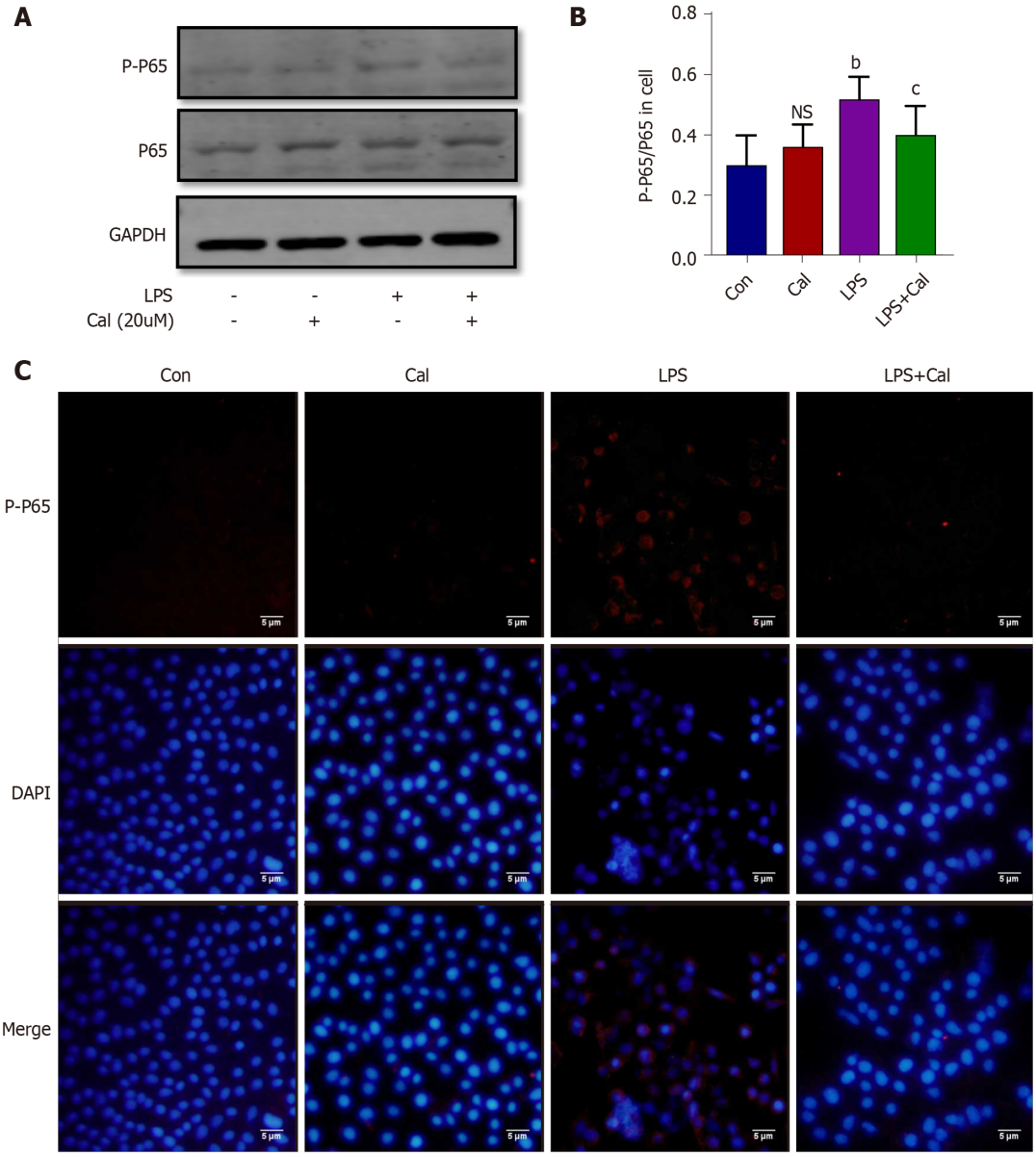


Figure 9 Effects of calycosin on the expression of phosphorylated nuclear factor-kappa B-p65 expression in the *in vitro* model of LPS-induced ALI using A549 cells. A: Western blot detection of phosphorylated nuclear factor-kappa B-p65 (NF-κB-p65) expression in A549 cells; B: Quantitative analysis of phosphorylated NF-κB-p65 in A549 cells; C: Immunofluorescence staining of phosphorylated NF-κB-p65 in A549 cells. Data represent mean ± SD values; $^{b}P < 0.01$ *vs* Con group; $^{c}P < 0.05$ *vs* LPS group. Ns: No significance *vs* Con group. p-p65: Phosphorylated nuclear factor-kappa B-p65; Con: Control group; Cal: Calycosin group; LPS: Lipopolysaccharide group.

ALI is the most common extra-pancreatic complication leading to death in SAP patients, and there is no consensus on the most effective treatment[3]. Therefore, we aimed to explore the effect of Cal on ALI in SAP. We showed that Cal extracted from the Chinese medicinal herb *Radix astragali* effectively protected mice against L-arg-induced ALI in SAP. L-arg-induced SAP is a well-established model of pancreatitis that reflects the pathological changes seen in humans[18]. We found that mice prophylactically treated with Cal exhibited a marked decrease in serum amylase and tissue MPO activity, pronounced reductions in pancreatic and pulmonary lung tissue damage, and significantly diminished pro-inflammatory cytokine production in part due to inhibition of the HMGB1/NF-κB signaling axis. The molecular docking analysis results suggest that Cal directly binds HMGB1 *via* hydrogen bonds and hydrophobic interactions *via* multiple residues on HMGB1. However, the implication of these residues on HMGB1 activity and function remains to be elucidated.

We first assessed the effect of Cal on the histopathological injury in pancreas and lung tissue. The results revealed severe pancreatic histoarchitectural changes, including pancreatic edema and vacuolization, acinar cell necrosis, and inflammatory cell infiltration. The incidence of lung inflammation, including histopathological

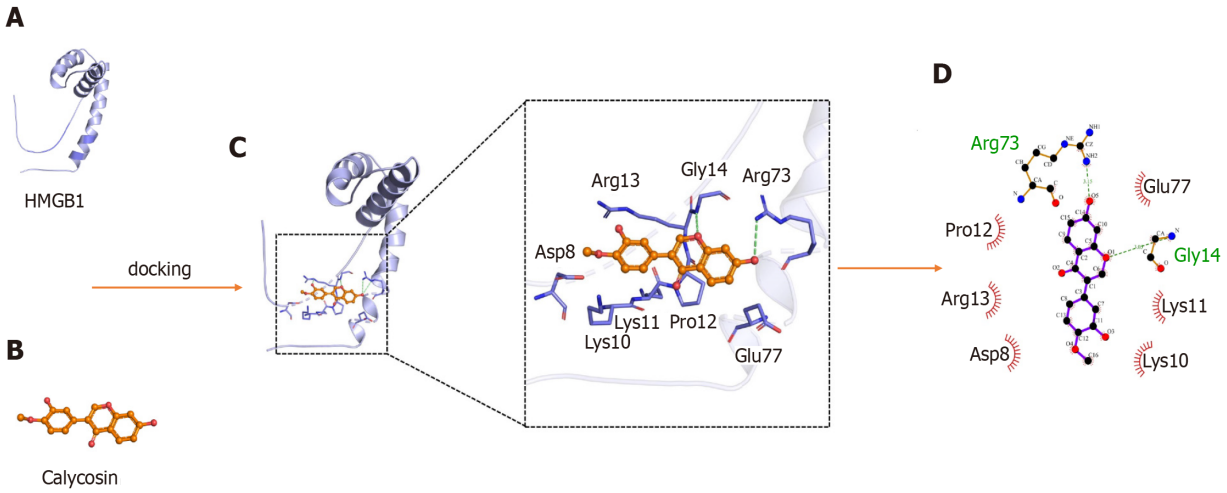


Figure 10 Theoretical binding mode of calycosin and high mobility group box 1 A-box. A: The 3D structure of high mobility group box 1 (HMGB1) A-box; B: The 3D structure of calycosin (Cal); C: 3D docking mode between Cal and HMGB1 A-box simulated by Discovery Studio and the amino acid of the active site; D: Two-dimensional schematic interaction diagram between Cal and HMGB1 A-box, the color of the amino acid residue is drawn by interaction. HMGB1: High mobility group box 1; Cal: Calycosin.

changes such as marked alveolar wall thickening, edema (demonstrated by increased lung wet/dry ratio), and pronounced inflammatory cell infiltration particularly by neutrophils (elevated lung MPO levels) was observed in SAP mice. In contrast, mice prophylactically treated with Cal showed dose-dependent amelioration of the severity of ALI in L-arg-induced SAP, as demonstrated by significant reductions in the histopathological manifestations and serum indices of SAP and ALI.

We then investigated the protective effect of Cal on the expression of inflammatory factors and neutrophil infiltration. In fact, the severity of local and systemic organ damage is dependent on the level of pro-inflammatory cytokine production[30]. Pro-inflammatory cytokines TNF-α, IL-1β and IL-6, play critical roles in the development and progression of SAP and perpetrators of systemic inflammatory response and organ damage, including ALI[31,32]. Blockade of serum TNF-α, IL-1β and IL-6 has been shown to attenuate the disease process[33,34]. Consistent with previous findings and our histopathological observations, serum levels of TNF-α, IL-6, and IL-1β were elevated in SAP mice when compared with controls as well as the mRNA levels in lung tissue, while Cal treatment reversed the change. In particular, the infiltration of inflammatory cells, mainly neutrophils, is a hallmark of tissue injury in SAP. Previous studies have demonstrated that HMGB1 and chemokine (CXC motif) ligand 1 (CXCL-1) play a role in the recruitment of neutrophils to the lungs, leading to tissue damage [8,35]. Functional inhibition of HMGB1 or CXC chemokines was shown to ameliorate tissue damage[11,12,36]. Similarly, we showed that Cal treatment suppressed the serum levels and gene expression of HMGB1 and CXCL-1 levels in the lung, with an associated reduction in neutrophil infiltration and MPO expression in lung tissue. This result may have partially contributed to the inhibitory effects of Cal on inflammatory factors and neutrophil infiltration.

Mechanistically, the HMGB1-dependent activation of NF-κB has been implicated in the development of ALI[37]. Studies have shown that the suppression of HMGB1 expression using siRNA can inhibit NF-κB activation, reduce inflammatory reactions, and protect mice against developing ALI in SAP[38,39]. Consistent with these reports, we demonstrated that Cal treatment dose-dependently inhibited the expression of HMGB1 and NF-κB signaling activation both *in vivo* and *in vitro*. In addition, studies have illustrated that HMGB1 mediates pancreatic pain by targeting RAGE and the CXCL12/CXCR4 signaling axis in mice with AP[40], therefore, pain may be relieved in animals after Cal treatment.

This study provides an experimental basis for the clinical application of Cal, which may be a candidate for the treatment of SAP-ALI patients in the future. However, there are limitations to the present study. For example, Cal inhibited the HMGB1/NF-κB signaling pathway *in vivo* and *in vitro* and validated the interaction through molecular docking. Therefore, the specific interaction between Cal and HMGB1 requires further study.

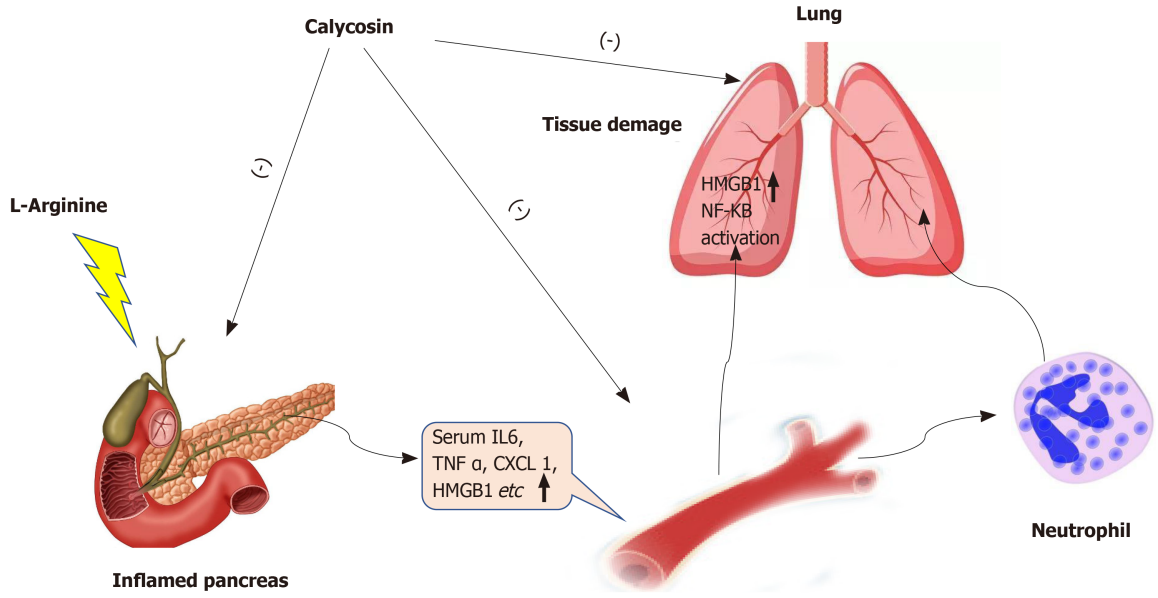


Figure 11 Calycosin attenuates acute lung injury in L-arginine induced severe acute pancreatitis by curtailing high mobility group box 1-induced inflammation.

CONCLUSION

In summary, our data clearly demonstrated that Cal exhibits protective and beneficial effects against ALI in SAP by averting local and systemic neutrophil infiltration and the inflammatory response in part *via* the suppression of HMGB1-NF-κB signaling activation. (Figure 11).

ARTICLE HIGHLIGHTS

Research background

Acute lung injury (ALI) is a common and life-threatening complication of severe acute pancreatitis (SAP). There are currently limited effective treatment options for SAP and associated ALI. Calycosin (Cal), a bioactive constituent extracted from the medicinal herb *Radix astragali* exhibits potent anti-inflammatory properties, but its effect on SAP and associated ALI has yet to be determined.

Research motivation

To determine the effect of Cal on the SAP-ALI and its underlying mechanism.

Research objectives

To identify the roles of Cal in SAP-ALI and the underlying mechanism.

Research methods

SAP was induced *via* two intraperitoneal injections of L-arginine (L-arg: 4g/kg). Cal-treated mice received intraperitoneal injections of Cal (25 or 50 mg/kg) 1 h prior to the first L-arg challenge. Mice were sacrificed 72 h after the second L-arg challenge and indices of SAP and associated ALI were examined histologically and biochemically. An *in vitro* model of lipopolysaccharide (LPS)-induced ALI was established using A549 cells. Cells were either fixed for immunofluorescence analysis or protein extracted for western blot assessment of High Mobility Group Box 1(HGMB1) and nuclear factor-kappa B (NF-κB) expression, respectively. Molecular docking analyses were conducted to examine the interaction of Cal with HMGB1.

Research results

Cal treatment significantly reduced serum amylase levels and alleviated histopathological injury associated with SAP and ALI. Neutrophil infiltration and lung tissue levels of the neutrophil mediator myeloperoxidase (MPO) were reduced in line with

the protective effects of Cal against ALI in SAP. Cal treatment also attenuated the serum levels and mRNA expression of pro-inflammatory cytokines in lung tissue. Cal treatment markedly suppressed the expression of HMGB1 and phosphorylated NF-κB p65 in lung tissues and in an *in vitro* model of LPS-induced ALI in A549 cells. Furthermore, molecular docking analysis provided evidence for the direct interaction of Cal with HGMB1.

Research conclusions

Cal protects mice against L-arg-induced SAP and associated ALI by attenuating local and systemic neutrophil infiltration and the inflammatory response *via* inhibition of HGMB1 and the NF-κB signaling pathway.

Research perspectives

Cal may be used as a potential medicine in SAP-ALI therapy.

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World J Gastroenterol 2021 November 28; 27(44): 7687-7704

DOI: 10.3748/wjg.v27.i44.7687

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

ORIGINAL ARTICLE

Basic Study

Prediction of genetic alterations from gastric cancer histopathology images using a fully automated deep learning approach

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Author contributions: Jang HJ and Lee SH were responsible for the study concept and design; Lee SH enrolled the cohorts and collected clinicopathological data from patients; Lee A, Kang J, Song IH and Lee SH performed the assays; Jang HJ , Lee A, Kang J, Song IH and Lee SH analyzed data; Jang HJ and Lee SH wrote the manuscript.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of the College of Medicine at the Catholic University of Korea (KC19SESI0787).

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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Abstract

BACKGROUND

Studies correlating specific genetic mutations and treatment response are ongoing to establish an effective treatment strategy for gastric cancer (GC). To facilitate this research, a cost- and time-effective method to analyze the mutational status is necessary. Deep learning (DL) has been successfully applied to analyze hematoxylin and eosin (H and E)-stained tissue slide images.

AIM

To test the feasibility of DL-based classifiers for the frequently occurring mutations from the H and E-stained GC tissue whole slide images (WSIs).

METHODS

From the GC dataset of The Cancer Genome Atlas (TCGA-STAD), wild-type/mutation classifiers for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes were trained on 360 × 360-pixel patches of tissue images.

RESULTS

The area under the curve (AUC) for the receiver operating characteristic (ROC) curves ranged from 0.727 to 0.862 for the TCGA frozen WSIs and 0.661 to 0.858 for the TCGA formalin-fixed paraffin-embedded (FFPE) WSIs. The performance of the classifier can be improved by adding new FFPE WSI training dataset from our institute. The classifiers trained for mutation prediction in colorectal cancer completely failed to predict the mutational status in GC, indicating that DL-based mutation classifiers are incompatible between different cancers.

CONCLUSION

Supported by the National Research Foundation of Korea funded by the Korea government (Ministry of Science and ICT), No. 2019R1F1A1062367.

Country/Territory of origin: South Korea

Specialty type: Pathology

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

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

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Received: July 29, 2021
Peer-review started: July 29, 2021
First decision: August 19, 2021
Revised: September 5, 2021
Accepted: November 13, 2021
Article in press: November 13, 2021
Published online: November 28, 2021

P-Reviewer: Kumar I, Nearchou IP, Salvi M
S-Editor: Wang LL
L-Editor: A
P-Editor: Wang LL

This study concluded that DL could predict genetic mutations in H and E-stained tissue slides when they are trained with appropriate tissue data.

Key Words: Gastric cancer; Mutation; Deep learning; Digital pathology; Formalin-fixed paraffin-embedded

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Core Tip: Recently, deep learning approach has been implemented to predict the mutational status from hematoxylin and eosin (H and E)-stained tissue images of diverse tumors. The aim of our study was to evaluate the feasibility of classifiers for mutations in the CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes in gastric cancer tissues. The area under the curves for receiver operating characteristic curves ranged from 0.727 to 0.862 for the The Cancer Genome Atlas (TCGA) frozen tissues and 0.661 to 0.858 for the TCGA formalin-fixed paraffin-embedded tissues. This study confirmed that deep learning-based classifiers can predict major mutations from the H and E-stained gastric cancer whole slide images when they are trained with appropriate data.

Citation: Jang HJ, Lee A, Kang J, Song IH, Lee SH. Prediction of genetic alterations from gastric cancer histopathology images using a fully automated deep learning approach. *World J Gastroenterol* 2021; 27(44): 7687-7704
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7687.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7687

INTRODUCTION

Molecular tests to identify specific mutations in solid tumors have improved our ability to stratify cancer patients for more selective treatment regimens[1]. Therefore, molecular tests to detect various mutations are recommended for some tumors, including EGFR mutations in lung cancer, KRAS in colorectal cancer, and BRAF in melanoma. However, it is not routinely applied to cancer patients because molecular tests are not cost- and time-efficient[2]. Furthermore, the clinical significance of many mutations is still not well understood. For example, mutation profiling of gastric cancer (GC) is still proceeding, and the meaning of each mutation is not clearly understood[3]. GC is the fifth most common cancer and the third leading cause of cancer-related deaths worldwide[4]. It is important to evaluate the relationship between the mutational status and clinical characteristics of GC to improve the clinical outcomes of GC patients. Furthermore, many targeted drugs for treating various tumors are not effective in GC therapy because GC is not enriched with known driver mutations[5]. Therefore, research to characterize the roles of GC-related genes on the clinical behavior of tumors and the potential response to targeted therapies will have immense importance for the improvement of treatment response in GC[6]. A cost- and time-effective method to determine the mutational status of GC patients is necessary to promote these studies.

Recently, deep learning (DL) has been increasingly implemented to predict the mutational status from hematoxylin and eosin (H and E)-stained tissue slides of various cancers[7-11]. The H and E-stained tissue slides were made for almost all cancer patients for basic diagnostic studies by pathologists[12]. Therefore, mutation prediction from the H and E-stained tissue slide based on a computational method can be a cost- and time-effective alternative tool for conventional molecular tests[13-15]. Although it has long been recognized that the morphological features of tissue architecture reflect the underlying molecular alterations[16,17], the features are not easily identifiable by human evaluators[18,19]. DL offers an alternative solution to overcome the limitations of a visual examination of tissue morphology by pathologists. By combining feature learning and model fitting in a unified step, DL can capture the most discriminative features for a given task directly from a large set of tissue images [20]. Digitization of tissue slides has been rapidly increasing after the approval of digitized whole-slide images (WSIs) for diagnostic purposes[21]. Digitized tissue data

are rapidly accumulating with their associated mutational profiles. Therefore, the DL-based analysis of tissue slides for the mutational status of cancer tissues has immense potential as an alternative or complementary method for conventional molecular tests.

Based on the potential of DL for the detection of mutations from digitized tissue slides, in a previous study, we successfully built DL-based classifiers for the prediction of mutational status of APC, KRAS, PIK3CA, SMAD4, and TP53 genes in colorectal cancer tissue slides[11]. This study investigated the feasibility of classifiers for mutations in the CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes in GC tissues. First, the classifiers were trained and tested for GC tissue slides from The Cancer Genome Atlas (TCGA). The generalizability of the classifiers was tested using an external dataset. Then, new classifiers were trained for combined datasets from TCGA and external datasets to investigate the effect of the extended datasets. The results suggest that it is feasible to predict mutational status directly from tissue slides with deep learning-based classifiers. Finally, as the classifiers for KRAS, PIK3CA, and TP53 mutations for both colorectal and GC were available, we also analyzed the generalizability of the DL-based mutation classifiers trained for different cancer types.

MATERIALS AND METHODS

Part I: Tests with The Cancer Genome Atlas whole-slide image datasets

Patient cohort: The Cancer Genome Atlas (TCGA) provides extensive archives of digital pathology slides with multi-omics test results to test the possibility of tissue-based mutation detection[22]. After a carefully review of all the WSIs in the TCGA GC dataset (TCGA-STAD), we eliminated WSIs with poor scan quality and very small tumor contents. We selected slides from 25, 19, 34, 64, and 160 patients, which were confirmed to have mutations in CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, respectively. There were more than two slides for many patients in the TCGA dataset, with a maximum of four slides for some patients. However, in many cases, one or two slides contained only normal tissues. We excluded normal slides and selected a maximum of two tumor-containing slides per patient. The final number of frozen tissue slides was 34, 26, 50, 94, and 221 and that of formalin-fixed paraffin-embedded (FFPE) tissue slides was 27, 19, 34, 66, and 174 for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, respectively. We selected 183 patients with wild-type CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes. Therefore, the same patients with wild-type genes for CDH1, ERBB2, KRAS, PIK3CA, and TP53 can be involved in the training of every classifier as a non-mutated group. This may help the comparison of the different classifiers more standardized because they all have the same group of patients as the wild-type group. The TCGA IDs of the patients in each group are listed in Supplementary Table 1. Our previous studies recognized that a DL model cannot perform optimally for both training and testing unless the dataset is forced to have similar amounts of data between classes[23]. Therefore, we limited the difference in patient numbers between the mutation and wild-type groups to less than 1.4 fold by random sampling. For example, only 35 of the 183 wild-type patients were randomly selected as the CDH1 wild-type group because there were only 25 CDH1 mutated patients. Ten-fold cross-validation was performed based on these randomly sampled wild-type patients. However, the classifiers yielded better results when the tumor patches from all wild-type patients other than the test sets were randomly sampled to match the 1.4 fold data ratio of wild-type/mutation groups for training, as this strategy could include a greater variety of tissue images. Therefore, we included all wild-type patients other than the test sets during training and randomly selected patients during testing.

Deep learning model: In general, a WSI is too large to be analyzed simultaneously using a deep neural network. Therefore, the analysis results for small image patches are integrated for conclusion. We divided a WSI into non-overlapping patches of 360 × 360 pixel tissue images at 20× magnification to detect mutational status. To make the classification process fully automated, artifacts in the WSIs such as air bubbles, compression artifacts, out-of-focus blur, pen markings, tissue folding, and white background should be removed automatically. A simple convolutional neural network (CNN), termed as tissue/non-tissue classifier, was trained to discriminate these various artifacts all at once. The structure of the tissue/non-tissue classifier was described in our previous study[11]. The tissue/non-tissue classifier could filter out almost 99.9% of the improper tissue patches. Then, tissue patches classified as "improper" by the tissue/non-tissue classifier were removed, and the remaining

"proper" tissue patches were collected. For the tumor or mutation classifiers described below, only proper tissue patches were analyzed (Figure 1).

Morphologic features reflecting mutations in specific genes might be expressed mainly in tumor tissues rather than normal tissues[24,25]. Therefore, tumor tissues should be separated from the WSI to predict the mutational status of the WSI. In a previous study, we successfully built normal/tumor classifiers for various tumors, including GC[26]. We concluded that frozen and FFPE slides should be separately analyzed using a deep neural network due to their different morphologic features. Thus, we adopted the normal/tumor classifiers for frozen and FFPE tissue slides from a previous study to delineate the normal/tumor gastric tissues for the frozen and FFPE slides of the TCGA-STAD dataset in the present study. Mutation classifiers were trained separately for the selected tumor patches for frozen and FFPE tissues. We selected tumor patches with a tumor probability higher than 0.9 to collect tissue patches with evident tumor features. We adopted a patient-level ten-fold cross-validation to completely characterize the TCGA-STAD dataset. Therefore, patients in each mutation/wild-type group for the five genes were separated into ten different folds, and one of the ten folds was used to test the classifiers trained with data from the other nine folds. Therefore, ten different classifiers were trained and tested for each group. The same label for all tumor tissue patches in a WSI as either 'wild-type' or 'mutated' were assigned based on the mutational status of the patient. Thereafter, the Inception-v3 model, a widely used CNN architecture, was trained to classify the tumor patches into 'wild-type' or 'mutated' tissues, as in our previous study on mutation prediction in colorectal cancer[11]. We fully trained the network from the beginning and did not adopt a transfer-learning scheme. The average probability of all tumor patches in a WSI was calculated to determine the slide-level mutation probability of a WSI. The Inception-v3 model was implemented using the TensorFlow DL library (http://tensorflow.org), and the network was trained with a mini-batch size of 128 and cross-entropy loss function as a loss function. For training, we used the RMSProp optimizer, with an initial learning rate of 0.1, weight decay of 0.9, momentum of 0.9, and epsilon of 1.0. Ten percent of the training slides were used as the validation dataset, and training was stopped when the loss for the validation data started to increase. Data augmentation techniques, including random horizontal/vertical flipping and random rotations by 90°, were applied to the tissue patches during training. Color normalization was applied to the tissue patches to avoid the effect of stain differences[27,28]. At least five classifiers were trained on each fold of mutation for the frozen and FFPE WSIs separately. The classifier with the best area under the curve (AUC) for the receiver operating characteristic (ROC) curves on the test dataset was included in the results. The ROC curves for fold with the lowest AUC, highest AUC, and the concatenated results for data from all ten folds are shown in the figures.

In summary, a WSI is analyzed as follows: 1. The whole slide is split into non-overlapping 360 × 360 pixel tissue patches, 2. Proper tissue patches are selected by tissue/non-tissue classifier, 3. Only tumor patches with tumor probability higher than 0.9 are selected, 4. High probability tumor patches are classified by each wild-type/mutation classifier, 5. The probabilities of tumor patches are averaged to obtain the slide-level mutation probability. The number of tissue patches used for the training of all mutation prediction models is summarized in Supplementary Table 2. The average number of training epochs for each classifier is summarized in Supplementary Table 3.

Part II: Tests on the external cohorts

Patient cohort: GC tissue slides were collected from 96 patients who had previously undergone surgical resection at Seoul St. Mary's Hospital between 2017 and 2020 (SSMH dataset). An Aperio slide scanner (Leica Biosystems) was used to scan the FFPE slides. The Institutional Review Board of the College of Medicine at the Catholic University of Korea approved this study (KC19SESI0787).

Mutation prediction on SSMH dataset: For CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, 6, 6, 12, 11, and 39 patients were confirmed to have the mutations, respectively. Thirty-eight patients had wild-type genes for all five genes. For CDH1, ERBB2, KRAS, and PIK3CA genes, we selected the number of wild-type patients to be 1.4 times that of mutated patients. For TP53, all 38 patients with wild-type genes were enrolled. The normal/tumor classifier for TCGA FFPE tissues was also used to discriminate the tumor tissue patches of SSMH WSIs. Our previous study showed that the normal/tumor classifier for TCGA-STAD was valid for SSMH FFPE slides[29]. First, the mutational status of the SSMH slides was analyzed by classifiers trained on TCGA-

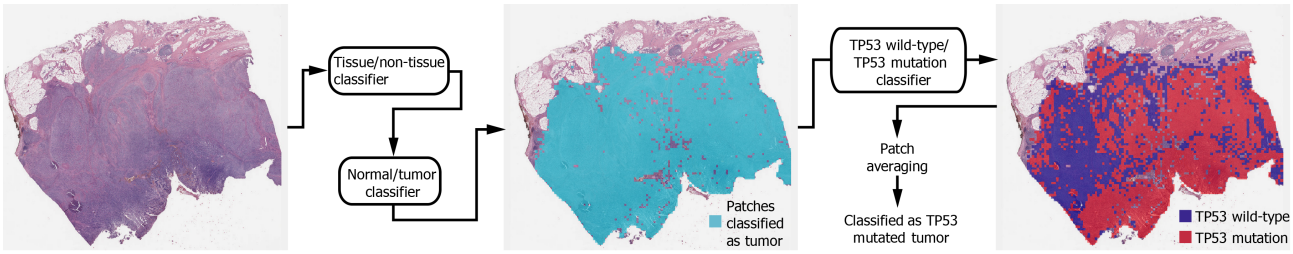


Figure 1 Workflow for the fully automated prediction of mutation. Tissue image patches with tumor probability higher than 0.9 were selected by sequential application of the tissue/non-tissue and normal/tumor classifiers. Then the tumor patches were classified into the wild-type or mutated patches. The patch-level probabilities of mutation are averaged to yield the slide-level probability.

STAD FFPE WSIs. Subsequently, new classifiers were trained using both TCGA and SSMH FFPE tissues. Patient-level three-fold cross validation was applied to the SSMH datasets because the number of mutated patients was not sufficient for ten-fold cross-validation.

Statistical analysis

To demonstrate the performance of each classifier, the ROC curves and their AUCs are presented in the figures. For the concatenated results from all ten folds, 95% confidence intervals (CIs) were also presented using the percentile bootstrap method. In addition, the accuracy, sensitivity, specificity, and F1 score of the classification results of mutation prediction models with cutoff values for maximal Youden index (sensitivity + specificity - 1) were presented. We used a permutation test with 1000 iterations to compare the differences between the two paired or unpaired ROC curves when a comparison was necessary[30]. Statistical significance was set at $P < 0.05$.

RESULTS

Tissue patches with high tumor probability were automatically collected from a WSI by sequentially applying the tissue/non-tissue and normal/tumor classifiers to 360 × 360 pixels tissue image patches (Figure 1). Then, classifiers to distinguish the mutational status of CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes in the tumor tissue patches from the TCGA-STAD frozen and FFPE WSI datasets were separately trained with a patient-level ten-fold cross-validation scheme.

The classification results of the TCGA-STAD WSIs are presented in Figures 2 to 6 for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes. Results for the frozen and FFPE tissues are presented in the upper and lower part of each figure, respectively. Panels A and C demonstrated the representative binary heatmaps of tissue patches classified as wild-type or mutated tissues. The WSIs with gene mutation correctly classified as mutation, with wild-type gene correctly classified as wild-type, with gene mutation falsely classified as wild-type, and with wild-type gene falsely classified as mutation are presented from left to right for panels A and C. The binary heatmaps were drawn with the wild-type/mutation discrimination threshold set to 0.5. We simply set the threshold to 0.5, because every classifier for different folds had different optimal thresholds. Slide-level ROC curves for folds with the lowest and highest AUCs are presented to demonstrate the differences in the performance between folds (left and middle ROC curves in each figure). Finally, the slide-level ROC curves for the concatenated results from all ten folds were used to infer the overall performance (right ROC curves). The results for the CDH1 gene are shown in Figure 2. The AUCs per fold ranged from 0.833 to 1.000 for frozen WSIs and from 0.833 to 1.000 for FFPE WSIs. The AUCs for the concatenated results were 0.842 (95%CI: 0.749-0.936) and 0.781 (95%CI: 0.645-0.917) for frozen and FFPE WSIs, respectively. For ERBB2 (Figure 3), the lowest and highest AUCs per fold were 0.667 and 1.000, respectively, for both frozen and FFPE WSIs. The concatenated AUCs were 0.751 (95%CI: 0.631-0.871) and 0.661 (95%CI: 0.501-0.821), respectively. For the KRAS gene (Figure 4), the AUCs per fold were between 0.775 and 1.000 for frozen WSIs and between 0.750 and 1.000 for FFPE WSIs. The concatenated AUCs were 0.793 (95%CI: 0.706-0.879) and 0.858 (95%CI: 0.738-0.979) for frozen and FFPE WSIs, respectively. For the PIK3CA gene (Figure 5), the concatenated AUC for the frozen WSIs was 0.862 (95%CI: 0.809-0.916), with a range of 0.705

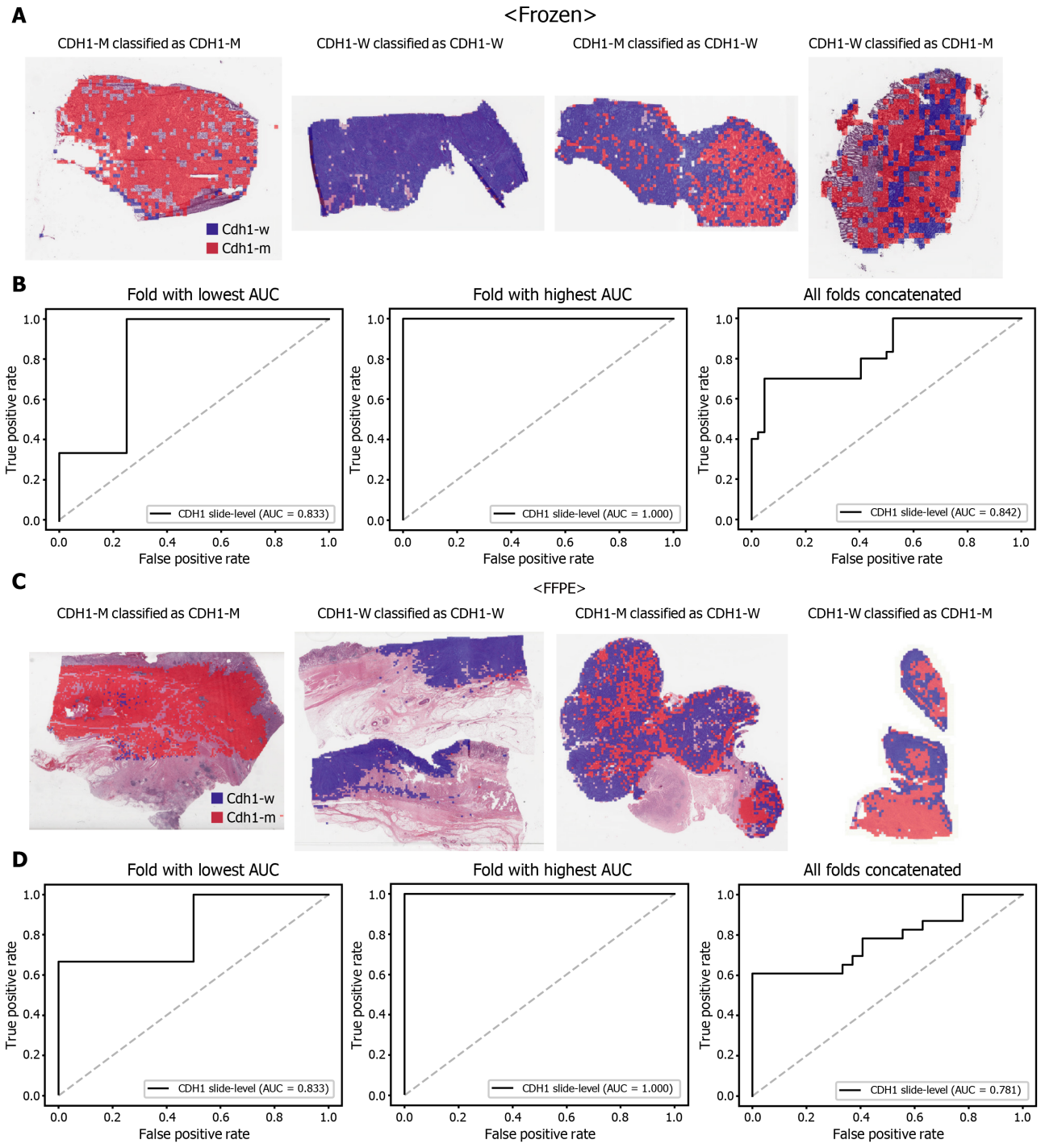


Figure 2 Classification results of CDH1 gene in the The Cancer Genome Atlas gastric cancer dataset. A: Representative whole slide images (WSIs) of the frozen slides with CDH1 gene mutation correctly classified as mutation, with wild-type gene correctly classified as wild-type, with gene mutation falsely classified as wild-type, and with wild-type gene falsely classified as mutation, from left to right; B: Receiver operating characteristic (ROC) curves for the fold with lowest area under the curve (AUC), for the fold with highest AUC, and for the concatenated results of all ten folds, from left to right, obtained with the classifiers trained with the frozen tissues; C and D: Same as A and B but the results were for the formalin-fixed paraffin-embedded WSIs. CDH1-M: CDH1 mutated, CDH1-W: CDH1 wild-type.

to 0.990. For FFPE WSIs, the lowest and highest AUCs per fold were 0.675 and 1.000, respectively, yielding a concatenated AUC of 0.828 (95%CI: 0.750-0.907). Lastly, the results for the TP53 gene are presented in Figure 6. The AUCs per fold were between 0.666 to 0.810 for frozen WSIs and between 0.702 to 0.847 for FFPE WSIs. The concatenated AUCs were 0.727 (95%CI: 0.683-0.771) and 0.727 (95%CI: 0.671-0.784) for frozen and FFPE WSIs, respectively. For the colorectal cancer dataset from TCGA, mutation classification results for frozen tissues were better than those for FFPE tissues in some genes[11]. However, there were no significant differences between the frozen and FFPE tissues in the TCGA-STAD dataset (P = 0.491, 0.431, 0.187, 0.321, and 0.613

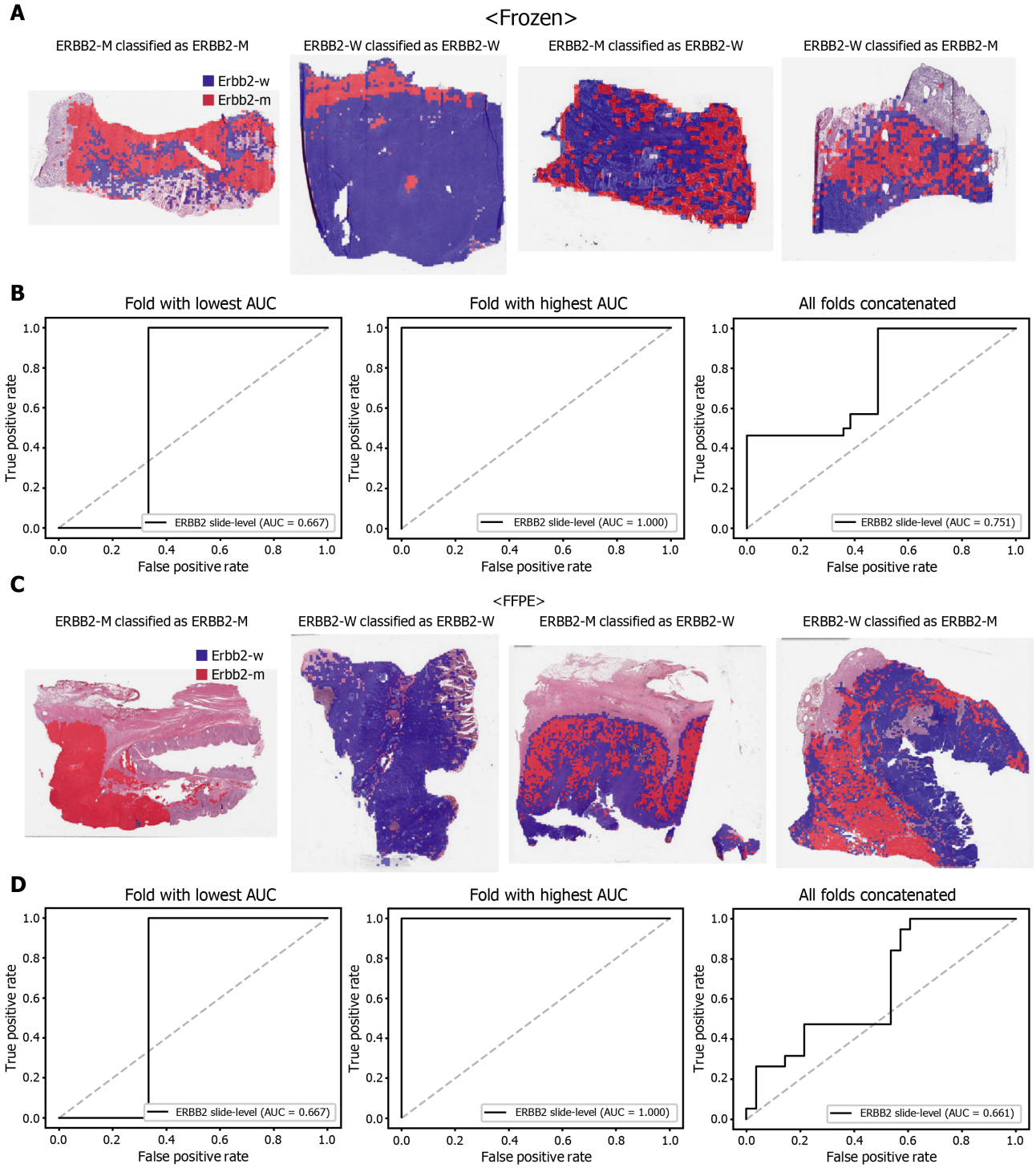


Figure 3 Classification results of ERBB2 gene in the The Cancer Genome Atlas gastric cancer dataset. The configuration of the figure is the same as in Figure 2. A and B: Upper panels are results for the frozen tissue and lower panels; C and D: Results for the formalin-fixed paraffin-embedded tissues. ERBB2-M: ERBB2 mutated, ERBB2-W: ERBB2 wild-type.

between the concatenated AUCs for the frozen and FFPE tissues by Venkatraman's permutation test for unpaired ROC curves for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, respectively. For a clearer assessment of the performance of each model, the accuracy, sensitivity, specificity, and F1 score of the classification results are presented in Table 1.

The performance of a DL model on an external dataset should be tested to validate the generalizability of the trained model. Therefore, we collected GC FFPE WSIs with matching mutation data from Seoul St. Mary's Hospital (SSMH dataset). The normal/tumor classifier for TCGA-STAD FFPE tissues was also applied to select tissue patches with high tumor probabilities. Thereafter, the mutation classifier for each gene

Table 1 Accuracy, sensitivity, specificity, and F1 score of the classification results of mutation prediction models for the The Cancer Genome Atlas datasets

	Accuracy	Sensitivity	Specificity	F1 score
TCGA Frozen Tissue Slides				
CDH1	0.847	0.700	0.952	0.792
ERBB2	0.716	1.000	0.512	0.746
KRAS	0.773	0.883	0.708	0.745
PIK3CA	0.834	0.771	0.884	0.806
TP53	0.667	0.743	0.602	0.673
TCGA FFPE Tissue Slides				
CDH1	0.820	0.608	1.000	0.756
ERBB2	0.574	1.000	0.285	0.655
KRAS	0.894	0.727	1.000	0.842
PIK3CA	0.803	0.629	0.923	0.723
TP53	0.673	0.678	0.668	0.659

TCGA: The Cancer Genome Atlas; FFPE: Formalin-fixed paraffin-embedded.

trained on the TCGA-STAD FFPE tissues was tested on the SSMH dataset. The slide-level ROC curves for the CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes are presented in Supplementary Figure 1. The AUCs for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes were 0.667, 0.630, 0.657, 0.688, and 0.572, respectively. For the KRAS, PIK3CA, and TP53 genes, the performance of the TCGA-trained mutation classifiers on the SSMH dataset were worse than that of the TCGA dataset ($P = 0.389$, $P = 0.849$, $P < 0.05$, $P < 0.05$, and $P < 0.05$ for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, respectively, by Venkatraman's permutation test for unpaired ROC curves). These results demonstrate that the mutation classifiers trained with TCGA-STAD WSI datasets had limited generalizability. It is of interest if the performance can be enhanced by training the classifiers with expanded datasets, including both TCGA and SSMH datasets. Cancer tissues from different ethnic groups can show different features[16,19]; therefore, the performance of the classifiers can be improved by mixing the datasets. When the classifiers trained with the mixed datasets were used, the performance on the SSMH dataset was generally improved because the SSMH data were included in the training data in this setting (Figures 7 and 8). The AUCs became 0.778, 0.833, 0.838, 0.761, and 0.775 for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, respectively ($P = 0.234$, $P < 0.05$, $P < 0.05$, $P = 0.217$, and $P < 0.05$ between the ROCs of classification results by classifiers trained on the TCGA-STAD dataset and mixed dataset for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, respectively, by Venkatraman's permutation test for paired ROC curves). Furthermore, the performance on the TCGA-STAD FFPE dataset was also generally improved by the new classifiers trained on both datasets, except for the PIK3CA gene, which showed worse results (Supplementary Figure 2). The AUCs were 0.918, 0.872, 0.885, 0.766, and 0.820 for the CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, respectively ($P < 0.05$, $P < 0.05$, $P = 0.216$, $P < 0.05$, and $P < 0.05$ compared with the TCGA-trained classifiers by Venkatraman's permutation test for paired ROC curves). The accuracy, sensitivity, specificity, and F1 score of the classification results of mutation prediction models trained with both SSMH and TCGA datasets are presented in Supplementary Table 4.

Another interesting question is whether the DL-based classifiers for mutational status can be compatible with other types of cancers. We already built the mutation classifiers for KRAS, PIK3CA, and TP53 genes in the colorectal cancer dataset of TCGA in a previous study[11]. Therefore, we tested whether the classifiers trained on colorectal cancer can distinguish the mutational status in GC. As shown in Figure 9, the classifiers trained to discriminate the mutational status of KRAS, PIK3CA, and TP53 genes in the FFPE tissues of colorectal cancer approximately failed to distinguish the mutational status in the FFPE tissues of the TCGA-STAD dataset, with AUCs of 0.458, 0.550, and 0.538 for the KRAS, PIK3CA, and TP53 genes, respectively. The results indicate that the tissue morphologic features reflecting the wild-type and

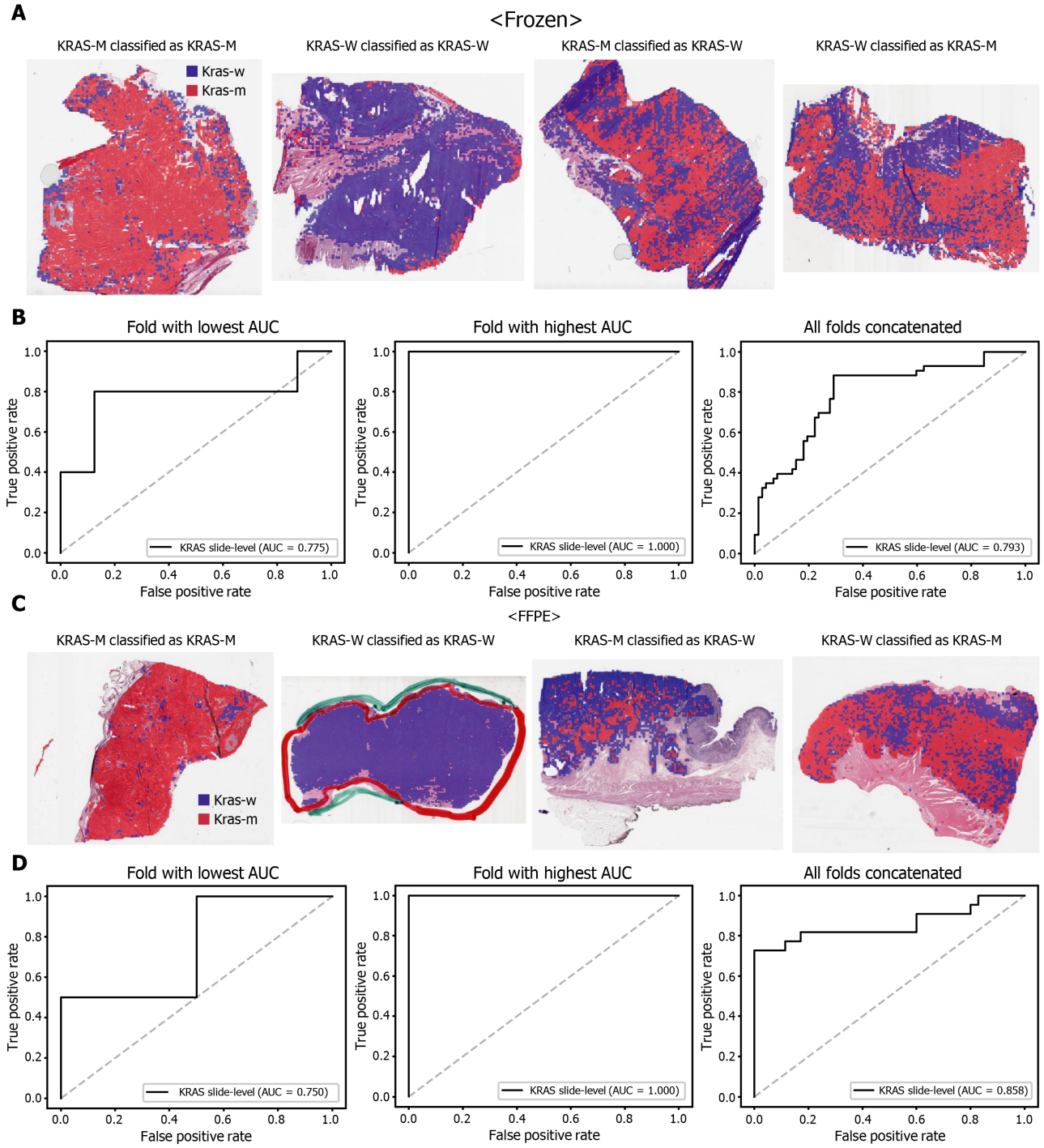


Figure 4 Classification results of KRAS gene in the The Cancer Genome Atlas gastric cancer dataset. The configuration of the figure is the same as in Figure 2. A and B: Upper panels are results for the frozen tissue and lower panels; C and D: Results for the formalin-fixed paraffin-embedded tissues. KRAS-M: KRAS mutated, KRAS-W: KRAS wild-type.

mutated genes are relatively different between cancers originating from different organs.

DISCUSSION

Recently, many drugs targeting specific biological molecules have been introduced to improve the survival of patients with advanced GC[31]. However, patient stratification strategies to maximize the treatment response of these new drugs are not yet well established. Targeted therapies can yield different responses depending on the mutational status of genes in patients with cancer[32]. To overcome this complexity,

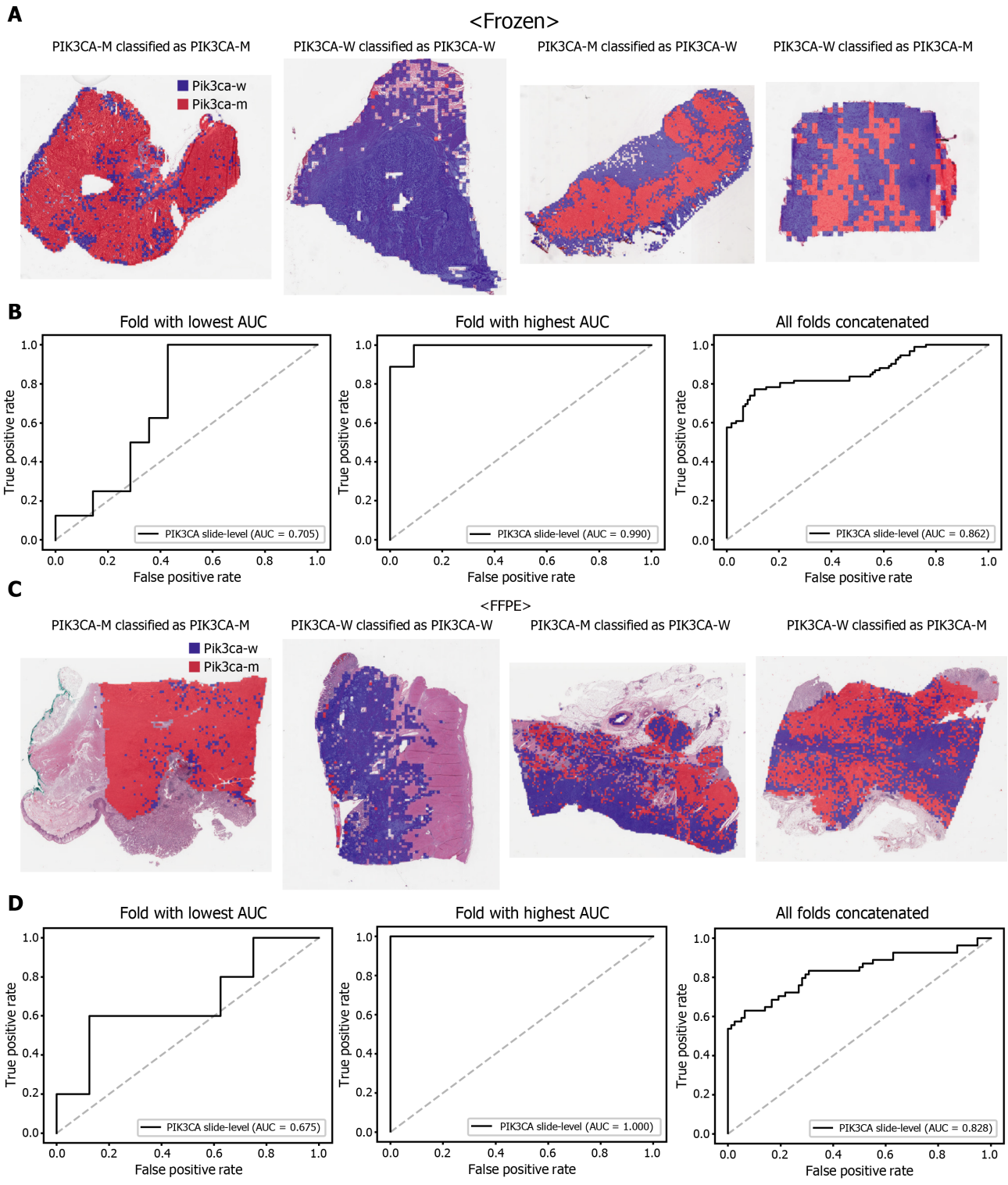


Figure 5 Classification results of PIK3CA gene in the The Cancer Genome Atlas gastric cancer dataset. The configuration of the figure is the same as in Figure 2. A and B: Upper panels are results for the frozen tissue and lower panels; C and D: Results for the formalin-fixed paraffin-embedded tissues. PIK3CA-M: PIK3CA mutated, PIK3CA-W: PIK3CA wild-type.

clinical trials for new drugs have begun to adopt the umbrella platform strategy, which assigns treatment arms based on the mutational status of cancer patients[6,33]. Therefore, data regarding the mutational status of cancer patients is essential for patient stratification in modern-day medicine. However, molecular tests to detect gene mutations are still not affordable for all cancer patients. If cost- and time-effective alternative methods for mutation detection can be introduced, it will promote prospective clinical trials and retrospective studies to correlate the treatment response with the mutational profiles of cancer patients, which can be retrospectively obtained from clinical data and stored tissue samples. Therefore, the new cost- and time-

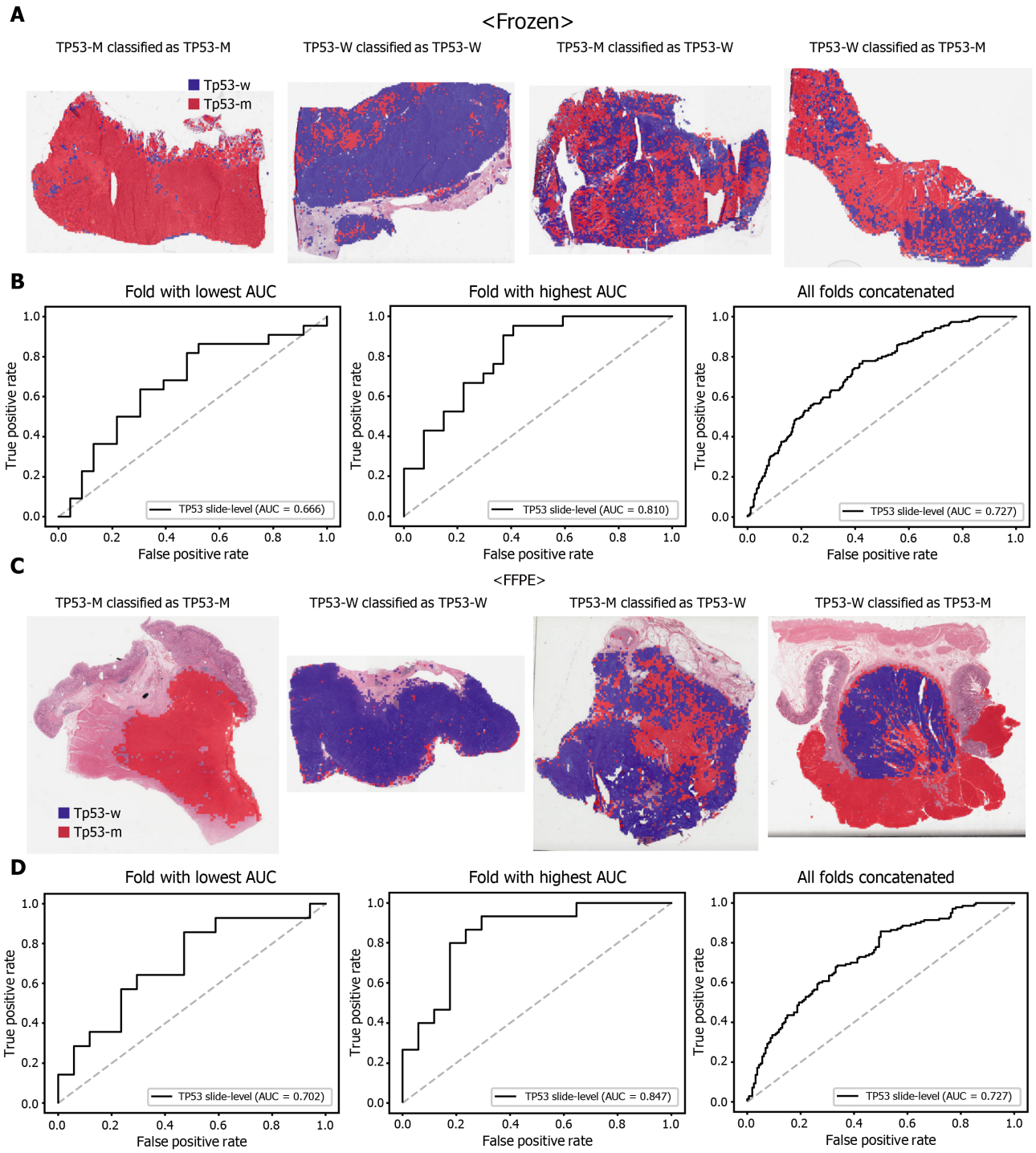
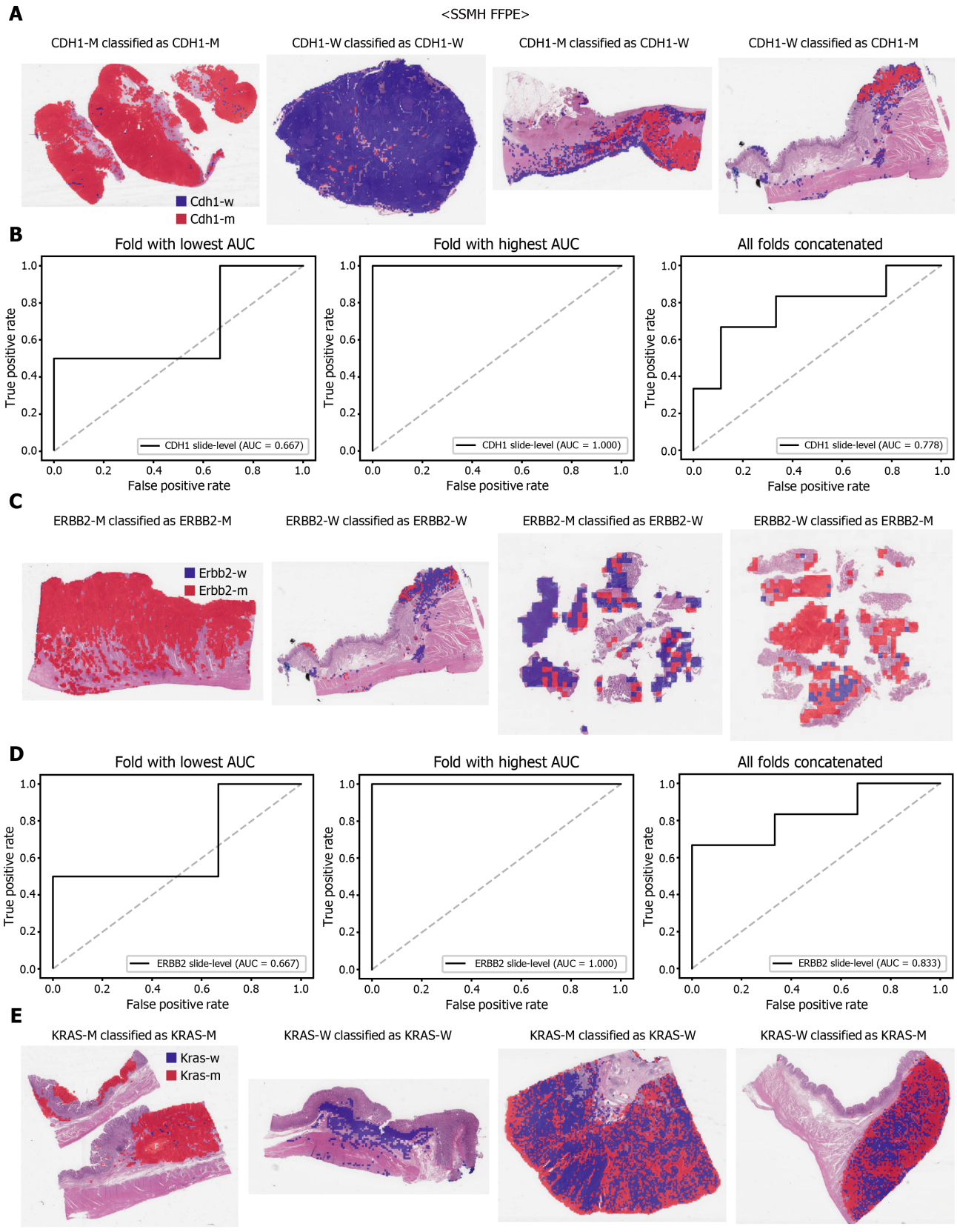


Figure 6 Classification results of TP53 gene in the The Cancer Genome Atlas gastric cancer dataset. The configuration of the figure is the same as in Figure 2. A and B: Upper panels are results for the frozen tissue and lower panels; C and D: Results for the formalin-fixed paraffin-embedded tissues. TP53-M: TP53 mutated, TP53-W: TP53 wild-type.

effective methods will help to establish molecular stratification of cancer patients that can be used to determine effective treatment and improve clinical outcomes[34].

Cancer tissue slides are made and stored for most cancer patients. As a result, DL-based mutation prediction from the tissue slides can be a good candidate for alternative methods. It has been well recognized that the molecular alterations are manifested as morphologic changes in tissue architecture[35]. For example, some morphological features in GC tissues have been associated with specific mutations, including CDH1 and KRAS genes[36,37]. Although it is impractical to quantitatively assess these features for the detection of mutations by visual inspection, DL can learn and distinguish subtle discriminative features for mutation detection in various cancer tissues[7-11]. This study demonstrated the feasibility of DL-based prediction of


A
<SSMH FFPE>
CDH1-M classified as CDH1-M
CDH1-W classified as CDH1-W
CDH1-M classified as CDH1-W
CDH1-W classified as CDH1-M
Cdh1-w
Cdh1-m
B
Fold with lowest AUC
Fold with highest AUC
All folds concatenated
True positive rate
False positive rate
CDH1 slide-level (AUC = 0.667)
CDH1 slide-level (AUC = 1.000)
CDH1 slide-level (AUC = 0.778)
C
ERBB2-M classified as ERBB2-M
ERBB2-W classified as ERBB2-W
ERBB2-M classified as ERBB2-W
ERBB2-W classified as ERBB2-M
Erbb2-w
Erbb2-m
D
Fold with lowest AUC
Fold with highest AUC
All folds concatenated
True positive rate
False positive rate
ERBB2 slide-level (AUC = 0.667)
ERBB2 slide-level (AUC = 1.000)
ERBB2 slide-level (AUC = 0.833)
E
KRAS-M classified as KRAS-M
KRAS-W classified as KRAS-W
KRAS-M classified as KRAS-W
KRAS-W classified as KRAS-M
Kras-w
Kras-m

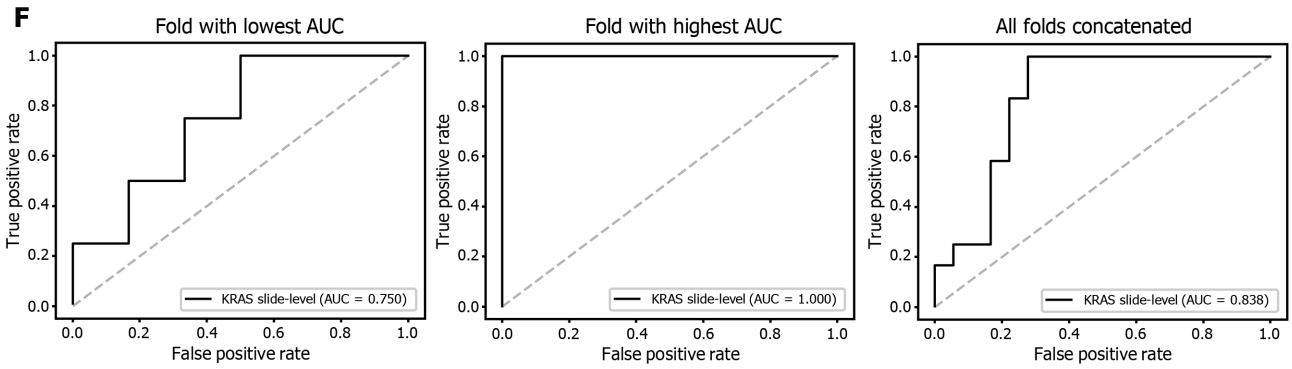


Figure 7 The classifiers trained with both The Cancer Genome Atlas and SSMH data were used to predict the mutation of CDH1 (A and B), ERBB2 (C and D), and KRAS (E and F) genes. Representative binary heatmaps of the whole slide images (WSIs) correctly classified as mutation, correctly classified as wild-type, falsely classified as wild-type, and falsely classified as mutation were presented. Receiver operating characteristic curves for the folds with the lowest and highest area under the curve and the concatenated ten folds were also presented for each gene. CDH1-M: CDH1 mutated, CDH1-W: CDH1 wild-type, ERBB2-M: ERBB2 mutated, ERBB2-W: ERBB2 wild-type, KRAS-M: KRAS mutated, KRAS-W: KRAS wild-type.

mutations in CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes, which are prevalent in both the TCGA and SSMH GC datasets, from tissue slide images of GC. Other studies have also shown that mutations in these genes are frequently observed in GC[3,5]. Furthermore, many studies have attempted to evaluate the prognostic value of these mutations[3,5,38]. However, the clinical relevance of these mutations for prognosis and treatment response has not been completely determined because the studies often presented discordant results. Various factors, including the relatively low incidence of mutation, small study size, and ethnicity of the studied groups, may have contributed to the inconsistent study results. Although it is still unclear how specific mutations are involved in the prognosis and treatment response in GC patients, further studies for the fine molecular stratification of patients based on mutational status are ongoing[6]. DL-based mutation prediction from the tissue slides could provide valuable tools to support these efforts because the mutational status can be promptly obtained with minimal cost from the existing H and E-stained tissue slides.

Furthermore, DL-based classifiers can provide important information for the study of tumor heterogeneity[39]. The heatmaps of classification results overlaid on the tissue images in figures showed that mutated and wild-type regions are aggregated into separated regions. For example, the rightmost tissues in Figure 6C showed clear demarcation between TP53-mutated and wild-type regions. These results indicated that a tumor tissue can contain molecularly heterogeneous regions which can be easily visualized with the help of DL-based classifiers. The clear demarcation of molecularly heterogeneous regions in a tissue slide is an important advantage of a DL-based system and it can help the studies for the understanding of the prognostic and therapeutic values of the tumor heterogeneity without the application of the very expensive molecular tests such as multi-point single-cell sequencing.

However, further studies are needed to build practical DL classifiers for mutation prediction. Our data showed that the performance was still unsatisfactory for verifying mutational status. The AUCs ranged from 0.661 to 0.862 for the TCGA dataset. The frequency of mutation in GC TCGA dataset was relatively low. The average mutation rate for CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes was 8.28%. In our previous study for the mutation prediction in colorectal cancer TCGA dataset, the average mutation rate for APC, KRAS, PIK3CA, SMAD4, and TP53 genes was 39.18%[11]. Furthermore, the classifiers showed limited generalizability to the external dataset. Because DL critically depends on data for learning prominent features, it is generally recommended to build a large multinational dataset[1,2]. Therefore, to test whether the expanded dataset can improve the performance of the classifiers, new classifiers were trained using mixed data from the TCGA and SSMH datasets. As a result, the AUCs generally increased with the larger multinational datasets. These results suggest that we could improve the performance of DL-based mutation classifiers if a large multi-national and multi-institutional dataset can be built. One exception was the PIK3CA gene, which showed worse performance for the TCGA FFPE slides by a classifier trained with the mixed dataset. Although the reason for the decreased performance is unclear, we speculate that there are some different tissue features for the wild-type and mutated PIK3CA gene between the two datasets due to different ethnicities, which could negatively affect the feature learning process for the

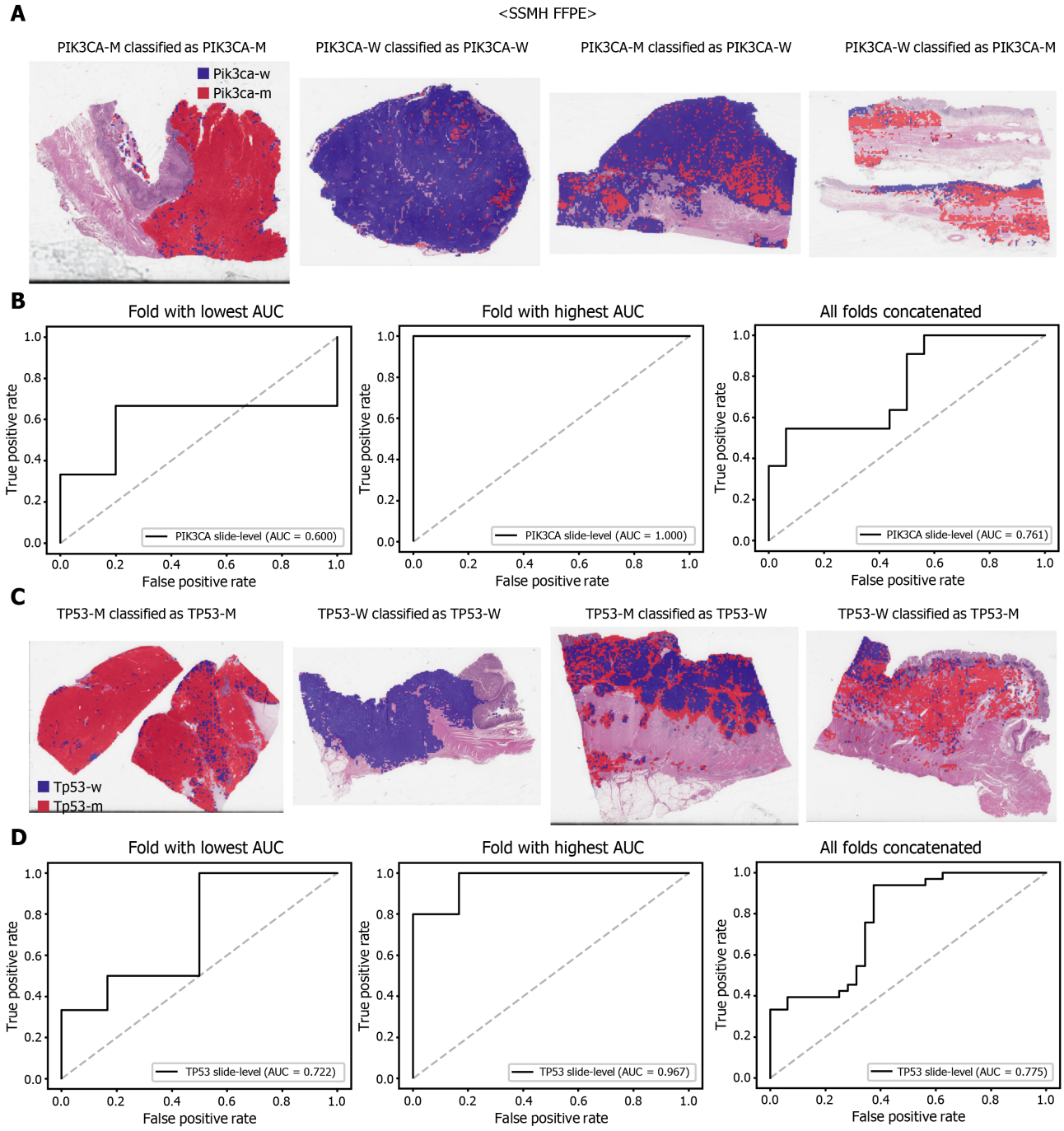


Figure 8 Mutation prediction of PIK3CA (A and B) and TP53 (C and D) genes for the SSMH gastric cancer tissue slides by the classifiers trained with both The Cancer Genome Atlas and SSMH data. The configuration of the figure is the same as in figure 7. PIK3CA-M: PIK3CA mutated, PIK3CA-W: PIK3CA wild-type, TP53-M: TP53 mutated, TP53-W: TP53 wild-type.

TCGA dataset. In addition, the numbers of patients with PIK3CA mutations were different; 64 and 11 for the TCGA and SSMH datasets, respectively. The different numbers of patients also hamper proper feature learning for the mixed dataset because data imbalance usually negatively affects the learning process. Furthermore, the studied tissues carry many additional mutations other than CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes. Because every tissue presented a different combination of mutations, the confounding effect of a mixture of different mutations on the tissue morphology would hamper the effective learning of features for the selected mutation. This factor also necessitates larger tissue datasets for proper learning of morphological features of specific mutations, irrespective of coexisting mutations. In our opinion, the datasets are still immature for building a prominent classifier for mutation prediction. Therefore, efforts to establish a larger tissue dataset with a mutation profile will help to understand the potential of DL-based mutation prediction systems. Recently, many

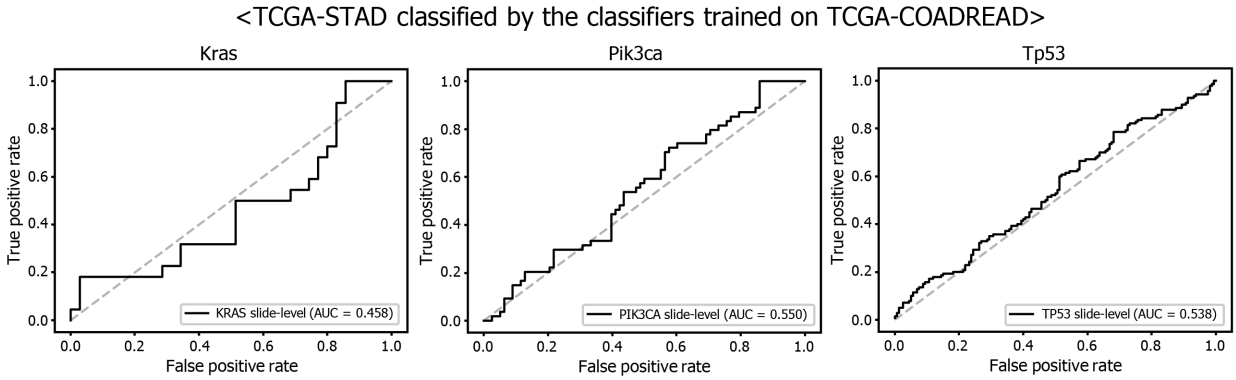


Figure 9 Mutation prediction of KRAS, PIK3CA, and TP53 genes for the The Cancer Genome Atlas gastric cancer tissue slides by the classifiers trained with The Cancer Genome Atlas colorectal cancer tissues. Receiver operating characteristic curves of the classification results for each gene were presented.

countries have started to build nationwide datasets of pathologic tissue WSIs with genomic information. Therefore, we expect that the performance of DL-based mutation prediction can be greatly improved.

Although we argued for the potential of DL-based mutation classifiers, there are important barriers to the adoption of DL-based assistant systems. First, the 'black box' nature of DL limits the interpretability of DL models and remains a significant barrier in their validation and adoption in clinics[18,40,41]. We could not trust a decision made by a DL model before we could clearly understand the basis of the decision. Therefore, a method for visualizing the features that determine the behavior of a DL model should be developed. Another barrier is the need for an individual DL system for an individual task. As described, separate systems should be built for tasks such as the classification of normal/tumor tissues for frozen and FFPE tissues. The classifier for each mutation should also be built separately. Furthermore, as shown in Figure 9, there was no compatibility between the different cancer types for the classification of genetic mutations. Therefore, many classifiers should be built to achieve optimal performance. It requires time to build many necessary classifiers to renovate current pathology workflows.

CONCLUSION

Despite these limitations, DL has enormous potential for innovative medical practice. It can help capture important information by learning features automatically from the data that are waiting to be explored in the vast database of modern hospital information systems. This information will be used to determine the best medical practice and improve patient outcomes. The tissue slides of cancer patients contain important information on the prognosis of patients[42]; therefore, DL-based analysis of tissue slides has enormous potential for fine patient stratification in the era of precision medicine. Furthermore, its cost- and time-effective nature could help save the medical cost and decision time for patient care.

ARTICLE HIGHLIGHTS

Research background

Studies correlating specific genetic mutations and treatment response are ongoing to establish an effective treatment strategy for gastric cancer (GC). With the increased digitization of pathologic tissue slides, deep learning (DL) can be a cost- and time-effective method to analyze the mutational status directly from the hematoxylin and eosin (H and E)-stained tissue whole slide images (WSIs).

Research motivation

Recent studies suggested that mutational status can be predicted directly from the H and E-stained WSIs with DL-based methods. Motivated by these studies, we invest-

igated the feasibility of DL-based mutation prediction for the frequently occurring mutations from H and E-stained WSIs of GC tissues.

Research objectives

To predict the mutational status of CDH1, ERBB2, KRAS, PIK3CA, and TP53 genes from the H and E-stained WSIs of GC tissues with DL-based methods.

Research methods

DL-based classifiers for the CDH1, ERBB2, KRAS, PIK3CA, and TP53 mutations were trained for the The Cancer Genome Atlas (TCGA) datasets. Then, the classifiers were validated with our own dataset. Finally, TCGA and our own dataset were combined to train a new classifier to test the effect of extended data on the performance of the classifiers.

Research results

The area under the curve (AUC) for receiver operating characteristic (ROC) curves were between 0.727 and 0.862 for the TCGA frozen WSIs and between 0.661 and 0.858 for the TCGA formalin-fixed paraffin-embedded WSIs. Furthermore, the results could be improved with the classifiers trained with both TCGA and our own dataset.

Research conclusions

This study demonstrated that mutational status could be predicted directly from the H and E-stained WSIs of GC tissues with DL-based methods. The performance of the classifiers could be improved if more data can be used to train the classifiers.

Research perspectives

Current molecular tests for the mutational status are not feasible for all cancer patients because of technical barriers and high costs. Although there is still room for much improvement, the DL-based method can be a reasonable alternative for molecular tests. It could help to stratify patients based on their mutational status for retrospective studies or prospective clinical trials with very low cost. Furthermore, it could support the decision-making process for the management of patients with GCs.

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World J Gastroenterol 2021 November 28; 27(44): 7705-7715

DOI: 10.3748/wjg.v27.i44.7705

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

ORIGINAL ARTICLE

Observational Study

Autosomal recessive 333 base pair interleukin 10 receptor alpha subunit deletion in very early-onset inflammatory bowel disease

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Author contributions: Lv JJ and Su W contributed equally to this work; Lv JJ and Su W performed the experiments, data acquisition, analysis and interpretation, and drafting of the article; Chen XY, Yu Y, Xu X, Xu CD, Deng X, and Huang JB analyzed the data and critically revised the article; Wang XQ and Xiao Y designed the project and critically revised the article for important intellectual content; and all authors have read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Ruijin Hospital (Shanghai).

Informed consent statement: All study participants or their legal guardians provided informed consent prior to study enrollment.

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Abstract

BACKGROUND

Interleukin 10 receptor alpha subunit (IL10RA) dysfunction is the main cause of very early-onset inflammatory bowel disease (VEO-IBD) in East Asians.

AIM

To identify disease-causing gene mutations in four patients with VEO-IBD and verify functional changes related to the disease-causing mutations.

METHODS

From May 2016 to September 2020, four young patients with clinically diagnosed VEO-IBD were recruited. Before hospitalization, using targeted gene panel sequencing and trio-whole-exome sequencing (WES), three patients were found to harbor a *IL10RA* mutation (c.301C>T, p.R101W in one patient; c.537G>A, p.T179T in two patients), but WES results of the fourth patient were not conclusive. We performed whole-genome sequencing (WGS) on patients A and B and reanalyzed the data from patients C and D. Peripheral blood mononuclear cells (PBMCs) from patient D were isolated and stimulated with lipopolysaccharide (LPS), interleukin 10 (IL-10), and LPS + IL-10. Serum IL-10 levels in four patients and tumor necrosis factor-α (TNF-α) in the cell supernatant were determined by enzyme-linked immunosorbent assay. Phosphorylation of signal transducer and activator of transcription 3 (STAT3) at Tyr705 and Ser727 in PBMCs was determined by western blot analysis.

RESULTS

Conflict-of-interest statement: There are no conflicts of interest to declare.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement checklist of items.

Supported by the National Natural Science Foundation of China, No. 81741103.

Country/Territory of origin: China

Specialty type: Gastroenterology and hepatology

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

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

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Received: May 31, 2021
Peer-review started: May 31, 2021
First decision: July 1, 2021
Revised: July 9, 2021
Accepted: November 9, 2021
Article in press: November 9, 2021
Published online: November 28, 2021

P-Reviewer: Nayudu SK

The four children in our study consisted of two males and two females. The age at disease onset ranged from 18 d to 9 mo. After hospitalization, a novel 333-bp deletion encompassing exon 1 of *IL10RA* was found in patients A and B using WGS and was found in patients C and D after reanalysis of their WES data. Patient D was homozygous for the 333 bp deletion. All four patients had elevated serum IL-10 levels. *In vitro*, IL-10-stimulated PBMCs from patient D failed to induce STAT3 phosphorylation at Tyr705 and only minimally suppressed TNF-α production induced by LPS. Phosphorylation at Ser727 in PBMCs was not affected by LPS or LPS + IL-10 in both healthy subjects and in patient D.

CONCLUSION
WGS revealed a novel 333-bp deletion of *IL10RA* in four patients with VEO-IBD, whereas the WES results were inconclusive.

Key Words: Interleukin 10 receptor alpha subunit mutation; Very early-onset inflammatory bowel disease; Whole-genome sequencing; Immunodeficiency; Crohn's disease; Whole-exon sequencing

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Core Tip: Children less than 6 years old with very early-onset inflammatory bowel disease (VEO-IBD) exhibit severe and refractory disease phenotypes, which indicate a monogenic type disease. Here, we report four cases clinically diagnosed with VEO Crohn's disease, of which three were compound heterozygous carriers for a 333-bp deletion and an additional single-nucleotide variant, and one was homozygous for the 333-bp deletion in *IL10RA*. Based on these cases with heterozygous pathogenic variants in *IL10RA*, the possibility of another large fragment deletion that can be missed by whole-exon sequencing or gene panels should be considered, particularly when serum IL-10 is increased in patients with VEO-IBD.

Citation: Lv JJ, Su W, Chen XY, Yu Y, Xu X, Xu CD, Deng X, Huang JB, Wang XQ, Xiao Y. Autosomal recessive 333 base pair interleukin 10 receptor alpha subunit deletion in very early-onset inflammatory bowel disease. *World J Gastroenterol* 2021; 27(44): 7705-7715
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7705.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7705

INTRODUCTION

Inflammatory bowel disease (IBD) in children < 6 years of age is known as very early-onset IBD (VEO-IBD)[1] and represents a specific disease course with a distinct phenotype that can be more severe and refractory than classic IBD[2-3]. Recent studies suggested that patients with VEO-IBD, particularly those with symptoms such as perianal disease soon after birth, suffer from failed treatment, indicating a monogenic type of disease[4-6].

By utilizing next-generation sequencing (NGS), many genetic disorders associated with epithelial defects or immunodeficiencies have been found in patients[7-9]. Notably, interleukin 10 receptor alpha subunit (IL10RA) dysfunction is the most common cause of the disease in East Asians, particularly in the Chinese, Japanese, and Korean populations[10-12].

According to our previous retrospective study, increased serum ferritin levels in VEO-IBD patients are indicative of monogenic disease, and very high serum levels of IL-10 suggest that patients with VEO-IBD are more likely to have *IL10RA* mutations [13].

We report four cases clinically diagnosed with VEO-Crohn's disease with high serum IL-10 levels, indicating *IL10RA* dysfunction. However, neither results of targeted gene panel sequencing (TGPS) nor those of whole-exome sequencing (WES) in the probands were conclusive. Whole-genome sequencing (WGS) was performed in two patients, and a novel 333-bp deletion in *IL10RA* was identified. The results of trio-WES for the other two patients were subsequently reanalyzed, and the same novel

S-Editor: Wang JJ
L-Editor: Wang TQ
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333-bp deletion was found.

MATERIALS AND METHODS

Patients

Four patients with VEO-IBD, including two boys and two girls, were enrolled in our study. The medical history and clinical characteristics of the patients are summarized in Table 1. All patients were of Chinese Han ethnicity and were born to parents who were non-consanguineous, presented disease at the age of less than 1 year (range: 11 d to 8 mo), and experienced severe diarrhea with fistulas in the perianal region; blood samples were collected from three healthy volunteers.

Written informed consent was obtained from the parents of the four patients who participated in the study. This study was approved by the Institutional Review Board of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine (No. 2019-15).

Whole-genome sequencing

Sample preparation and WGS were carried out by Beijing Berry Genomics Co., Ltd. (Beijing, China). The quality of the isolated genomic DNA was verified using the following two methods: (1) DNA degradation and contamination were monitored by electrophoresis on 1% agarose gels; and (2) DNA concentration was measured using the Qubit DNA Assay Kit and Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, United States).

A total of 1 μg DNA per sample was used as the input material for DNA library preparation. The DNA sequencing library was generated using the CLEANNGS DNA kit following the manufacturer's recommendations, and indexing codes were added to each sample. Briefly, genomic DNA samples were enzymatically disrupted to a size of 350 bp. The DNA fragments were then end-polished, a-tailed, and ligated with a full-length adapter for Illumina sequencing, followed by further polymerase chain reaction (PCR) amplification. After the PCR products were purified (AMPure XP system, Beckman, Brea, CA, United States), libraries were analyzed to determine theirsize distribution using an Agilent 2100 Bioanalyzer (Santa Clara, CA, United States) and quantified by qPCR.

Clustering of the index-coded samples was performed on a cBot Cluster Generation System using the NovaSeq 5000/6000 S4 Reagent Kit (Illumina, San Diego, CA, United States) according to the manufacturer's instructions. After cluster generation, the DNA libraries were sequenced on an Illumina NovaSeq 6000 platform, and 150-bp paired-end reads were generated.

The pathogenicity of all mutations was further evaluated according to the American College of Medical Genetics and Genomics guidelines.

Isolation and stimulation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated according to a previous study, with minor modifications[14]. Briefly, blood was drawn from patient D and healthy controls by standard venipuncture in our pediatric ward and collected into a tube containing ethylenediamine tetraacetic acid. Blood (4 mL) was diluted 1:1 with sterile RPMI 1640 medium (Hyclone, Logan, UT, United States) at room temperature (RT) and carefully dropped into a 15-mL tube (Corning, Inc., Corning, NY, United States) containing 4 mL Ficoll-Paque Plus (GE Healthcare, Little Chalfont, United Kingdom). Notably, diluted blood was present on the surface of the Ficoll gradient. The 15-mL tube was centrifuged at 800 × g at RT for 20 min (brake off), after which the buffy coat was carefully aspirated and transferred to another sterile 15-mL tube. After washing the cells with 5 mL RPMI 1640 medium three times by centrifugation at 400 × g for 15 min at RT, most of the supernatant, as much as possible, was pipetted off.

Cells were aspirated with complete RPMI 1640 (10% fetal bovine serum and 1% penicillin-streptomycin) and cultured in 6-well plates at a density of 2×10^6 cells/well. Four groups of PBMCs from patients or healthy controls were established as follows: Unstimulated phosphate buffered saline (PBS), lipopolysaccharide (LPS) (100 ng/mL), LPS (100 ng/mL) + IL-10 (20 ng/mL), and IL-10 (20 ng/mL)[15]. Cells were cultured in the indicated milieu for 12 h at 37 °C. Proteins in PBS-, LPS-, and IL-10-stimulated PBMCs were collected in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors for western blot analysis. The supernatants of the PBS-, LPS-, and LPS + IL-10-stimulated PBMCs were collected to determine tumor necrosis factor-α (TNF-α) level.

Table 1 Clinical and laboratory characteristics of patients

	Patient A	Patient B	Patient C	Patient D	Normal range
Sex	Male	Female	Male	Female	
Consanguinity	-	-	-	-	
Disease onset	3 mo	9 mo	11 d	18 d	
Diarrhea (times/d)	1	8	20	6	
Bloody stool	+	-	+	+	
Weight (kg) SDS	7.65 (-1.475)	6.2 (-1.4)	8.2 (-1.05)	6.3 (-1.15)	
Height (cm) SDS	76 (-1.7)	65.2 (-3.65)	78 (0.25)	67 (1)	
Perianal disease	+	+	+	+	
Extragastro-intestinal manifestations	Fever, UTI, Sepsis	Recurrent otitis media	Fever, respiratory infetion	Fever, UTI, eczama	
WBC ($\times 10^9$)	12.4	19.44	15.34	24.5	3.69-9.16
Hemoglobin (g/L)	101	77	107	96	113-151
Platelet ($\times 10^9$)	624	621	424	387	101-320
IL-10 (pg/mL)	87.3	106	43.4	80.4	< 12.9
TNF-α (pg/mL)	20.7	30.5	3.9	19.8	< 16.5
ESR (mm/h)	6	55	19	23	F: 0-20; M: 0-15
Albumin (g/L)	28	29	33	23	35-55
Ferritin	17	47.3	46.5	68.2	11-306.8
Identified mutation	*IL10RA* (c.301C>T, p.R101W): Exon 1 del	*IL10RA* (c.537G>A, p.T179T): Exon 1 del	*IL10RA* (c.537G>A, p.T179T): Exon1 del	*IL10RA* (exon.1 del): Exon 1 del	

SDS: Standard deviation score; UTI: Urinary tract infection; TNF-α: Tumor necrosis factor α; IL10RA: Interleukin-10 receptor α subunit; del: Deletion; WBC: White blood cell.

Western blot analysis

Western blotting was performed as described previously[16]. Polyvinylidene fluoride membranes were blotted with monoclonal antibodies against phos-signal transducer and activator of transcription 3 (STAT3) (Tyr705), phos-STAT3 (Ser727), STAT3 (Cell Signaling Technology, Danvers, MA, United States), and glyceraldehyde-3-phosphate dehydrogenase (Servicebio, Wuhan, China). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit (Cell Signaling Technology) secondary antibodies were detected using a chemiluinescent substrate (Millipore, Billerica, MA, United States). Images were captured using an automatic chemiluminescence image analysis system (Tanon, Shanghai, China).

Enzyme-linked immunosorbent assay

The supernatant of PBMCs after stimulation with PBS, LPS or LPS + IL-10 was collected. IL-10 and TNF-α levels were determined using sandwich ELISA kits (DAKEWE, Shenzhen, China) according to the manufacturer's instructions.

Statistical analysis

Continuous variables are presented as the mean ± SEM, and the unpaired two-tailed Student's *t*-test or analysis of variance was used to compare the differences between groups as appropriate. Bonferroni correction was used for pairwise comparisons (GraphPad Prism v.5.0 software; GraphPad, Inc., La Jolla, CA, United States). Statistical significance was set at $P < 0.05$.

RESULTS

Clinical characteristics of four patients with VEO-IBD

All four patients had severe diarrhea (> 6 times/d) and hematochezia during the first year of life. Patients C and D suffered from the disease during the newborn period. In addition to gastrointestinal symptoms, all cases exhibited extraintestinal manifestations, such as perianal abscesses, skin tags (Figure 1A), rectoperineal fistula, failure to thrive, recurrent otitis, urinary tract or respiratory infection, folliculitis, and even sepsis (Table 1). Elevated numbers of peripheral white blood cells and platelets and decreased hemoglobin and albumin levels were found in each patient. Remarkably, immune-related investigations showed that all patients had high serum levels of IL-10 (Table 1). All patients underwent colonoscopy and intestinal biopsy under general anesthesia, revealing erosive lesions (Figure 1B), and were diagnosed with Crohn's disease.

Identification of a novel compound heterozygous mutation in IL10RA

Before admission, all patients underwent TGPS or trio-WES. Two heterozygous pathogenic variants of *IL10RA* were detected in three patients (patient A: c.301C>T, p.R101W; patients B and C: c.537G>A, p.T179T) (Figure 1C and Supplementary Figures 1 and 2). Mutation of c.537G>A occurred at the exon-intron boundary of exon 4, which is a variant hotspot and disrupts RNA splicing (Figure 2A). No pathogenic or likely pathogenic variants were found in patient D.

As *IL10RA* mutation causes infantile IBD in an autosomal recessive manner and serum levels of IL-10 were very high in the four infantile patients with IBD, which is a valuable clinical indicator for identifying infantile IBD as a monogenic disease as we demonstrated previously[13], we suspected that mutations had been overlooked in WES owing to the techniques' limitations. After performing WGS in patients A and B, the breakpoints of the novel deletion were identified by manual review and correction. The deletion was located at chr11:117857030 upstream of exon 1 and chr11:117857362 in intron 1, which contains the 5'-untranslated region (UTR), all of exon 1, and part of intron 1 in *IL10RA* (Figure 1D and Figure 2B). PCR revealed a paternally-inherited 333-bp deletion in addition to the point mutations mentioned above (Figure 1E). The deletion and point mutations were inherited from both parents and eventually constituted compound heterozygotes in patients B (Figure 1F) and A (Supplementary Figure 1).

After identifying the 333-bp deletion in the gene in patients A and B, we reanalyzed the trio-WES data for patients C and D, specifically in the region from 117857030 and 117857362 on chromosome 11 and detected the same deletion. Patient C was a compound heterozygous carrier for c.537G>A, p.T179T (maternal), and the 333-bp deletion (paternal) (Supplementary Figure 2), and patient D was homozygous for the 333-bp deletion.

Histological and functional analysis of patient DIL10RA deletion

Histological findings in a specimen from the colon obtained during colonoscopy revealed oval-shaped intramural abscesses in the submucosa (Figure 3A). Figure 3B shows a higher magnification of inset 1 in Figure 3A, depicting intramural microabscesses.

To determine whether the novel 333-bp deletion in *IL10RA* caused IL-10R dysfunction and subsequently inhibited TNF-α production, supernatants of cultured PBMCs were collected and used to determine TNF-α levels. In healthy controls, LPS stimulation caused a remarkable increase in TNF-α production, whereas addition of IL-10 significantly decreased its abundance. Although LPS led to increased TNF-α production in patient D, this phenomenon was not reversed by the addition of IL-10, as observed in the healthy controls (Figure 3C).

To clarify the exact mechanisms involved, PBMCs were isolated from patient D because the patient was homozygous for the *IL10RA* deletion. PBMCs were stimulated with LPS in the presence or absence of IL-10. The results of western blot analysis showed that in PBMCs from healthy controls, both LPS and IL-10 stimulation caused an increase in the phosphorylation of STAT3 at Tyr705 but not at Ser727 (Figure 3D). In PBMCs from patient D, LPS stimulation also induced increased phosphorylation of STAT3 at Tyr705 but not at Ser727. However, IL-10 stimulation failed to significantly increase phosphorylation of STAT3 at Tyr705 in PBMCs of patient D compared with that in PBS-stimulated PBMCs. No significant differences in STAT3 phosphorylation at Ser727 were observed among the PBS-, LPS-, and IL-10-stimulated PBMCs from patient D (Figure 3D).

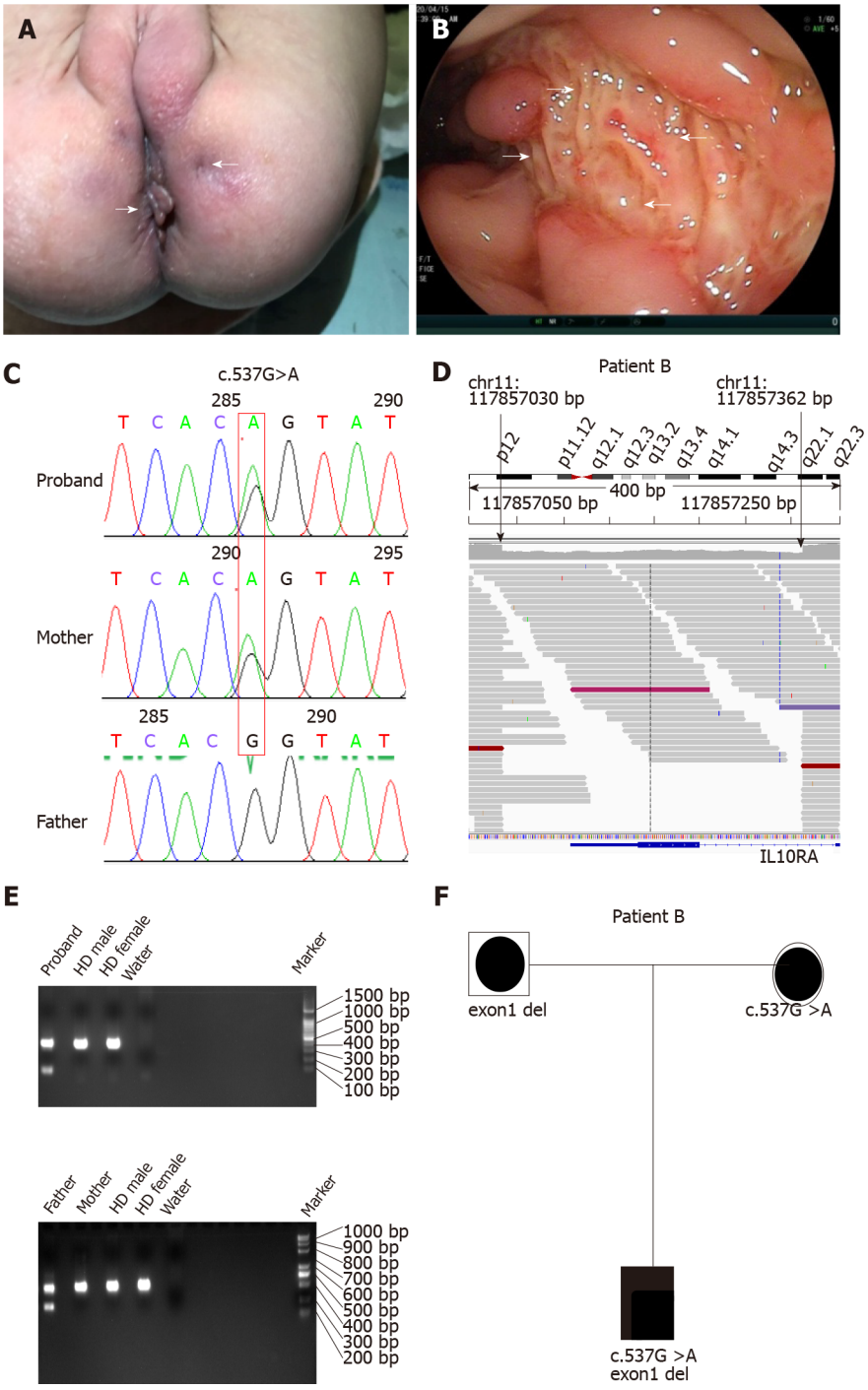


Figure 1 Identification of a novel 333-bp deletion spanning interleukin 10 receptor alpha subunit exon1. A: Perianal skin tag; B: Endoscopic image of ulcerations; C: Sanger DNA sequencing verified a compound heterozygous variant (c.537G>A) inherited from the mother in patient B; D: Whole-genome sequencing (WGS) data showing sequencing read pairs at breakpoints chr:117857030 and chr:117857362 of interleukin 10 receptor alpha subunit (*IL10RA*); E: Polymerase chain reaction validated the heterozygous deletion of 333 bp spanning exon1 inherited from the father; F: WGS revealed compound heterozygous variants of *IL10RA* in patient B with very early-onset inflammatory bowel disease. bp: Base pair; HD: Healthy donor.

DISCUSSION

VEO-IBD is challenging to diagnose and treat because the patients are critically ill and exhibit numerous potential mono-genic defects. Approximately 56 Mendelian genetic defects that can lead to IBD-like colitis have been identified, some of which show almost 100% penetrance, such as defects in *IL-10*, *IL10RA*, *IL10RB*, *FoxP3*, and *XIAP*[1]. NGS has led to breakthroughs in the diagnosis of genetic diseases, including monogenic VEO-IBD. According to a recent single-center study performed by Crowley *et al*[17], 7.8% of VEO-IBD (141 patients) and 13.8% of infantile-onset IBD (29 patients) cases had rare variations associated with monogenic genes. This prevalence was lower

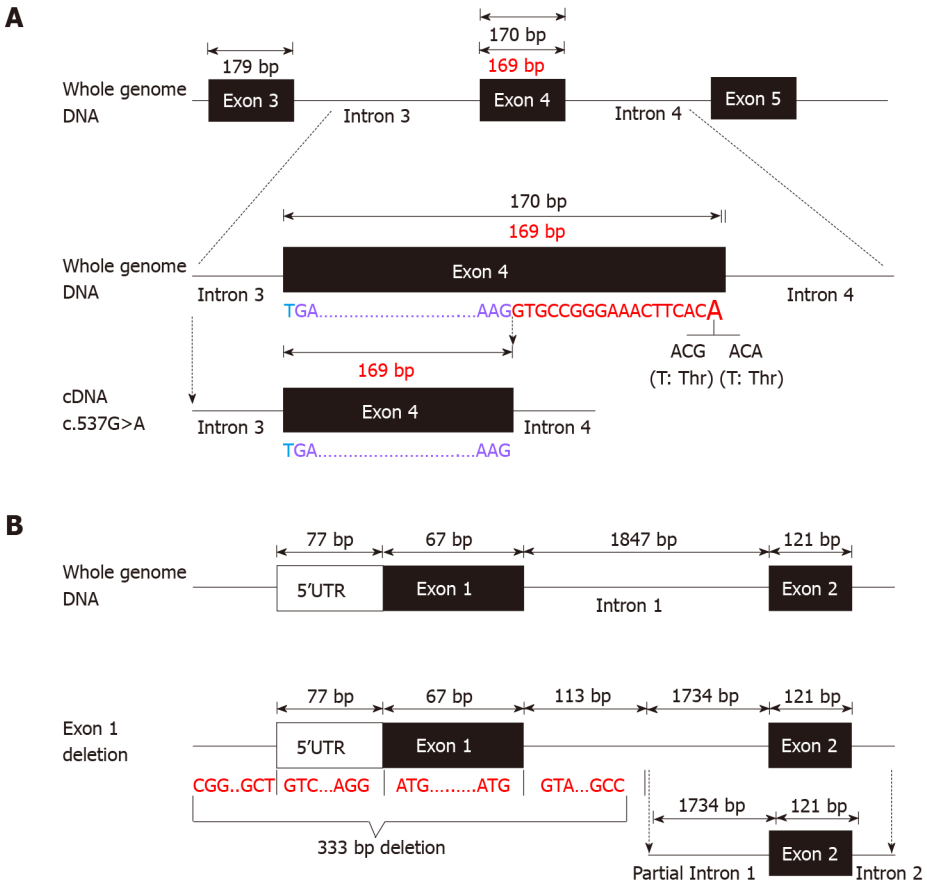


Figure 2 Schematic diagram of disrupted RNA splicing and 333-bp deletion in interleukin 10 receptor alpha subunit. A: Schematic diagram of disrupted splicing caused by c.537G>A in interleukin 10 receptor alpha subunit (*IL10RA)* between the boundary of exon4 and intron4; B: Schematic diagram of *IL10RA* 333-bp deletion. bp: Base pair.

than that reported in Chinese or European studies, which was approximately 31.9%-45.2%[12,18]. In our center, we found that 60.3% of patients with infant-onset IBD had monogenic disease, with mutations in *IL10RA* identified as the most common defect [13].

We evaluated four patients who had Crohn's disease from early infancy and exhibited failure to thrive, severe perianal disease, and resistance to medication. These characteristics indicate the presence of underlying genetic conditions[7]. Although the patients underwent NGS in a local hospital, neither TGPS nor WES revealed conclusive results. However, all patients showed very high serum IL-10 levels, and three patients had a disease-causing heterozygous mutation in *IL10RA*. According to our previous research, serum IL-10 levels > 33.05 pg/mL in patients with VEO-IBD strongly indicates the presence of IL10RA dysfunction[13]. Thus, we predicted that additional *IL10RA* mutations were missed during TGPS or WES. We did not detect the 333-bp deletion in *IL10RA* in two patients until WGS was performed. We then analyzed the same deletion in the other two patients by reanalyzing the trio-WES data. Finally, all patients were precisely diagnosed with VEO-IBD owing to compound heterozygous mutations in *IL10RA* in three patients and homozygous deletion involving *IL10RA* in one patient.

NGS, including TGPS and WES, is a powerful tool for identifying Mendelian genetic diseases in patients with VEO-IBD. The position paper on VEO-IBD by NASPGHAN/ESPGHAN suggests that NGS combined with the patient clinical history represents a vital component of the diagnostic approach[1]. A previous multicenter study showed that molecular diagnosis was achieved in 32% of patients with VEO-IBD when NGS was employed[18]. However, clinical NGS applications have limitations such as short read lengths, relatively high error rates, and incomplete coverage. Non-coding, yet potentially functional regions, and approximately 5% of exons are poorly covered in WES[19]. It is difficult to detect variants involving extensive deletions/insertions or short tandem repeats[20]. Charbit-Henrion *et al*[18] reported three WES-negative cases harboring large deletions in *LRBA* and *NCF1*. Compared to WES, WGS can detect all single-nucleotide variants, small indels, large

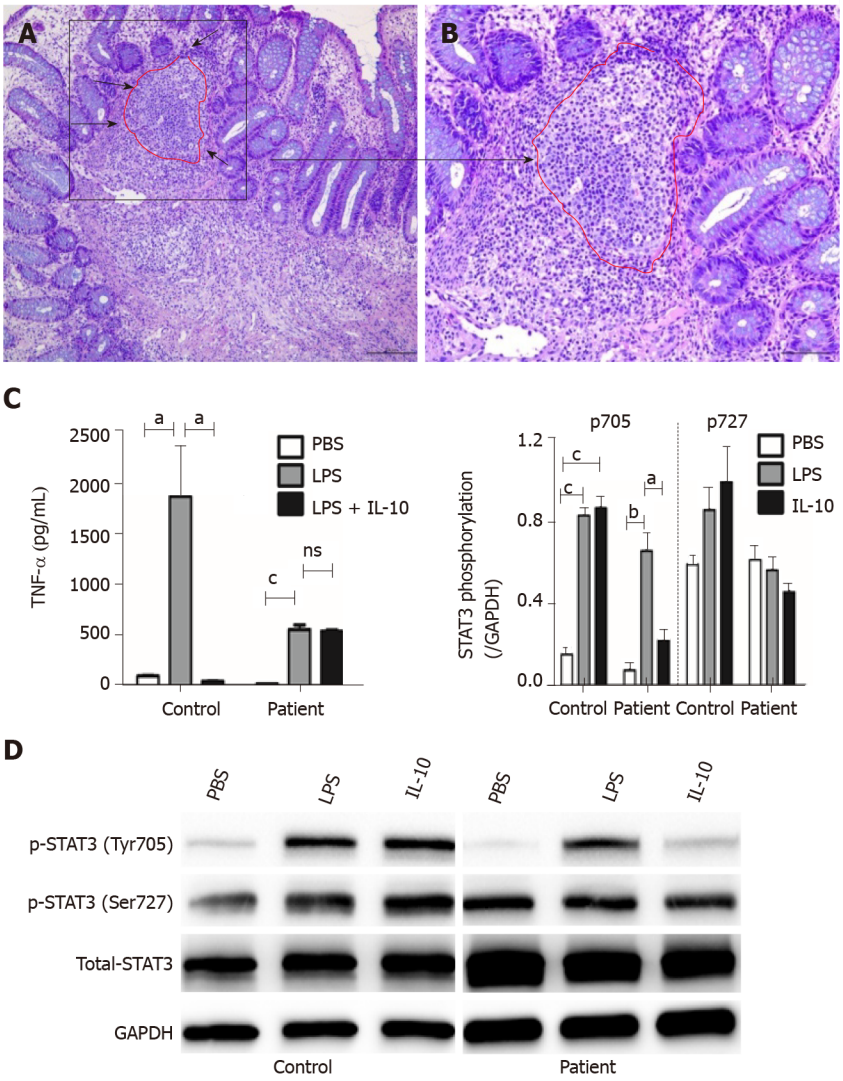


Figure 3 Histopathochemistry and functional results of homozygous interleukin 10 receptor alpha subunit mutation. A: Histological findings in a colonic specimen obtained during colonoscopy showing oval-shaped intramural abscesses; B: Higher magnification of inset A; C: Determination of TNF-α levels in the supernatant of cultured PBMCs from patient D after stimulation with PBS, LPS, or LPS + IL-10 *in vitro*; D: Western blot results of PBMCs isolated from patient D after stimulation with LPS or IL-10. Phosphorylation of STAT3 at Tyr705 and Ser727 and total STAT3 protein were detected (n = 3). $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ were considered as statistically different.

indels, and copy number variants. In our cases, the 333-bp *IL10RA* deletion contained a 5′-UTR, exon 1, and part of intron 1. This large deletion was easy to overlook when WES was used because of its technical limitations and insufficient bioinformatics analysis. After detecting this deletion, we requested re-analysis of the WES data for patients C and D, specifically for the 333-bp region spanning *IL10RA* exon 1. As expected, these two patients harbored the deletion. These results indicate that WES can detect the 333-bp deletion, which was easily overlooked in bioinformatics analysis because of algorithm defects and insufficient experience. WGS compensates for the limitations of WES. Our results indicated that WGS should be performed in VEO-IBD cases with negative WES results, particularly in those with infantile-onset IBD and treatment failure.

A comprehensive range of defects may be associated with VEO-IBD. It is difficult to differentiate every patient based on these defects and their underlying genetic disorders. A small number of patients show distinct phenotypes associated with specific functions, such as IL-10/IL-10R defects, IPEX, CGD, and XIAP[1]. *IL10RA* mutation-induced VEO-IBD shows specific characteristics such as refractory pan-colitis, perianal defects, fistulas, and growth failure, which occur during the neonatal period. An assay that can detect the lack of IL-10 inhibition in LPS can confirm receptor mutations[1]. Nevertheless, this assay is complicated and not routinely available in most hospitals in China. In a retrospective study, we found that the assay was useful for diagnosing IL10RA defects when the serum level of IL-10 was > 33.05

pg/mL. The assay sensitivity was very close to 100%, and the specificity was approximately 84%[13]. Elevated serum levels of IL-10 in patients with VEO-IBD indicated that even such high level of IL-10 could not inhibit TNF-α release and alleviate inflammation. Thus, serum IL-10 level may be a substitute for determining IL-10 inhibition in LPS functional testing. Therefore, when classic symptoms and laboratory results indicate that patients may have IL-10/IL-10R dysfunction but WES is inconclusive, *IL-10/IL10RA/IL-10RB* must be investigated and analyzed. It is recommended to use WGS or specific PCR to detect whether a large deletion involving these genes has occurred.

Interestingly, TNF-α production in LPS-stimulated PBMCs was not as robust in patient D as in control subjects; in our study, patient D was administered with anti-TNF-α antibody (Infliximab) before blood collection, which may have led to relatively low TNF-α production in the supernatant of LPS-stimulated PBMCs compared to that in healthy controls. Another possible reason is that increased TNF-α in the blood of patient D led to activation of the TNF-α receptor, resulting in JNK signaling-dependent inhibition of Bcl-2 expression, which acts as a major anti-apoptosis protein[21].The lack of a comprehensive functional test for *IL10RA* mutation is the major limitation of our study, although we conducted western blotting to determine the possible mechanism of *IL10RA* mutation-induced dysfunction of IL10RA signaling. Further studies are needed to explore other potential differences in IL10RA signaling.

CONCLUSION

Using WGS, we identified a novel 333-bp deletion in *IL10RA* that contributed to four cases of clinically diagnosed VEO-IBD with inconclusive *IL10RA* mutations. Most importantly, we confirmed that typical clinical manifestations and increased serum levels of IL-10 strongly indicate the existence of IL-10R dysfunction even when WES results are negative.

ARTICLE HIGHLIGHTS

Research background

Interleukin 10 receptor alpha (*IL10RA*) gene mutations constitute the most common monogenic disease in East Asia, affecting the health of children. However, identifying disease-causing mutant sites or copy number variants remains challenging in the clinic.

Research motivation

According to the results of our previous study, severe clinical symptoms as well as significantly increased serum IL-10 indicate IL10RA dysfunction, a monogenic phenotype of very early-onset inflammatory bowel disease (VEO-IBD). In addition, such very early-onset IBD showed a heterozygous *IL10RA* gene mutation by whole exon sequencing, leading to the employment of WGS and subsequent identification of 333-bp deletions in *IL10RA*.

Research objectives

We investigated the potential disease-causing gene mutations missed by WES and target gene panel sequencing (TGPS). Our results may contribute to monogenic disease diagnosis.

Research methods

Four patients clinically diagnosed with VEO-IBD during the past 5 years were recruited for this study. Based on their severe clinical phenotypes and the fact that before hospitalization, three patients harbored an *IL10RA* mutation (c.301C>T, p.R101W in one patient; c.537G>A, p.T179T in two patients), as detected by TGPS and trio-WES, and because WES did not show conclusive results in the fourth patient, we performed whole-genome sequencing (WGS) on patients A and B and reanalyzed the trio-WES data from patients C and D. To verify the functional change caused by the novel mutation, peripheral blood mononuclear cells (PBMCs) from patient D were isolated and stimulated *in vitro* with lipopolysaccharide (LPS), IL-10, and LPS + IL-10. Serum IL-10 levels in four patients and tumor necrosis factor-α (TNF-α) in the cell

supernatant were determined by ELISA. Phosphorylation of signal transducer and activator of transcription 3 (STAT3) at Tyr705 and Ser727 in PBMCs was determined by western blot analysis.

Research results

Results of WGS revealed a novel 333-bp deletion encompassing exon 1 of *IL10RA* in patients A and B, which was also found in patients C and D after reanalyzing their WES data. Patient D was homozygous for the 333-bp deletion. All four patients showed elevated serum IL-10 levels. *In vitro*, IL-10-stimulated PBMCs from patient D failed to induce STAT3 phosphorylation at Tyr705 and minimally suppressed TNF-α production induced by LPS. Phosphorylation at Ser727 in PBMCs was not affected by LPS or LPS + IL-10 in both healthy subjects and patient D.

Research conclusions

Genome-wide uniformity of coverage of WGS identified a novel 333-bp deletion in *IL10RA* in four patients with VEO-IBD, whereas the results of initially performed WES were inconclusive. WGS, which was more informative than WES, is the most important comprehensive second-tier genomic test for monogenic diseases in the clinic.

Research perspectives

We will customize a multiplex ligation-dependent amplification probe of the 333-bp deletion in *IL10RA* to help diagnose *IL10RA* mutation-related monogenic diseases.

ACKNOWLEDGEMENTS

The authors would like to thank the members of the Department of Pediatrics and Pediatric Laboratory.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 28; 27(44): 7716-7733

DOI: 10.3748/wjg.v27.i44.7716

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

SYSTEMATIC REVIEWS

Proton pump inhibitors and colorectal cancer: A systematic review

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Conflict-of-interest statement: All the authors declare that they have no competing interests.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 checklist, and the manuscript was prepared in accordance with the PRISMA guidelines.

Country/Territory of origin: Poland

Specialty type: Gastroenterology and hepatology

Provenance and peer review:

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Abstract

BACKGROUND

The use of proton pump inhibitors (PPI) is common worldwide, with reports suggesting that they may be overused. Several studies have found that PPI may affect colorectal cancer (CRC) risk.

AIM

To summarize current knowledge on the relationship between PPI and CRC from basic research, epidemiological and clinical studies.

METHODS

This systematic review was based on the patients, interventions, comparisons, outcome models and performed according to PRISMA guidelines. MEDLINE, EMBASE, Scopus, and Web of Science databases were searched from inception until May 17, 2021. The initial search returned 2591 articles, of which, 28 studies met the inclusion criteria for this review. The studies were categorized as basic research studies (n = 12), epidemiological studies (n = 11), and CRC treatment studies (n = 5). The quality of the included studies was assessed using the Newcastle-Ottawa Scale or Cochrane Risk of Bias 2.0 tool depending on the study design.

RESULTS

Data from basic research indicates that PPI do not stimulate CRC development *via* the trophic effect of gastrin but instead may paradoxically inhibit it. These studies also suggest that PPI may have properties beneficial for CRC treatment. PPI appear to have anti-tumor properties (omeprazole, pantoprazole), and are potential T lymphokine-activated killer cell-originated protein kinase inhibitors

Unsolicited article; Externally peer reviewed.

Peer-review report's scientific quality classification

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

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Received: July 4, 2021
Peer-review started: July 4, 2021
First decision: July 13, 2021
Revised: July 14, 2021
Accepted: September 8, 2021
Article in press: September 8, 2021
Published online: November 28, 2021

P-Reviewer: Yoshimatsu K, Zhang Y
S-Editor: Wang JJ
L-Editor: Webster JR
P-Editor: Li JH

(pantoprazole, ilaprazole), and chemosensitizing agents (pantoprazole). However, these mechanisms have not been confirmed in human trials. Current epidemiological studies suggest that there is no causal association between PPI use and increased CRC risk. Treatment studies show that concomitant PPI and capecitabine use may reduce the efficacy of chemotherapy resulting in poorer oncological outcomes, while also suggesting that pantoprazole may have a chemosensitizing effect with the fluorouracil, leucovorin, oxaliplatin (FOLFOX) regimen.

CONCLUSION

An unexpected inhibitory effect of PPI on CRC carcinogenesis by way of several potential mechanisms is noted. This review identifies that different PPI agents may have differential effects on CRC treatment, with practical implications. Prospective studies are warranted to delineate this relationship and assess the role of individual PPI agents.

Key Words: Colorectal cancer; Proton pump inhibitor; Carcinogenesis; Cancer epidemiology; Capecitabine; Translational medicine

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Core Tip: Proton pump inhibitors (PPI) are a widely, often inappropriately, used class of drugs. Through various mechanisms, they are suspected to increase the risk of gastrointestinal cancers, including colorectal cancer (CRC). The aim of this review is to summarize existing literature on the effect of PPI on CRC. The review assessed basic research studies to identify mechanisms at play in this relationship, observational studies to determine if a causal association exists between PPI use and CRC incidence, and clinical studies to examine if PPI use during chemotherapy influences treatment efficacy and oncological outcomes.

Citation: Patel A, Spychalski P, Antoszewska M, Regula J, Kobiela J. Proton pump inhibitors and colorectal cancer: A systematic review. *World J Gastroenterol* 2021; 27(44): 7716-7733
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7716.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7716

INTRODUCTION

Proton pump inhibitors (PPI) are among the most widely prescribed medications globally[1,2]. Since their development in the 1980s, these drugs have been used for conditions such as peptic ulcer disease, gastroesophageal reflux disease, stress gastritis, and gastrinomas[3]. PPI are available by prescription, but are also sold over-the-counter resulting in frequent use without appropriate indication[4,5]. The mechanism of action of PPI involves irreversible, long-lasting binding to and inhibition of the hydrogen-potassium adenosine triphosphatase (ATPase) enzyme system on gastric parietal cells[6]. These ATPase pumps are responsible for secreting H^+ ions into the gastric lumen, resulting in the production of gastric acid. Suppression of gastric acid production by PPI lowers the acidity of gastric contents while causing feedback hypergastrinemia.

Gastrin, in turn, is a potent growth factor involved in several physiological and pathological processes, including neoplastic transformation[7]. One hypothesis suggests that gastrin may have pro-inflammatory properties and can stimulate the tumor microenvironment *via* macrophage activation and chemotaxis. It is therefore possible that PPI and the resultant hypergastrinemia have a cancer-promoting effect [8].

Some studies, however, suggest that PPI may also exert anti-tumor properties. These drugs might paradoxically inhibit the proliferative effects of hypergastrinemia while demonstrating anti-oxidant, anti-inflammatory, and pro-apoptotic activity[9]. PPI could also have a potential chemotherapeutic role by reducing tumor resistance to chemotherapeutics. De Milito *et al*[10] reported that manipulating cancer pH may

sensitize them to certain chemotherapeutics. In contrast, the TRIO-013/LOGiC trial demonstrated that PPI may negatively affect the efficacy of some cytotoxic drugs, possibly due to alkalinization of the gastric environment[11].

Overall, concerns are increasing regarding the safety of PPI use because of induced hypergastrinemia and a possible association with gastrointestinal (GI) cancer risk, including colorectal cancer (CRC). Many current patients with CRC may have a history of PPI use, but precise epidemiological data are not available. Ahn *et al*[12] and Ma *et al*[13] have summarized observational studies assessing the association of PPI use with the risk of developing CRC. Ahn *et al*[12] found no significant effect of PPI on CRC risk, whereas Ma *et al*[13] found a weak association between long-term PPI use (> 5 years) and increased CRC risk. However, there are no systematic reviews summarizing the evidence from basic research studies exploring mechanisms by which PPIs may affect CRC and from human epidemiological and clinical studies examining PPI use in the context of CRC survival and treatment. As a systematic review might identify important epidemiological and clinical findings, our aim was to provide a comprehensive report on the association of PPI use and CRC based on recent basic research and human studies.

MATERIALS AND METHODS

Literature search

This systematic review was performed in accordance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement using PICO (patients, interventions, comparisons, outcomes)-based questions. Following a predefined search strategy, we searched the MEDLINE, EMBASE, Web of Science, and Scopus online databases to identify suitable articles. No filters were applied during the search, and we also performed backward citation chaining of eligible full-text studies.

Evidence acquisition

On May 17, 2021, two independent researchers (AP, PS) performed a search of the target online databases for eligible studies. The search string was ("*proton pump inhibitors*" or "*proton pump inhibitor*" or "*ppis*" or "*ppi*" or "*omeprazole*" or "*pantoprazole*" or "*esomeprazole*") and ("*CRC*" or "*colorectal cancer*" or "*colon cancer*" or "*rectal cancer*"). The preliminary search returned 2591 articles, which two independent researchers (AP, PS) screened. The entire protocol is presented in a PRISMA flowchart (Supplementary Figure 1).

Inclusion and exclusion criteria

We used PICO framework-based research questions for this review (Supplementary Table 1). If articles met predefined criteria, they were included and categorized as basic research (animal and cell studies), epidemiological (incidence and mortality studies), and treatment studies. Articles were excluded if the full text was not available or was not in English, were not original articles, or did not conform with PICO.

Evidence synthesis and Quality Assessment:

Two independent researchers (Patel A and Spychalski P) retrieved and summarized information from the eligible studies in tables. The authors (Patel A, Spychalski P, Antoszewska M and Kobiela J) discussed conflicts regarding inclusion of studies and resolved them by consensus. Two independent researchers (Patel A and Antoszewska M) assessed the quality of included case-control and cohort studies using the Newcastle-Ottawa scale (NOS)[14]. This scale awards a maximum of nine points for each of the following items: Selection (four stars), comparability (two stars) and outcomes (three stars). Studies were considered of high quality if they scored seven or more stars on NOS assessment. Additionally, the Cochrane Risk of Bias 2.0 tool was used to assess bias in randomized controlled studies included in the retrospective post-hoc analysis reports[15]. The results of quality assessment are described in Supplementary Material along with Supplementary Table 2.

RESULTS

A total of 28 studies were included in the review: Basic research studies (n = 12) [animal models (n = 5), CRC cell lines (n = 1), or both (n = 6)]; epidemiological studies (

n = 11) [analyzing CRC risk (n = 9) and survival (n = 2) associated with PPI use]; and treatment studies (n = 5), examining the effects of PPI on CRC chemotherapy regimens.

Basic studies

The included basic studies examined two primary themes: (1) Trophic effects of PPI-induced hypergastrinemia; and (2) Potential chemotherapeutic role of PPI as cytostatic drugs, chemosensitizing drugs, or T lymphokine-activated killer cell-originated protein kinase (TOPK) inhibitors. The information from the basic studies is summarized in Tables 1 (animal models) and 2 (CRC cell lines).

Trophic studies: The trophic effects of PPI-induced hypergastrinemia were investigated in six studies[16-21]. Four animal studies demonstrated that PPI-induced hypergastrinemia did not influence growth and invasiveness of CRC[16-19]. These studies showed that omeprazole treatment resulted in significantly higher serum or plasma gastrin levels (4- to 20-fold across studies) in comparison to control groups. However, the treated and control groups were similar in terms of tumor burden and/or invasiveness of CRC. Graffner *et al*[16] found omeprazole-treated and control mice to be similar in terms of tumor size, survival and distant metastasis rate. Pinson *et al*[17] compared low-dose and high-dose omeprazole, ranitidine (histamine-2 receptor antagonist), and control exposure in rats. They found that overall tumor burden and survival were similar among these groups but documented significantly lower mean tumor number, volume, and total mass in the ranitidine group (multiple comparisons, all $P < 0.05$). Hurwitz *et al*[18] reported concordant findings in their study, additionally noting no significant differences in DNA, RNA or protein concentration in tumor-free colonic tissues of treated *versus* control rats. Chen *et al*[19] performed sham operation, colostomy and/or fundectomy, omeprazole treatment, or fasting with refeeding to assess the short-term and long-term effects of hypergastrinemia. None of the groups demonstrated growth of CRC tumors, but the fundectomy group showed suppressed tumor growth.

Two studies indicated that PPI treatment resulted in suppression of CRC growth[20, 21]. Penman *et al*[20] found a significantly lower incidence of CRC tumors in omeprazole-treated rats than controls (63% *vs* 95%, $P < 0.02$). They hypothesized that omeprazole possibly influenced metabolism of the carcinogen (azoxymethane) by affecting either intestinal microflora or P450 isoenzymes, therefore resulting in lower CRC growth. Working with the NCI-H719 human colon cancer cell line, Tobi *et al*[21] demonstrated a dose-dependent decrease in proliferation (cytostatic effect) with omeprazole, but noted no such effect in two other cell lines (DLD-1 and LCC-18). These researchers found that the cytostatic effect of omeprazole persisted even when omeprazole was combined with gastrin, suggesting a potential paradoxical inhibition of gastrin's trophic influence on CRC.

Chemotherapeutic studies: Six studies addressed the potential chemotherapeutic role of PPI in CRC[22-27]. Three studies assessed the cytotoxic effects – anti-proliferative, pro-apoptotic, and anti-inflammatory properties – of PPI on CRC and found that PPI (omeprazole, pantoprazole) dose-dependently inhibited proliferation and induced apoptosis in CRC models[22-24]. Patlolla *et al*[22] reported that omeprazole resulted in upregulation of p21waf1/cip1 and downregulation of cyclin A, Bcl-2, Bcl Xl, and survivin expression, leading to induction of cell apoptosis. Kim *et al*[23] reported on the anti-inflammatory activities of PPI, describing reduced tumor necrosis factor-alpha (TNF-α), nitric oxide (NO), colon thiobarbituric acid-reactive substance (TBA-RS), and expression of cyclooxygenase-2 (COX-2) and NO synthetase. These authors also suggested a potential anti-proteolytic and anti-mutagenic action of PPI, reporting decreased levels of matrix metalloproteinase (MMP)-9, MMP-11, and MT1-MMP and decreased beta-catenin accumulation in omeprazole-treated mice as compared to controls.

Han *et al*[24] reported similar findings on the pro-apoptotic, anti-inflammatory, and anti-proliferative properties of PPI, along with a potential anti-angiogenic effect. They found that PPI treatment reduced expression of angiogenic factors such as interleukin (IL)-8, platelet-derived growth factor, vascular endothelial growth factor, and hypoxia-inducible factor 1-alpha. Moreover, Kim *et al*[23] and Han *et al*[24] demonstrated that PPI may paradoxically inhibit the trophic effect of gastrin on CRC cells. Kim *et al*[23] found cell proliferation to be significantly ($P < 0.05$) reduced in cells treated with both omeprazole and gastrin compared to with gastrin only. Han *et al*[24] reported similar findings and found that PPI antagonized gastrin's binding to cholecystokinin B receptor (CCKBR), both alone and in combination with gastrin.

Table 1 Summary of basic research studies (animal models)

Author	Aim of study	PPI investigated	Species strain, gender	Methods of CRC induction	PPI treatment	Experimental/ control group	Outcome measure	Main findings	Mechanism studied	Role of PPI in CRC
Graffner *et al*[16] 1992	To determine the influence of PPI-induced endogenous hypergastrinemia on growth in CRC-implanted mice	OME	BALB/C mice, M	MC-26 tumor cells injected SC in epigastric region	Daily for 19 d, 400 μmol/kg, PO	18/18	Tumor size, survival	5-fold higher serum gastrin levels in OME-treated animals than controls. No differences in tumor size, tumor weight, survival and metastatic potential (61% *vs* 72%, P = NR) between tumor-bearing treated and control group	Trophic effect of gastrin	NE
Penman *et al*[20] 1993	To assess the influence of OME-induced hypergastrinemia on CRC development in animal models	OME	Sprague-Dawley rats, F	12 (weekly) SC azoxymethane (10 mg/kg/wk)	Daily for 27 wk, 40 μmol /kg, PO	19/20	Number of tumors, position, volume; metastatic disease	9-10-fold higher gastrin levels in OME-treated groups than control groups. Significantly fewer OME-treated rats developed tumors compared to control group (63% *vs* 95%, $P < 0.02$). Number of tumors were also significantly lower in OME-treated rats. Average tumor size and invasiveness of CRC was similar for both groups	Trophic effect of gastrin	PE
Hurwitz *et al*[18] 1995	To evaluate effect of omeprazole-induced hypergastrinemia on carcinogen-induced CRC in rats	OME	Sprague-Dawley rats, M	Six (weekly) IP methylazoxymethanol (30 mg/kg)	Daily for 10 wk, 40 mg/kg, gastric gavage	NR	Number of tumors, volume and total tumor burden, biochemical and histological analysis	Serum gastrin levels were elevated 6-fold in OME-treated animals *vs* controls. No differences in tumor number, tumor volume, and total tumor burden between treated and control group. No histological (crypt/mucosal height) or biochemical features in CRC-free regions of colon	Trophic effect of gastrin	NE
Pinson *et al*[17] 1995	To assess if hypergastrinemia enhances progression or invasiveness of CRC	OME	Sprague-Dawley rats, M	Six (weekly) IP methylazoxymethanol (30 mg/kg)	Daily for 10 wk, 14 or 40 mg/kg, gastric gavage	162/108	Number of tumors, volume and total tumor burden, histological analysis	Plasma gastrin levels in the treated groups (low-dose OME, high-dose OME, ranitidine) were 3–5-fold higher than controls. Crypt height/mucosal height ratio of CRC-free colonic mucosa was similar between all groups. No significant differences in tumor number, tumor burden and invasiveness between OME-treated and control groups.	Trophic effect of gastrin	NE
Chen *et al*[19] 1998	To examine trophic effects of endogenous hypergastrinemia colonic mucosa and transplanted colon adenocarcinoma in rats	OME	Sprague-Dawley rats, M	Injection of K-12 cell line (Established in syngeneic BDIX rats *via* induction using 1,2-dimethylhydrazine)	Daily for 10 d, 400 μmol/kg, PO	NR	Tumor weight and volume, histological analysis, labelling index	OME treatment and fundectomy raised serum gastrin levels by 4-5-fold. OME-treatment did not stimulate growth of transplanted tumor (K-12) cells, while fundectomy suppressed CRC growth (decreased labelling index, weight and volume of tumor) Sustained hypergastrinemia did not affect the thickness and labelling index of normal colon mucosa	Trophic effect of gastrin	NE
Kim *et al* [23] 2010	To evaluate chemo-preventive properties of	OME	C57BL/6 mice, F	Colitis induction - 15 cycles of 0.7% DSS in drinking	NR, 10 mg/kg, IP	12/24	Tumor burden, biochemical and	OME-treated group developed significantly lower number of colon tumors than control	Cytostatic properties	PE

	omeprazole in a colitis-associated CRC mouse model			water			histological analysis	groups. OME administration also resulted in decreased inflammatory markers (TNF-α, serum NO, and colon TBA-RS levels), attenuated expression of MMP, COX-2, NO synthase, and β-catenin, and greater apoptotic index		
Patlolla *et al*[22] 2012	To assess chemo-preventive effects of omeprazole	OME	F344 rats, M	Two (weekly) SC azoxymethane (15 mg/kg)	9 wk, 200/400 ppm, PO	30/18	Aberrant crypt foci incidence	Omeprazole inhibited the AOM-induced colonic foci formation in a dose-dependent manner	Cytostatic properties	PE
Han *et al* [24] 2014	To study the effects of PPI on colitis-associated carcinogenesis	PAN	APCMin/+ mice, M	Genetically engineered mutation in APC gene	Thrice weekly for 10 wk, 8 mg/kg, IP	NR/8	Number and size of intestinal polyps	Gastrin + PPI exerted significant anti-polyposis effect through β-catenin inactivation, increased apoptosis, anti-angiogenic, and MAPK inactivation relevant to decreased levels of pro-inflammatory mediators	Cytostatic properties	PE
Zeng *et al*[26] 2016	To evaluate the effect of pantoprazole as TOPK inhibitor *in vivo* and *in vitro*	PAN	Non-obese diabetic-SCID mice	HCT 116 cells inoculated SC into left flank	Every 2 d for 19 d, 100 mg/kg, IP	8/8	Tumor volume, immunohistochemical analysis	Tumors treated with PAN grew significantly more slowly, and the size of tumors was smaller compared with the control group. PAN-treated group had lower average tumor volume per mouse compared to controls (111 mm^3 *vs* 285 mm^3, $P < 0.05$). Average body weight was similar throughout the study indicating no toxic effects of PAN in the mice IMHC for phosphorylated histone H3 revealed substantially decreased expression in PAN-treated group compared to control	TOPK inhibition	PE
Zheng *et al*[27] 2017	To evaluate the effect of PPI as a TOPK inhibitor *in vivo* and *in vitro*	ILA	CB-17/Icr-scid mice	HCT 116 cells inoculated SC into left flank	Daily for 19 d, 150 mg/kg, PO	8/8	Tumor volume, immunohistochemical analysis	Estimated tumor volumes of treatment groups were less than that of the control group. No toxicity or differences in body weight were observed. Expression levels of phosphorylated histone H3 were substantially decreased in ilaprazole-treated groups compared with the control group	TOPK inhibition	PE
Wang *et al*[25] 2017	To investigate the chemosensitizing potential of PPI in CRC	PAN	BALB/C mine, F	HT29 cells injected SC	Weekly for 4 wk, 30 mg/kg, IP	NR	Tumor burden	PAN combined with 5-FU demonstrated greater inhibition of tumor growth and smaller tumor sizes compared to 5-FU alone	Chemosensitizing properties	PE

AOM: Azoxymethane; CRC: Colorectal cancer; COX-2: Cyclooxygenase-2; F: Female; 5-FU: 5-Fluorouracil; ILA: Ilaprazole; IMHC: Immunohistochemistry; IP: Intraperitoneal; M: Male; MAPK: Mitogen-activated protein kinase; MMP: Matrix metalloproteinase; NO: Nitric oxide; NE: No effect; NR: Not reported; OME: Omeprazole; PAN: Pantoprazole; PO: Per os; PPI: Proton pump inhibitors; PE: Protective effect; SC: Subcutaneous; TOPK: T lymphokine-activated killer cell-originated protein kinase; TNF-α: Tumor necrosis factor-alpha; TBA-RS: Thiobarbituric acid-reactive substance.

Wang *et al*[25] found that PPI increased the chemosensitivity of human colon cancer cells (HT29 and RKO lines) as PPI combined with 5-fluorouracil (5-FU) resulted in significantly higher cell inhibition rates than 5-FU alone (*in vitro* experiment: $P = 0.04$; *in vivo* experiment: $P = 0.03$).

Table 2 Summary of basic research studies (colorectal cancer cell lines)

Author	Aim of study	PPI investigated	Cell lines studied	Outcome measure	Main finding	Mechanisms	Role of PPI in CRC
Tobi *et al*[21] 1995	To assess the direct effects of gastrin and OME on growth of CRC origin cells separately and in combination	OME	NCI-H716, LCC-18, DLD-1	Proliferation of cell lines	OME treatment resulted in cytostatic effect on 1 of the 3 cell (NCI-H716) lines tested. Dose-dependent decrease in cell proliferation noted compared to controls ($P < 0.05$). Effect seen with gastrin (low concentration), OME, or both in combination. Gastrin increased proliferation of NCI-H716 cells only at high concentrations	Trophic effect of gastrin	PE
Kim *et al* [23] 2010	To evaluate chemo-preventive properties of omeprazole in a colitis-associated CRC mouse model	OME	HT29	Cell viability and growth	Significant cleavage of capsase-3 in presence of 500 μmol/L omeprazole, but effect attenuated with gastrin pre-treatment, signifying that gastrin could attenuate the cytotoxicity of PPI by decreasing apoptosis. Compared with the gastrin-treated group, cell proliferation was significantly attenuated in the presence of omeprazole ($P < 0.05$), suggesting that PPI could offset the trophic action of gastrin on colon cells	Cytostatic properties	PE
Patlolla *et al*[22] 2012	To assess chemo-preventive effects of OME	OME	HCA-7, HCT-116	Cell viability, cytotoxicity assays, apoptotic assays	Dose-dependent suppression of cell growth and induction of apoptosis seen in both cell lines	Cytostatic properties	PE
Han *et al* [24] 2014	To study the effects of PPI on colitis-associated carcinogenesis	PAN	HCT116	Proliferation rate, apoptosis, and molecular analysis	PPI antagonizes trophic actions of gastrin, causes dose-dependent suppression of cellular viability. Combination of PPI and gastrin had higher cytotoxic activity than PPI alone. PPI alone or in combination with gastrin induces apoptosis and blocks gastrin-CCKBR binding. PPI may possess anti-angiogenic activity, which inhibits the expression of angiogenic factors induced by gastrin	Cytostatic properties	PE
Zeng *et al*[26] 2016	To evaluate the effect of pantoprazole as TOPK inhibitor *in vivo* and *in vitro*	PAN	HCT116, SW480, WiDr	Cell viability, TOPK assay analysis, cytotoxicity assays	Pantoprazole had different cytotoxicity toward different colon cancer cells. It inhibits anchorage-independent growth of colon cancer cells. Cell line with high TOPK activity (HCT116) was more sensitive to pantoprazole. The study suggests that TOPK is a direct target for pantoprazole to suppress colon cancer cell growth	TOPK inhibition	PE
Zheng *et al*[27] 2017	To evaluate the effect of PPI as TOPK inhibitor *in vivo* and *in vitro*	ILA	HCT116	Cell viability, TOPK assay analysis, cytotoxicity assays	Ilaprazole exhibited potent inhibitory effect on growth and induced apoptosis in HCT116 cells in a dose-dependent manner. The study suggests that TOPK was a direct target for ilaprazole to suppress cancer cell growth and its anticancer activities were dependent on the TOPK expression. Inhibition of TOPK by ilaprazole is dependent on TOPK abundance in cancer cells	TOPK inhibition	PE
Wang *et al*[25] 2017	To investigate the chemosensitizing potential of PPI in CRC	PAN	HT29, RKO	Cell inhibition rate	PPI in combination with 5-FU had a higher inhibitory effect on CRC cell line growth compared to controls. The study suggests that PPI may increase sensitivity of CRC tumors to 5-FU *in vitro*	Chemosensitizing properties	PE

CCKBR: Cholecystokinin-B receptor; CRC: Colorectal cancer; ILA: Ilaprazole; OME: Omeprazole; PAN: Pantoprazole; PPI: Proton pump inhibitors; PE: Protective effect; TOPK: T lymphokine–activated killer cell–originated protein kinase.

Zeng *et al*[26] and Zheng *et al*[27] investigated pantoprazole and ilaprazole, respectively, as potential TOPK inhibitors. Both groups found that PPI inhibited CRC cell growth *via* TOPK inhibition *in vitro* and *in vivo*. Among the PPI, ilaprazole and pantoprazole showed the strongest affinity for TOPK. Zeng *et al*[26] examining three colon cancer cell lines with different TOPK expression levels reported that pantoprazole had a growth-inhibiting effect through interaction with TOPK. Zheng *et al*[27] described similar results for ilaprazole, with PPI treatment resulting in decreased phosphorylation of histone, a TOPK-mediated process, suggesting that TOPK may be a direct target for these drugs. Furthermore, the authors found ilaprazole to be an inducer of apoptosis *via* activation of caspases and cleavage of poly-(ADP-ribose) polymerase.

Epidemiological studies

Six case-control studies[28-33], two prospective studies[34,35], and one retrospective study[36] addressed the incidence of CRC in PPI-users *versus* non-users. Two retrospective cohort studies assessed the survival of CRC patients in relation to PPI use.

Incidence studies: The information from the six included case-control incidence studies is abstracted in Table 3[28-33]. The time definition of PPI use varied across studies. The included studies analyzed information from healthcare databases or registries of different regions – Denmark, the Netherlands, United Kingdom, San Francisco (United States), and Washington (United States). A total of 31829 CRC patients matched with 276647 controls were included in this review. After adjustment for confounders, none of the studies revealed an increased risk of CRC in current or ever PPI-users in comparison to non-users. Furthermore, most (5/6) of the studies found that the duration of PPI use or average daily dose of PPI did not influence CRC risk[28-30,32,33]. However, Lee *et al*[31] reported that the risk of CRC increased significantly with ≥ 10 years of PPI use compared to no use [odds ratio (OR) = 1.28, 95% confidence interval (CI): 1.15-1.44]. Robertson *et al*[28], Yang *et al*[30] and Kuiper *et al*[33] did not find any significant increase in risk in recent or former PPI-users. However, Kuiper *et al*[33] found that current PPI-users were at an increased risk of developing CRC (OR = 1.30, 95%CI: 1.16-1.47), especially with concomitant non-steroidal anti-inflammatory drugs use (OR=1.57, 95%CI: 1.27-1.93).

Three cohort studies assessed the hazard of developing CRC in PPI-users and non-users[34-36] (Table 4). The review included a total of 108107 PPI-users and 609800 non-users identified through healthcare databases in Korea, United States, and Taiwan. Hwang *et al*[34] and Babic *et al*[35] found no significant association between PPI exposure and CRC development, but Lei *et al*[36] reported a significantly increased risk of CRC among PPI-users [hazard ratio (HR) = 2.03, 95%CI: 1.56-2.63, $P < 0.05$]. Hwang *et al*[34] reported that PPI use was associated with increased CRC risk in individuals at low risk for CRC (non-obese, non-diabetics, female, aged < 50 years, no history of alcoholism, receiving ≥ 180 daily defined dose of PPI) (HR = 12.30, 95%CI: 1.71-88.23, $P < 0.01$). Babic *et al*[35] found that the period of PPI use had no effect on CRC risk but that current PPI use was associated with a decreased risk (HR = 0.82, 95%CI: 0.68-0.98). In contrast, Lei *et al*[36] reported a time-dependent and dose-dependent relationship between PPI use and CRC development, with patients at higher risk if they were using PPI for ≥ 1 year and increasing doses of PPI. On further analysis, Lei *et al*[36] found that the risk of CRC was increased with esomeprazole, lansoprazole, and omeprazole, but no such association was seen with pantoprazole and rabeprazole.

Survival studies: Survival of CRC patients was assessed in two retrospective studies. Graham *et al*[37] included 1304 CRC (117 PPI-users at diagnosis) patients with similar baseline characteristics, but greater cardiac comorbidities in PPI-users ($P < 0.05$). The authors found similar overall survival (OS) rates at 1-, 2- and 5-years between PPI-users and non-users, but the cumulative survival of PPI-users was significantly shorter than non-users (1775 *vs* 2279 d, $P = 0.048$). Furthermore, after controlling for known risk factors, the risk of mortality was significantly higher in CRC patients using PPI (HR = 1.34, 95%CI: 1.01-1.78, $P = 0.04$). Tvingsholm *et al*[38] analyzed cancer-specific mortality for nine cancers in a cohort of 347919 patients, including 47188 CRC patients. They found that the risk of mortality in CRC patients was approximately 12 times higher in PPI-users as compared to non-users (HR = 11.8, 95%CI: 11.3-12.4).

Treatment studies

The effects of PPI use concurrently with chemotherapeutic treatment of CRC was

Table 3 Summary of epidemiological studies assessing the exposure of proton pump inhibitors in colorectal cancer patients

Author, year, place	Accrual year	Study design	Grouping	Number	Exposed	Unexposed	OR (95%CI)	Adjustments	Risk of CRC
Robertson *et al*[28] 2007 (Denmark)	1989-2005	CC	CRC patients	5589	295	5294	1.11 (0.97-1.27)	Age, sex, place of residence (matched), H2 blocker use, aspirin/NSAIDs, statins/diabetics use, history of cholecystectomy, alcohol	No increased risk
			Non-CRC control	55890	2692	53198			
Van Soest *et al*[29] 2008 (Netherland)	1996-2005	CC	CRC patients	594	53	541	0.85 (0.63-1.16)	Age, sex, calendar time, follow-up duration (matched), comorbidities	No increased risk
			Non-CRC control	7790	725	7065			
Yang *et al*[30] 2007 (United Kingdom)	1987-2002	CC	CRC patients	4432	769	3663	1.2 (0.8-1.9)	Age, sex, alcohol, smoking, BMI, H2 blocker use, aspirin/NSAID use, calendar time, follow-up, general practice site (matched), HRT use, history of colonoscopy/flexible sigmoidoscopy	No increased risk
			Non-CRC control	44292	5133	39159			
Lee *et al*[31] 2020 (San Francisco, United States)	1996-2016	CC	CRC patients	18595	1406	17189	NR	Age, sex, ethnicity, general practitioner site, enrolment duration, smoking, alcoholism, BMI, history of colonoscopy, family history of CRC, Crohn's disease, Ulcerative colitis	No increased risk
			Non-CRC control	160122	10813	149309			
Chubak *et al*[32] 2009 (Washington State, United States)	2000-2003	CC	CRC patients	641	16	482	1.7 (0.8-4.0)	Age, sex, calendar time, follow-up duration (matched)	No increased risk
			Non-CRC control	641	9	471			
Kuiper *et al*[33] 2020(Netherlands)	2007-2014	CC	CRC patients	1978	1041	937	1.08 (0.97-1.21)	Age, sex, calendar time, H2 blocker use, aspirin, NSAIDs, statins, antidiabetics use	No increased risk
			Non-CRC control	7912	4161	3751			

BMI: Body mass index; CC: Case-control; PPI: Proton pump inhibitors; CRC: Colorectal cancer; CI: Confidence interval; H2: Histamine-2 receptor; HRT: Hormone replacement therapy; NSAIDs: Nonsteroidal anti-inflammatory drugs; NR: Not reported; OR: Odds ratio.

assessed in three retrospective studies, two post-hoc analyses of randomized controlled trials (RCTs)[39-43]. These studies cumulatively examined 7065 patients and are summarized in Table 5.

Zhang *et al*[39] examined 125 patients with stage II-III rectal cancer dichotomizing them as eligible omeprazole users (EOU, 20 mg per os at least once/day for 6 d and/or 40 mg IV infusion daily during adjuvant chemotherapy) or non-EOU, and an effective omeprazole group (EOG, OME ≥ 200 mg total during the study period), or a non-EOG. The authors found that 5-year disease-free survival (DFS) was significantly decreased in the EOG *vs* non-EOG group (P = 0.032), but OS was similar among the groups (P = 0.092). Additionally, the recurrence of rectal cancer was more common in the non-EOG group than in the EOG group (31.3% *vs* 10.3%, P = 0.025). A comparison of EOU and non-EOU patients revealed similar DFS and OS at 3 and 5 years.

In a cohort of 298 patients with stage I-III CRC, Sun *et al*[40] identified 77 patients who used PPIs concurrently during adjuvant capecitabine therapy. PPI-users were found to have significantly lower 5-year recurrence-free survival (RFS) (74% *vs* 83%, P = 0.03), but similar OS (81% *vs* 78%, P = 0.7) compared to non-users. Multivariate analysis revealed similar RFS between the groups (HR = 1.65, 95%CI: 0.93-2.94, P =

Table 4 Summary of epidemiological studies assessing the effect of proton pump inhibitors exposure on the risk of developing colorectal cancer

Author, year, place	Accrual year	Study design	Grouping	Number of patients	Developed CRC	Did not develop CRC	HR (95%CI)	Adjustments	PPI use and CRC risk
Hwang *et al* 2017[34] (Korea)	2007-2013	P	PPI users	49520	Total cases (including PPI users and non-PPI users) 5304	NR	NR	Sex, age, smoking, alcohol, BMI, consumption, physical activity, type 2 diabetes, CCI score, aspirin use, metformin use, stain use, socioeconomic status	No association
			Non-PPI users	401764					
Babic *et al* 2020[35] (United States)	1988-2015	P	PPI users	13205	83	13122	0.84 (0.67-1.04)	Age, physical activity, BMI, family history of CRC, alcohol, smoking, history of lower endoscopy, caloric intake, vitamin D, calcium intake, regular aspirin use, folate intake, menopausal hormone therapy use, and red meat	No association
			Non-PPI users	162654	1172	161482			
Lei *et al* 2020[36] (Taiwan)	1999-2011	R	PPI users	45382	172	45210	2.03 (1.56-2.63)	Sex, age, year of index date, diabetes, coronary artery disease, HTN, dyslipidemia, COPD, cirrhosis, CCI, aspirin/NSAID use, statin use, antidiabetic use	Increased risk
			Non-PPI users	45382	93	45289			

BMI: Body mass index; CCI: Charlson comorbidity index; COPD: Chronic obstructive pulmonary disease; CRC: Colorectal cancer; CI: Confidence interval; HR: Hazard ratio; HTN: Hypertension; NSAID: Nonsteroidal anti-inflammatory drugs; NR: Not reported; PPI: Proton pump inhibitor; P: Prospective study; R: Retrospective study.

0.09).

Wong *et al*[41] studied PPI use with adjuvant CapeOx (capecitabine, intravenous oxaliplatin), or adjuvant FOLFOX (intravenous 5-FU, leucovorin, oxaliplatin) therapy. Of 389 patients with stage II-III CRC, 214 underwent CapeOx therapy and 175 had FOLFOX therapy. The proportions of patients taking PPI in both groups were similar (23.4% CapeOx *vs* 28% FOLFOX, P = 0.3). Comparing PPI-users and non-users, the authors found 3-year RFS to be significantly lower in CapeOx-treated PPI-users (P = 0.029) but similar between the two groups in FOLFOX-treated patients (P = 0.66). Multivariate analysis showed that PPI use was associated with increased risk of CRC recurrence in the CapeOx-treated group (HR = 2.20, 95%CI: 1.14-4.25, P = 0.018). The use of PPI in combination with either adjuvant treatment regimen did not affect 3-year OS in this study (CapeOx, P = 0.35; FOLFOX, P = 0.929).

Kichenadasse *et al*[42] analyzing data from six RCTs including metastatic CRC patients reported that PPI-users had significantly poorer OS (HR = 1.20, 95%CI: 1.03-1.40, P = 0.02) and PFS (HR = 1.20, 95%CI: 1.05-1.37, P = 0.009). The subgroup analysis revealed that chemotherapy type, use of capecitabine or 5-FU, line of therapy and VEGF inhibitor use, across studies, did not influence oncological outcomes between users and non-users. Kim *et al*[43] described post-hoc analysis of data relating to PPI use from the AXEPT trial. The authors reported that PPI users in the FOLFIRI (fluorouracil, leucovorin, irinotecan) arm had significantly better OS (HR = 0.5, 95%CI: 0.30-0.85; P = 0.011) and PFS (HR = 0.55, 95%CI: 0.33-0.91, P = 0.02) compared to non-users, while there were no differences noted in the mXELIRI (capecitabine, irinotecan) arm.

DISCUSSION

This systematic review of 26 articles is the first in the literature to summarize the evidence on the association between PPI and CRC from basic research, epidemiological, and clinical treatment studies. Previously published meta-analyses by Ahn *et*

Table 5 Summary of treatment studies

Author	Center	Study design	Cancer stage and type	Cancer treatment	PPI use (definition)	No. of patients	Results
Zhang *et al* [39] 2017	Guangzhou, China	R	Stage II-III Rectal cancer	LCRT (46 Gy, Oxaliplatin + Capecitabine (2 cycles)	EOU = OME: 20 mg PO, min. OD for 6 d / 40 mg IVI, daily). EOG = total OME dose ≥ 200 mg[1]	125	EOG *vs* non-EOG: [1]DFS (3-year) = 77.1% *vs* 96.6%, *P* = 0.032, DFS (5-year) = 69.6% *vs* 46.7%, *P* = 0.032, OS (3-year) = 82.3% *vs* 96.6%, *P* = 0.092, OS (5-year) = 76.9% *vs* 89.5%, *P* = 0.092 EOU *vs* non-EOU: [1]DFS (3-year) = 85.5% *vs* 77.8%, *P* = 0.658, DFS (5-year) = 75.6% *vs* 74.6%, *P* = 0.658, OS (3-year) = 90.3% *vs* 82.5%, *P* = 0.754, OS (5-year) = 82% *vs* 77.6%, *P* = 0.754
Sun *et al*[40] 2016	Edmonton, Canada	R	Stage I-III CRC	Adjuvant Capecitabine monotherapy	Any use during treatment (based on prescription data)	298	PPI-user *vs* non-users: RFS (5 years) = 74% *vs* 83%, *P* = 0.03; OS (5-year) = 81% *vs* 78%, *P* = 0.7. Multivariate RFS (5-year): HR (95%CI) = 1.65 (0.93-2.94), *P* = 0.09
Wong *et al*[41] 2019	Alberta, Canada	R	Stage II-III CRC	Adjuvant CapeOx or FOLFOX	Any use during treatment (based on prescription data)	389	PPI-users *vs* non-users, RFS (3-year): CapeOX = 69.5% *vs* 82.6%, *P* = 0.03; FOLFOX = 82.9% *vs* 61.7%, *P* = 0.7; Multivariate RFS: HR (95%CI) = 2.20 (1.14-4.25) *P* = 0.018; OS (3-year): CapeOX = 90.1% *vs* 91.2%, *P* = 0.345, FOLFOX = 77.4% *vs* 80.1%, *P* = 0.929
Kichenadasse *et al*[42] 2021	6 clinical trials	Retrospective post-hoc analysis of RCT	Stage IV CRC	Fluoropyrimidine-based chemotherapy (± additional agents). Regimens differed across included trials	Minimum 7 d of use during study period	5633	OS: Significantly worse in PPI-users [HR (95%CI) = 1.20 (1.03-1.40)], *P* = 0.02; PFS: Significantly worse in PPI-users [HR (95%CI) = 1.20 (1.05-1.37)], *P* = 0.009 Various treatment subgroups did not influence OS and PFS
Kim *et al*[43] 2021	China, Japan, South Korea (98 centers)	Retrospective post-hoc analysis of RCT	Stage IV CRC	mXELIRI or FOLFIRI (± Bevacizumab)	Use for ≥ 20% of study period	620	mXELIRI arm: No difference in OS or PFSFOLFIRI arm: Significantly better OS [HR (95%CI) = 0.5 (0.3-0.85), *P* = 0.11] and PFS [HR (95%CI) = 0.55 (0.33-0.91), *P* = 0.20] in PPI users

[1]Definitions of EOU and EOG are stated in the results section. CapeOX: Capecitabine plus oxaliplatin; CRC: Colorectal cancer; CI: Confidence interval; DFS: Disease free survival; EOU: Eligible omeprazole users; EOG: Effective omeprazole group; FOLFOX: 5-Fluorouracil, leucovorin, and oxaliplatin; FOLFIRI: 5-Fluorouracil, leucovorin, and irinotecan; HR: Hazard ratio; LCRT: Long-course chemoradiotherapy; mXELIRI: Capecitabine plus irinotecan; OME: Omeprazole; OS: Overall survival; PPI: Proton pump inhibitors; PFS: Progression free survival; RFS: Recurrence free survival; R: Retrospective cohort study; RCT: Randomized controlled trials.

al[12] and Ma *et al*[13] primarily focused on the epidemiological aspect, assessing the risk of CRC with PPI exposure. In this systematic review, we describe evidence from basic research studies on the potential pro-tumor (proliferative) and anti-tumor (therapeutic) effects of PPI, assess if these findings are translatable into human studies, and discuss future clinical and research aspects related to the use of PPI in patients with CRC.

Although primarily responsible for gastric acid secretion, gastrin and its precursors are also potent growth factors for normal and malignant GI tissues[44]. Gastrin exerts its trophic effect through interaction with CCKBR, resulting in activation of growth-promoting downstream pathways[24,44]. As noted, long-term PPI use causes hypergastrinemia, raising concerns regarding the effects of PPI-induced hypergastrinemia on GI cancers. Recent reviews on the association of PPI use and various GI cancers such as pancreatic, hepatocellular, esophageal, and gastric cancer have yielded conflicting evidence[45-47]. Previous reviews addressing PPI and CRC suggested that there may not be any causative association between them[12-13]. However, Ma *et al* [13] suggested that long-term PPI use (> 5 years) may increase CRC risk.

Of the six basic research studies on PPI-induced hypergastrinemia, four demonstrated that PPI did not influence CRC growth and progression, whereas two suggested that PPI may even have a protective effect against CRC[16,17,19-21]. The

two publications reporting a suggested protective effect, by Penman *et al* and Tobi *et al* [21], demonstrated a lower CRC tumor burden in PPI-treated animal models and a dose-dependent decrease in CRC cell line (NCL-H716) proliferation, respectively. These findings may be suggestive of an anti-tumor effect of this drug class or may be explained by a possible interaction with the carcinogen (azoxymethane) used for tumor induction (as Penman *et al*[20] hypothesized).

The included human epidemiological studies do not present compelling evidence of a causative relationship between PPI use and CRC. Seven of nine studies demonstrated no significant risk of CRC development in patients previously or currently using PPI[28-35]. However, of these, Lee *et al*[31] reported an increased incidence with long-term use (≥ 10 years), whereas Hwang *et al*[34] found increased cases in a specific cohort of patients using PPI and at low risk of developing CRC. Of the remaining two studies, Lei and colleagues[36], found that the risk of CRC was significantly increased in PPI users while Kuiper *et al*[33] found significantly increased risk only in current PPI users, especially those using NSAIDs concomitantly. These results from human studies may be corroborative of the basic research findings that PPI do not have a growth-promoting effect on CRC. However, two included retrospective analyses examining survival among CRC patients, found that mortality risk was significantly higher in those using PPI compared to non-users[37,38]. In their cohort, Graham *et al*[37] found any comorbidities, advanced tumor stage, and poor tumor differentiation to be significant predictors of mortality. Such data may depict a potential pro-tumor influence of PPI on the CRC microenvironment, in contrast to findings from basic research mentioned above. Another explanation for poorer survival seen in PPI-using patients with CRC could be drug-drug interactions between PPI and commonly used chemotherapeutics, such as capecitabine. However, neither study describes information on CRC treatment of their cohorts.

Capecitabine is rapidly and predominantly absorbed from the upper GI tract[48]. It is thought that the dissolution and absorption of capecitabine may be reduced with increasing gastric pH (an effect produced by PPI)[49]. Sun *et al* and Wong *et al* studied the drug interaction between PPI and capecitabine in patients diagnosed with CRC. After adjustment for confounders, these authors found conflicting evidence: Sun *et al* [40] reported similar RFS, but Wong *et al*[41] found significantly lower 3-year RFS in the cohort concomitantly treated with CapeOx and PPI. These studies did not account for several potential confounders, such as concomitant drug use (statins, aspirin, anti-diabetics), serious comorbidities, and treatment modifications, making it difficult to draw firm conclusions. Furthermore, a recent study by Sekido *et al*[50] concluded that rabeprazole does not influence the plasma concentration of capecitabine and its metabolites, and subsequently their inhibitory effect on CRC cell proliferation.

Several basic research studies have also focused on identifying potential anti-tumor mechanisms of PPIs in CRC. Three basic research studies revealed that PPI may exert anti-tumorigenic effects through several mechanisms: Reducing pro-inflammatory signaling molecules (TNF-α, COX-2, and IL-6), oxidative stressors (NO and TBA-RS), and proteolytic enzymes (MMP-9, MMP-11, and MT1-MMP); exerting anti-mitogenic effects (inhibition of MAPKs) and anti-angiogenic effects (hypoxia-inducible factor 1-alpha, vascular endothelial growth factor, platelet-derived growth factor, IL-8); and inducing apoptosis *via* upregulating pro-apoptotic molecules (p21waf1/cip1) and downregulating anti-apoptotic molecules (cyclin A, Bcl-2, Bcl-xl and survivin)[23,24]. Additionally, these studies also found that PPI could exert anti-tumor properties even when co-administered with gastrin. It seems that instead of enhancing the trophic effects of gastrin, PPI may paradoxically inhibit these effects by interfering with the interaction between gastrin and CCKBR[24].

These results may explain the earlier findings of Penman *et al*[20] and Tobi *et al*[21], who also used omeprazole in their work and found a protective effect of PPI on CRC. Zeng *et al*[26] and Zheng *et al*[27] identified another potential action of specific PPI agents (pantoprazole, ilaprazole), as inhibitors of TOPK, a kinase highly expressed in rapidly proliferating tissues of embryological and cancerous origin[51]. The overexpression of TOPK in cancers has been associated with aggressive tumor behavior and poor clinical outcomes. Therefore, this kinase has been speculated to be a viable target for inhibiting downstream growth-promoting pathways[51]. Considering that no specific TOPK-inhibiting drugs have been approved for clinical use and the reported findings, further examination of these properties of pantoprazole and ilaprazole may be worthwhile.

It has also been suggested that PPIs have chemosensitizing ability. This was highlighted by Wang *et al*[25] demonstrating that pantoprazole enhanced the cytostatic effect of FOLFOX in CRC. Chemoresistance has been associated with an acidic tumor microenvironment, which results from the increased production of lactic acid

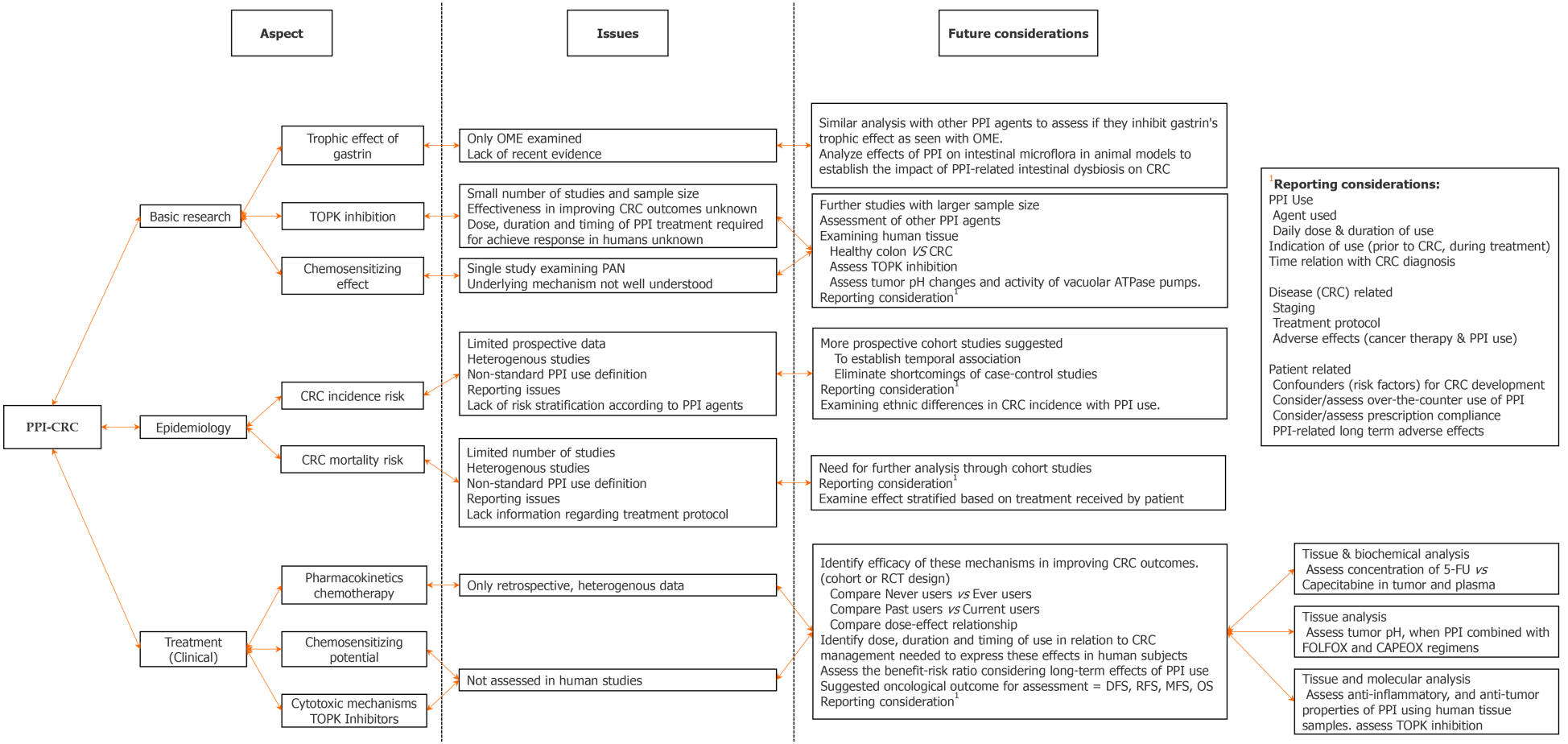


Figure 1 Figure outlining areas for future research to establish a better understanding of the relationship between proton pump inhibitors and colorectal cancer. CapeOx: Capecitabine plus oxaliplatin; CRC: Colorectal cancer; DFS: Disease free survival; FOLFOX: Fluorouracil, leucovorin, oxaliplatin; 5-FU: 5-flourouracil; MFS: Metastasis free survival; OME: Omeprazole; OS: Overall survival; PAN: Pantoprazole; PPI: Proton pump inhibitors; RCT: Randomized controlled trials; RFS: Recurrence free survival; TOPK: T lymphokine–activated killer cell-originated protein kinase.

(Warburg effect) and/or overexpression of vacuolar-ATPase pumps[52,53]. It is thought that this microenvironment neutralizes the effects of chemotherapeutic agents while decreasing their uptake into cancer cells. PPI appear to inhibit the activity of vacuolar-ATPase pumps, thereby increasing the pH of cancer cells and sensitizing them to chemotherapeutics[54]. This mechanism may suggest a potential role for PPI as adjuvants during chemotherapy, not only for the symptomatic treatment of side

effects but also to improve oncological outcomes. Zhang *et al*[39] further provided evidence to support this rationale by demonstrating lower recurrence rates and better chemoradiotherapy efficacy in patients using omeprazole concomitantly during chemotherapy compared with those did not.

Various PPI agents have been developed on the basis of the prototype PPI, omeprazole. They all share structural similarities and are generally effective and safe in the treatment of acid-related disorders[6]. However, differences in pharmacokinetics and pharmacodynamics exist among them, with the newer agents offering several advantages[55]. Although these differences are primarily related to the onset of action and degree of acid suppression, they also include reduced potential for drug interaction and other potential mechanisms of action that could make them effective in the treatment of diseases other than acid-related disorders.

Finally, it is important to mention that long-term PPI use also results in intestinal dysbiosis, with reduced abundance and diversity of gut microbiota and an increase in pathogenic bacteria[56]. Pathogenic bacteria implicated in the carcinogenesis of CRC, such as *Fusobacterium nucleatum*, *Escherichia coli*, *Enterococcus faecalis etc.* are more prevalent in PPI users[57]. These bacteria form a special microenvironment in the colorectal tissue that is conducive to neoplastic transformation and progression. They produce toxins that can damage the intestinal cell barrier, dysregulate immune cell function, induce a chronic inflammatory state, and cause DNA damage and genomic instability, all of which increase cell proliferation and contribute to the development of CRC[57,58]. One group has found that the abundance of *Fusobacterium nucleatum* may be associated with chemoresistance in CRC, resulting in poor response to 5-FU and oxaliplatin and higher recurrence rates[59]. This review did not identify any studies assessing the effect on CRC of intestinal dysbiosis resulting from PPI use. Studies focusing on the interaction of PPI-induced dysbiosis in CRC are needed to resolve the inconsistencies between the basic research and human studies.

A major limitation of this review is the heterogeneity among the included studies. The basic research studies describe experiments with different animal models and cell lines, using different PPI doses and exposure periods, whereas the human (epidemiological and treatment) studies varied in accounting for potential confounding factors and inclusion criteria for PPI exposure. Additionally, most of the human studies used prescription databases to ascertain PPI use, which may fail to accurately determine PPI use because they do not account for prescription non-adherence and possible over-the-counter use. Moreover, stratification of epidemiological and treatment-related evidence based on the individual PPI agents was lacking in all but one included study. These limitations make it difficult to present conclusive evidence on the question of whether PPI are an adversary or an ally in relation to CRC. Nonetheless, this review is the first to systematize the entirety of current evidence on the topic, and summarize data from different levels and aspects of the relationship.

In light of the evidence, we suggest that PPI use should continue when appropriately indicated, while a cautious approach should be implemented when combining them with capecitabine-based chemotherapy. Patients must be educated regarding the potential adverse effects of long-term PPI use and advised to avoid over-the-counter use for improper indications, with physicians being more diligent not to overprescribe. There are several aspects of this relationship which require further, high-quality investigation as outlined in Figure 1.

CONCLUSION

In conclusion, this review highlights an unexpected potential beneficial role of specific PPI agents in relation to CRC. First, PPI instead of promoting CRC growth *via* trophic effects of hypergastrinemia, may paradoxically inhibit them. Second, current evidence suggests that individual PPI agents may affect CRC differently: Pantoprazole and ilaprazole as TOPK inhibitors; rabeprazole with lower drug interaction capability with capecitabine; and pantoprazole and rabeprazole with little impact on CRC incidence (as evidenced by Lei *et al*[36])[26,27,50]. These findings warrant further studies to better understand these mechanisms and possibly facilitate use of PPI differently in clinical practice.

ARTICLE HIGHLIGHTS

Research background

Proton pump inhibitors (PPI) are one of the most widely used medications globally. Several reports have raised concerns that they may be inappropriately or even overused. Several adverse effects of PPI have been reported such as increased risk of gastrointestinal cancers including colorectal cancer (CRC).

Research motivation

There is no systematic review covering the entire body of evidence on the influence of PPI on CRC carcinogenesis. Previous reviews have primarily focused on the epidemiological aspect, in terms of CRC incidence, of their relationship. A comprehensive review analyzing the association between PPI use and CRC may yield findings, which may have practical implications. Therefore, this systematic review aimed to summarize evidence from basic research studies on potential mechanisms of PPI, as well as from human epidemiological and clinical studies assessing the influence of PPI use on survival and treatment outcomes of CRC patients.

Research objectives

To summarize evidence from basic research, epidemiological and clinical studies focusing on the relationship between PPI and CRC.

Research methods

This systematic review performed according to the PRISMA guidelines was based on patients, interventions, comparisons, and outcomes. Using a predetermined search strategy, MEDLINE, EMBASE, Scopus, and Web of Science electronic databases were searched from inception until May 17, 2021. The initial search returned 2591 articles. Twenty-eight studies were included in this review and categorized as basic research studies (n = 12), epidemiological studies (n = 11), and CRC treatment studies (n = 5). The Newcastle-Ottawa Scale or Cochrane Risk of Bias 2.0 tool were utilized to assess the quality of the included studies depending on the study design.

Research results

Basic research studies show that PPI may paradoxically inhibit the trophic effect of gastrin rather than stimulating CRC development through it. Additionally, PPI may possess several anti-tumor properties (omeprazole, pantoprazole) while also being potential T lymphokine-activated killer cell-originated protein kinase inhibitors (pantoprazole, ilaprazole) and chemosensitizing agents (pantoprazole). Based on data from epidemiological studies, it appears that no causal association between PPI use and increased CRC risk exists. Treatment studies suggest that concomitant use of PPI with capecitabine use may reduce the efficacy of chemotherapy and result in poorer oncological outcomes. These studies also suggest that pantoprazole may have a chemosensitizing effect with the FOLFOX regimen.

Research conclusions

This systematic review identifies an unexpected inhibitory effect on CRC carcinogenesis by way of several potential mechanisms. Moreover, it appears that different PPI agents may have differential effects on CRC treatment, which may have practical implications. Further prospective studies are warranted to delineate this relationship as well as assess the role of individual PPI agents.

Research perspectives

PPI do not appear to have a growth promoting effect on CRC, however, a cautious approach should be adopted while concomitantly administering PPI and capecitabine-based chemotherapy. Recent evidence suggests that individual PPI agents have a differential effect on CRC carcinogenesis, with newer agents such as pantoprazole, ilaprazole and rabeprazole possessing beneficial characteristics, which may have a role in the treatment of CRC.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 November 28; 27(44): 7734-7738

DOI: 10.3748/wjg.v27.i44.7734

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

LETTER TO THE EDITOR

SARS-CoV-2 infection in liver transplant recipients: A complex relationship

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Author contributions: All authors contributed to the conception, writing, and review of the article and approved the submitted version.

Conflict-of-interest statement: The authors declare having no conflicts of interest.

Supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), No. 19/02679-7 and No. 20/13148-0.

Country/Territory of origin: Brazil

Specialty type: Gastroenterology and hepatology

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

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

Open-Access: This article is an open-access article that was

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Abstract

The recent manuscript reviewed investigations involving liver damage in coronavirus disease 2019 (COVID-19) patients, and COVID-19 in patients with previous chronic hepatological diseases, such as patients with liver graft. The literature presents several conflicting results concerning the anti-SARS-CoV-2 response in patients with solid organ transplants, in liver transplant recipients. Therefore, we would like to humbly state a few points for consideration involving liver transplant recipients and COVID-19, such as the time since transplantation, comorbidities, and immunosuppressive regimens.

Key Words: COVID-19; SARS-CoV-2; Liver transplant; Immunosuppression; Infection; Comorbidities

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Core Tip: There is not a consensus whether solid organ transplant recipients present increased severity or death rates due to coronavirus disease 2019 (COVID-19) compared with the general population. In particular, liver allograft has a low risk of rejection, therefore enabling treatment with relatively less immunosuppressive regimens. The reduction in the production of proinflammatory cytokines, without a drastic suppression of the immune response, may benefit liver transplant recipients during COVID-19. Further investigations should compare different organ transplant

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Received: July 20, 2021
Peer-review started: July 20, 2021
First decision: August 6, 2021
Revised: August 14, 2021
Accepted: November 17, 2021
Article in press: November 17, 2021
Published online: November 28, 2021

P-Reviewer: Han J
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR



recipients, elapsed time from the organ transplant, different immunosuppressive treatments, and their anti-SARS-CoV-2 response.

Citation: Alberca RW, Benard G, Alberca GGF, Sato MN. SARS-CoV-2 infection in liver transplant recipients: A complex relationship. *World J Gastroenterol* 2021; 27(44): 7734-7738
URL: https://www.wjgnet.com/1007-9327/full/v27/i44/7734.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i44.7734

TO THE EDITOR

We read with great interest the article entitled "Liver dysfunction and SARS-CoV-2 infection", recently published by Gracia-Ramos *et al*[1] in the World Journal Gastroenterology[1]. Gracia-Ramos *et al*[1] performed a review on patients with liver dysfunction and coronavirus disease 2019 (COVID-19), highlighting investigations involving liver injury in COVID-19 patients, and COVID-19 in patients with previous chronic liver disease, such as cirrhosis and liver transplant recipients. Nevertheless, we would like to raise a few considerations regarding liver transplant recipients and COVID-19.

Transplantation is a treatment for organ failure and end-stage organ illnesses, requiring patients to undergo regular use of immunosuppressive treatment to avoid organ rejection[2]. There is no consensus regarding the increase in the incidence or severity of COVID-19 on solid organ transplant (SOT) recipients[3]. A few reports have identified an increase in severe COVID-19 and mortality rate in SOT recipients[4,5], while others failed to do so[3,6].

SOT patients may respond differently to COVID-19 due to associated comorbidities, the organ grafted, elapsed time from the transplant surgery, drugs used to prevent organ rejection, or drugs used to treat SARS-CoV-2 infection[7-9]. The identification of the organ grafted is usually described in the manuscripts[6], but only one manuscript with comparison between the different organ transplant recipients has been made so far, identifying an increase in mortality in kidney and heart transplant recipients in comparison to liver transplant recipients[5].

A systematic review identified a similar death rate in liver transplant recipients and non-transplant recipients, but a higher percentage of non-transplant recipients were obese or had cardiovascular or respiratory diseases[10]. Hospitalization in the intensive care unit presented mixed results, and only one investigation reported the need for mechanical ventilation, and liver transplant recipients presented a greater need for it, in comparison to non-SOT patients[10]. Consequently, it is not possible to confirm if liver transplant recipients have a lower risk for severe illness or death risk in comparison to non-transplant recipients during COVID-19.

The liver allograft has a lower risk of rejection in comparison to heart and kidney allograft. Therefore, it is usually treated with reduced immunosuppressive regimens compared with the other SOT patients[11,12]. Different immunosuppressive regimens can affect the detection of SARS-CoV-2 RNA in nasopharyngeal swab tests[13]. Tacrolimus, a commonly used drug by SOT patients, has been shown to display anti-coronavirus effects in vitro[14] and putative protective properties in liver transplant recipients with COVID-19[15]. In contrast, another report identified that tacrolimus has no significant effect on the mortality risk[16].

We hypothesize that the inhibition of the calcineurin by tacrolimus could curb the production of proinflammatory cytokines, similarly to the observed in other inflammatory models[17-19], and reduce or prevent the development of the cytokine storm in COVID-19[20]. A recent report associated tacrolimus therapy with a protective effect in liver transplant recipients with COVID-19[15]. Importantly, the use of tacrolimus in association with dexamethasone in immunocompetent COVID-19 patients is currently being tested in a Spanish clinical trial (clinicaltrials.gov/ct2/show/NCT04341038).

Importantly, SOT recipients (including liver, kidney, heart, lung, and others) have an increased risk of mortality due to infections in the first year after the transplant[21], especially respiratory infections[22]. A recent review identified a higher mortality rate in patients with over a year of organ draft[23]. Another report identified no difference in elapsed time from transplant in survival and non-survival COVID-19 patients, but

patients presented a high incidence of other comorbidities[24]. In a recent report, liver transplant was not associated with greater mortality risk, while the association with other comorbidities (mainly diabetes, hypertension, obesity, and cardiovascular disease) posed a higher death risk[25]. It is reasonable to assume that comorbidities associated with poor outcomes in non-SOT patients will also influence the COVID-19 disease course in liver recipients, such as co-infection[26], respiratory disorders[27], and alcohol consumption[28]. In another report, liver transplant recipients presented similar mortality to non-SOT patients and reduced mortality in comparison with patients with liver cirrhosis[29].

On the other hand, liver transplant recipients developed lower levels of anti-SARS-CoV-2 IgG, and a more pronounced reduction in SARS-CoV-2-specific IgG levels, 6 mo after COVID-19[30]. Therefore, a long-term follow-up is necessary to fully determine the duration of the anti-SARS-CoV-2 immune response and the long-term protection offered by COVID-19 vaccines in liver transplant recipients. A recent report has identified SARS-CoV-2 infections in fully vaccinated SOT patients, and, importantly, the death of a fully vaccinated heart transplant patient due to COVID-19[30].

In conclusion, Gracia-Ramos *et al*[1] presented interesting points concerning liver transplant recipients with COVID-19. However, further investigations are needed to better understand the impact of comorbidities, elapsed time since the organ transplant, immunosuppressive regimen, and vaccination on COVID-19 in liver transplant patients.

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

World Journal of *Gastroenterology*

World J Gastroenterol 2021 December 7; 27(45): 7739-7865



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World Journal of Gastroenterology

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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: *Ying-Yi Yuan*; **Production Department Director:** *Xiang Li*; **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, Subrata Ghosh

EDITORIAL BOARD MEMBERS

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

PUBLICATION DATE

December 7, 2021

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World Journal of Gastroenterology

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World J Gastroenterol 2021 December 7; 27(45): 7739-7747

DOI: 10.3748/wjg.v27.i45.7739

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

EDITORIAL

Orosomucoid in liver diseases

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Author contributions: Elpek GO performed the design of the article, obtained, analyzed and interpreted the data, and wrote the article.

Conflict-of-interest statement: There is not any conflict of interest.

Country/Territory of origin: Turkey

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
Grade D (Fair): 0
Grade E (Poor): 0

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Abstract

In this editorial, the roles of orosomucoid (ORM) in the diagnoses and follow-up assessments of both nonneoplastic diseases and liver tumors are discussed with respect to the publication by Zhu *et al* presented in the previous issue of *World Journal of Gastroenterology* (2020; 26(8): 840-817). ORM, or alpha-1 acid glycoprotein (AGP), is an acute-phase protein that constitutes 1% to 3% of plasma proteins in humans and is mainly synthesized in the liver. ORM exists in serum as two variants: ORM1 and ORM2. Although the variants share 89.6% sequence identity and have similar biological properties, ORM1 constitutes the main component of serum ORM. An interesting feature of ORM is that its biological effects differ according to variations in glycosylation patterns. This variable feature makes ORM an attractive target for diagnosing and monitoring many diseases, including those of the liver. Recent findings suggest that a sharp decrease in ORM level is an important marker for HBV-associated acute liver failure (ALF), and ORM1 plays an important role in liver regeneration. In viral hepatitis, increases in both ORM and its fucosylated forms and the correlation of these increases with fibrosis progression suggest that this glycoprotein can be used with other markers as a noninvasive method in the follow-up assessment of diseases. In addition, similar findings regarding the level of the asialylated form of ORM, called asialo-AGP (AsAGP), have been reported in a follow-up assessment of fibrosis in chronic liver disease. An increase in ORM in serum has also been shown to improve hepatocellular carcinoma (HCC) diagnosis performance when combined with other markers. In addition, determination of the ORM level has been useful in the diagnosis of HCC with AFP concentrations less than 500 ng/mL. For monitoring patients with AFP-negative HCC, a unique trifucosylated tetra-antennary glycan of ORM may also be used as a new potential marker. The fact that there are very few studies investigating the expression of this glycoprotein and its variants in liver tissues constitutes a potential limitation, especially in terms of revealing all the effects of ORM on carcinogenesis and tumor behavior. Current findings indicate that ORM2 expression is decreased in tumors, and this is related to the aggressive course of the disease. Parallel to this finding, in HCC cell lines, ORM2 decreases HCC cell migration and invasion, supporting reports of its tumor suppressor role. In conclusion, the levels of ORM and its different glycosylated

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Received: March 15, 2021
Peer-review started: March 15, 2021
First decision: June 3, 2021
Revised: June 14, 2021
Accepted: November 25, 2021
Article in press: November 25, 2021
Published online: December 7, 2021

P-Reviewer: Shahini E, Xiol X
S-Editor: Gao CC
L-Editor: A
P-Editor: Gao CC

variants are promising additional biomarkers for identifying ALF, for monitoring fibrosis in viral hepatitis, and for diagnosing early HCC. Although there is evidence that the loss of ORM2 expression in HCC is associated with poor prognosis, further studies are needed to support these findings. Additionally, investigations of ORM expression in borderline dysplastic nodules and hepatocellular adenomas, which pose diagnostic problems in the differential diagnosis of HCC, especially in biopsy samples, may shed light on whether ORM can be used in histopathological differential diagnosis.

Key Words: Orosomucoid; Alpha-1-acid glycoprotein; Viral hepatitis; cirrhosis; Hepatocellular carcinoma; Downregulation

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Core Tip: Orosomucoid (ORM) has been suggested as a noninvasive marker in the diagnosis and follow-up of liver diseases. Currently, the results support the hypothesis that ORM can be used together with other markers to diagnose acute liver failure, monitor the development of cirrhosis, and detect early hepatocellular carcinoma (HCC). Although its role in carcinogenesis has not been entirely determined, the fact that decreased ORM2 expression is associated with carcinogenesis and poor prognosis warrants further study with the aim of better understanding the role of ORM in tumor behavior. The use of ORM expression to distinguish HCC from other neoplastic lesions and its role in differential diagnosis await investigation.

Citation: Elpek GO. Orosomucoid in liver diseases. *World J Gastroenterol* 2021; 27(45): 7739-7747
URL: https://www.wjgnet.com/1007-9327/full/v27/i45/7739.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i45.7739

INTRODUCTION

Orosomucoid (ORM), also known as alpha-1 acid glycoprotein (AGP), is an unusual protein with a carbohydrate content of 45% and a very low pI (2.8-3.8) that was identified more than a century ago[1]. Its variation in molecular weight between 37 and 54 kDa is closely related to the differences in its glycosylation content. Although the level of ORM varies according to species, it constitutes 1% to 3% of plasma proteins in humans[2]. This level is slightly higher for men than for women[3]. It is mainly synthesized in the liver; hence, it is also considered a hepatokine. However, smaller amounts can also be produced by breast epithelial cells, type II alveolar epithelial cells, endothelial cells, granulocytes, monocytes, and macrophages[2].

As the name implies, ORM is mainly composed of polypeptide chains and carbohydrate segments. The structures of genes encoding the polypeptide chain of ORM can vary within a broad spectrum. It is located in a cluster of 3 neighboring genes located on the long arm of the 9th chromosome in humans[4]. These neighboring genes are known as AGP-A, AGP-B, and AGP-B'. Among these, AGP-A, which has 3 different alleles (ORM1 * S, ORM1 * F, and ORM1 * F2), encodes ORM-1, and the distribution in the frequency of these alleles in humans may differ according to geographical region[3,5]. AGP-B and AGP-B' encode ORM2. Therefore, ORM exists in human serum as two variants: ORM1 and ORM2[4]. Notably, most individuals carry a mixture of these variants. Although these variants are reported to share 89.6% sequence identity and similar biological properties, ORM1 is the main component of serum ORM, as it is present in plasma at a fivefold higher concentration than ORM2[6,7]. ORM contains 5 N-linked glycans linked to the polypeptide structure, each of which can be a bi-, tri-, or tetra-antennary glycan and can exhibit varying degrees of fucosylation and sialylation and branching[8]. These different glycosylation sites change the protein's biological properties.

ORM is one of the major acute-phase proteins in humans, and its levels increase 2-fold to 6-fold in plasma in most disease states, including inflammation and cancer. Although the biological role of ORM remains unclear, it can regulate immunity and

play roles in both pro- and anti-inflammatory responses, can bind to endogenous ligands such as steroids, and has the ability to bind and transport large numbers of basic and neutral lipophilic drugs[2,8]. Recent studies have shown evidence that it can affect muscle tissue through glycogen metabolism, act as an adipokine in adipose tissue, and be involved in bile metabolism[9-11]. The general properties of ORM are summarized in Table 1.

Because ORM levels vary in many diseases and since their biological effects depend on the glycosylation pattern, this glycoprotein is an attractive target for use in diagnosis and treatment[3]. Accordingly, many current studies are exploring different organs, including the liver, with the aim of using ORM as a potential biomarker for the diagnosis and monitoring of diseases and of revealing its roles in carcinogenesis and tumor behavior.

In this article, the role of ORM in the diagnosis and follow-up of liver diseases is presented.

ORM IN ACUTE LIVER DAMAGE AND REGENERATION

Loss of liver function caused by viral hepatitis, cirrhosis, alcohol, and drug-induced liver damage is a life-threatening condition. Therefore, many studies have been conducted to better understand liver regeneration mechanisms, mainly to shed light on possible clinical applications, such as in the treatment of acute liver failure (ALF), which is a lethal disease characterized by sudden hepatic metabolic and immunological function loss. Liver regeneration is a complicated but coordinated multistep process that is mediated by the integration of multiple factors. Among these factors, proliferation plays a crucial role in the initiation of regeneration, as suggested by a large amount of data on the activation of the cell cycle and the proliferation of quiescent hepatocytes[12,13]. Therefore, eliminating arrest-promoting mechanisms affects the growth of differentiated hepatocytes; in other words, proliferation plays a key role in overcoming liver failure. Although some mitogens, such as growth factors, have been shown to affect proliferation, in recent years, paracrine mediators and nonmitogenic cytokines, including ORM1, have also been shown to participate in the control of this process in a coordinated manner[14]. Indeed, ORM1 expression increases during regeneration following liver resection in both humans and mice[15].

Moreover, the knockdown of ORM1 downregulates the signaling pathways controlling chromatin replication, supporting the notion that ORM1 plays a role in the cell proliferation involved in liver regeneration[16]. This finding is also in line with previous findings related to STAT-3, which is one of the transcription factors involved in liver injury and is associated with proliferation, that show that ORM synthesis is induced in liver injury due to the use of drugs that cause oxidative stress[17,18]. In proteomic analyses, it has been suggested that the sharp decrease in serum ORM levels in HBV-induced ALF (HBV-ALF) patients, as indicated by comparisons between liver tissue samples obtained from HBV-ALF and healthy individuals, may be a valuable biomarker in the diagnosis of ALF in patients with chronic liver disease[19]. Parallel to these findings, the results from a recent study on HBV-ALF showed the downregulation of four genes involved in the immune response and the complement and coagulation cascades, including ORM1 and ORM2; this suggests that both can be potential treatment targets for ALF[20]. However, the tissue expression level of ORM in the liver of patients with HBV-ALF was found to be 4.595-fold higher than that in the liver of healthy patients[21]. This finding is partially explained by the hypothesis that blood ORM accumulates in the liver in response to ALF.

Although further studies that involve a large number of patients and analyze both the blood and tissue levels in the same patients are needed to clarify the role of ORM in the diagnosis and treatment of ALF, current evidence indicates that ORM can be a useful marker in the diagnosis of this lethal disease.

ORM AND NONNEOPLASTIC LIVER DISEASES

As an acute-phase reactant, the ORM level in plasma, which is normally between 4% and 6%, can vary in many inflammatory diseases, including that of the liver. Recently, Oguz *et al*[22] showed that patients with HCV hepatitis have higher ORM levels than healthy individuals. Moreover, evidence has shown that the ORM level fluctuates with fibrosis progression and increases with the development of cirrhosis. Regarding the treatment of HCV hepatitis, no significant difference was found in the responding

Table 1 A brief overview of the general properties of orosomucoid protein

General properties of orosomucoid protein		
Chromosome location	9	
Genes	AGP-A	AGP-B and AGP-B′
Product	ORM1	ORM2
Alleles	ORM1*S, ORM*F, ORM*F2	Monomorphic, except in Japan
Structure		
Polypeptide chain	Single, 183 amino acids with disulfide bonds; There are 22 amino acid differences between ORM 1 and ORM2	
Carbohydrate parts	Five N-linked potential glycosylation sites: Sialic acid, neutral hexoses, mannose, fructose, galactose and hexosamine. Alterations in fucosylation, sialylation, and branching affect its biological properties	
Synthesis	Predominantly by hepatocytes and parenchymal cells. Extrahepatic secretion is rare (breast, endothelial cells, and tumor cells)	
Secretion		
Inflammatory mediators	Glucocorticoids, TNF-α, Interleukins: 1, 6, 8, 11	
Exogenous factors	Phenobarbital, Rifampicin, Retinoic acid, Macrolides	
Biological activities		
Acute-phase reactant	Concentration is elevated 1-10 times during several pathological conditions. Infection, inflammation, tumor, surgery, tissue injury, sepsis, and necrosis	
Immunomodulation	Inhibit leukocyte rolling/adhesion and migration and lymphocyte proliferation. Vitamin D-mediated macrophage deactivation. Agalacto/asialo derivative suppresses the immune response. ORM1 contributes to both anti- and proinflammatory signals to mediate mechanisms activated by the acute-phase response	
Transporting protein	Drug-binding and transporting in the serum. The existence of two forms in the blood also has an influence the binding affinity. ORM1 binds warfarin, prazosin, imatinib, quinidine, and dipyridamole. ORM2 binds methadone, disopyramide, propafenone, and amitriptyline	
Endothelial functions	Maintain the barrier function of capillaries. Regulate injury-induced angiogenesis. Enhance blood-brain barrier functional integrity. Beneficial effect on the glomerular barrier	
Metabolism	ORM1 increases glucose uptake activity in adipocytes. A potential biomarker in distinguishing obese women with metabolic syndrome from those without metabolic disturbances	

ORM: Orosomucoid; TNF-α: Tumor necrosis factor alpha.

group and the nonresponding group, suggesting that ORM levels can only be used as adjuvants to monitor early treatment response. Although not significant, the remarkable increase observed in the early phase of treatment has been attributed to the association of ORM with IFN. In contrast to these findings, another study found that the ORM level in HCV hepatitis was lower than that in healthy subjects and was significantly increased with fibrosis progression[23]. It has been suggested that this decrease may be due to HCV proteins suppressing C3 synthesis. Additionally, variations in the level of ORM were associated with neither necroinflammatory activity nor viral genotype.

High levels of ORM have also been reported in HBV hepatitis and associated cirrhosis[19-21]. In contrast, ORM levels were observed to be within normal limits in NASH and chronic alcoholic liver disease[24,25].

Since the glycosylation pattern of ORM can be modified throughout diseases, these alterations were analyzed for any correlation with the severity of liver diseases to determine the use of ORM as a surrogate marker of fibrosis. In recent years, the fucosylated form of ORM has been observed at a higher level in patients with both HBV and HCV hepatitis than in healthy individuals[26-29]. Moreover, it was emphasized that the fucosylated ORM might be useful in monitoring fibrosis because of it increased with the progression of fibrosis toward cirrhosis[27]. Another interesting finding indicates that during this progression, the increase in ORM fucosylation is associated with a concordant decrease in sialylation. These results support the idea that its glycosylation is modified by the severity of fibrosis and might be useful in disease monitoring[30].

In light of the accumulated data on the modification of the sialylation content of ORM, a few recent studies have been performed to evaluate the diagnostic performance of asialo-AGP (AsAGP) in the detection of cirrhosis in patients with chronic

liver disease. Increasing serum levels of AsAGP were correlated with the degree of fibrosis, and this level was highest in cirrhosis[31,32]. There have also been findings indicating that an increased level of AsAGP shows an inverse correlation with albumin but a positive correlation with the stage of fibrosis, and it may be a positive predictor of cirrhosis[31]. However, in these studies, similar results were not found in comparisons with liver stiffness, a noninvasive method for detecting fibrosis. Thus, further studies are warranted.

ORM IN LIVER TUMORS

Hepatocellular carcinoma (HCC) continues to be one of the major causes of cancer deaths worldwide due to its lack of specific clinical findings at the early stages and the lack of efficient screening methods[33]. To prevent HCC from being diagnosed in advanced stages where treatment options are limited, many efforts have been made to identify new diagnostic and screening methods. For this purpose, several tumor markers have been proposed for use in HCC diagnosis. Because evidence has established that ORM is associated with carcinogenesis and behavior in many organs, changes in the ORM serum level in HCC have been investigated as diagnostic markers. The results of many studies have indicated that the ORM level is increased in patients with HCC and is significantly higher than that in patients with chronic hepatitis and cirrhosis[34-37]. ORM has also been shown to improve HCC diagnostic performance when combined with other markers, such as des-g-carboxy prothrombin (DCP)[38].

Furthermore, determination of the ORM level has been observed to be useful in the diagnosis of HCC in which the AFP values are less than 500 ng/mL[39]. In light of these data, it can be concluded that monitoring ORM levels together with AFP and/or other biomarker levels may be useful in the early detection of HCC. Additionally, it has recently been shown that the combination of urinary ORM-1 levels with urinary AFP levels has a high sensitivity (85%) in the diagnosis of HCC and that this noninvasive method can also be used[40].

Few studies have evaluated the role of ORM in the evolution and prognosis of cholangiocarcinoma (CCC). An experimental study showed that the ORM2 Level increases before tumor onset and tends to be upregulated during tumor progression [41]. The levels of ORM2 were also investigated in patients with CCA. The sensitivity and specificity of ORM2 in distinguishing CCC patients from healthy individuals were 92.86% and 73.68%, respectively[42].

It has been proposed that alterations in the glycosylation pattern of ORM can be used to detect the progression and metastasis of many types of cancer[43]. Indeed, the aberrant glycosylation of ORM in liver cancer progression has received considerable attention in biomarker studies. Previous studies revealed that although there was an increase in the ORM levels of patients with liver cirrhosis and HCC compared to healthy controls, different degrees of fucosylation may distinguish HCC cases from cirrhosis cases[44-47]. Performing an elegant study, Liang *et al*[48] demonstrated a unique trifucosylated tetra-antennary glycan of ORM predominantly identified in HCCs. However, this glycan was absent in both healthy subjects and the majority of cirrhosis patients, as determined by matrix-assisted laser desorption ionization-mass spectrometry, providing a new potential marker for monitoring AFP-negative HCC patients. Although the level of fucosylated ORM differs significantly in advanced stages, determining whether these markers can be used to determine HCC behavior requires further studies in large series.

Similar to nonneoplastic diseases of the liver, the efficacy of AsAGP in the diagnosis of HCC has been investigated in some studies. The AsAGP level is higher in cirrhosis and HCC[49,50]. Kim *et al*[50] revealed that these increases in both cirrhosis and HCC might be related to damaged asialoglycoprotein receptors on the hepatic cell surface, as demonstrated in human and animal studies. The release of extra neuraminidase into the circulation during cellular transformation and the production of incomplete asialoglycoproteins in hepatic cells are also hypothesized to explain the increase in AsAGP in these patients.

In liver diseases, the vast majority of studies addressing ORM were performed in body fluids. However, there are very few studies that investigated ORM expression at the tissue level that have yielded significant results. Although ORM1 and ORM2 are mostly observed in liver tumors compared to other organ cancers, their expression levels are significantly decreased compared with those in neighboring liver tissue, suggesting that ORM genes are downregulated in liver tumors[37,51,52]. The down-

regulation of ORM2 inversely correlates with intrahepatic metastasis and histological grade; in other words, ORM2 is negatively correlated with aggressive tumor behavior [51]. Parallel to these findings, studies using HCC cell lines showed that ORM2 decreased HCC cell migration and invasion, supporting its tumor suppressor role[52]. In addition, Zhu *et al*[52] observed that the prognosis of patients with low ORM2 expression was worse than that of patients with higher ORM2 expression. Therefore, it has been suggested that ORM2 may be a new prognostic factor for liver cancer patients. Moreover, in this study, the inverse association between downregulated ORM2 expression and pathways involved in hepatocarcinogenesis, such as the G2/M checkpoint, E2F target signaling, Wnt/β-catenin, and hedgehog signaling pathways, also supported the use of ORM2 as a marker of liver cancer[51]. The observation of the involvement of ORM2 in tumor-associated macrophage infiltration and the T-cell mediated checkpoint in liver tumor tissue also suggested that its downregulation may be another marker for efficiently predicting the need to apply immune checkpoint therapy. It should be noted that since none of the three current guidelines on the management of HCC include the use of ORM in diagnosis, further studies are needed before recommending its use as a diagnostic marker[53-55]. Moreover, these findings should also be supported by further studies in not only HCC but also CCC. In addition to the relationship of ORM with the immune checkpoint, its relationships with other pathways that may be potential therapeutic targets need to be investigated more comprehensively. The role of ORM expression in differential diagnosis, especially in biopsy samples, to distinguish other neoplastic lesions, such as borderline dysplastic nodules and adenomas, from HCC has not been reported. Furthermore, considering that metastatic tumors of the liver are more common than primary tumors, the role of ORM in these tumors is unknown.

CONCLUSION

In addition to being an acute-phase reactant, ORM is a potential biomarker that can be used with other liver markers for the diagnosis of ALF in the follow-up assessment of fibrosis in viral hepatitis. Similarly, it can be used together with other noninvasive methods to detect early stages of HCC. Recent studies suggest that ORM expression may be useful in determining the behavior of HCC and tumor progression.

However, in HCC, the relationship of ORM with other pathways targeted for treatment in addition to immune checkpoint pathways should be clarified.

Furthermore, the relationship between metastatic tumors and ORM expression, if any, and its role in the histopathological differential diagnosis of HCC require further investigation.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 7; 27(45): 7748-7770

DOI: 10.3748/wjg.v27.i45.7748

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

FRONTIER

Novel frontiers of agents for bowel cleansing for colonoscopy

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Author contributions: Di Leo M and Di Leo A planned the study; Di Leo M, Iannone A, Arena M, Losurdo G wrote the manuscript; Palamara MA, Iabichino G, Consolo P, Rendina M, Luigiano C, Di Leo A supervised the study; all authors read and approved the final version.

Conflict-of-interest statement: 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 report's scientific quality classification

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

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Abstract

The incidence of colorectal cancer (CRC) is characterized by rapid declines in the wake of widespread screening. Colonoscopy is the gold standard for CRC screening, but its accuracy is related to high quality of bowel preparation (BP). In this review, we aimed to summarized the current strategy to increase bowel cleansing before colonoscopy. Newly bowel cleansing agents were developed with the same efficacy of previous agent but requiring less amount of liquid to improve patients' acceptability. The role of the diet before colonoscopy was also changed, as well the contribution of educational intervention and the use of adjunctive drugs to improve patients' tolerance and/or quality of BP. The review also described BP in special situations, as lower gastrointestinal bleeding, elderly people, patients with chronic kidney disease, patients with inflammatory bowel disease, patients with congestive heart failure, inpatient, patient with previous bowel resection, pregnant/lactating patients. The review underlined the quality of BP should be described using a validate scale in colonoscopy report and it explored the available scales. Finally, the review explored the possible contribution of bowel cleansing in post-colonoscopy syndrome that can be related by a transient alteration of gut microbiota. Moreover, the study underlined several points needed to further investigations.

Key Words: Colonoscopy; Bowel preparation; Cleansing agents; Polyethylene glycol; Adequate cleansing; Constipation

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Received: March 17, 2021
Peer-review started: March 17, 2021
First decision: April 15, 2021
Revised: April 23, 2021
Accepted: November 24, 2021
Article in press: November 24, 2021
Published online: December 7, 2021

P-Reviewer: Gweon TG, Katada K, Magalhaes RDS
S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H



Core Tip: Colonoscopy is the best modality for colorectal cancer (CRC) screening, preventing death from CRC through removal of adenomatous polyps and early detection of CRC. The accuracy of colonoscopy is related to quality of bowel preparation (BP). International guidelines underlined the methods to improve BP. In this review, we aimed to summarize the current strategy to increase bowel cleansing before colonoscopy.

Citation: Di Leo M, Iannone A, Arena M, Losurdo G, Palamara MA, Iabichino G, Consolo P, Rendina M, Luigiano C, Di Leo A. Novel frontiers of agents for bowel cleansing for colonoscopy. *World J Gastroenterol* 2021; 27(45): 7748-7770
URL: https://www.wjgnet.com/1007-9327/full/v27/i45/7748.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i45.7748

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in both genders[1] with an incidence characterized by rapid declines in the wake of widespread screening. Colonoscopy is considered the gold standard for CRC screening. Colonoscopy with removal of adenomatous polyps prevents death from CRC[2].

To perform screening colonoscopy, the high quality of endoscopic procedure is mandatory. An adequate bowel preparation (BP) is one of the most important factors, ensuring a high accuracy of procedure, thought an optimal visualization of colonic mucosa increasing adenoma detection rate (ADR)[3-5]. ADR is defined as the percentage of screening colonoscopies in which one or more conventional adenomas are detected[6]. ADR is inversely associated with the risks of interval and lower long-term CRC incidence and mortality[7,8].

Moreover, an inadequate BP is associate to prolonged procedures, higher cost leaded to repeat colonoscopy (longer hospital stay and no cost/efficacy of screening program), lower cecal intubation rates, higher risk of electrocautery and unsatisfactory patient experience with an increased likelihood of repeat procedure.

Despite European Society of Gastrointestinal Endoscopy (ESGE) Guidelines recommended a minimum of 90% procedure with adequate BP, with a target of > 95% [9], suboptimal BP is still encountered in clinical practice[10].

Other review papers regarding bowel cleansing were published[11,12]. However, new evidences change same feature of bowel cleansing process.

The aim of the present review is to describe the current literature regarding BP options, in order to explore factors that can be improved.

BP QUALITY SCALE

ESGE guidelines recommend recording the BP quality using a validated scale[9]. Validity refers to how well the scale measures what it is aimed to assess. For BP, validity could be assessed by comparison of different scales or with another parameter of colonoscopy quality. Another essential attribute of a scale is the reliability that indicates the reproducibility of the results in the same operator (intrarater reliability) or between different endoscopist (interrater reliability).

Several scales were proposed in the last decades to describe the quality of BP of colonoscopy.

The first one was the Aronchick Scale[13] and it is still one of the most commonly used validated BP quality scales in clinical trials and clinical practice.

The quality of the preparation is described as the percentage of entire colonic mucosa covered by stool, before washing or suctioning, ranging from 1 (excellent) to 5 (inadequate). No study has evaluated a threshold to define adequate the BP described by Aronchick Scale.

Validity was not evaluated in clinical studies, while inter-observer reliability was assessed in one study (coefficient was 0.77 in the total colon)[14].

The second developed scale was the Ottawa Bowel Preparation Quality Scale (OBPQS)[14]. This scale is composed by two separate scores. One score is assigned

according to global fluid quantity in the entire colon, from 0 (small amount of fluid) to 2 (large amount of fluid). The second score quantifies the visibility of three separate colon segments (right colon, mid colon and rectosigmoid colon) and also the amount of washing or suctioning required achieving optimal visualization and it ranges from 0 to 4. The total score is obtained by adding the score of each segment and total colon fluid score, ranging from 0 (excellent) to 14 (poor), before washing or suctioning. In one study, the value of at least 8 was proven to be an optimal cut-off value to define inadequate BP because of the inability to detect a 5 mm polyp[15].

The validity was also demonstrated in two study comparing OBPQS with visual analogue scale[16] and with Boston Bowel Preparation Scale (BBPS)[17].

The study of Martinato *et al*[16] detected also a good agreement between nurses and physicians, [r = 0.6010 (95%CI for r 0.4877 to 0.6944)]. One prospective study demonstrated the high interobserver agreement and reliability of OBPQS compared to Aronchick Scale[14] with no statistically significant differences between segment evaluations. Intra-observer reliability and clinical relevance were not evaluated.

BBPS described the colonic mucosa that can be evaluated. The advantages of this scale are multiple. First of all, it is a numeric score ranging from 0 (unprepared colon mucosa) to 9 (entire mucosa well seen) for the entire colon, avoiding the use of qualitative and subjective terms. Second vantage is that a score is assigned for each colonic segment (right colon, transverse, left colon-each one from 0 to 3), allowing a detailed description of BP. Third, the score is assigned after washing and suctioning as recommended by United States Multi-Society Task Force on Colorectal Cancer[18]. Finally, the validity and the reliability of this score has been evaluated in several studies.

The validity of the score was proven in several studies, demonstrating the association with polyp detection rate, insertion and withdrawn times, needed to repeat colonoscopy for inadequate BP. Lai *et al*[19], including 633 screening colonoscopies (22 clinicians), found an association with BBPS ≥ 5, higher polyp-detection rate, an inversely correlation with BBPS and insertion and withdrawal times, and an inverse relation between BBPS and the need to repeat colonoscopy for an inadequate preparation. The latest inverse correlation was confirmed in the study of Calderwood *et al*[20] and in the study of Kim *et al*[21] that also confirmed a correlation with polyp detection rate (PDR). Calderwood conducted a second study with a very large sample size (74 endoscopists performed 2516 colonoscopies) finding that a total score of ≥ 6 and score of ≥ 2 for each segment is the definition of adequate BP[22]. The best cut-off of 2 in each segment as definition of adequate BP is proven also by Clark *et al*[23].

The reliability was determinate in different studies demonstrating a good interobserver agreement, quantified as intraclass correlation coefficient or weighted kappa (ranging between 0.67-0.93)[19-21,24,25]. Indeed, a good intraobserver agreement were found in three different studies (weighted kappa = 0.77; 95%CI: 0.66-0.87[19]; weighted kappa = 0.78; 95%CI: 0.73-0.84)[20] and weighted kappa = 0.67; 95%CI: 0.51-0.84)[24].

The results of these large and very well conducted studies corroborating the validity and the reliability of BBPS, allowed to suggest the routine use of BBPS in the clinical practice as proposed by Parmar *et al*[26].

Promising data come from artificial intelligence, as recently described by Zhou *et al* [27]. They developed a deep convolution neural network called ENDOANGEL to assign BBPS, with a 91.9% of accuracy. In the unique study on this topic, so further data are needed to support the routinely use of this system.

Bubbles scale

None of the previous scales provided an adequate evaluation of presence of bubbles that can impact on mucosa evaluation. This inadequacy affects also the strength of the conclusions of two recent meta-analyses reporting a benefit of added oral simethicone to increase BP[28,29].

The amount of foam/bubble interfering with colonic visualization was also measured in different studies regarding BP[30-42].

Parente *et al*[30] evaluated the presence of bubble in terms of the overall impact on mucosal visualization, as excellent (clear imaging, no or minimal amount of bubbles or foam that can be easily removed), fair (modest amount of bubbles and foam that can be cleared, with some waste of time) and insufficient (a large amount of foam and bubbles that reduces significantly the clear visualization of the mucosa) in each bowel segment.

A Bowel Bubble Scale, a four-point scoring system (0, no bubbles; 1, minimal or occasional bubbles; 2, moderate or obviously present; and 3, severe or many bubbles that vision is obscured) was developed by McNally *et al*[32] and used by Guo *et al*[31] and Yuanchao *et al*[33].

Another intraluminal Bubbles Scale was used in studies performed by Matro *et al* [34] graded 4 segments of the colon (cecum, right colon and hepatic flexure, transverse colon and splenic flexure, and colon distal to the splenic flexure) and each colon segment was graded using a 3-point scale (A = no/minimal bubbles, B = moderate bubbles/interfere with detecting a 5 mm polyp, and C = severe bubbles/interfere with detecting a 10 mm polyp).

Repici *et al*[35] measured the bubble score according the overall mucosal visibility using a 3-grading scale from grade 0 (optimal) to grade 2 (insufficient), the same scale was used by Spada *et al*[38] to asses mucosal visibility.

Yoo *et al*[36,37] used a scale assigned the bubble score in accordance with the degree of obscuration by bubbles, bile, or debris from 0 (severe obscuration) to 3 (no obscuration), applied also by Zhang *et al*[40].

A revised version of this scale was adopted by Rishi *et al*[39], who assigned the score (from 1 to 4) according the percent circumference of colonic mucosa clear of all bubbles/foam, not divided between segments of the colon.

Movareji *et al*[41] used a bubble scale used adapted from the one previously described by Sudduth *et al*[42], evaluating the entire colon by adding each individual segment score (from 0, no or minimal bubbles, to 3, bubbles filling the entire lumen).

In the two latest studies, the authors failed to validate and establish the reliability of the colon bubble scales. In particular, the interobserver agreement for bubble scale score was moderate (kappa = 0.537[41], kappa = 0.4024[39]).

Recently a new scale, named Colon Endoscopic Bubble Scale (CEBuS) was developed and its reliability was determined in a multicentre prospective observational study[43]. The scale CEBuS ranged from 0 (no or minimal bubbles, covering < 5% of the surface) to 2 (bubbles covering > 50%). A high intraobserver reliability [kappa 0.82 (95%CI: 0.75-0.88) *vs* 0.86 (95%CI: 0.85-0.88)] and high interobserver agreement [ICC 0.83 (0.73-0.89) *vs* 0.90 (0.86-0.94)] were reported in both experts group and mix expert/non-expert group. These encouraging preliminary results needed to be confirmed with a larger study.

CLEANSING AGENTS FOR BP

Four-liter high-volume polyethylene glycol (PEG)-based preparations were the first formulations introduced for bowel cleansing prior to colonoscopy. These isosmotic solutions provide rates of adequate BP > 90%[44-47] , without producing relevant fluid shifts or electrolyte imbalances[11]. Despite high efficacy and safety, the large volume of liquids and poor solution taste may decrease patients' compliance to the assumption of these preparations[12].

PEG-based and non-PEG-based low-volume solutions have been developed in order to reduce the total volume of preparation and improve patients' acceptability. The hyperosmotic 2 L PEG-based agents (containing PEG plus ascorbate, citrate, or bisacodyl) showed similar efficacy in bowel cleansing with higher patients' tolerability and willingness to repeat the preparation compared to high-volume PEG-based solutions in meta-analyses[44,45] and randomized trials[38,48-51]. Additionally, comparable adenoma detection rates were found between 2 L PEG plus ascorbate and 4 L PEG solutions[49,50]. A recently developed low-volume solution of 1 L PEG plus ascorbate had similar quality of BP, adenoma detection rate, and safety profile compared to 2 L PEG plus ascorbate in a randomized trial[52]. This preparation showed higher rate of adequate colon cleansing compared to 4 L high-volume PEG (84.3% *vs* 77.4%, P = 0.039) in hospitalized patients, with no differences in electrolyte imbalances, creatinine and haematocrit[53]. However, these results are based on a post-hoc analysis of an observational study. The non-PEG-based hyperosmotic low-volume preparations include magnesium citrate with sodium picosulfate, oral sulfate solution (*i.e.* trisulfate), and oral sodium phosphate. As the PEG-based low-volume solutions, these formulations showed non-inferiority in terms of efficacy and better safety profile as well as patients' tolerability compared to 4 LPEG[54-58].

On these bases, current ESGE guidelines recommend low-volume PEG-based and non-PEG-based solutions as alternatives of equal efficacy to high-volume PEG-based formulation for routine BP, with the exception of oral sodium phosphate for the relevant risk of kidney injury[59]. However, safety concerns have been raised on hyperosmotic low-volume agents in patients at risk for hydroelectrolyte imbalances, such as those suffering from severe renal insufficiency or congestive heart failure. Moreover, ascorbate-containing solutions are contraindicated in people with phenylketonuria or glucose-6-phosphate dehydrogenase deficiency[60]. Thus, the

choice of the adequate preparation for bowel cleansing prior to colonoscopy should be individualized, especially in specific categories of patients at high risk of adverse events.

TIMING OF BP

Timing of consuming BP is highly important. The last dose of BP should be started in the 5 h before colonoscopy and ended 2 h before the scheduled time of the procedure [59]. This recommendation is translate in clinical practice in two different timing for colonoscopy of the morning and colonoscopy of the afternoon.

For morning colonoscopy, both American and European Guidelines strongly recommend split-dose regimens[59,61,62].

Split-dose regimen is defined as assuming half of the BP the day before the colonoscopy and half on the day of the colonoscopy. Several evidences provided the superiority of split dose regimens over a day-before preparation to achieve a better colon cleaning, regardless the cleansing agent[48,63-69]. Moreover, the split-dose preparation showed better patient tolerability and higher proportion of patients willing to repeat the regimen[47,70].

Effectiveness of colonoscopy is highly dependent on the quality of BP. Different observational studies and also a recent meta-analysis found that split dose preparations increase adenoma detection rate[69,71-75]. The meta-analysis demonstrated also an increase rate of advance adenomas and sessile serrated polyps in split-dose regimen, including seven trials comparing split-dose *vs* day-before BP regimens. No differences in the same variables were found comparing spilt-dose and same-day BPs [75]. Another meta-analysis did not confirm the increase of ADR with split dose regimen, but it included only 4 randomized controlled trials (RCTs)[70] with moderate overall quality of evidence.

For afternoon colonoscopy, the same-day BP is recommended[59].

Considering studies including higher number of colonoscopies scheduled in the afternoon, same-day BP showed similar rate of adequate bowel cleaning, with no difference in tolerability and patient willingness to repeat it, comparing to split-dose regimens. The ADR was similar for the two regimens as showed by two different meta-analysis[75,76]. Moreover, patients in same-day regimens reported better sleep quality (OR 0.44, 95%CI: 0.24-0.82)[77].

Instead, the same-day regimen showed a significantly lower quality of BP considering studied including only morning colonoscopies[78], or lower patient tolerability and compliance[79,80], with lower willingness to repeat the same preparation in the future[79].

DIET BEFORE COLONOSCOPY

Diet restriction has traditionally been recommended before colonoscopy because it can reduce the amount of stools in the intestines, but adherence is low. The European and American Societies of Gastrointestinal Endoscopy actually recommended the use of a low residue diet (LRD) for colonoscopy defined as a diet with a total fiber intake inferior of 10 g/day[59,81].

Two meta-analysis[82,83] including studies comparing LRD with clear liquid diet (CLD) on the day before colonoscopy examination, found a significantly higher odds of tolerability and willingness to repeat preparation with no differences in adequate BPs or adverse effects.

In the last year two new meta-analysis comparing LRD *vs* CLD for BP before colonoscopy were published[84,85].

Zhang *et al*[84] performed a systematic literature search until September 2019 and they included twenty RCTs. Adequacy of bowel cleansing and polyps detection rate were similar in both groups (P = 0.79 and P = 0.68 respectively). There were significantly fewer adverse events in individuals in LRD group: nausea (P = 0.02) vomiting (P = 0.04), hunger (P < 0.001), and headache (P = 0.02). In addition, significantly more individuals in the LRD group found it easy to complete the diet (P = 0.01) and showed willingness to repeat it (P = 0.005).

Chen *et al*[85] included 16 studies and found a significantly better tolerability and willingness to repeat intestinal preparation in patients with LRD compared with CLD (both P < 0.05), but no differences with adequate intestinal preparations, detected polyp or overall adverse reactions.

These latest evidences showed that LRD is a promising approach for BP before colonoscopy with comparable adequacy of BP with that of CLD.

A recent study of Gimeno-Garcia *et al*[86] aimed to assess if a 3 d LRD is better regarding bowel cleansing than a single day LRD regimen, concluded that there is not a concrete advantage.

Recently, Avalos *et al*[87] performed a meta-analysis of randomized trials comparing BP outcomes between a LRD or regular diet (RD) compared with a CLD. Twelve RCTs, grouped patients taking a LRD (8 RCTs) or a RD (4 RCTs) and compared them to patients taking a CLD. In the 7 high-quality studies included, they no found differences in BP quality among the LRD/RD and CLD groups (RR 0.98; 95%CI: 0.93-1.04). Tolerability and willingness to repeat were better in the liberalized diet arm. There was no significant difference in the adenoma detection rate, whereas hunger was more common in the CLD group (RR 1.93, 95%CI: 1.13-3.3)[87]. Further studies are needed to confirm other findings, Table 1.

ADJUNCTIVE DRUGS

Various adjuvant drugs have been added to standard BP regimens to increase quality of BP by direct action (as simethicone) or by the improving of patient experience.

Simethicone

Simethicone is an antifoaming agent using to reduce excessive gas, abdominal discomfort, and bubble formation in the gastrointestinal tract.

Several RCTs have investigated the effect of oral simethicone on bowel cleansing.

Since 2011, four meta-analyses were conducted. The first one[88] included 7 RCTs (714 patients) comparing purgative plus Simethicone with purgative alone for colonoscopy. The air bubbles were significantly decreased, while no difference in adequate colon preparation was found.

The role of added oral simethicone on ADR was investigated in meta-analysis of Pan *et al*[89]. Such meta-analysis included 6 RCTs (1855 patients) and found an increase of ADR in simethicone group. Different result was found by another meta-analysis[28], including 12 randomized controlled studies (6003 participants) that found no difference in ADR between the groups with or without simethicone.

The last meta-analysis by Moolla *et al*[29] aimed to determine the effect that simethicone has on bowel cleanliness, ADR and tolerability, and included 16 RCTs (5630 patients) using PEG for bowel agent cleaning. Authors found an increase rate of adequate BP in PEG cohort with simethicone compared with PEG alone (OR 1.48), considering all 16 RCTs.

This finding was confirmed in three subgroup analysis: (1) Excluding RCT with bisacodyl or with different volume preparation; (2) Including only preparation with PEG 2 L; and (3) PEG single dosing the day before. On the other hand, considering patients with split dose regimen, no difference was found in adequate bowel colonoscopy rate between PEG group and PEG + simethicone group.

Regarding ADR, no difference was found considering all studies evaluating ADR (7 studies). However, ADR was significantly higher increase in simethicone group and in the subgroup analysis considering single-dosing preparations (3 RCTs). Moreover, the authors found an increase of bloating in PEG alone group, while no differences were found in the incidence of nausea, vomiting and abdominal pain.

Currently, ESGE guidelines suggest the use of oral simethicone for BP[59].

Instead, the routine use of simethicone through the working channel is advised against by ESGE guidelines[90], due to evidence that simethicone may contribute to biofilm formation in the endoscope working channel, reducing reprocessing effectiveness[91].

Recently,vmulti-society guideline[92] underlined factors associated to simethicone persistence in the endoscope channel. The first is the concentration, the second is the modality of delivering. So, when simethicone is needed, the guideline suggested the use of lowest concentration (less the 5%) and the smallest volume needed avoiding the simethicone delivering *via* water bottle/irrigation jet channel.

Similar recommendations were reported by Gastroenterological Society of Australia [93], despite allowing the administration of simethicone the endoscope irrigation channel.

Both guidelines recommended a strict adherence to manufactures' instruction for each passage of simethicone use (way for simethicone administration, cleaning and disinfection of the scope).

Table 1 Low fiber diet on the day preceding colonoscopy

Type of food	Allow	Avoid
Milk and milk products	Skim or low-fat milk; Buttermilk; Low-fat cheeses; Low-fat ice cream; Sherbet; Yogurt without seeds, berries, rinds or nuts	Yogurt with seeds, berries, rinds or nuts
Vegetables	Any well-cooked vegetables without seeds (*e.g.*, carrots, pumpkin); Lettuce; Potatoes without skin; Strained vegetable juice	All raw vegetables, except lettuce; Broccoli; Brussels sprouts; Cabbage and sauerkraut; Cauliflower; Corn; Fried vegetables; Greens (mustard, turnip, collards); Mushrooms; Okra; Onions; Peppers; Potato skins
Meats and other protein foods	Eggs; Smooth nut butters; Tofu; Tender, well-cooked meat, poultry and fish	Chunky nut butters; Legumes; Nuts or seeds; Tough or chewy cuts of meat
Grains	Bread, bagels, rolls, crackers, pasta and cereals made from white or refined flour (*e.g.*, crispy rice cereal and cornflakes); Cooked cereals (farina and creamy rice); White rice	Brown rice and wild rice; Cereals made from whole grains; Grain products made with seeds or nuts; Whole-wheat or whole-grain breads, rolls, crackers or pasta
Fruits	Fruit juice without pulp (except prune juice); Most canned, soft and pureed fruit without skin (except pineapple); Peeled apple; Ripe banana or melons	All raw fruits except peeled apple, ripe bananas and melon; Canned berries, canned cherries; Dried fruits, including raisins; Prunes and prune juice
Beverages	Coffee, tea, chamomile; Sports drinks; Water	
Condiments	Ketchup and mustard; Margarine, butter, oils, mayonnaise, sour cream and salad dressing; Plain gravies; Spices, cooked herbs, bouillon, broth, and soups made with allowed vegetables; Sugar, clear jelly, honey and syrup	

Agents improving patient experience

To increase the quality of BPs, several adjuncts were evaluated. All of them act through the increasing of tolerability and palatability of bowel cleaning agents.

Four studies evaluated the role of drinks different from water. One study evaluated the BP, the palatability and the adverse effects of Coca-Cola (Coke) Zero as solvent for PEG comparing with water. The authors found a better quality of BP and palatability in Coke group, with no difference in rate of adverse events neither in PDR[94]. The palatability is also increased with orange juice intake before drinking 2 L of PEG plus ascorbic acid[95], while no differences were found in quality of BP. Also, pineapple juice was tested to increase palatability of BP. In one single randomized study[96], patients were assigned to one of the following regimens: 4 L PEG or 2 L PEG or 2 L PEG plus 1 L of pineapple juice. The third group had better quality of bowel cleansing in the right side and in transverse colon, but no difference in tolerability.

A prospective, randomized controlled recent study of Hao *et al*[97] aimed to evaluate the effectiveness and safety of concomitant use of green tea (GT) with 2 L PEG in BP for colonoscopy. Adding GT increased the compliance, reduced adverse events with comparable bowel cleanliness in BP.

Five studies evaluated the role of tablets and gum chewing in the BP. The study of Lan *et al*[98] compared two groups of patients received 2 L PEG alone or plus citrus reticulata peel in form of "buccal tablet" eaten between drinks. The second group had higher acceptable taste, lower rate of swallowing difficulty and adverse events with no differences in quality of colonic cleansing. Three randomized studies evaluation the contribution of gum chewing[99-101] and in all of them, patients' tolerability was better in gum chewing group than the other group. In one study[99], better quality was reached in gum chewing group, no difference was found in the other two studies.

The menthol candy drops[102] were used in one randomized study demonstrating the better grade preparation in candy drops-added group, with no difference in side effects.

A recent systematic review and meta-analysis was performed including 6 single-blind RCTs (1187 patients)[103]. The included adjuncts were citrus reticulata peel, orange juice, menthol candy drops, simethicone, Coke Zero and sugar-free chewing gum. The study concluded that the adjunct improved palatability and willingness to repeat BP, with fewer side effects as bloating, vomiting, but no difference in nausea or abdominal pain. Moreover, the rate of adequate BP was higher in the adjunct group.

EDUCATIONAL INTERVENTION

In the effort to improve BP, several methods emphasizing the importance of BP quality and the instructions for BP were evaluated. Different methods were tested, including

pictures, cartoon visual aids, booklets, video, instructions by the nurse, short message service, smartphone applications were evaluated separately with conflicting results.

Seven meta-analyses[104-110] were conducted to compare the adequacy of BP in patients who received enhanced instructions and patients who received standard ones. All of them demonstrated that enhanced instructions are useful to improve the quality of BP, and in the same time to increased ADR.

So, both European and United States guidelines suggested the enhanced instruction before colonoscopy[18,59].

SPECIFIC CATEGORIES OF PATIENTS

Lower gastrointestinal bleeding

Colonoscopy has an important role for optimal management of acute lower gastrointestinal bleeding (LGIB), with diagnostic and therapeutic potential[111].

Colonoscopy should be performed after hemodynamic stabilization. Moreover, adequate colon cleansing is crucial to achieve before performing colonoscopy for LGIB, because of the increasing risk of perforation, and major risk of missed bleeding mucosal lesions in poorly prepped colon and properly evaluation of the entire mucosa [112].

However, cleansing the colon from stool, clots and blood is difficult to accomplish [111].

According to the latest European guidelines, preparation for colonoscopy should include 4-6 L of a polyethylene glycol solution or the equivalent, administered over 3-4 h until the rectal effluent is clear. A nasogastric tube can be placed to facilitate colon preparation in intolerant to oral intake patients. Prokinetic/anti-emetic agent immediately prior to initiating the colon preparation may reduce nausea and facilitate gastric emptying[59].

Although colonoscopy has several advantages in the management of LGIB (identification of bleeding sources, multiple therapeutic options, definitive diagnosis, reduction of hospital length of stay and safety), it also has several disadvantages (need for colon preparation and sedation, experienced staff and endoscopy facilities, low prevalence of stigmata of hemorrhage, invasive nature, and rare but serious complications)[111].

A higher risk of urgent colonoscopy adverse events may occur in elderly patients with comorbidities or on antithrombotic therapy[113,114]. BP may increase the risk of vomiting, aspiration pneumonia a volume overload[111].

Niikura *et al*[115] in a retrospective review investigated adverse events and hemodynamic instability during BP and colonoscopy in hospitalized patients with acute LGIB. They showed that during BP, the 9% of LGIB patients experienced an adverse event. None of them experienced volume overload, aspiration pneumonia or loss of consciousness; however, 7% had hypotension and 2% vomited. There were no significant differences in the five BP-related adverse events between LGIB and non-GIB patients.

The use of lower volume or alternative colon preparation solutions in LGIB patients is not well defined, only preliminary data are available and seems encouraging[116].

The American College of Gastroenterology, ESGE and British Society of Gastroenterology recommends against un-prepped colonoscopy in the setting of acute LGIB[59, 117,118].

A prospective pilot study of Repaka *et al*[119] in severe LGIB subjects reported the feasibility and safety of unprepared hydroflush colonoscopy that combined three 1-L tap water enemas, a water-jet pump irrigation system, and a mechanical suction device to cleanse the colon. Cecal intubation was performed in 69.2% of patients and definitive bleeding sources of 38.5% of patients were detected. However, localization of diverticular bleeding, can be difficult in the setting of residual blood and stool and poor visualization may also increase the risk of perforation.

A recent single-center study performed on elderly patients with severe LGIB investigated the efficacy, safety and outcomes of unprepared polyethylene glycol-flush retrograde colon cleansing colonoscopy[120]. In this study cecal intubation was 100%, the rate of definitive bleeding sources was 90.9%. They concluded that this approach was safe, effective and reduced the time of hospital stay, therefore further data are necessary.

Although, the international guidelines recommend BP of this cohort of patients, the best modality to achieve the cleaning of the colon is still an open problem.

Chronic kidney disease and hemodialysis

The assessment of renal function is a key point in the choice of the most adequate and safe bowel cleansing agent prior to colonoscopy, since the assumption of hyperosmotic solutions may lead to dehydration and electrolyte imbalances in people with pre-existing chronic renal disease[59]. Although the relevance of the issue, high quality evidence on different preparations for this high-risk population is lacking, with available data deriving from observational studies. Lee *et al*[121] found no difference in electrolytes or estimated glomerular filtration rate (eGFR) between 4 L PEG and 2 L PEG plus ascorbate in patients with an eGFR < 60 mL/min before colonoscopy. A transient > 30% rise in creatinine levels was recorded in 7.5% and 11.5% of high-volume and low-volume group, respectively (P > 0.05). In a similar population, Russman *et al*[122] showed that oral sodium phosphate was associated with a 12.6 (95%CI: 1.5-106.5) times increased risk of renal function worsening compared to 4 L PEG. Frazzoni *et al*[53] compared 1 L PEG plus ascorbate with 4 L PEG, including 52 patients with chronic kidney disease, showing no different shift in serum electrolytes levels and creatinine. Considering these results, PEG-based preparations may be a safe choice in people with pre-existing mild to moderate chronic kidney disease (eGFR ranging from 89 to 30 mL/min), whereas current international guidelines do not recommend hyperosmotic low-volume PEG-based agents in people with severe renal insufficiency (eGFR < 30 mL/min) for the high risk of electrolyte imbalances. However, high quality randomized trials are needed to better clarify the safety profile of PEG-based solutions in the setting of chronic kidney disease. On opposite, the use of non-PEG-based low volume preparations should be avoided in this population due to possible magnesium toxicity or acute phosphate nephropathy[53,123].

Some warnings have been raised on the safety of bowel cleansing agent administration in people on haemodialysis[124]. Indeed, potential intravascular depletion following bowel cleansing agent intake may lead to hypotension and thrombosis of the arteriovenous fistula. Moreover, the association of BP assumption and hemodialysis treatment may cause severe hypovolaemia. Additionally, high-volume PEG-based solutions may produce fluid overload in these anuric patients. Despite these relevant concerns, there is currently no high quality evidence on the safety of the different formulations of bowel cleansing agents in this population, which has been systematically excluded from randomized trials. Only two studies explored the efficacy and safety of PEG-based preparations prior to colonoscopy in patients with pre-existing chronic kidney disease, including a cohort of people receiving hemodialysis[121,125]. The authors found no significant variation in serum electrolyte levels after the assumption of PEG-based formulations. However, these studies have a retrospective observational design and enrolled a total of 37 patients on hemodialysis. On these bases, specific recommendations on the use of bowel cleansing agents in this at-risk population are not provided. The first randomized trial comparing the efficacy and safety of 4 L PEG *vs* 2 L PEG plus citrate prior to colonoscopy in people receiving hemodialysis is currently ongoing (NCT04709770).

Inflammatory bowel disease

Adequate BP is crucial to assess disease activity in patients with inflammatory bowel disease (IBD). Moreover, the widespread promotion of dye-based and virtual chromoendoscopy as appropriate diagnostic techniques for neoplasia surveillance in this population at high risk of CRC further emphasizes the relevance of achieving high quality BP[126-128].

Evidence from randomized trials showed comparable efficacy between high-volume and low-volume PEG-based solutions in people with IBD. Manes *et al*[129] found no significant difference in adequate bowel cleansing between 4 L PEG and 2 L PEG plus bisacodyl in 216 patients with ulcerative colitis (75.0% *vs* 81.5%, respectively). Kim *et al*[130] demonstrated comparable rates of satisfactory BP between 4-liter PEG and 2-liter PEG plus ascorbate (96.2% *vs* 92.9%; P = 0.68) in a cohort of 109 participants with ulcerative colitis. Similarly, Kato *et al*[131] showed the non-inferiority of 2 L PEG plus ascorbate in terms of bowel cleansing compared to 4 L PEG in 70 patients with ulcerative colitis or Crohn's disease. In these trials low-volume formulations had higher patients' tolerability and willingness to repeat the preparation than 4 L PEG. Based on these results, both high-volume and low-volume PEG-based BPs are recommended in patients with IBD before colonoscopy[59], although low-volume agents may be a more advisable choice in people undergoing a considerable number of colonoscopies during their lifetime[132]. Conversely, low-volume non-PEG-based preparations should be avoided in this population, since they may cause mucosal alterations mimicking IBD[18,59]. Lawrance *et al*[133] showed a 10-fold higher rate of

preparation-induced mucosal inflammation with magnesium citrate plus sodium picosulfate and sodium phosphate compared to 4 L PEG in a randomized trial enrolling 634 participants without pre-existing or suspected IBD. Sodium phosphate-related inflammatory abnormalities were detected in 3.3% of patients in a prospective observational study including 730 participants without previous diagnosis of IBD and not using non-steroidal anti-inflammatory drugs[134].

Inpatient

Previous evidence underlined that inpatient status is one of the associated factors with inadequate BP[135-138]. In this cohort of patients, the percentage of colonoscopy with adequate preparation is between 50% and 75%[135,139,140], thus increasing the hospital length and costs[138].

It is crucial to identify predictive factors associated with inadequate BP in this cohort of patients, and in the same time, to found the best bowel cleansing agent.

The explanation could be the worse American Society of Anesthesiologists status in inpatient setting[141] prolonged immobility and the use of concomitant drugs that can impair bowel motility[142], as opiate drug[139].

The multicenter observational study of Fuccio *et al*[143] identified the factors associated with a more proper colon cleansing (physicians' meetings to optimize BP, written and oral instructions to patients, admission to gastroenterology unit, split-dose regimens, a 1 L polyethylene glycol-based bowel purge, and 75% or more intake of BP). The authors, also, found factors associated to an increased risk of inadequate colon cleansing(bedridden status, constipation, diabetes mellitus, use of anti-psychotic drugs, and 7 or more days of hospitalization).

Considering the modifiable factors, Gkolfakis *et al*[140] evaluated the role of education interventions to increase adequate BP in a recent meta-analysis. In the six included studies, the adequacy was achieved in 77% (62%-91%) of patients with education interventions *vs* 50% (32%-68%) of patients with no intervention. However, this strategy is not enough to reach to 90% of adequate colonoscopy as required by ESGE guidelines.

Regarding the choice of BP, only one study was aimed to assess the role of low volume PEG solution in inpatient cohort. In a retrospective post-hoc propensity matching score analysis of a previously prospective observational study, Frazzoni *et al*[53] found a higher rate of adequate bowel cleansing in group prepared with 1 L-PEG plus ascorbate hyperosmolar preparation than patients with the 4 L-PEG preparation. A specifically designed study is needed to better investigate the efficacy and the safety of low volume bowel agent in this setting of patients.

Elderly people

Patients with more than 65 years require special attention during the BP before colonoscopy, due to fragile equilibrium and/or increase incidence of concomitant diseases. Large volume of BP has a better risk profile, causing less electrolyte abnormalities and low risk of dehydration, but requires a high patient's compliance. Low volume cleaning agents with magnesium citrate or bisacodyl or sodium phosphate should be avoided in this fragile category of patients, due to an increased risk of electrolyte unbalance, ischemic colitis and renal function impairment, respectively [144-146].

Only two RCTs were specifically designed to evaluate BP in elderly people. Jung *et al*[48] enrolled 230 patients aged > 65 years with normal renal function and electrolytes, randomly assigned to one of 3 arms (single-dose 4 L-PEG on the day before colonoscopy; split-dose 4 L-PEG; or split-dose 2 L-PEGA). The rate of adverse events did not differ among the 3 groups, however, patients in 2 L-PEGA group had higher willingness to repeat the same preparation than other groups. The second study[57] evaluated the efficacy safety and efficacy, safety, and acceptability of the oral sulfate solution (OSS) preparation, comparing to 4 L–PEG, in elderly patients. This RCT, enrolling 193 patients, concluded that OSS with a split-dose regimen has greater acceptability and comparable efficacy in bowel cleansing compared to 4 L PEG.

So, despite low evidence, ESGE guidelines[59] suggested the use of PEG solution in elderly patients, and ASGE guidelines[147] recommended to avoid sodium phosphate preparations in these patients.

Congestive heart failure

People with congestive heart failure are at high risk of electrolyte imbalances following the intake of BPs. Indeed, this clinical condition is associated with a decrease in renal blood flow and eGFR. This may lead to acute phosphate nephropathy, due to the reduction in phosphate excretion, or hyponatraemia, linked to hypovolaemia and

high-volume water assumption[124]. Despite the substantial lack of evidence on the efficacy and safety of BPs in this population, high-volume isotonic PEG-based solutions may represent the most adequate option for their reduced risk of causing electrolyte imbalances and fluid shifts[59,124]. Low-volume PEG-based solutions may be an alternative approach due to the reduction in the total volume of liquid intake, although they are currently not recommended in patients with significant congestive cardiac failure (New York Heart Association class III or IV) for the potential harms linked to the osmotically active components included in the formulations[59]. Regardless of the preparation used, strict monitoring is advocated when PEG-based bowel cleansing agents are administered in people with congestive heart failure. On the other hand, low-volume non-PEG based solutions should be avoided in patients with congestive cardiac failure, especially oral sodium phosphate for the risk of causing acute phosphate nephropathy[59,124].

Randomized trials comparing high-volume *vs* low-volume PEG-based preparations in people with congestive heart failure are needed to assess the efficacy and safety of these agents and inform clinical decisions.

Patients with constipation

Constipation is a common gastrointestinal disorder in the community with a global prevalence of 12%–17%[148]. It has been found that constipation exists in 11.9%–17.5% of patients undergoing colonoscopy[137,149] and it is considered one of the risk factors for inadequate BP[137,149-151].

Chen *et al*[152] investigated the efficacy, tolerance, and safety of oral sodium phosphate compared with PEG in patients with chronic constipation and demonstrated that oral sodium phosphate provides better quality BP, despite a smaller amount of intestinal air bubbles than standard 4-L PEG.

Another study of Pereyra *et al*[153] compared the efficacy of different doses of sodium phosphate (NaP) and PEG alone or with bisacodyl for colonic cleansing in constipated and non-constipated patients. In constipated patients the combination of NaP plus bisacodyl presented higher rates of satisfactory colonic cleansing than PEG (95% *vs* 66%; $P = 0.03$).

Although NaP has been shown to be effective in BP of patients with constipation, its use may be causally related to serious organ toxicity (*i.e.* renal damage and permanent renal failure). Therefore, its routine use is not recommended[59].

Despite the low quality of the evidence, additional bowel purgatives are often considered in patients with chronic constipation[59].

An Italian RCT[30] compared bowel cleansing efficacy, tolerability and acceptability of 2 L polyethylene-glycolcitrate-simethicone (PEG-CS) plus 2 d bisacodyl (reinforced regimen) *vs* 4 L PEG in patients with chronic constipation undergoing colonoscopy. There was no statistically significant difference in bowel-cleansing efficacy between the enhanced regimen 2 L PEG-CS plus 2-d bisacodyl and split-dose 4 L PEG in patients with chronic constipation. However, the low-volume PEG preparation containing simethicone showed greater patient acceptability and compliance and was associated with a reduced amount of foam and bubbles over the colonic mucosa.

In a study of Lu *et al*[154] 90 patients with constipation were enrolled and randomly divided into study group (lactulose oral solution and polyethylene glycol electrolyte powder), and control group (polyethylene glycol electrolyte powder only) with 45 patients in each group. Cleansing was significantly better in the study group than in the control group ($P < 0.05$).

One nonrandomized study of Kunz *et al*[155], including 372 patients, compared the effectiveness of high-volume (4 L) PEG solution with low-volume (2 L) PEG solution with ascorbate in constipated and non-constipated adults: no statistically significant difference between the two group was found.

A prospective, randomized, investigator-blinded trial[156] randomized 227 patients with constipation into three groups; enema before purgative use, enema after purgative use, and no enema. The authors found a statistically significant better colon cleansing in the female patients in the enema before purgative group and they concluded that use of enemas before purgatives in patients with constipation significantly improves adequacy of right colon cleansing.

In a multicenter, retrospective cohort study of Yoshida *et al*[157], the efficacy of short duration of polyethylene glycol plus electrolytes (PEG + E Movicol) in improving BP with highly concentrated PEG for colonoscopy in patients with chronic constipation was analyzed. Two or four sachets of PEG + E were prescribed for 1 wk before colonoscopy. They found an improvement rate of BP of 72.6%, regardless of gender, age, and underlying diseases. Also, insertion time and pain score were improved.

Recently, Dang *et al*[158] performed a systematic review of the literature aimed to determine the ideal BP regiment for patients with chronic constipation. Patients receiving NaP had a higher chance of a successful BP than patients receiving PEG (P = 0.003). So, they concluded that, in chronically constipated patients undergoing colonoscopy, the use of NaP may result in superior colonic cleanliness when compared to PEG, however quality of evidence was low. In summary, evidence that would allow recommendation of a special regimen or supplemental treatment for BP in patients with chronic constipation is still lacking. Further studies are needed to establish patient-specific colonoscopy preparation protocols, indeed ESGE does not suggest any specific BP in patients with constipation[59].

Patient with previous bowel resection

Patients with previous bowel resection for neoplasia need to undergo a strictly follow up with colonoscopy to detect anastomotic recurrence and prevent metachronous lesion[159]. A good BP is extremely important in these cohorts of patients. Unfortunately, the history of colorectal surgery is a risk factor for inadequate colon preparation. In fact, the study performed by Lim *et al*[160] is the first one demonstrating that the percentage of inadequate BP is higher in the resection group (gastric or colonic resection) than in the control group. This data was confirmed by Pontone *et al*[161] using the same cleaning agent (4 L PEG).

However, other evidence did not support this data. Indeed, In Yoo *et al*[162] did not found a statistically significant difference in adequate cleansing between patients with colonic resection and control group. So, if the patients with colonic resection are a category of patients "hard to prepare" is still debated.

Moreover, the right bowel cleaning agent is still an open problem for this cohort. In a study by Yoo *et al*[162], the BP was performed using two types of agents (2 L and 4 L). In the resection group, the univariate analysis showed a better bowel cleansing in patients who received 2 L of PEG-Asc (1 L at 8:00 PM the day before the colonoscopy, the second 1 L 5 h before the procedure).

A specifically designed study to assess the better cleaning agent in this cohort of patients was performed by Mussetto *et al*[163]. The authors did not find any difference in adequate BP between patients with prior colorectal resection using low volume *vs* high volume preparation; however, the first preparation was better tolerated. The authors demonstrated as well a greater efficacy of low volume preparation in the right colon. However, this finding needs to be taken with caution because the study was not adequately sized and powered to specifically assess this issue. So, a larger study is needed to investigate the better cleaning agent for these patients.

Recently, a prospective, single-center, randomized controlled, endoscopist-blinded study was performed aiming to compare morning-only 2 L PEG group or a split-dose 4 L PEG in patients with previous colorectal surgery for CRC[164]. Adequate BP rate and patients' satisfaction were higher in the 4 L PEG group than in the other one.

No significant differences were found in PDR, ADR, patient compliance, tolerance, willingness to repeat the preparation or difficulty of the BP process.

Pregnant/lactating patients

Colonoscopy should be performed only if is strongly indicated in pregnant/breast-feeding women. According to ESGE guidelines there are insufficient evidence to determine for or against the use of specific regimens. PEG regimens may be preferred and tap water enemas may be considered for sigmoidoscopy[59].

Limited information is available about the safety of bowel cleansing agents during pregnancy. The systemic absorption of PEG is minimal and abdominal bloating and gas symptoms are infrequent. However, polyethylene glycol solutions have not been studied during pregnancy. Sodium phosphate solutions should be avoided during pregnancy because of it may cause fluid and electrolyte disturbance and may be associated with the risk of phosphate nephropathy. In addition, newborns may have bone demineralization and bone growth failure because of maternal phosphate overload. BP with phosphate enemas before flexible sigmoidoscopy may be safe, but has not been studied in pregnancy; instead, sigmoidoscopy with tap water enemas may be sufficient.

Therefore, flexible sigmoidoscopy with tap water enemas is preferred instead of colonoscopy[165,166]. To our knowledge, no study in the publicly available literature has yet reported the safety profiles of the various BP agents/regimens in lactating women. Interrupting breastfeeding during and after BP with cathartic agents or application of a tap water enema for sigmoidoscopy it would seem the more careful choice[167].

BP AND POST-COLONOSCOPY SYNDROME

Post-colonoscopy syndrome is a condition characterized by persistent abdominal pain, discomfort and bloating after the procedure. In more than 30% of patients, the symptoms affected the normal activity and became persistent for at least 48 h after the procedure[168].

It is more common in females and when the procedural time is long, and may be predicted by conscious sedation and irritable bowel syndrome diagnosis[169].

In this regard, it has been speculated that a transient alteration of gut microbiota, induced by bowel cleansing, could partially concur to its pathogenesis.

It is easily hypothesizable that a profound cleansing induced by ingestion of a purgative solution rich in minerals and PEG may induce a change in microbiota composition. Several studies have tried to address this issue.

In a study on ten adult patients receiving a 4 L-PEG solution, after one month a reduction in *Firmicutes* and an increase in *Proteobacteria* was observed; in particular gamma-proteobacteria were 2.5 times more abundant. At family level, an increase of *Enterobacteriaceae* and a suppression of *Lactobacillaceae* was recorded; overall, authors concluded that this profile change was hallmarked by a reduction of beneficial species [170]. In another study conducted on a pediatric population of 31 children receiving sodium picosulphate, magnesium citrate and senna, a lower diversity in microbial communities was observed after preparation, with increased *Faecalibacterium* and decreased *Ruminococcus*, *Escherichia*, *Pseudobutyrivibrio* and *Subdoligranum*[171]. Chen *et al*[172] enrolled twenty male overweight adults undergoing bowel cleansing with water and sodium phosphate and checked microbiota composition 28 d after the procedure. They identified two different microbiota phenotypes at baseline: *Bacteroides* -dominant and *Prevotella*-dominant. In the first group, preparation induced *Bulleida* appearance, while in the second one an increase in *Akkermansia* was noted. Interesting, authors underlined that both *Bulleida* and *Akkermansia* are associated with type 2 diabetes and obesity.

It is a debated topic whether the change in microbiota composition is transient. Mai *et al*[173] have demonstrated that these alterations may persist for several weeks.

On the other hand, a study[174] conducted on 23 healthy adults receiving 2 L-PEG and ascorbate showed a 31-fold reduction of microbiota load. However, within 14 d, normalization of such imbalance was observed. Interestingly, a single dose (instead of split preparation) implied more profound changes with increase of *Proteobacteria* and *Fusobacteria*. Additionally, it was demonstrated that the preparation increased pH, thus lowering species producing short chain fatty acids and reducing mucous layer. Someone has speculated that in this study the reversion to microbiota normality could have been justified by the fact that only young patients have been enrolled, thus prompting the need of studies on a more variegated population[175].

However, some studies did not find radical difference in taxonomic abundance after BP[176]. For example, in a Japanese study[177] on eight young adults receiving sodium picosulphate and sennosides, it was observed only a transient modification with increase of *Streptococcus* that reverted after 14 d. However, in this study, a more evident change in microbiota-derived metabolites was found, with increase in alanine, carnitine, choline and others. Similarly, O'Brien *et al*[178] recruited 15 adults who were given 2 L-PEG plus bisacodyl, and, after 3 mo, only four patients did not return to pre-colonoscopy microbiota state.

Only one study was specifically aimed to evaluate the microbiota composition in post-colonoscopy syndrome. In a South Korean study[179], 24 patients underwent colonoscopy after 2 L-PEG plus ascorbate bowel cleansing with evaluation of microbiota composition. Five out of 24 experienced abdominal pain, discomfort, distension, constipation or diarrhea after the endoscopy. It was found that these patients had a high ratio *Firmicutes*/*Bacteroidetes* compared to those without post-colonoscopy syndrome. Moreover, they exhibited a higher alpha diversity, which progressively improved after the colonoscopy, paralleling the regression of symptoms.

Two RCT studies evaluated the role of probiotic administration after colonoscopy in the resolution of bloating, abdominal pain and altered bowel function post colonoscopy. In the first one probiotic group had a lower number of pain day after colonoscopy performed with air insufflation[180]. The same group did not found significant difference in post-procedural discomfort, bloating nor time to return of normal bowel function between probiotic and placebo groups, after colonoscopies performed with CO_2 insufflation[181].

Therefore, despite the evidences are scarce and worth of investigation in the future, these researches could represent a hint about the involvement of microbiota, BP and insufflation in pathogenesis of minor complications after colonoscopy.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 7; 27(45): 7771-7783

DOI: 10.3748/wjg.v27.i45.7771

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

FRONTIER

Chronic rejection after liver transplantation: Opening the Pandora's box

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Author contributions: Angelico R, Sensi B, Manzia TM, Tisone G, Grassi G, Signorello A, Milana M, Lenci I acquisition of data; Angelico R did the analysis and interpretation, drafting of manuscript, critical revision; Sensi B, Manzia TM, Tisone G, Grassi G, Signorello A, Milana M, Lenci I critical revision; Baiocchi L proposal of study, study conception, correction of manuscript, critical revision.

Conflict-of-interest statement: None to disclose by all authors.

Country/Territory of origin: Italy

Specialty type: Gastroenterology and hepatology

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

Peer-review report's scientific

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Abstract

Chronic rejection (CR) of liver allografts causes damage to intrahepatic vessels and bile ducts and may lead to graft failure after liver transplantation. Although its prevalence has declined steadily with the introduction of potent immunosuppressive therapy, CR still represents an important cause of graft injury, which might be irreversible, leading to graft loss requiring re-transplantation. To date, we still do not fully appreciate the mechanisms underlying this process. In addition to T cell-mediated CR, which was initially the only recognized type of CR, recently a new form of liver allograft CR, antibody-mediated CR, has been identified. This has indeed opened an era of thriving research and renewed interest in the field. Liver biopsy is needed for a definitive diagnosis of CR, but current research is aiming to identify new non-invasive tools for predicting patients at risk for CR after liver transplantation. Moreover, the minimization or withdrawal of immunosuppressive therapy might influence the establishment of subclinical CR-related injury, which should not be disregarded. Therapies for CR may only be effective in the "early" phases, and a tailored management of the immunosuppression regimen is essential for preventing irreversible liver damage. Herein, we provide an overview of the current knowledge and research on CR, focusing on early detection, identification of non-invasive biomarkers, immunosuppressive management, re-transplantation and future perspectives of CR.

Key Words: Liver transplantation; Chronic rejection; Immunosuppression; T cell-mediated rejection; Antibody-mediated rejection; Donor-specific antibody; Re-transplantation; Graft loss; Complications; Outcomes

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

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Received: April 27, 2021
Peer-review started: April 27, 2021
First decision: June 13, 2021
Revised: June 25, 2021
Accepted: November 20, 2021
Article in press: November 20, 2021
Published online: December 7, 2021

P-Reviewer: Gencdal G, Tavabie OD, Xu X
S-Editor: Wu YXJ
L-Editor: A
P-Editor: Wu YXJ



Core Tip: Chronic rejection (CR) still represents a cause of graft loss after liver transplantation. Recent advances in understanding the pathways leading to CR, through a T cell-mediated or antibody mediated injury, are opening new strategies for its management. Early detection of CR, tailored immunosuppressive regimen and strict monitoring are essential to prevent graft loss rejection-related requiring re-transplantation. The current perspectives aim to identify non-invasive biomarkers predicting patients at risk for CR in order to prevent irreversible liver damage by adequate immunosuppressive regimen and improving long-term outcomes after liver transplantation.

Citation: Angelico R, Sensi B, Manzia TM, Tisone G, Grassi G, Signorello A, Milana M, Lenci I, Baiocchi L. Chronic rejection after liver transplantation: Opening the Pandora's box. *World J Gastroenterol* 2021; 27(45): 7771-7783
URL: https://www.wjgnet.com/1007-9327/full/v27/i45/7771.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i45.7771

INTRODUCTION

Chronic rejection (CR) of a liver allograft is an immunologically mediated insult to the parenchyma, resulting in damage to vessels and bile ducts[1]. The prevalence of CR after liver transplantation (LT) has been declining over the last years, mainly due to the introduction of new potent immunosuppressive (IS) regimens. However, when CR occurs, it may be successfully treated only in the early phases, while irreversible injury may lead to graft loss requiring re-transplantation[1]. Despite CR being a post-transplantation complication that has been recognized for a long time, its pathogenesis, as well as its clinical implications and treatment, are fields that still require full elucidation.

CR is the consequence of the activation of the host's immune system against the liver allograft and can occur through two general mechanisms: (1) T cell-mediated and (2) Antibody-mediated pathways[1,2]. T cell-mediated chronic rejection (TCMCR) is a long-known complication of LT. Antibody-mediated chronic rejection (AMCR) instead has been observed initially for heart, kidney and pancreas transplants. Yet, it was thought to spare liver allografts, because the liver is a relatively immune-privileged organ and is commonly not targeted by antibodies. Today, it is known that AMCR affects liver allografts as well, albeit rarely, and with not-so-clear clinical consequences. Nonetheless, the condition can evolve to graft failure and death. Therefore, it is of paramount importance to progress in the prevention, early diagnosis, and specific management of CR.

IS drugs play a crucial role in influencing the onset of CR. After LT, the "adequate" IS is considered as this which maintains a viable graft and healthy patients, balancing the risk of rejection (maintaining the target drugs' blood trough levels) and the risk of IS-associated side effects. Yet, different strategies of IS regimens are currently adopted depending of a large number of variables (such as recipient's renal function, cardiovascular disease, risk of infections and tumours, recurrence of underling liver disease). Nevertheless, there is not a unique definition for the optimal IS regimen since it should be tailored to each recipient.

In this narrative review, we highlight the current knowledge and hint to future perspectives on CR in LT, which today represents a growing field for the long-term outcomes of LT recipients.

IMMUNE PRIVILEGES OF THE LIVER ALLOGRAFT

The liver allograft is uniquely immunologically privileged organ and can be transplanted successfully despite positive crossmatch, irrespective of HLA matching. LT requires less amount of induction therapy and maintenance IS compared to other organs, and features of CR rates are low even when IS regimens are minimized or absent.

The mechanisms underlying LT tolerance have not been fully elucidated yet, but appear to be related to particular characteristics of the liver micro-environment. Firstly, the liver contains both innate immune cells expressing undetectable levels of major histocompatibility complex antigens and a large population of migratory immune cells, which have immunoregulatory activity inducing its privileged status[1, 2]. Secondly, the liver has evolved to clear high concentrations of bacterial antigens gut-derived coming from the portal circulation, without inducing useless and potentially harmful immune activation. In particular, with high intrahepatic antigen load, T-cells activated in the liver can induce to a dysfunctional response or to a regulatory phenotype. This may be due to high concentrations of interleukin 10 produced by Kupffer cells or due to antigen presentation by sinusoidal endothelial cells or stellate cells, which have poor costimulatory capacity. Therefore, the intravascular compartment of the liver (composed by sinusoidal antigen presenting cells and endothelial cells, Kupffer cells and dendritic cells) is also recognized as an anatomical structure that supports the induction of tolerance.

DEFINITION OF CHRONIC REJECTION

The diagnosis of CR is made on the basis of clinical, laboratory, histologic and radiological criteria (Table 1); among these, the histological recognition of CR is a fundamental step in diagnosis.

The most widely accepted histologic criteria for the diagnosis of CR are those proposed by the Banff Working Group, an international expert panel, which are periodically refined and updated[1,2].

T cell-mediated chronic rejection

The criteria for TCMCR include the presence of three features: Bile duct atrophy/pyknosis affecting the majority of bile ducts, bile duct loss in more than 50% of portal tracts and foam cell obliterative arteriopathy[2]. The latter feature is considered pathognomonic. Yet unfortunately, it is rarely found in needle biopsy specimens, while it has traditionally been observed in lost allografts at re-transplantation or autopsy. Therefore, the diagnosis relies mainly on the detection of bile duct atrophy and bile duct loss. Both of these features, instead, are rather unspecific, often making CR a diagnosis of exclusion, which requires a thorough exclusion of other causes, including arterial stenosis or biliary strictures, drug-mediated injury and cytomegalovirus infection. An important point in the differential diagnosis is the general absence of "ductular reactions" in TCMCR specimens, in contrast to what is common in other biliary diseases. Small arterial branches may also be missing in TCMCR, making the identification of portal tracts difficult, as well as a distinction between bile ducts and ductular reactions. Staining for cytokeratin may be helpful, as well as epithelial membrane antigen, which preferentially stains bile ducts, as opposed to ductules[3].

Pathologists have also developed TCMCR grading criteria, which are particularly useful, as they correlate with the reversibility of the condition and with prognosis[2]. TCMCR is distinguished into early and late stages according to the Banff schema[2], as summarized in Table 2. Histologically, the most important characteristic in the differentiation between early and late CR is the loss of bile ducts, which occurs in less than 50% of portal tracts (with associated degenerative changes in other ducts), early rejection and greater than 50% in late rejection. Other diagnostic criteria are bridging perivenular fibrosis and small arterial loss, which have all been correlated with a high rate of graft failure. The staging distinction of TCMCR is clinically important; early CR is potentially reversible, whereas late-stage CR is generally irreversible.

Antibody-mediated chronic rejection

The existence of AMCR has been elusive and a strict definition has only been attained in recent years (Table 1)[4]. This has been mainly explored through studies on suboptimally immunosuppressed patients, in those undergoing IS weaning and in the paediatric population, as most primary paediatric diseases requiring LT are not immune-mediated and do not recur[5]. The development of AMCR seems to be multifactorial. It has been proposed that a first independent liver insult (*i.e.*, acute rejection and low immunosuppression) may be necessary to precipitate AMCR by inducing up-regulation of class II HLA molecules and consequently rendering the liver vulnerable to donor-specific antibody (DSA)-mediated damage.

Table 1 Histological definition and clinical features of chronic rejection

	T cell-mediated chronic rejection	Antibody-mediated chronic rejection
Histological definition (according to the 2016 Banff Group [1])	Presence of bile duct atrophy/pyknosis affecting the majority of bile ducts; OR Bile duct loss in more than 50% of the portal tracts; OR Foam cell obliterative arteriopathy	At least mild mononuclear portal and/or perivenular inflammation with interface and/or perivenular necroinflammatory activity; AND At least moderate portal/periportal, sinusoidal or perivenular fibrosis; AND Positive C4d staining in at least 10% of the portal tracts; AND Circulating DSAs in serum samples collected within 3 months of biopsy; AND Other causes have reasonably been excluded
Incidence	2%-5%	Unknown
Risk factors	(1) History of T cell-mediated *acute* rejection episodes; (2) Autoimmune aetiology of the primary liver disease; (3) Non-compliance with IS therapy; (4) Cyclosporine-based IS regimens as opposed to tacrolimus-based regimens; (5) Previous re-transplantation for rejection; (6) Donor/recipient gender mismatch; and (7) Donor age greater than 40	(1) Donor-specific antibodies (especially *de novo* anti-HLA class II antigens); (2) Inadequate IS (cyclosporine regimens or low CNI concentrations); (3) MELD score > 15; (4) Young age at transplantation; and (5) Re-transplantation
Clinical implications	15%-20% graft loss	Increased fibrosis and graft failure in an unknown percentage of patients

CNI: Calcineurin inhibitors; DSA: Donor-specific antibody; IS: Immunosuppressive; HLA: Human leukocyte antigen; MELD: Mayo End-Stage Liver Disease.

Table 2 Histological features of early and late chronic T cell-mediated rejection according to the Banff schema[2]

Structure	Early CR	Late CR
Small bile ducts (< 60 μm)	(1) Degenerative changes involving the majority of ducts: Eosinophilic transformation of the cytoplasm; Increased nucleus: Cytoplasm ratio; nuclear hyperchromasia; uneven nuclear spacing; ducts only partially lined by biliary epithelial cells; and (2) Bile duct loss in < 50% of the portal tracts	(1) Degenerative changes in remaining bile ducts; and (2) Loss in > 50% of the portal tracts
Terminal hepatic venules and zone 3 hepatocytes	(1) Intimal/luminal inflammation; (2) Lytic zone 3 necrosis and inflammation; and (3) Mild perivenular fibrosis	(1) Focal obliteration; (2) Variable inflammation; and (3) Severe (bridging) fibrosis
Portal tract hepatic arterioles	Occasional loss involving < 25% of the portal tracts	Loss involving > 25% of the portal tracts
Other	So-called "transition" hepatitis with spotty necrosis of hepatocytes	Sinusoidal foam cell accumulation and marked cholestasis
Large perihilar hepatic artery branches	Intimal inflammation and focal foam cell deposition without luminal compromise	(1) Luminal narrowing by subintimal foam cells; and (2) Fibrointimal proliferation
Large perihilar bile ducts	Inflammation damage and focal foam cell deposition	Mural fibrosis

CR: Chronic rejection.

The difficulty in identifying this condition is due to the lack of specific histologic findings. According to the 2016 Banff conference report, AMCR is histologically defined as inflammation with an interface of necroinflammatory activity, moderate fibrosis and evidence of C4d-positive staining in at least 10% of the portal tracts in a patient with circulating human leukocyte antigen (HLA) DSAs in serum samples collected within 3 mo of biopsy and when other causes have reasonably been excluded [1].

Finally, a combination of the two types of rejection (AMCR and TCMCR) is also possible. Suspicion of combined AMCR should be raised when late TCMCR presents with severe fibrosis and/or is resistant to therapy. In these cases, C4d staining and DSA dosing should be performed.

For the time being, a definitive diagnosis of CR can only be made with histologic confirmation and therefore requires a liver biopsy, which is a fairly invasive procedure. Therefore, identification of non-invasive biomarkers to predict or identify patients with CR is highly desirable and has become the object of investigation. However, at present, no non-invasive tests have been identified for the diagnosis of CR, which hence, still remains a histological diagnosis.

INCIDENCE AND RISK FACTORS FOR CHRONIC REJECTION

Nowadays, CR is relatively uncommon in liver allografts, and its incidence has declined vertiginously in the last decades due to improved IS, especially after the introduction of tacrolimus (TAC)-based IS regimens. Incidence rates of 15%-20% documented in the first LT series have now been minimized to 2%-5% in the adult population after a median of 5 years, while it has been recorded in up to 16% of the paediatric population[6,7]. These figures primarily refer to TCMCR, as the incidence of AMCR today remains unknown. In fact, AMCR develops in a fraction of patients with persistent or *de novo* DSAs, which themselves account for around 15% of recipients.

After LT, the incidence of CR is significantly lower compared to other solid organ transplants, such as heart (25%-60%), combined kidney and pancreas (20%-40%), pancreas alone (30%-70%) and lung (28%-45%) transplantation. Notably, in contrast to different organ transplants, the risk of CR is highest during the first year and seems to decrease thereafter[6].

Risk factors for the development of TCMCR have been extensively studied. The most important and consistently reported risk factor is the number and severity of preceding T cell-mediated acute rejection episodes (Figure 1)[1,2]. Other frequently cited risk factors include autoimmune aetiology of the primary liver disease, non-compliance with IS therapy, cyclosporine-based IS therapy (as opposed to TAC-based regimens), previous re-transplantation for rejection, donor/recipient gender mismatch and donor age greater than 40 years[6,7]. The risk factors themselves seem to be influenced by specific IS regimens, with patients on TAC exhibiting loss of association with most risk factors, with the exception of acute rejection episodes[6]. A newly identified risk factor is donor/recipient mismatch in the minor histocompatibility antigen glutathione S-transferase T1.

DSAs are considered a risk factor for the development of AMCR, as they are a necessary, although insufficient, requirement for the diagnosis of AMCR[8]. Other risk factors for AMCR include possibly inadequate IS therapy [cyclosporine regimens or low concentrations of calcineurin inhibitors (CNIs)], recipient's Mayo End-Stage Liver Disease (MELD) score > 15, young age at transplantation and re-transplantation.

CLINICAL IMPLICATIONS OF CHRONIC REJECTION

TCMCR can develop at any time after LT and no specific time parameter exists, although most cases occur more than 90 d after LT. It can manifest as a spectrum of disease severity, ranging from mild and inconsequential alterations in blood tests to liver failure and death. The most typical presentation is that of a cholestatic-pattern in liver function tests (LFTs), with the preferential elevation of gamma glutamyl-transpeptidase and alkaline phosphatase, in a patient with previous episodes of acute T cell-mediated rejection. At other times, this scenario may develop as a consequence of untreated or therapy-refractory acute rejection. Rarely, it may evolve insidiously with worsening of LFTs in patients without previous acute rejection.

Early TCMCR can also be found in protocol biopsies without clinical or laboratory alterations, which is a rare event in adults, but found in up to 20% of cases of paediatric LT[9,10]. The clinical implication of this last situation is uncertain. Symptoms are rarely present and unspecific, such as fatigue, abdominal pain and fever. Biochemical dysfunction may regress or further evolve into progressive jaundice and other signs heralding liver failure, such as coagulopathy and malnutrition.

Progression or regression of TCMCR can be predicted based on the histological grading and timing of occurrence[2,9]. Early TCMCR can be reversible with prompt adoption of an adequate therapy. On the other hand, late TCMCR is associated with a much higher chance of evolution into liver failure. Deterioration may be rapid and allograft failure may typically occur within the first year[6]. TCMCR occurring more than 1 year after LT is usually seen in inadequately immunosuppressed patients, either as a result of non-compliance, intolerable side effects or failed IS weaning attempts. Overall, around 15%-20% of patients with TCMCR will ultimately face graft loss and require re-transplantation.

The clinical implication of AMCR is particularly controversial. Its existence has been debated for a long time, yet in the last years, there is mounting evidence that AMCR can portend poor graft outcomes after LT.

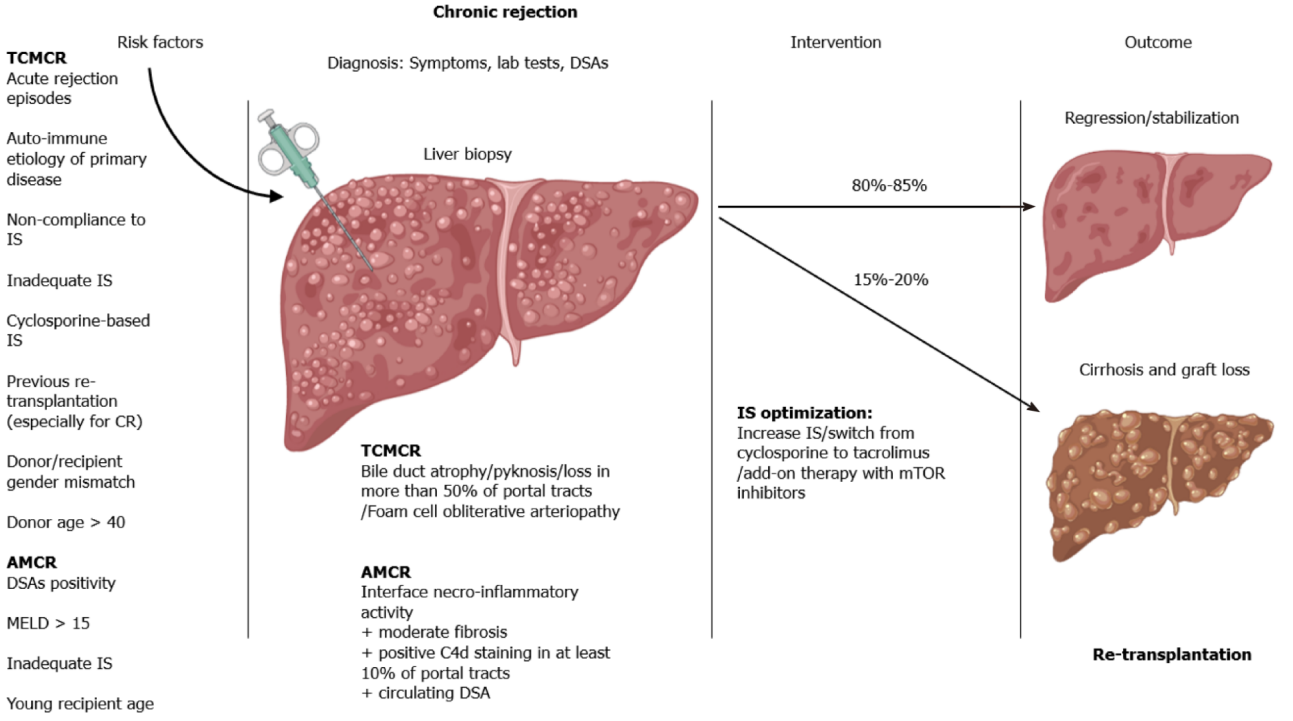


Figure 1 Chronic rejection in a nutshell.

ROLE OF DONOR SPECIFIC ANTIBODIES

DSAs can either be existent before LT (preformed), and may disappear or persist, or they can be generated *de novo* after LT. Generally, preformed DSAs are more closely correlated to acute rejection, while *de novo* DSAs are more tied to AMCR[8,11]. *De novo* DSAs may appear in 0.4%-8% of patients 1 year after LT. O'Leary *et al*[4] showed that the prevalence of *de novo* DSAs was 62% in patients with CR and 38% in patients without CR (P = 0.047). In the same study, the difference was even more significant within the first year after LT, where DSAs were found in 44% and 13% of patients with and without CR, respectively (P = 0.004)[4]. The positivity of DSAs have been associated with higher mortality after LT. On the other hand, recently Feng *et al*[5] published the long-term results of IS withdrawal in 12 paediatric patients and reported the absence of fibrosis or inflammation in protocol biopsies, despite the presence of DSAs in nine recipients.

In this scenario, nowadays, a major goal in the field of transplant immunology is to understand the role of serum DSAs in LT recipients with normal LFTs. Recently, Höfer *et al*[12] correlated DSA testing with liver allograft histological findings in patients with normal graft function, aiming to screen for subclinical rejection/fibrosis in a prospective biopsy-based program. Their results indicated that DSA positivity was associated with greater graft inflammation [odds ratio (OR): 5.4] and fibrosis (OR: 4.2) and to histological criteria that excluded the possibility of the minimization of immunosuppression (in 92%-97% of cases). On the other hand, DSA seronegativity could predict the absence of fibrosis in 87%-99% of cases. Therefore, the authors suggest that DSA testing can help to preselect patients for IS reduction in case of DSA negativity, while DSA positivity should prompt further studies using elastography or liver biopsy for the assessment of subclinical graft injury. In the same German study, another non-invasive biomarker, namely cytokeratin-18 cell death marker (M65), was investigated for liver damage; however, it had no diagnostic value for the detection of subclinical liver graft injury. Interestingly, studies have suggested that DSA seropositivity can also accelerate the progression of fibrosis in patients with hepatitis C virus (HCV) recurrence[13].

These data indicate that serum DSA monitoring in post-liver transplant follow-up may be highly helpful, especially in patients in whom IS minimization is attempted, as in those with long-term graft dysfunction.

In 2017, the Consensus on Managing Modifiable Risk in Transplantation (COMMIT) Group, an expert panel of transplant clinicians who provide practical recommendations for the identification and management of modifiable risk factors to maximize

graft and patient survival after transplantation, recommended testing for serum DSAs, when available, in all patients with a well-functioning graft at 1, 5 and 10 years after transplantation. According to the COMMIT Group, DSA positivity should warrant protocol graft biopsies or, at least, the use of non-invasive testing for fibrosis[14]. However, these recommendations are based on a low level of evidence from the currently available literature. Moreover, despite there being growing evidence for the possible benefit of measuring serum DSAs in LT, routine screening for DSAs is not yet available in all transplant centres.

All these data suggest that DSA testing should be implemented both in adult and paediatric LT recipients during long-term follow-up to better understand their role in the post-transplant liver injury process.

IMMUNOSUPPRESSION AND CHRONIC REJECTION

Judicious management of immunosuppression is crucial for the long-term outcomes and quality of life of LT recipients. The perfect management of IS regimens rests on a thin line, with rejection-mediated graft damage on one side and drug-related side effects on the other. The adverse effects of immunosuppression should never be underestimated and may include the development of kidney injury, metabolic syndrome, neurological complications, malignancies, cytomegalovirus and other opportunistic infections. Furthermore, the aetiology of primary liver dysfunction should always be kept in mind as immunosuppression may have impact on its recurrence. Recurrence of hepatocellular carcinoma (HCC) or unresolved HCV infection are favoured by immunosuppression, while autoimmune aetiologies, such as autoimmune hepatitis, primary sclerosing cholangitis or primary biliary cirrhosis, often warrant an increase of IS therapy. Therefore, it is clear that IS management is highly complex and must be tailored to the particular patient, taking into consideration a great number of variables.

Generally, after solid organ transplantation, the IS regimen is manipulated mainly for drug-related toxicity or for oncologic prevention (*de novo* or recurrent tumours) and different strategies can be adopted, such as: (1) Switch to another IS regimen; (2) IS minimization; or (3) IS withdrawal. However, all these actions may affect the development of CR, a possibility which should not be disregarded. In the following paragraphs, we will analyse how IS management might influence the establishment of CR-related injury.

Standard immunosuppressive regimens

TAC-based IS therapy is currently the most commonly used regimen after LT, as there is evidence that TAC decreases the chance of graft failure. Moreover, there is consistent proof in the literature that TAC-based IS regimens also decrease the risk of CR[6].

To prevent graft rejection, initial TAC trough levels should be kept between 6 and 10 ng/mL. A large meta-analysis has demonstrated that levels < 10 ng/mL guarantee no increase in rejection episodes, while halving the incidence of nephrotoxicity, compared to levels > 10 ng/mL. The first month after transplantation, the TAC dosage may be decreased with a target of 4-8 ng/mL. Thus, while initial TAC target levels are largely endorsed in the literature, there is no consensus on the optimal level in the long term. The COMMIT Group states that "lower" TAC levels may be acceptable in combination therapy, when TAC-related toxicity is an issue[14]. Also, the International Liver Transplant Society (ILTS) suggests that, from year 1 after LT onward, TAC trough levels can be dropped to 3 ng/mL; furthermore, after the fifth year, keeping drug levels "above detectable" is fine as long as graft function is optimal[15].

TAC is often initially used with other IS drugs, the most common of which are corticosteroids and mycophenolate mofetil (MMF). During the first 90 d after LT, steroids should be gradually tapered, leading to suspension after the third month. When aiming for TAC monotherapy, higher trough levels should be maintained, while dual therapy permits lower levels to be acceptable. Indeed, in a retrospective study aiming to assess CR in LT recipients receiving low-dose CNI monotherapy, TAC levels < 5 ng/mL were not associated with silent CR on protocol liver biopsies[16].

When TAC needs to be reduced or suspended due to adverse events, the guidelines recommend adjunct or substitution with MMF or other IS agents, such as mammalian target of rapamycin (mTOR) inhibitors (everolimus and sirolimus), to keep a low threshold for suspicion of rejection[15].

Nephrotoxicity is the major side effect of TAC requiring dose adjustments of the drug. When nephrotoxicity is anticipated to be an issue before transplantation, induction with basiliximab plus MMF and delayed TAC introduction may be used to decrease renal impairment in the first weeks, as demonstrated by the DIAMOND study[17]. In this multicentre prospective randomized trial, delayed initiation of high-dose TAC significantly reduced renal function impairment compared with immediate post-transplant administration, while the risk of rejection was similar[17].

For later appearing nephrotoxicity, renal-sparing regimens are often used, with most being common alternative agents to CNIs. Several studies have shown that switching from TAC to mTOR inhibitor-based regimens, such as everolimus monotherapy, significantly improved renal function after LT. Everolimus significantly increases the estimated glomerular filtration rate (amounting to 5.6 mL/min), with a low incidence of histologically proven rejection (5%) and side effects[18]. In contrast, for sirolimus, a randomized trial comparing *ab initio* standard TAC with low-dose TAC plus sirolimus found an excess of graft loss mortality in the combination therapy arm, which was due to hepatic artery/portal vein thrombosis or sepsis.

TAC-based IS regimens are usually also later replaced with mTOR inhibitor regimens in patients transplanted for HCC. In fact, TAC trough levels above 10 ng/mL during the first 30 d post-LT seem to increase the rate of HCC recurrence; hence, mTOR inhibitors, which have an anti-proliferative effect, may offer protection from tumour recurrence, maintaining the same risk of post-transplant graft rejection.

Immunosuppressive minimization

As rejection is considered a relatively uncommon cause of graft failure after LT, current guidelines suggest to employ IS minimization strategies in an effort to limit the morbidity associated with excessive immunosuppression, which represents the main cause of late mortality after LT[14]. In fact, IS minimization has been recognized to be feasible and is nowadays incorporated in existing recommendations[14,19]. However, IS minimization protocols need to be rigorous in order to avoid the risk of favouring acute and chronic rejection[19].

First, IS minimization strategies should be entertained after the third post-transplantation month, in patients with stable laboratory exams for a minimum of 4 wk and under tight control. Liver allograft biopsy is not required prior to IS minimization, but any sign of rejection, including rising LFTs, should prompt returning to previous IS doses and performing an eventual biopsy, even in the setting of a clinically-well patient. As clinical concordance in whether a patient needs biopsy is quite low among transplant clinicians, a model to predict acute rejection on biopsy has been proposed[20]; yet, no specific model for anticipating CR has been developed yet.

However, in LT recipients under an IS minimization regimen, insufficient TAC serum concentrations have been linked with AMCR. In a retrospective cohort of 749 adult LT recipients, Kaneku *et al*[21] reported that *de novo* serum DSA formation (incidence rate: 8%) was favoured by low CNI levels (TAC < 3 ng/mL or cyclosporine < 75 ng/mL) and by the use of cyclosporine in place of TAC, while it was inversely related to age > 60 years old and a MELD score > 15[21]. Patients with *de novo* DSAs were found at significantly higher risk for graft loss and death. Hence, serum DSA monitoring in patients who are candidates for IS minimization may be useful. Accordingly, the ILTS guidelines encourage clinicians to screen for serum DSAs before minimization attempts and to consider biopsy if strong DSA positivity is detected[15].

Additionally, an association between variability in TAC levels and late CR has been implied, especially in paediatric LT recipients. The largest of these studies included a heterogeneous population (n = 144) of children (age: 8-18 years) transplanted with different solid organ grafts (liver, kidney, heart or lung), where high variability in TAC blood levels increased the risk of late CR and graft loss, hampering the generalization of results to adult liver grafts[22]. Therefore, until new evidence emerges, excess variability in TAC levels should probably be best avoided.

Immunosuppressive withdrawal

Nonetheless, many studies have gone further and investigated the possibility of IS withdrawal, a unique chance offered to LT recipients[23]. The aim of IS withdrawal is to avoid long-term IS-related side effects, which influence patient/graft survival, as well as the quality of life of LT recipients. Overall, among IS withdrawal trials, CR had a very low incidence, accounting for 0%-3%[24].

Initial pioneering studies demonstrated the feasibility and safety of IS discontinuation after LT, but they lacked sample size assessments, clear withdrawal protocols, histopathologic surveillance and long-term follow up[25]. In these studies, acute rejection episodes could generally be managed successfully, and only three cases

of CR were reported, two of which culminated in graft loss and re-transplantation.

A prospective trial in 102 patients found that 42 (41.17%) LT recipients could be weaned off completely and did not suffer rejection, as assessed by protocol liver biopsies[22]; moreover, the time elapsed from transplantation was the key variable influencing the success of IS weaning, with high chances 10 years from transplantation, while success was rare when 3-5 years from LT had elapsed and was achieved only in patients > 50 years of age.

Recently, Jucaud *et al*[26] described the prevalence and impact of *de novo* DSAs during a multicentre IS withdrawal trial in adult LT recipients. Patients who were IS-free had a high (66.7%) prevalence of *de novo* DSAs, in particular for HLA-I. Yet, these *de novo* DSAs did not seem to be deleterious to the graft. However, their significance in the long term is still unknown, as well as their possible association with CR in patients who achieved a successful and sustained suspension of IS therapy.

Nowadays, around 20% of LT recipients can ultimately be weaned off IS therapy completely[24]. Yet, IS withdrawal is not a common practice and should only be attempted in the controlled setting of a clinical trial. There is accumulating evidence that under-immunosuppression may lead to *de novo* DSA formation, and in turn, DSA formation may lead to AMCR or mixed CR and eventually to graft loss[10,24]. Therefore, further IS weaning studies should include protocol biopsies and DSA testing[24].

TREATMENT OF CHRONIC REJECTION

Immunosuppressive management

The treatment of LT recipients who have developed CR is difficult and not entirely defined, as in general, there is still a low level of evidence. Thus, most recommendations are only based on expert opinions[14,15].

Generally speaking, patients with TCMCR may benefit from increased IS blood levels. Nonetheless, the increase in dosage should be gradual. High variability in TAC concentrations should be avoided, especially when graft function is impaired, as this may increase mortality through over-IS-related events[14]. Therefore, TAC dosage modifications should be carried out progressively and with special caution in patients with liver dysfunction[14].

Despite there being limited evidence to support a specific IS regimen for CR, patients taking a cyclosporine-based IS regimen should be switched to a TAC-based one[6]. The conversion from cyclosporine to TAC is associated to a 70% response rate and graft survival; moreover, an "early" conversion to TAC, before bilirubin levels exceed 10 mg/dL, seems to be fundamental to achieve good outcomes[6].

In a recent report, 23 patients with biopsy-proven CR were treated with a "rescue therapy" consisting of addition of an mTOR inhibitor (either everolimus or sirolimus) to the baseline IS regimen[27]. In 12 patients (52%), this strategy was successful and reversed CR, while the other patients had a poor outcome and most of them required re-transplantation. Although evidence is scarce and further data are needed, these preliminary data suggest that mTOR inhibitor add-on therapy may be valuable in treating TCMCR, and its potential benefits should be further explored in this setting.

While acute antibody-mediated rejection has different potential treatment strategies, most of which are transposed from the kidney transplantation experience, none of these is indicated for AMCR. So far, the treatment of AMCR remains an unmet clinical need, with no study to date on the subject, to the best of our knowledge.

In case of a strong positivity for serum DSAs is detected (MFI > 5000), irrespective of the class of anti-HLA antibodies, and the histological pattern is consistent with AMCR, the COMMIT Group suggested to reinforce the baseline IS regimen by increasing the CNI trough level (if tolerated) or by the introduction of MMF or other agents in patients receiving CNI monotherapy[14]. Moreover, if the histological pattern is suggestive of *de novo* autoimmune hepatitis with positive DSAs, the addition of corticosteroids is recommended. In all cases, strict follow-up and evaluation of therapeutic changes need to be based on repeated liver biopsies and DSA/MFI monitoring. Yet, with the new recent international definition of AMCR and the evidence that its incidence is expanding, a new field of study has been opened, and it is likely that the treatment of AMCR will be the object of many investigations in the near future.

In summary, despite the lack of evidence based on randomized trials, so far literature data suggest that when CR is diagnosed early (before the development of significant ductopenia, perivenular fibrosis and obliterative arteriopathy), CR is

potentially reversible by increasing or changing the IS regimen.

Improved histology and resolution of liver function abnormalities can be seen in successfully treated patients. In contrast, late CR is usually unresponsive to increased IS therapy and generally requires re-transplantation.

Re-transplantation

When CR is severe enough to compromise graft function, the chances of regression are quite low and most patients will ultimately face graft loss. In such cases of advanced CR, the only chance for survival is therefore re-transplantation.

In the early series, CR represented the most frequent indication for re-transplantation. Yet, this rate has significantly decreased after the introduction of CNIs. In a recent multicentre study analysing data from the European Liver Transplant Registry, including LT performed from 1988 to 2016, CR was found to be the cause of around 16% of all re-transplants[28]. Furthermore, according to the Clinical Practice Guidelines in Liver Transplantation of the European Association for the Study of the Liver, the rate of graft loss due to ductopenic CR has significantly decreased in recent years to less than 2%[29].

On the other hand, re-transplantation is a technically challenging procedure with significantly worse outcomes than primary LT. The technical difficulty mainly lies in a more complex dissection, with a large percentage of patients necessitating vascular and biliary reconstructions[30-32]. In analyses of the UNOS database, the 1-year risk of graft failure was 37.8% and patient survival at 1, 3 and 5 years was 67%, 60% and 53%, respectively, which is far below the corresponding rates for primary LT[30]. In fact, to avoid futility of liver re-transplantation, many studies have proposed stringent predictive scores be used to select appropriate candidates[33].

Among patients candidate for liver re-transplantation, most deaths are due to sepsis and are directly related to the MELD score, time from the primary LT (liver re-transplantation within 1 year from the first LT has the highest mortality), and warm ischaemic time. Other studies have found a correlation with donor age above 60, recipient age above 50, bilirubin levels and intraoperative transfusions[34]. Moreover, MELD scores higher than 30 are associated with prohibitive mortality risks (42% at 1 year and 21% at 5 years)[33-34]. However, no increased postoperative mortality has been reported in liver re-transplantation for CR, as opposed to other indications[33]. IS in re-transplantation for CR remains an issue as to date there is no data to guide choice of therapeutic regimens. Induction therapy with antibodies such as basiliximab is reasonable yet controversial. In particular, some studies suggest lower rates of acute rejection episodes while others do not confirm such results[35-37].

FUTURE PERSPECTIVES

The future of CR prevention, diagnosis and management holds promise. The recent recognition of AMCR in liver allografts has opened a whole new area of research. One of the main objectives would be to recognize patients at high risk for rejection. Many studies are currently aiming to identify useful biomarkers for rejection. Yet, translation of the results into clinical practice is still lagging behind. Despite new promising biomarkers, such as intracellular IFN-γ and IL-2, being proposed to select patients at higher risk for acute rejection, no biomarker has yet been recognized to identify CR [38].

Another area of interest is the definition of non-invasive methodologies for the diagnosis of CR. Lutz *et al*[39] have shown how liver ultrasound, with calculation of the right hepatic vein resistance index, and transient elastography have a high correlation with graft fibrosis. Unfortunately, although fibrosis is indeed a component of CR, it is not specific; however, detection of significant fibrosis may represent a valuable tool to select asymptomatic patients to be biopsied.

Another potential benefit of identifying non-invasive biomarkers for CR would be to drive a tailored IS regimen according to the individual patients risk to develop CR [38]. For example, they could predict a response or adverse effects of diverse drugs in the individual patient. Nowadays, as a similar experience is growing in kidney transplant recipients in this field, we foresee that it will also occur for LT recipients.

Finally, rejection prevention would be the ultimate goal. Schlegel *et al*[40] have demonstrated in a mouse model that graft preconditioning with hypothermic oxygenated perfusion can induce immune tolerance and prevent graft failure, even in the absence of IS therapy. Their results suggest that one day operational tolerance could be "induced" rather than depend strictly on the so far unpredictable graft-host

interaction, reducing or abolishing the risk of CR.

CONCLUSION

CR of liver allografts has become a relatively rare entity due to stronger IS regimens, but it still represents a diagnostic and management challenge to the transplant clinician. In the last decade, the definition of antibody-mediated rejection has opened the way for an entire new research field in the study of CR, effectively unleashing a Pandora's box. The next few years hold promise to improve CR management by the identification of non-invasive biomarkers predicting patients at risk for CR, which could hopefully be prevented by the use of tailored IS regimens, thus no longer becoming an issue after LT.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 December 7; 27(45): 7784-7791

DOI: 10.3748/wjg.v27.i45.7784

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

OPINION REVIEW

Humans have intestinal bacteria that degrade the plant cell walls in herbivores

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Author contributions: Fujimori S contributed to the writing of this paper.

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

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

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Abstract

The cell walls of plants are mainly made of cellulose and contain a large number of calories. However, the main component, cellulose, is an indigestible plant fiber that is thought to be difficult for humans to use as energy. Herbivores acquire energy through the degradation of cell wall-derived dietary fiber by microorganisms in the digestive tract. Herbivores, especially horses, have a highly developed cecum and large intestine, and plants are fermented for their efficient use with the help of microorganisms. Humans also have an intestinal tract with a wide lumen on the proximal side of the large intestine, in which fermentation occurs. The digestive process of horses is similar to that of humans, and many of the intestinal bacteria found in horses that degrade plants are also found in humans. Therefore, it is thought that humans also obtain a certain amount of energy from cell wall-derived dietary fiber. However, the intake of dietary fiber by modern humans is low; thus, the amount of calories derived from indigestible plant fiber is considered to be very low. Cellulose in the plant cell wall is often accompanied by hemicellulose, pectin, lignin, suberin, and other materials. These materials are hard to degrade, and cellulose is therefore difficult for animals to utilize. If the cell wall can be degraded to some extent by cooking, it is thought that humans can obtain calories from cell wall-derived dietary fiber. If humans can use the calories from the cell wall for their diet, it may compensate for human food shortages.

Key Words: Intestinal flora; Human; Herbivore; Indigestible plant fiber; Cell wall; Calorie

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Core Tip: The plant cell wall is mainly composed of cellulose and contains a high number of calories. However, it is classified as an indigestible dietary fiber, and its

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Received: May 3, 2021
Peer-review started: May 3, 2021
First decision: June 12, 2021
Revised: June 22, 2021
Accepted: November 24, 2021
Article in press: November 24, 2021
Published online: December 7, 2021

P-Reviewer: Belkova N
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

energy utilization is difficult. Many of the intestinal bacteria found in herbivorous horses that degrade plants are found in humans. Therefore, it is thought that humans can also utilize plant cell walls for energy to some extent. If cell wall-derived dietary fiber can be cooked to make it easier for humans to use, it may compensate for human food shortages.

Citation: Fujimori S. Humans have intestinal bacteria that degrade the plant cell walls in herbivores. *World J Gastroenterol* 2021; 27(45): 7784-7791
URL: https://www.wjgnet.com/1007-9327/full/v27/i45/7784.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i45.7784

INTRODUCTION

Cell walls support the structure of plants and are mainly composed of cellulose. Cellulose contains a large amount of potential energy, although only the method of binding glucose molecules makes it different from starch. Therefore, it is beneficial for organisms to use cell walls as energy. However, with the exception of a few multicellular organisms, such as termites, the main organisms that are capable of digesting the cell wall with their own cellulases are microorganisms, and most higher organisms cannot digest the cell wall without the assistance of microorganisms.

Some bacterial groups have enzymes that degrade cell wall components through fermentation, and many herbivores use this fermentation ability in the digestive tract to obtain energy from plant cell walls. Plant digestion is divided into various methods according to the evolution of animals. Many herbivores have a large fermenting organ in their digestive tract, but there are also herbivores, such as giant pandas that eat bamboo as their staple food, that have a digestive tract similar to that of carnivorous animals[1]. Therefore, herbivores do not necessarily need fermenting organs.

Humans are classified as omnivorous animals and eat plants. Plants consumed by humans are utilized *via* the decomposition and absorption of easily available plant cell contents. Most components of the cell wall are classified as indigestible insoluble dietary fibers and are thought to contribute little to the energy of humans. However, in the human intestinal flora, there are many bacterial species that degrade the cell wall that are also found in the gastrointestinal flora of herbivorous animals. Therefore, intestinal bacteria can ferment cell wall components that are classified as insoluble dietary fibers. It is thought that the components degraded by bacteria can be utilized by humans.

The utilization of energy derived from cell walls can improve the increasing eating habits of humans. In this paper, we examine the similarities in intestinal bacteria between humans and herbivores and summarize the findings on the utilization of energy derived from plant cell walls by humans.

CELL WALL DIGESTION

Plant cell walls consist of two layers. The outer layer is formed first and is called the primary cell wall. The primary cell wall is mainly composed of cellulose. Cellulose has a strong molecular bond with glucose, and crystalline cellulose is very hard and wiry. Hemicellulose and pectin are bound to hard wire-like cellulose. Therefore, cellulose is considered to be difficult to degrade. In the secondary cell wall formed at the site where plant growth has stopped, lignin binds to cellulose to increase its strength. Lignin is a polymer that can have completely different structures depending on the plant species and the growing environment. This lignin binds to cellulose and interferes with cellulase action[2]. Furthermore, it is very difficult to degrade cell walls with attached waxy suberin. Cork is rich in suberin and lignin; however, the fact that cork retains its morphology for a long period of time suggests that it is difficult to degrade, even by bacteria.

Moreover, the cell wall is made of hard cellulose solidified with other molecules to further increase its strength. For this reason, a large amount of effort is required to degrade the cell wall. Thus, most organisms do not degrade the cell wall themselves and instead utilize a method to mainly consume cell contents that are easily absorbed.

For example, although butterfly or moth larvae actively eat the leaves of plants, there are few bacteria in their gastrointestinal tracts. They chew the leaves and absorb the cytoplasm in them, and the cell walls are excreted from the body. In other words, except for a few kinds of insects, such as termites, it is difficult even for insects to utilize the cell wall.

If organisms consume a large number of plants, then enough of the contents of the cells can be ingested. However, plant abundance is not always sufficient. Large amounts of plants are required, especially for large herbivorous organisms. Herbivores are animals that use bacteria to degrade plant cell walls to efficiently ingest and digest plants. If the cell wall can be converted into energy, efficient energy intake can be achieved with fewer plants. Herbivores have bacteria in their gastrointestinal tracts that can degrade cell walls. There are several ways in which bacteria degrade the cell wall and absorb cell wall components.

DIGESTION OF PLANTS BY HERBIVORES

Herbivorous mammals can be broadly divided into three types according to digestion. Type 1 herbivores have a large fermenting organ in front of the stomach, and this type includes cows and goats. Type 2 herbivores have a large intestine and cecum with a large volume in which fermentation occurs, and this type includes rabbits and horses. This group is further divided into two subtypes: a subtype that conducts coprophagia, such as rabbits (2a), and a subtype that does not conduct coprophagia, such as horses (2b). Type 3 herbivores, such as giant pandas, have a digestive tract that is not much different from that of carnivorous animals. This type of herbivore does not have a large fermenting organ. The features of each digestion type are briefly described below.

Type 1 has a large fermenting organ in front of the stomach

Cows are known to have four stomachs. The rumen (1st stomach) is a very large organ where bacteria ferment food. It produces alcohols and volatile fatty acids, such as acetic acid, propionic acid and butyric acid, which are then absorbed. Fermentative bacteria increase exponentially at this site. The reticulum (2nd stomach) is located at the base of the esophagus, near the entrance to the rumen, and is a ruminant motor organ that returns poorly chewed food to the mouth. The ruminant returns the food to the mouth where it is chewed again before being sent to the rumen. Well-degraded food passes through the reticulum and is sent to the omasum (3rd stomach). The omasum is located on the anal side of the reticulum and has a file-like mucosa that provides the final mechanical crushing of food. The first to third stomachs are thought to be derived from the esophagus. The final stomach, the abomasum (4th stomach), produces gastric acid. Here, the food is chemically degraded, and the increased bacterial cells are destroyed by gastric acid. Then, in the small intestine, both the gastric residue and the degraded bacterial cell components are digested and absorbed.

Type 2 uses the cecum and the large intestine as large fermenting organs

Type 2a herbivores conduct coprophagia: In rabbits, food that is consumed orally passes through the stomach and into the small intestine, similar to the process in humans. First, the stomach absorbs nutrients that can be digested and absorbed. Indigestible polysaccharides, mainly cell walls, are degraded and fermented by bacteria in the dilated cecum and the oral side of the large intestine. The absorbable nutrients such as short-chain fatty acids (SCFAs), mainly volatile fatty acids, are absorbed instantly. Nutrients that are difficult to absorb by the large intestine, such as vitamins synthesized by intestinal bacteria, pass through the stomach again by coprophagia and are absorbed by the small intestine.

Type 2b herbivores do not conduct coprophagia: In horses, food that is consumed orally passes through the stomach and into the small intestine, similar to the process in rabbits. First, the stomach absorbs nutrients that can be digested and absorbed. Indigestible substances are degraded and fermented by bacteria in the cecum and oral side of the dilated large intestine to absorb nutrients. However, since coprophagia is not normally conducted, the efficiency of decomposition and absorption of nutrients is considered lower than that of rabbits and other animals engaging in coprophagia. This type of gastrointestinal tract is long, but it is similar to that in humans. Since the analysis of horse intestinal flora has been sufficiently advanced, we compare horse and human intestinal bacteria in the subsequent section.

Type 3 does not have a large fermenting organ

Giant pandas are herbivores that eat bamboo as their staple food; however, their digestive tract is said to be similar to that of carnivores. The intestinal bacteria found in giant pandas have been reported to be distinct[3].

THE INTESTINAL BACTERIA OF HORSES COMPARED TO THOSE OF HUMANS

Humans do not have a large cecum like horses, although the ascending colon to transverse colon is wide, and fermentation by intestinal bacteria occurs in this area. In humans, bacteria that are ingested orally are killed by gastric acid (pH: 1.5-2.0), which is more acidic than that in horses (pH: 4.4)[4]. Thus, the number of intestinal bacteria is very small on the oral side of the small intestine. However, intestinal bacteria increase rapidly from the proximal end to the distal end of the small intestinal tract. Moreover, gut bacteria progress from aerobic bacteria to anaerobic bacteria with increasing distance in the small intestine. By the ileocecal valve, the bacterial count increases to 10^7-10^9/mL and eventually increases to approximately 10^{10}-10^{12}/mL in the colon[5]. It is estimated that this flora contains 500-1000 different species of bacteria[6], with nearly 80% belonging to the two bacterial phyla *Bacteroidetes* and *Firmicutes*[7].

Table 1 shows a modified report of the horse gut microbiota compiled by Kauter *et al*[8]. This table summarizes whether microorganisms found in the intestinal tract of horses are also found in humans. The gram-negative bacilli *Bacteroides thetaiotaomicron* and *B. ovatus*, which metabolize complex dietary polysaccharides, have been found in the human intestine and have been reported to be involved in the degradation of plant cell walls[9]. The genus *Bacteroides* has been reported to increase in abundance in the large intestines of horses that consume increased proportions of grass[10]. *Bacteroides* is also a cell wall-degrading bacterium that accounts for a large proportion of the intestinal flora in both humans and horses[7]. In addition, some bacterial species reported in humans have been identified as cell wall-degrading bacteria. Therefore, these bacteria were added to create a table. From this table, it can be seen that many species of horse bacteria are also present in the human colonic flora. In other words, the intestinal bacteria of humans can degrade plant cell walls and synthesize SCFAs, including volatile fatty acids. These SCFAs can be absorbed by the large intestine[11]. Horses can obtain energy from this system. This raises the question of how much energy humans obtain from the cell wall.

CALORIES DERIVED FROM THE CELL WALL IN HUMANS

In general, a reference for the current caloric contribution of food was determined by S ánchez-Peña *et al*[12]. This reference includes the inputs of carbohydrates 4 kcal/g, lipids 7 kcal/g, and proteins 4 kcal/g, and the input of alcohols 7 kcal/g was recently added. In recent years, it has been recommended to display these values in joules, *i.e.*, carbohydrates 17 kJ/g, lipids 37 kJ/g, proteins 17 kJ/g, and alcohols 29 kJ/g. One calorie is approximately 4.186 J, and there is a slight difference between the cal display and the J display. The standards are set by the national institutions of each country. There are differences between countries regarding items, but there are no differences in the basic amount of heat. The Food and Agriculture Organization has proposed adding dietary fiber to the calorie calculation, and 2 kcal/g and 8 kJ/g have been adopted in countries that add dietary fiber calories to the calculation[13]. Dietary fiber has fewer calories than carbohydrates because watery dietary fiber is calculated as calories. However, insoluble dietary fiber, which is the main component of plant cell walls, is not used in calorie calculations.

We further considered how much dietary fiber actually becomes calories. We examined the extent to which dietary fiber becomes calories. Dietary fiber that is fermented is generally considered to be soluble dietary fiber. However, since bacteria that degrade insoluble dietary fiber, such as cellulose, also exist in humans, as described above, insoluble dietary fiber is slightly degraded by fermentation in the intestinal tract of humans. The caloric content of cellulose was reported to be 4.16 kcal/g[14]. However, it is estimated that there will be large individual differences in the acquisition of calories based on not only whether the cellulose is raw or cooked but also the influence of cell wall strength, the intestinal flora and intestinal residence time. Elia and Cummings[15] suggested that 70% of dietary fiber is fermented. A total of

Table 1 Enterobacteria that break down fibers derived from cell walls

Family	Genus		Species	Putative effects	Horse		Human	
						Ref.		Ref.
Acidaminococcaceae	*Phascolarctobacterium*	spp.		Fiber fermenters	+	[21]	+	[22]
Bacteroidaceae	*Bacteroides*	spp.		Plant wall degradation	+	[7]	+	[7]
			ovatus	Polysaccharide decomposition; Plant wall degradation	N/A		+	[9]
			thetaiotaomicron	Complex polysaccharide decomposition	N/A		+	[9]
Clostridiaceae	*Clostridium*	spp.		Cellulolytic, fibrolytic	+	[8]	+	[23]
Eubacteriaceae	*Eubacterium*	spp.		Cellulolytic, fibrolytic	+	[8]	+	[23]
Fibrobacteraceae	*Fibrobacter*	spp.		Plant wall degradation	+	[7]	N/A	
			intestinalis	Plant wall degradation	+	[24]	N/A	
Lachnospiraceae	*Butyrivibrio*	spp.		Cellulolytic, fibrolytic	+	[8]	+	[25]
	Blautia	spp.		Fiber fermenters	+	[26]	+	[27]
Prevotellaceae	*Prevotella*	spp.		Fiber fermenters	+	[26]	+	
			copri	Fiber fermenters	N/A		+	[28]
Ruminococcaceae	*Ruminococcus*	spp.		Cellulolytic, fibrolytic bacteria	+	[8]	+	
			albus	Plant wall degradation	+	[8]	+	[29]
			bromii	Plant wall degradation	N/A		+	[30]
			champanellensis sp.nov.	Plant wall degradation	N/A		+	[31]
			flavefaciens	Plant wall degradation	+	[32]	+	[33]
			sp.nov.	Plant wall degradation	N/A		+	[34]

Based on the intestinal flora of horses compiled by Kauter *et al*[8], we selected microorganisms that decompose cell walls and summarized whether they exist in the human large intestine. Several bacterial species reported in humans as cell wall-degrading bacteria are added to the Table. N/A: Not available.

51.0% of the energy initially present is lost through feces, and 3.5% is lost through H_2 and CH_4 gaseous products. It is assumed that the remaining 45.5% is energy that can be absorbed and metabolized by human tissues[15]. Based on this calculation, the calories of dietary fiber were set to approximately 2 kcal/g and 8 kJ/g.

In the 1980s, when it was thought that cellulose could not be digested by humans, tests were conducted on humans using radioactive isotopes to examine the degradation of cellulose. ^{14}C-cellulose was given to each of 10 healthy subjects, and stool and breath samples were collected for 7 d. A wide range of variations in ^{14}C recovery were recorded, with 57% in feces, 16% in respiration as $^{14}CO_2$. The total recovery rate of ^{14}C was reported to be 73%[16]. Some of the $^{14}CO_2$ recovered by respiration was metabolized by intestinal bacteria and absorbed through the large intestinal mucosa. The remaining 27% did not recover and may have been incorporated into the body. At the time, it was suspected that the study did not use pure cellulose but used cellulose containing starch. However, it has since been proven that cellulose is degraded by gut bacteria. Therefore, this study is considered to provide valuable human data. The wide range of variations in ^{14}C recovery may be related to differences in intestinal bacteria and different lengths of stay in the gastrointestinal tract. This study showed that 27%-43% of cellulose could have been used by humans. As mentioned above, the actual cell wall is made of more than indigestible cellulose alone, and it is estimated that the actual amount utilized by humans is below this value. Therefore, it is understandable that the cell wall, which is classified as water-insoluble dietary fiber, is not added to the caloric calculation.

Ninety to ninety-five percent of the SCFAs produced in the large intestine by the fermentation of dietary fiber, including watery dietary fiber, are absorbed by the large intestine[17]. It has been reported that SCFAs absorbed in the colon contribute 6% to

10% of the total energy requirement of humans and that this contribution is probably increased in humans who consume more fiber[18,19]. Currently, the United States Food and Drug Administration (FDA) has set the recommended daily intake (RDA) of fiber at 38 g/d and 25 g/d for healthy men and women, respectively, aged 19-50 years. However, most Americans are reportedly not consuming the recommended dietary fiber intake. In other words, even if the encouraged fiber intake is met and is completely converted into energy, it amounts to approximately 150-100 kcal. Therefore, the proportion of dietary fiber in the total amount of energy in the current diet is minimal [20].

CONCLUSION

Water-insoluble dietary fiber derived from plant cell walls is considered to be indigestible dietary fiber, and it is difficult to ferment and degrade. However, many species of bacteria present in the intestines of herbivores are also found in the digestive tract of humans. Therefore, it is possible that the dietary fiber derived from plant cell walls is degraded to some extent, even in the digestive tract of humans, and used as energy. Since the amount of dietary fiber in the total food intake is minimal, the calorie intake is minimal. The main reason why it is difficult to degrade dietary fiber derived from plant cell walls is that cellulose, which is difficult to degrade, is in a firmly solidified state integrated with hemicellulose, pectin, lignin, suberin, and other components. If cellulose can be separated from these other components and degraded, it is possible that plant cell walls can contribute more calories in humans than when cellulose is combined with the other cell wall components. The cell wall contains a large amount of potential energy. Thus, if the amount of energy utilized from the cell wall by cell wall-degrading bacteria can be increased, the food situation in food-deficient areas can be expected to improve. Currently, bioethanol is being developed, and its dietary use in the degradation of cell walls is anticipated.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 7; 27(45): 7792-7800

DOI: 10.3748/wjg.v27.i45.7792

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

MINIREVIEWS

Gut microbiome in allogeneic hematopoietic stem cell transplantation and specific changes associated with acute graft *vs* host disease

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Author contributions: Le Bastard Q, Chevallier P, and Montassier E have been involved equally and have read and approved the final manuscript; all authors meet the criteria for authorship established by the International Committee of Medical Journal Editors and verify the validity of the results reported.

Conflict-of-interest statement: There is no conflict of interest associated with any of the senior author or other coauthors who contributed their efforts in this manuscript.

Country/Territory of origin: France

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

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Abstract

Allogeneic hematopoietic stem cell transplantation (aHSCT) is a standard validated therapy for patients suffering from malignant and nonmalignant hematological diseases. However, aHSCT procedures are limited by potentially life-threatening complications, and one of the most serious complications is acute graft-versus-host disease (GVHD). During the last decades, DNA sequencing technologies were used to investigate relationship between composition or function of the gut microbiome and disease states. Even if it remains unclear whether these microbiome alterations are causative or secondary to the presence of the disease, they may be useful for diagnosis, prevention and therapy in aHSCT recipients. Here, we summarized the most recent findings of the association between human gut microbiome changes and acute GVHD in patients receiving aHSCT.

Key Words: Gut microbiome; DNA sequencing technologies; Allogeneic hematopoietic stem cell transplantation; Transplants; Acute graft *vs* host disease; Biomarkers; Composition; Function

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Core Tip: This review reports the compositional and functional changes in gut microbiome of allogeneic hematopoietic stem cell transplantation recipients associated with acute graft-versus-host disease that could serve a biomarker for diagnosis and

Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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Received: April 20, 2021
Peer-review started: April 20, 2021
First decision: July 27, 2021
Revised: August 5, 2021
Accepted: November 20, 2021
Article in press: November 20, 2021
Published online: December 7, 2021

P-Reviewer: Hegazy MAE, Sameer S
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

prevention in patients receiving allogeneic hematopoietic stem cell transplantation.

Citation: Le Bastard Q, Chevallier P, Montassier E. Gut microbiome in allogeneic hematopoietic stem cell transplantation and specific changes associated with acute graft *vs* host disease. *World J Gastroenterol* 2021; 27(45): 7792-7800
URL: https://www.wjgnet.com/1007-9327/full/v27/i45/7792.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i45.7792

INTRODUCTION

In human health, DNA sequencing technologies, including 16S rRNA gene-based amplicon sequence analysis and whole genome shotgun metagenomic analysis were used to discover how exogenous and intrinsic host factors influence gut microbiome composition[1,2]. In large cohorts, scientists investigated the impact of factors such as lifestyle, dietary information, anthropometrics and drugs on the gut microbiome communities[3,4]. They found that age, gender, dietary factors and intrinsic parameters were highly correlated with composition and function of the gut microbiome. They also observed that several drug categories, such as antibiotics, proton-pump, metformin, statins, and laxatives, had a strong effect on the gut microbiome. On the other hand, gut microbiome can affect the bioavailability of oral drugs[5]. Thus, microorganisms can impact drug absorption and metabolism, that may explain, in part, inter-individual heterogeneity in drug response and disposition[6].

In addition, DNA sequencing technologies were used to investigate associations between composition or function of the gut microbiome and various disease states. Studies performed cross sectional comparisons between subjects with and without disease, and reported changes in the gut microbiome of patients with inflammatory bowel diseases, colorectal cancer, diabetes, obesity and metabolic disease, or autoimmune diseases compared to controls[7]. Even if it remains unclear whether these microbiome alterations are causative or secondary to the presence of the disease, gut microbiome could be viewed as a diagnostic biomarker[8].

Allogeneic hematopoietic stem cell transplantation (aHSCT) is a standard validated therapy, which every year allows an increasing number of patients suffering from malignant and nonmalignant hematological diseases, to benefit from an HSC transplant. The donor can be related (called geno-identical if HLA matched or haploidentical if sharing only one haplotype) or unrelated (called pheno-identical if HLA matched and including cord blood). However, aHSCT procedures are limited by potentially life-threatening complications, and one of the most serious complications is acute graft-versus-host disease (GVHD). GVHD occurs when immune competent T cells in the donated tissue recognize the recipient as foreign[9], that induces tissue damages in target organs of the host[10], including skin, liver and the gut, and typically occurs 3 to 6 wk after transplantation in nearly 60% of related donor transplants and 80% of unrelated donor transplants[11]. Hahn *et al*[12] assessed acute GVHD risk factors in 1960 adults after HLA-identical sibling myeloablative transplant in 226 centers worldwide. They found that cyclophosphamide and total-body irradiation, blood cell *vs* bone marrow grafts in patients age 18 to 39 years, recipient age 40 and older, chronic myeloid leukemia, Karnofsky performance score less than 90, and recipient/donor cytomegalovirus-seronegative were independent factors significantly associated with grade 2 to 4 acute GVHD[12].

Based on preclinical data demonstrating reduced acute GVHD severity in germ-free or antibiotic-treated mice (*i.e.*, gut microbiome with reduced diversity and richness), the gut microbiome was suggested to play a pivotal role in the pathogenesis of acute GVHD following aHSCT[13]. These findings are in contradiction with the common paradigm that loss of microbiome diversity is associated with diseases[14]. Most recent studies, based on microbiome sequencing, however, reported that higher diversity of intestinal microbiota at the time of neutrophil engraftment was associated with lower mortality in patients undergoing allogeneic hematopoietic-cell transplantation[15].

Here, we summarized the most recent findings of the association between human gut microbiome changes and graft *vs* host disease of the intestinal tract in patients receiving aHSCT.

ALTERATIONS OF THE GUT MICROBIOME DURING aHSCT

Several studies reported the profound alteration of the gut microbiota during the aHSCT procedure, as summarized in Table 1. Following various conditioning regimens, both myeloablative or of reduced-intensity, a recent 16S rRNA gene-based amplicon sequence analysis that profiled 8767 fecal samples from 1362 patients undergoing aHSCT at four different centers observed a significant gut microbiota disruption characterized by loss of diversity and domination by single taxa[15], defined as occupation of at least 30% of the gut microbiota by a single predominating bacterial taxon. They also identified an association between lower intestinal diversity and higher risks of transplantation-related death. Moreover, samples collected prior transplantation also showed evidence of microbiome disruption, and lower diversity before transplantation was associated with poor survival. In another study, mortality outcomes were significantly worse in patients with lower intestinal diversity, with an overall survival at 3 years of 36% in low gut microbiome diversity patients compared to 67% in high diversity groups. Overall, low diversity showed a strong effect on mortality after multivariate adjustment for other clinical predictors. Furthermore, in subjects with lower diversity, the gut microbiota was generally dominated by a single bacterial genus, including *Enterococcus*, *Streptococcus*, Enterobacteriaceae (*Escherichia* and *Kluyvera*), and *Lactobacillus*[16].

Taur *et al*[16] analyzed a total of 439 fecal specimens obtained from 94 patients during their transplant hospitalization and reported that over the course of aHSCT, gut microbiome diversity index decreased and remained low until the end of the observation period (Day 35 post-transplant). Patients also demonstrated significant changes in gut microbiome composition, and in most patients, the microbial composition became dominated by a single bacterial taxon: *Enterococcus* (40% of the patients), Streptococcus (37%) and the phylum Proteobacteria (13%). Interestingly, they demonstrated that patients with enterococcal domination in the gut had a 9-fold increased risk of Vancomycin Resistant *Enterococcus* bacteremia, and intestinal domination by Proteobacteria increased the risk of bacteremia with aerobic gram-negative bacilli 5-fold[17]. In a recent study using metagenomic shotgun, able to achieve bacterial identification to species and strain level, Ilett *et al*[10] reported that, in addition to a significant loss of gut microbiota diversity, post-aHSCT samples were enriched in *Staphylococcus*, *Eggerthella*, *Streptococcus*, *Enterococcus* and *Lactobacillus* compared to pre-aHSCT samples, and several samples were dominated by a single micro-organism [*Enterococcus* (n = 41 of 112) or *Streptococcus* (n = 10 of 112)]. At species level, they observed an enrichment of *Enterococcus faecium*, *Lactobacillus delbrieckii*, *Staphylococcus epidermidis*, and *Streptococcus thermophilus* in the post-aHSCT samples compared to pre-aHSCT samples[10].

In patients receiving intensive chemotherapy regimen used as myeloablative conditioning treatment to prepare patients for aHSCT, gut microbiota after chemotherapy exhibited significant decrease in Firmicutes and Actinobacteria, and significant increase in Proteobacteria compared to samples collected before chemotherapy. At the genus level, gut microbiota after chemotherapy was significantly depleted in *Ruminococcus*, *Oscillospira*, *Blautia*, *Lachnospira*, *Roseburia*, *Dorea*, *Coprococcus*, *Anaerostipes*, *Clostridium*, *Collinsella*, *Adlercreutzia* and *Bifidobacterium*, and with significant increase in *Citrobacter*, *Klebsiella*, *Enterococcus*, *Megasphaera* and *Parabacteroides* compared with samples collected before chemotherapy. In addition, functional composition assessed using PICRUSt[18] revealed that following conditioning regimen, patients had reduced capacity for nucleotide metabolism, energy metabolism, metabolism of cofactors and vitamins, and increased capacity for glycan metabolism, signal transduction and xenobiotics biodegradation[19].

Thus, overall, aHSCT procedure is associated with a loss of gut microbiota diversity, a decrease in micro-organisms associated with health-promoting effects[20], and with a significant increase or domination by potentially pathobionts (Figure 1).

ALTERATIONS OF THE DIVERSITY OF THE GUT MICROBIOME AND ACUTE GVHD

Studies reported that decreased gut microbiota diversity and richness was associated with the onset of acute intestinal GVHD. Jenq *et al*[21], in 2015, reported in a cohort of 115 patients receiving aHSCT, that increased bacterial diversity was associated with reduced GVHD-related mortality[21]. Moreover, in their recent 16S rRNA gene-based amplicon sequence analysis, Peled *et al*[15] found that higher intestinal diversity was

Table 1 Gut microbiome diversity, composition and function changes in gut microbiota during allogeneic hematopoietic stem cell transplantation procedure

Ref.	Sequencing technology	Change in diversity	Changes in composition	Change in functions
Peled *et al* [15]	16S rRNA gene-based amplicon sequence	Loss of diversity	Gut microbiota dominate by single taxa (occupation of at least 30% of the gut microbiota by a single predominating bacterial taxon), including *Enterococcus*, *Streptococcus*, Enterobacteriaceae (*Escherichia* and *Kluyvera*), and *Lactobacillus*	
Taur *et al* [16]	16S rRNA gene-based amplicon sequence	Decrease of the gut microbiome diversity index	Gut microbiota composition frequently dominated by a single bacterial taxon, including *Enterococcus*, *Streptococcus* or Proteobacteria	
Ilett *et al*[10]	Metagenomic shotgun	Loss of gut microbiota diversity	Post-aHSCT samples enriched in *Staphylococcus*, *Eggerthella*, *Streptococcus*, *Enterococcus* and *Lactobacillus* compared to pre-aHSCT samples, &t species level enrichment in *Enterococcus faecium*, *Lactobacillus delbrieckii*, *Staphylococcus epidermidis*, and *Streptococcus thermophilus*	
Montassier *et al*[19]	16S rRNA gene-based amplicon sequence	Loss of gut microbiota diversity	Decreases in abundances of Firmicutes and Actinobacteria, and significant increases in abundances of Proteobacteria	Reduced capacity for nucleotide metabolism, energy metabolism, metabolism of cofactors and vitamins, and increased capacity for glycan metabolism, signal transduction and xenobiotics biodegradation

HSCT: Hematopoietic stem cell transplantation.

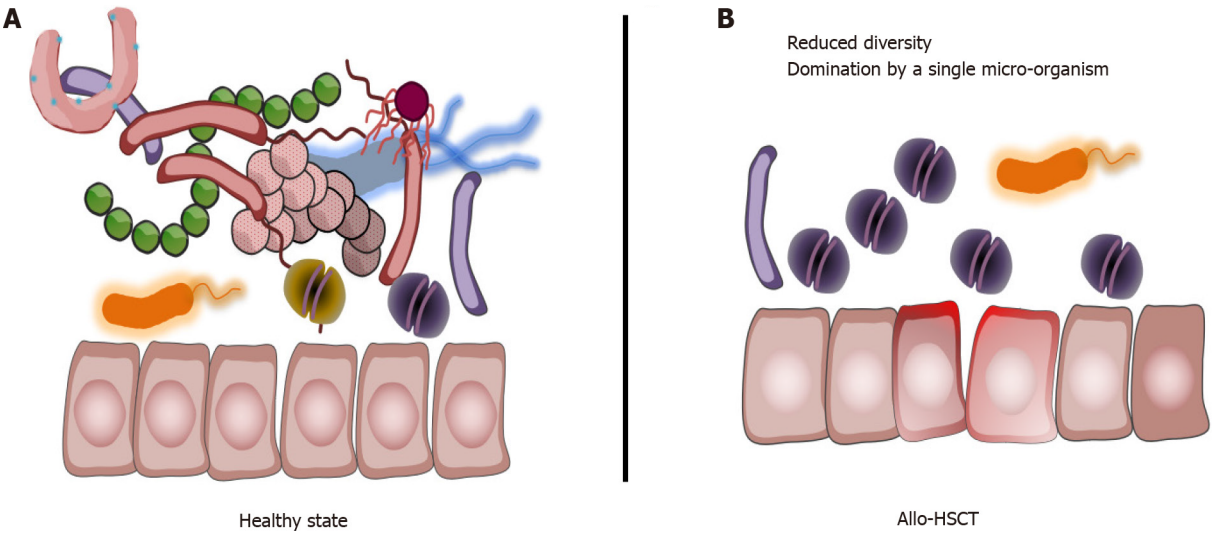


Figure 1 Gut microbiota changes during the allogeneic hematopoietic stem cell transplantation procedure. A: Healthy state with diverse and rich gut microbiota; B: Following conditioning regimen, gut microbiome alterations are marked by a drastic loss of diversity, a significant decrease in micro-organisms associated with health-promoting effects, and with a significant increase or domination, defined as occupation of at least 30% of the microbiota by a single predominating bacterial taxon, by potentially pathobionts. Allo-HSCT: Allogeneic hematopoietic stem cell transplantation.

associated with decreased risk of deaths attributable to GVHD (17 GVHD-related deaths among 244 patients in the higher-diversity group *vs* 26 such deaths among 184 patients in the lower-diversity group; hazard ratio, 0.49; 95%CI: 0.26-0.90)[15].

In a cohort of 44 patients, in which 16 (36%) experienced acute intestinal GVHD (median time to diagnosis: 53 d), Galloway-Peña *et al*[22] found that lower Shannon diversity index, popular diversity index in the ecological literature, of fecal samples collected at the time of engraftment was significantly associated with increased incidence of acute GVHD[22]. Golob *et al*[23] also found that diversity was statistically significantly lower in patients with acute GVHD when compared to those with no acute GVHD[23]. In another cohort of 57 patients, Liu *et al*[24] reported that decreased gut microbiota recipients' diversity was not associated with decreased risk of aGVHD. However, they found that high gut microbiota donor diversity was associated with

decreased risk of acute GVHD[24]. In a cohort of 70 patients, Payen *et al*[25] reported that patients with severe aGVHD had reduced gut microbiota diversity at disease onset, whereas patients with mild aGVHD had gut microbiota diversity more similar to those of controls[25].

Ilett *et al*[10] applied shotgun metagenomic sequencing to study a large cohort of adults (n = 150) undergoing aHSCT. Among them, 36 developed acute GVHD (median time to development: 34 d (interquartile range, 26-50 d post-aHSCT). They did not find significant association between diversity measures and acute GVHD in samples collected from the pre-aHSCT period. However, in samples collected in the early post-aHSCT period, patients who later developed acute GVHD had a significantly lower gene richness compared with those who did not develop acute GVHD[10].

Altogether, these findings clearly associate decreased diversity and richness of the gut microbiota, known to be associated with enhanced inflammation and impaired immunity[26], to onset of acute GVHD (Table 2).

COMPOSITION CHANGES IN GUT MICROBIOME AND ACUTE GVHD

Using a 16S rRNA-based sequencing analysis, Jenq *et al*[21] reported that genus *Blautia* were most significantly associated with reduced GVHD-related mortality, whereas genus *Veillonella*, was associated with increased GVHD-related mortality[21].

Holler *et al*[27] reported that post-transplant samples were increased in enterococci, and that the increase was more pronounced in patients developing acute GVHD compared to patients who did not develop aGVDH. They confirmed this trend using enterococcal polymerase chain reaction (PCR) and observed a predominance of *Enterococcus faecium* and *Enterococcus faecalis* in samples collected in patients who developed acute GVHD. Moreover, post-transplant samples collected in patients with acute GVHD were significantly depleted in Clostridia and *Eubacterium rectale*[27]. Stein-Thoeringer *et al*[28] also reported that fecal domination by *Enterococcus* (*i.e.*, relative genus abundance ≥ 30% in samples collected during early post-transplant period) was associated with increased risk of acute GVHD, and increased GVHD-related mortality [28]. Importantly, in a in gnotobiotic model, the authors demonstrated that *Enterococcus* growth is dependent on disaccharide lactose, and that dietary lactose depletion attenuates *Enterococcus* outgrowth and reduces the severity of GVHD[28].

In their cohort of 44 patients, Galloway-Peña *et al*[22] reported that only one taxon at the time of engraftment, *Coriobacteriia*, a class of Gram-positive bacteria within the Actinobacteria phylum, was negatively correlated with the incidence of aGVHD[22]. In another cohort of 107 patients, Doki *et al*[29] reported patients who developed acute GVHD exhibited a significantly higher abundance of phylum Firmicutes than patients who did not develop aGVHD[29].

Golob *et al*[23] found in a cohort of 66 patients, that in samples collected at neutrophil recovery post-HCT, the presence of Actinobacteria and Firmicutes was positively correlated with subsequent acute GVHD, whereas Lachnospiraceae were negatively correlated. In detail, *Butyricicoccus*, *Bacteroides luti*, *Bacteroides thetaiotaomicron*, *Bacteroides ovatus*, and *Bacteroides caccae* were negatively correlated with subsequent acute severe GVHD, while *Rothia mucilaginosa*, *Solobacterium moorei*, *Veillonella parvula*, and *Bacteroides dorei* were positively correlated[23]. Payen *et al*[25] reported that *Lachnoclostridium*, *Blautia*, *Sellimonas*, *Anaerostipes*, *Faecalibacterium*, *Flavonifractor*, *Erysipelatoclostridium* and *Lactococcus* were negatively associated with subsequent acute severe GVHD, whereas *Prevotella* and *Stenotrophomonas* were considered positive biomarkers of severe aGVHD. Moreover, using qPCR, they observed a significant depletion of the *Blautia coccoides* group (cluster XIVa) in patients with aGVHD compared with controls and patients with no aGVHD[25].

Based on a shotgun metagenomic sequencing analysis in 150 patients, Ilett *et al*[10] found that no bacteria were associated with acute GVHD in samples collected during the pre-aHSCT period. In samples collected during the early post-aHSCT period, they found that *Blautia*, *Akkermansia*, and *Campylobacter*, as well as the specific species *Akkermansia muciniphila*, *Blautia obeum*, *Blautia hydrogenotropica*, and *Blautia hansenii* were all significantly associated with reduced risk of a GVHD[10]. Still using shotgun metagenomic sequencing analysis, Turner assessed samples collected in nine patients with aGVHD and treated with standard-of-care high-dose steroids. Three of these patients were steroid-refractory, whereas six had a response. They showed that *Dorea longicatena* was associated with response to high-dose steroids treatment whereas *Akkermansia muciniphila* was associated with refractoriness. They also reported that maintenance of a stable *Dorea/Akkermansia* ratio predicted steroid response, whereas a

Table 2 Gut microbiome diversity, composition and function changes in patients receiving allogeneic hematopoietic stem cell transplantation procedure and developing acute graft versus host disease

Ref.	Sequencing technology	Change in diversity	Changes in composition	Change in functions
Ilett *et al*[10] (150 patients)	Shotgun metagenomic	Decrease in gene richness during the early post-aHSCT period in patients with aGVHD	Decrease in *Akkermansia muciniphila*, *Blautia obeum*, *Blautia hydrogenotropica*, and *Blautia hansenii* during the early post-aHSCT period in patients with aGVHD	Increase in toxin named PetZ, that triggers bacterial autolysis in pathological bacteria during the pre-aHSCT and the early post-aHSCT period in patients with aGVHD
Holler *et al*[27] (31 patients)	16S rRNA V3 sequencing		Increase in enterococci in patients who subsequently developed acute GVHD	
Galloway-Peña *et al*[22] (44 patients)	16S rRNA V4 sequencing	Lower Shannon diversity index in fecal samples collected at the time of engraftment in patients with subsequent aGVHD	*Coriobacteriia* negatively correlated with the incidence of acute GVHD	Fecal metabolites (Fecal indole and butyrate levels determined using liquid chromatography tandem mass spectrometry) associated with acute GVHD
Liu *et al*[24] (57 patients)	16S rRNA V4 sequencing	High gut microbiota donor diversity associated with decreased risk of aGVHD in recipient		
Doki *et al*[29] (107 patients)	16S rRNA V4 sequencing		Higher abundance of phylum Firmicutes in samples collected before aHSCT in patients with acute GVHD	
Golob *et al*[23] (66 patients)	16S rRNA V3-V4 sequencing	Diversity significantly lower in patients with GVHD	*Butyricicoccus*, *Bacteroides luti*, *Bacteroides thetaiotaomicron*, *Bacteroides ovatus*, and *Bacteroides caccae* negatively correlated with subsequent acute GVHD, *Rothia mucilaginosa*, *Solobacterium moorei*, *Veillonella parvula*, and *Bacteroides dorei* positively correlated with subsequent onset of GVHD	
Michonneau *et al*[31] (99 patients)	Metabolomics			Significant decrease in tryptophan metabolites, including microbiota-produced compounds, such as 3-indoxyl sulfate, indoleacetate, indoleacetylglutamine, and indolepropionate in patients with aGVHD
Payen *et al*[25] (70 patients)	16S rRNA V3-V4 sequencing	Decreased diversity in acute GVHD	*Lachnoclostridium*, *Blautia*, *Sellimonas*, *Anaerostipes*, *Faecalibacterium*, *Flavonifractor*, *Erysipelatoclostridium* and *Lactococcus* negatively associated with subsequent acute GVHD	

HSCT: Hematopoietic stem cell transplantation; GVHD: Graft-versus-host disease.

decline in this ratio preceded refractory disease. Importantly, Shono *et al*[30] previously demonstrated in a mouse model an increased in *Akkermansia muciniphila*, a commensal bacterium with mucus-degrading capabilities, raising the possibility that mucus degradation may contribute to murine GVHD[30].

Further studies are needed to confirm or not the controversial role of *Akkermansia* in acute GVHD. Moreover, some species of the genus *Blautia* should be investigated as a potential biomarker: High relative abundance at the time of engraftment being protective against GVHD, while low relative abundance could be considered a risk factor for secondary development of GVHD.

FUNCTION CHANGES IN GUT MICROBIOME AND ACUTE GVHD

The functional alterations of the intestinal microbiome in patients receiving aHSCT are currently poorly described because the majority of studies have used 16S ribosomal RNA gene-based technics, which boil down to providing bacterial taxonomy only at the genus level, but are ineffective in obtaining functional information. In a cohort of 44 patients, Galloway-Peña *et al*[22] reported that fecal metabolites (fecal indole and butyrate levels) were not associated with aGVHD[22]. In another study, Michonneau *et al*[31] reported that aGVHD was characterized by specific metabolomics changes in

two cohorts of patients (n = 99). They found that bile acids, plasmalogens, tryptophan, and arginine metabolites were the main contributors involved, especially the aryl hydrocarbon receptor ligand 3-indoxyl sulfate. In addition to host-derived metabolites, they also identified significant variation in microbiota-derived indole compounds, especially in aryl hydrocarbon receptor ligands. The authors suggested that allogeneic immune response during aGVHD might be influenced by bile acids and by the decreased production of aryl hydrocarbon receptor ligands by gut microbiome that could limit indoleamine 2,3-dioxygenase induction and influence allogeneic T cell reactivity[31]. To assess if altered composition of the gut microbiota may result in an altered metabolome, which potentially disrupts functionalities at the onset of aGVHD, Payen *et al*[25] quantified the Short Chain Fatty Acids (SCFA) content in fecal samples with measurement of total SCFAs and acetate, propionate, and butyrate. They found that the fecal amount of all SCFAs was drastically diminished at aGVHD onset. In detail, they observed that total SCFAs, acetate and butyrate respectively decreased by 80%, 75% and 95% in severe aGVHD patients as compared with controls[25]. Importantly, in a mouse model, Mathewson *et al*[32] demonstrated that butyrate restoration improved intestinal epithelial cells junctional integrity and mitigated aGVHD[32].

Based on a shotgun metagenomic sequencing analysis, Ilett *et al*[10] found that a total of 1267 and 1289 genes were present in significantly different amounts among those who developed aGVHD *vs* those who did not develop aGVHD during pre-HSCT and early post-aHSCT, respectively. Of these genes, 24 overlapped between the 2 time periods, all being significantly higher in abundance among those who did not develop aGVHD. They pointed out that 1 gene (O2.CD1-0-PT_GL0039283) had a 3-log fold-change in both periods and is known to function as a toxin named Petz, that triggers bacterial autolysis in pathological bacteria[10].

CONCLUSION

The investigations described in this mini review provide an understanding of the role of the gut microbiome in the pathophysiology of aGVHD in patients receiving aHSCT. Observational studies have shown that a decrease in diversity of the gut microbiome and specific species or metabolic pathways were associated with aGVHD. These specific changes could serve as biomarkers for diagnosis and prevention in patients receiving aHSCT. Moreover, functional alterations of the gut microbiome during aHSCT should be more investigated so that modulations of the gut microbiome could be tested to prevent this potentially life-threatening complication.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 December 7; 27(45): 7801-7812

DOI: 10.3748/wjg.v27.i45.7801

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

ORIGINAL ARTICLE

Clinical and Translational Research

MicroRNAs expression influence in ulcerative colitis and Crohn's disease: A pilot study for the identification of diagnostic biomarkers

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Author contributions: All authors contributed to this manuscript; Quaglio AEV, Rodrigues AMM, Sassaki LY and Di Stasi LC contributed to the conception of the study; Quaglio AEV and Santaella FJ did the acquisition; Di Stasi LC revised the manuscript critically for important intellectual content and submitted the final approval version; all authors contributed to the interpretation of data, drafting of the article and approved the final version of the article.

Institutional review board statement: This study was approved by the Botucatu Medical School Research Ethics Committee, No. 71379417.2.0000.5411.

Conflict-of-interest statement: There is no conflict of interest.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The

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Abstract

BACKGROUND

Inflammatory bowel disease (IBD) comprises two distinct diseases, Crohn's disease (CD) and ulcerative colitis (UC), both of which are chronic, relapsing inflammatory disorders of the gastrointestinal tract with a mostly unknown etiology. The incidence and prevalence of IBD are continually increasing, indicating the need for further studies to investigate the genetic determinants of these diseases. Since microRNAs (miRNAs) regulate protein translation *via* complementary binding to mRNA, discovering differentially expressed miRNAs (DE) in UC or CD patients could be important for diagnostic biomarker identification, assisting in the appropriate disease differentiation progressing the understanding of IBD pathogenesis.

AIM

To determine the miRNA expression profile in UC and CD patients and the potential pathophysiological contributions of differentially expressed miRNA.

METHODS

A total of 20 formalin-fixed paraffin-embedded colonic samples were collected

authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Supported by São Paulo Research Foundation (FAPESP) grants, No. 2017/03959-8 and No. 2015/15267-8.

Country/Territory of origin: Brazil

Specialty type: Gastroenterology and hepatology

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

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

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Received: April 28, 2021
Peer-review started: April 28, 2021
First decision: June 13, 2021
Revised: June 14, 2021
Accepted: November 20, 2021
Article in press: November 20, 2021
Published online: December 7, 2021

P-Reviewer: Corsello A
S-Editor: Wu YXJ
L-Editor: A
P-Editor: Wu YXJ

from the Pathology Department of Botucatu Medical School at São Paulo State University (Unesp). The diagnosis of UC or CD was based on clinical, endoscopic, radiologic, and histological criteria and confirmed by histopathological analysis at the time of selection. The TaqMan™ Array Human MicroRNA A+B Cards Set v3.0 (Applied Biosystems™) platform was used to analyze 754 miRNAs. Targets of DE-miRNAs were predicted using miRNA Data Integration Portal (mirDIP) and the miRNA Target Interaction database (MiRTarBase). All statistical analyses were conducted using GraphPad Prism software. Parametric and nonparametric data were analyzed using *t*-tests and Mann-Whitney *U* tests, respectively.

RESULTS
The results showed that of the 754 miRNAs that were initially evaluated, 643 miRNAs were found to be expressed in at least five of the patients who were diagnosed with either CD or UC; the remaining 111 miRNAs were not considered to be expressed in these patients. The expression levels of 28 miRNAs were significantly different between the CD and UC patients ($P \leq 0.05$); 13 miRNAs demonstrated a fold-change in expression level greater than 1. Five miRNAs with a downregulated expression were selected for enrichment analysis. The miRNAs whose expression levels were significantly lower in UC patients than in CD patients were enriched in certain signaling pathways that were mostly correlated with cancer-related processes and respective biomarkers.

CONCLUSION
MiRNAs could be used to differentiate UC from CD, and differently expressed miRNAs could help explain the distinct pathophysiology of each disease.

Key Words: Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; miRNA; Differential diagnosis; Biomarker

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Core Tip: This study identified 27 microRNAs (miRNAs) with significantly different expression levels between Crohn's disease (CD) and ulcerative colitis (UC) patients. Five miRNAs whose expression levels were significantly lower in the UC patients than in the CD patients were selected for enrichment analysis, which revealed enrichment in certain signaling pathways that were mostly associated with cancer-related processes. The characterization and comparison of the differentially expressed miRNAs in this study could lead to novel diagnostic biomarkers to differentiate UC from CD. These markers might also predict prognosis, help elucidate the distinct pathophysiology of each disease, and lead to novel therapeutic target identification.

Citation: Quaglio AEV, Santaella FJ, Rodrigues MAM, Sassaki LY, Di Stasi LC. MicroRNAs expression influence in ulcerative colitis and Crohn's disease: A pilot study for the identification of diagnostic biomarkers. *World J Gastroenterol* 2021; 27(45): 7801-7812
URL: https://www.wjgnet.com/1007-9327/full/v27/i45/7801.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i45.7801

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), the two main phenotypes of inflammatory bowel disease (IBD), are chronic, relapsing inflammatory disorders of the gastrointestinal (GI) tract whose etiologies remain unknown[1,2]. Although its pathophysiological mechanisms are unclear, IBD has been linked to an inappropriate and exacerbated immune response to commensal bacteria in genetically susceptible hosts, as well as with environmental factors, defects of the epithelial barrier, and dysregulation of the innate and adaptive immune responses at the level of the intestinal mucosa[1,2].

IBD affects 6 to 8 million people worldwide, with a prevalence of 84.3 people (79.2-89.9) per 100000 in 2017 and a mortality rate of 0.51[3]. The incremental increase in the incidence and prevalence of IBD globally is indicative of the need for further population-based genetic studies of those affected by these diseases. Differences in miRNA expression between these two diseases could lead to the identification and validation of diagnostic biomarkers, facilitating differential diagnosis and improving the understanding of IBD pathogenesis[4].

MiRNAs are a class of small (18-25 nucleotides in length), endogenous, non-coding, single-stranded RNA molecules that can negatively regulate target gene expression at the post-transcriptional level through binding to the 3′untranslated regions of the target mRNAs and promoting mRNA degradation or translational repression[4]. Overall, there is evidence that miRNAs contribute to the regulation of at least one-third of all protein-coding mRNAs, including those involved in the development, metabolism and cell cycle control[5]. Although studies have shown that miRNA plays an important role in both CD and UC and that they are differentially expressed in these disease states, there may have been potential confounding factors that were not considered. For example, the patients in these studies were treated with various medication classes, such as immunosuppressants, corticosteroids, and/or aminosalicylates[5-8]. Therefore, the aim of the present study was to investigate miRNA expression patterns in the tissues of treatment-naïve CD and UC patients to assess changes in miRNAs without the confounding influence of pharmacological agents.

MATERIALS AND METHODS

Ethics statement

The study was approved by the Botucatu Medical School Research Ethics Committee (protocol No. 71379417.2.0000.5411).

Sample collection

A total of twenty formalin-fixed paraffin-embedded (FFPE) colonic samples were collected from patients in the Pathology Department of Botucatu Medical School at São Paulo State University (Unesp). IBD diagnosis was based on clinical, endoscopic, radiologic, and histological criteria[9]. After sample selection, the diagnoses of UC or CD were confirmed by histopathological analysis of the biopsied colonic samples collected from each patient during a diagnostic colonoscopy; therefore, none of the patients received any medication at the time of the examination.

RNA isolation, reverse transcription, pre-amplification, and qRT-PCR analyses

Total RNA from each colon biopsy was isolated using MagMAX™ FFPE DNA/RNA Ultra Kit (Thermo Fisher Scientific, Massachusetts, EUA). For reverse transcription, Megaplex™ Reverse Transcription Primer Pools and Taqman miRNA reverse transcription kits (Thermo Fisher Scientific) were used. Briefly, 3 μL of RNA (1-350 ng) were reverse-transcribed by combining the Megaplex RT Primer (Thermo Fisher Scientific) with the TaqMan® miRNA Reverse Transcription Kit (Thermo Fisher Scientific). A volume of 2.5 μL of the reverse transcription product was used for pre-amplification with Megaplex™ PreAmp Primers (Thermo Fisher Scientific) and TaqMan® PreAmp Master Mix (Thermo Fisher Scientific). For qRT-PCR, 9 μL of the pre-amplified product was mixed with 441 μL of H_2O and 450 μL of TaqMan® Universal PCR Master Mix II without uracil-N-glycosylase (Thermo Fisher Scientific). Subsequently, 100 μL of each sample was loaded into each fill reservoir of the TaqMan microfluidic array cards. Real-time-PCR analysis was performed using a QuantStudio™ Real-Time PCR System (with TaqMan® Array Block) (Thermo Fisher Scientific) with universal cycling conditions [95°C/10 min, then (95°C/15 s, 60°C/60 s) for 40 cycles]. The TaqMan miRNA array output data (.sds files) were uploaded to the ThermoFisher Cloud App (https://www.thermofisher.com/mysso/LoginDisplay) and analyzed *via* the relative quantification (ΔΔCt) method using defined threshold settings for each miRNA. The geometric means of the threshold cycle (Ct) values of three small nucleolar RNAs (snRNAs), RNU44, RNU48, and U6, were used as the endogenous controls. Briefly, the change in quantification cycle (ΔCq) value was calculated as:

Cq (miRNA of interest) - mean Cq (endogenous control).

The ΔΔCq was subsequently calculated as:

ΔCq (miRNA of interest) - mean of ΔCq (miRNA of interest in the reference group - CD).

The relative quantification (Rq or gene expression fold-change) was calculated as $2^{-(\Delta\Delta Cq)}$. Subsequently, the FC was calculated using the Rq and the FC values, and *P* values were log2- and log10-transformed, respectively, and plotted as a volcano plot, displaying the -log 10(*P* value) *vs* the log2 (FC) for each target in the UC group relative to that in the CD group. The volcano plot was generated using an online server (https://paolo.shinyapps.io/ShinyVolcanoPlot/) after defining the statistical cutoffs as a log2 fold-change > 1 and a *P* value < 0.05. A heat map was created using a web tool (http://www.heatmapper.ca/expression/).

Target prediction and gene enrichment analysis

The targets of the DE miRNAs were predicted using miRNA Data Integration Portal (mirDIP) (http://ophid.utoronto.ca/mirDIP/index.jsp)[10,11] and the miRNA Target Interaction database (MirTarBase) (http://miRTarBase.mbc.nctu.edu.tw/)[12]. The combined gene list of each miRNA was uploaded to the Enrichr database (http://amp.pharm.mssm.edu/Enrichr/), a web server for comprehensive gene set enrichment analysis[13,14]. Duplicate susceptibility genes were excluded prior to analysis. The CD and UC susceptibility genes were identified from the National Human Genome Research Institute-European Bioinformatics Institute (NHGRI-EBI) Catalog of human genome-wide association studies (GWAS)[15].

Statistical analysis

The statistical methods of this study were reviewed by Ana E. V. Quaglio from the Laboratory of Phytomedicines, Pharmacology, and Biotechnology (PhytoPharmaTec), Department of Biophysics and Pharmacology, São Paulo State University (Unesp). All statistical analyses were conducted using GraphPad Prism (GraphPad Software, San Diego, CA, United States). To determine if data is from a Gaussian distribution were performed D'Agostino-Person normality test. Parametric and non-parametric data were analyzed using unpaired and two-tailed *t*-tests and Mann-Whitney *U* tests, respectively. Statistical significance was considered for $P \leq 0.05$.

RESULTS

This study included 20 patients with IBD (10 with UC and 10 with CD), with a mean age at the time of diagnosis of 36.1 and 31.6 years for those with UC and CD, respectively. In terms of sex, 40% of the UC and 30% of the CD patients were male, and 60% and 70% of the UC and CD patients were female, respectively. Other characteristics of the patients are presented in Table 1.

In this study, 377 miRNAs and four controls were analyzed in each plate, totalizing 754 miRNAs of interest. Of these, 643 miRNAs were expressed in at least five patients diagnosed with UC or CD, whereas 111 miRNAs were not considered to be expressed in these patients. The expression levels of 27 miRNAs significantly differed between the CD and UC patients. Relative to the expression levels in CD patients, the five miRNAs that were downregulated in UC patients with a fold-change greater than 1 were selected for the subsequent enrichment analysis: miR-192-3p/5p, miR-378a-3p/5p, and miR-429 (Figure 1).

Target prediction and gene enrichment analysis of miRNAs

To assess the potential functions of miRNAs in UC or CD, the targets of the differentially expressed miRNAs (Supplementary Table 1) were predicted using an online database. Relative to the expression levels in CD patients, the miRNAs that were decreased in UC patients were those that were enriched in signaling pathways, such as forkhead box protein O (FOXO), transforming growth factor-beta (TGF-β), and mitogen-activated protein kinase (MAPK), as well as in pathways associated with cancer, particularly colorectal cancer (CRC) (Table 2).

Several of the miRNA gene targets have been previously flagged as CD or UC susceptibility genes. Using the IBD susceptibility gene list from the 2019 GWAS[17], the miRNA targets that had already been linked to one of the two diseases were identified. There are 619 and 474 genes that have been proposed as CD and UC susceptibility genes, respectively. Of the predicted targets of the miRNAs that were downregulated, 24 and 11 overlapped with the proposed CD and UC susceptibility genes, respectively. A total of 328 genes were common to both diseases, and 54 were common to the two diseases and the predicted targets (Figure 2 and Supplementary Table 2).

Table 1 Demographic characteristics and clinical features of inflammatory bowel disease patients

	Ulcerative colitis	Crohn's disease
Number of patients	10	10
Age at onset (yr, mean)	36.1 ± 18.31	31.6 ± 14.6
Sex, (%)		
Male	40	30
Female	60	70
Race, (%)		
Caucasian	100	90
Non-caucasian	0	10
Alcoholism, (%)	10	20
Smoking, (%)	20	10
Family history of IBD, (%)	10	0
Site of UC, (%)		
Proctitis	20	-
Left-sided	30	-
Extensive	50	-
Site of CD, (%)		
Ileal	-	20
Colonic	-	20
Ileocolonic	-	60

IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease.

DISCUSSION

MiRNAs are short single-stranded, post-transcriptional regulatory RNAs that are involved in major cellular processes. The mature miRNA species may be derived from either the 5′ or 3′ arms of the precursor duplex and are referred to as the miRNA-5p and -3p species, respectively. Initially, it was believed that only one of these strands was functional and that the other strand, known as miRNA*, was destined for degradation[16]. However, recent reports have indicated that both the miRNA and miRNA* species often co-exist, and both are functional[16,17]. Based on this finding, the miRNA-5p and -3p nomenclature are used solely based on the 5′- or 3′-arm derivation of the miRNA species[16]. This study described, for the first time, the co-existence of miR-192-3p and miR-192-5p and, miR-378a-3p and miR-378a-5p in the colonic samples of IBD patients. In addition to miR-429, the expression levels of both miRNAs were decreased in UC compared with CD patients.

Most of the altered pathways identified following the enrichment analysis are related to cancer processes, alterations in the extracellular matrix (ECM), inflammatory mediators like TGF-β, members of the heat shock protein (HSP) family, and MAPKs. The decrease in the expression of miRNAs related to all these pathways leads to dysregulation. Proteoglycans are important components of the ECM that have multiple functions depending on both their protein and carbohydrate constituents[18]. Heparan sulphate proteoglycans on the cell membrane may play diverse roles in cancer-related processes, acting as either inhibitors or promoters of tumor progression depending on the tumor type and stage of progression[19]. In CRC, for example, the cell surface proteoglycan syndecan-2 (SDC2 gene) is upregulated, leading to increased cell migration[20]. Moreover, high SDC2 expression was shown to be related to tumorigenic behaviors mediated through the regulation of cell adhesion, proliferation, and migration[21]. Recently, chronic inflammatory hypoxia-mediated SD2 expression was reported to be correlated with CRC development[21]. In addition, acute inflammation could also induce SDC2 expression predominantly in the proximal colon, indicating its potential as a biomarker for acute colonic inflammation[22].

Table 2 Gene enrichment analysis of decreased microRNAs in ulcerative colitis patients compared with Crohn's disease patients

Pathway	*P* value	FDR
Proteoglycans in cancer	2.27E-11	6.99E-09
FoxO signaling pathway	2.37E-11	3.64E-09
Pathways in cancer	7.49E-11	7.69E-09
Colorectal cancer	1.25E-07	5.51E-06
TGF-β signaling pathway	1.47E-07	4.53E-06
Signaling pathways regulating pluripotency of stem cells	4.72E-07	1.32E-05
Autophagy	5.58E-07	1.32E-05
ErbB signaling pathway	9.95E-07	2.19E-05
mTOR signaling pathway	3.70E-06	7.61E-05
MAPK signaling pathway	1.66E-05	2.22E-04

FoxO: Forkhead box protein O; TGF: Transforming growth factor-β; ErbB: Erythroblastic leukemia viral oncogene homolog; mTOR: Mammalian target of rapamycin; MAPK: Mitogen-activated protein kinase; FDR: False discovery rate-adjusted.

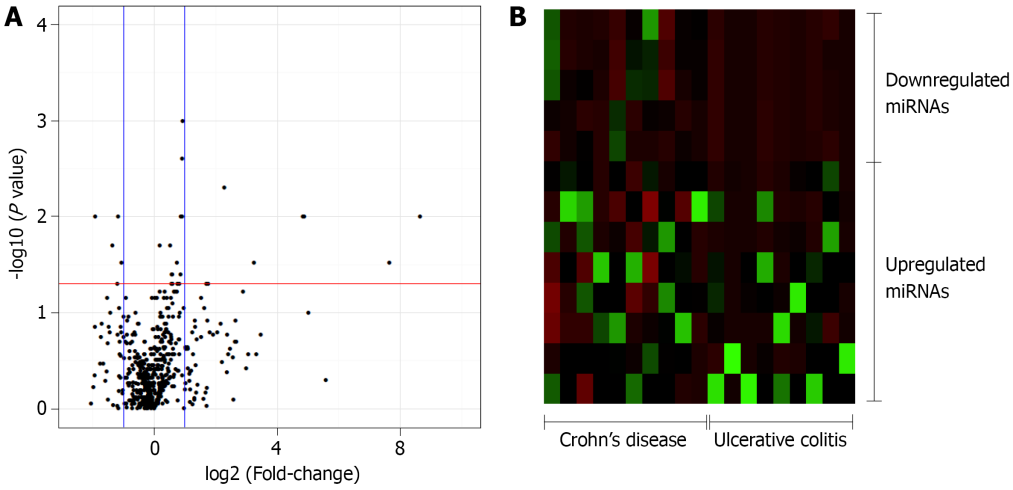


Figure 1 Volcano plot and heat map of the patients. A: Volcano plot of the 754-microRNA (miRNA) analyzed in inflammatory bowel disease patients. Control group: Crohn's disease (CD). Threshold x: fold change = 1; Threshold y: $P \leq 0.05$; B: Heat map of the 13 significantly different miRNA between the CD and ulcerative colitis patients with a fold-change in expression level greater than 1 relatively to the expression levels in CD patients (8 upregulated and 5 downregulated). miRNA: MicroRNA.

In the present study, SDC2 was predicted to be regulated by miR-429, which was proven to be related to cancer progression and metastasis, likely due to dysregulated SDC2 expression, increasing cell migration and proliferation. It has been reported that miR-429 becomes downregulated in esophageal squamous carcinoma cells, and its expression could predict poor prognosis for patients[23]. Moreover, they showed that miR-429 inhibited cellular proliferation through the nuclear factor kappa B (NF-κB) pathway and inhibited cell migration-mediated epithelial-mesenchymal transition (EMT) processes[23].

In nasopharyngeal carcinoma, miR-429 functions as a tumor suppressor by downregulating talin-1 (TLN1), a protein that enhances the migration and invasion of various carcinomas[24]. This miRNA is also related to an antimetastatic function, as it can regulate the metastasis of hepatocellular carcinoma by directly targeting Crk-like protein, which is involved in processes related to EMT, as well as the progression, development, invasion, metastasis, and apoptosis of a variety of cancers[25]. Most functional studies have reported that miR-429 plays an oncogenic role in CRC, and its expression is downregulated as the disease progresses[26,27]. In studies involving a dextran sodium sulphate-induced model of intestinal inflammation, miR-429 was downregulated[28], corroborating the findings of the present study. Others have

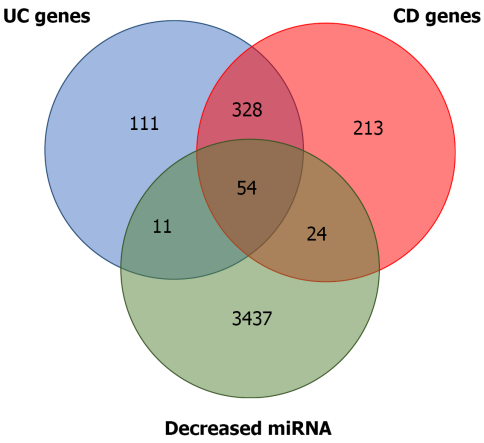


Figure 2 Overlap between predicted microRNA targets for microRNA differentially expressed and ulcerative colitis and Crohn's disease susceptibility genes. Detailed information is provided in Supplementary material 1. miRNA: MicroRNA; UC: Ulcerative colitis; CD: Crohn's disease.

reported that miR-429 modulates mucin secretion in human CRC cells and mouse colon tissues *via* upregulation of myristoylated alanine-rich protein kinase C substrate expression confirming that miR-429 is a candidate for intestinal anti-inflammatory therapy in human UC[28].

Another family of proteins with an important correlation with miRNA function is the HSP family. HSPs comprise several classes of constitutively active and/or stress-induced molecular chaperones that assist in proper polypeptide folding, the refolding of denatured proteins, protein transport, and stabilization of native protein structures, and numerous HSPs are overexpressed in inflamed tissues[29]. A recent review by Hoter & Naim (2019)[30] that analyzed the roles of HSPs in IBD found accumulating evidence linking the upregulation of HSP90 and/or HSP70 expression to the pathogenesis of IBD in the intestinal mucosa of patients with UC at the time of diagnosis, with the expression levels decreasing following the initiation of pharmacological therapy. Similar results were reported in a pre-clinical model of intestinal inflammation in which the colonic expression of HSP70 increased following the induction of intestinal inflammation[31]. Several authors have reported that HSPs are subject to post-transcriptional regulation by miRNAs[32]. The selected downregulated miRNAs found in the UC patients in the present study are predicted to regulate several members of the HSP70 family, such as HSPA1B and HSPA2, as well as HSP90B1, a member of the HSP90 family that plays critical roles in the folding of proteins such as Toll-like receptors and integrins[33], suggesting that the decrease in these miRNAs may have been related to the increased levels of the altered HSPs observed in the UC patients. Dysregulation on HSP70/90 Levels has also been related to intestinal inflammation associated with CRC. For example, Hoter & Naim (2019)[30] found that HSP90 was highly expressed and was implicated in the progression from UC to UC-associated CRC. Furthermore, HSP90 inhibition has been actively investigated as a treatment for gastrointestinal and CRC arising from IBD progression.

MiR-378a-3p/5p, two of the miRNAs correlated with HSP downregulation, have also been shown to be altered during intestinal inflammation[34]. The involvement of miR-378 in IBD patients has also been reported[35-37], indicating an upregulation in UC or a downregulation in CD. These differences may have resulted from the small number of patients included in these previous studies or the use of pharmacological agents by the participants, which could reflect treatment rather than disease effects. The patients in the present study were treatment naïve; therefore, the differences in expression resulted from the disease itself. A recent study conducted by Dubois-Camacho *et al*[38] in 2019, corroborates the present findings, as they showed that a decrease in miR-378a expression in UC patients was associated with higher levels of interleukin 33 and tumor necrosis factor-alpha (TNF-α), and that miR-378a expression levels increased following treatment with an anti-TNF-α monoclonal antibody[38].

In CRC, the overexpression of miR-378 inhibits the proliferation of colon cancer cells *in vitro* by inducing apoptosis and preventing migration and invasion[39]. For example, miR-378a also alleviated the malignant phenotypes of colon cancer cells by inhibiting the Wnt/β-catenin signaling pathway[39]. In addition, CRC patients with low miR-378a expression experienced a shorter survival time than those with high miR-378a expression, indicating that miR-378a may serve as an important diagnostic biomarker[40]. Collectively, it is possible to assume that the decrease in miR-378a-

3p/5p levels during inflammation and the later progression to CRC can increase HSP90 Levels. Thus, increased HSP90 and decreased miR-378a-3p/5p levels could act as important biomarkers and potential targets of pharmacological interventions for UC and UC-related CRC.

Among the downregulated miRNAs observed in IBD patients, miR-192-3p/5p, which was first cloned in 2003[41], is a tumor-related miRNA, as its dysregulation has been reported in several types of cancer[42]. Overexpression of miR-192 has been shown to induce apoptosis in bladder cancer cells and arrest breast cancer cells' growth[42,43]. Moreover, in CRC, miR-192 regulates the enzyme dihydrofolate reductase and cellular proliferation through the p53 tumor suppressor network, decreasing cancer progression and metastases[42,43]. Decreased levels of miR-192 are also related to IBD. For example, Wu *et al*[6] reported a link between chronic IBD and the altered expression of certain miRNAs, demonstrating that miR-192 was downregulated in the colonic epithelial cells of active UC patients which corroborates the findings of the present study.

An inverse correlation exists between the expression of miR-192 and MPI-2α, an epithelial cell-expressed chemokine previously implicated in IBD[6]. In addition, miR-192 was shown to be induced by TGF-β, suggesting that miR-192 plays a key role in inflammatory, fibrotic processes[6]. Moreover, other putative miR-192 targets identified in the enrichment analysis include mediators of inflammation and fibrosis, such as nucleotide-binding oligomerization domain-containing protein 2, TNF receptor-associated factor-interacting protein 3, 4, and 5, as well as matrix metalloproteinase 16 and 20 (Supplementary material 1). Changes in miR-192 expression have also been shown in a 2,4,6-trinitrobenzenesulfonic acid-induced intestinal inflammation model[44]. In that elegant study, the EMT was activated as a result of intestinal inflammation, along with a simultaneous increase in the early growth response protein 1 and fibroblast growth factor 2 expression levels (based on mesenchymal markers); on the other hand, miR-192 expression was decreased[44].

Conversely, elevated levels of miR-192 are associated with tumor suppression and cell proliferation[45-49]. Ji *et al*[45] demonstrated that miR-192 suppresses the growth of bladder cancer cells *via* targeting Yin Yang 1, a transcription factor that plays an important role in regulating development and cell proliferation. Similarly, Flammang *et al*[48], in a study involving pancreatic ductal adenocarcinoma, described miR-192 as a marker with prognostic value, as increased miRNA expression exerted a suppressive effect. In CRC, miR-192 also acts as a tumor suppressor, inhibiting CRC invasion[46, 47]. Furthermore, Huang *et al*[49] found that certain metabolites of normal intestinal microflora were capable of upregulating miR-192 expression, suppressing the proliferation of colon cancer cells through a decrease in bone morphogenic protein type 2 receptor levels; thus, cell proliferation, migration, and invasion ability are diminished, and the rate of cellular apoptosis is improved[49]. Collectively, these data indicate a potential role of miR-192-3p/5p as a biomarker for UC activity and a target for pharmacological treatment.

These results provide information that may explain the differences in CRC and mortality rates between UC and CD patients. Two cohort studies from Olén *et al*[50,51] (2020a; 2020b) compared CRC mortality and incidence in UC and CD patients. In the first study, which evaluated UC patients, the authors found that UC patients had a 36% higher incidence of CRC than that of reference individuals [hazard ratio (HR) = 1.66][51]. Among CD patients, the incidence was 21.9% higher than that of the reference group (HR = 1.4)[50]. These findings demonstrated a tendency toward a higher probability of developing CRC in UC compared with CD, which was associated with higher mortality[50,51]. The lower CRC incidence in CD could be explained by the early removal of this portion of the intestine[52]. Nevertheless, there was differential expression of these selected miRNAs between those with CD and UC. The decreased expression levels of miR-378a-3p/5p, miR-429, and miR-192-3p/5p in UC patients could represent a possible mechanism responsible for increasing their likelihood of developing CRC.

Although the overlap between DE-miRNAs targets and IBD susceptibility genes (Figure 2; Supplementary Table 1) suggested some involvement with the disease onset, progression and future cancer development, the data are preliminary and the sample size is small, so further studies are required to better comprehension of miRNA role in IBD pathogenesis. Future studies should aim to address some of the limitations by increasing the number of patients and investigating the potential links between these miRNAs and pathological UC and UC-associated CRC mechanisms.

CONCLUSION

In conclusion, this study highlighted the potential involvement of miR-192-3p, miR-192-5p, miR-378a-3p, miR-378a-5p and miR-429 as players in IBD pathology, UC differentiation and UC-associated CRC, indicating the potential use of these miRNAs as specific biomarkers for UC. Moreover, these miRNAs could be also useful as biomarkers for UC-associated CRC. Since samples of treatment-naïve patients were used, the distinct expression found in this study was a result from the disease itself with no medication effect. This way, the DE-miRNAs may represent a new pharmacological target for UC treatment.

ARTICLE HIGHLIGHTS

Research background

Inflammatory bowel disease (IBD) is a chronic and relapsing disorder of the gastrointestinal tract including two distinct phenotypes, ulcerative colitis (UC) and Crohn's disease (CD). IBD pathophysiological mechanisms are unclear.

Research motivation

IBD affects 6 to 8 million people worldwide and the incremental increase in the incidence and prevalence globally is indicative of the need for population-based genetic studies including microRNAs (miRNAs) expression profiles.

Research objectives

The present study aimed to investigate the miRNA expression patterns in tissue of treatment-naïve CD and UC patients and the potential pathophysiological contributions of differentially expressed (DE) miRNA in IBD.

Research methods

A total of 20 formalin-fixed paraffin embedded colonic samples were used in a TaqMan™ Array Human MicroRNA (Applied Biosystems™) platform aiming to analyze 754 miRNAs. After that, targets of DE-miRNAs were predicted using miRNa data integration portal (miRDIP) and the miRNA target interaction database (miRTarBase).

Research results

A total of 643 miRNAs were found to be expressed in both diseases but only 13 miRNAs were significantly different between the CD and UC patients ($P \leq 0.05$; fold-change > 1). The miRNAs whose expression levels were significantly lower in UC patients than in CD patients (miR-192-3p/5p, miR-378a-3p/5p and miR-429) were enriched in signaling pathways that were mostly correlated with cancer-related processes and respective biomarkers.

Research conclusions

Ulcerative colitis and Crohn's disease presented distinct patterns of miRNA expression that could be useful as new pharmacological targets besides acts as biomarkers for UC-associated CRC.

Research perspectives

New studies should be done with the DE-miRNAs using a large number of patients aiming to confirm the differences found in this pilot study. With this confirmation, the DE-miRNAs will be able to be used as pharmacological targets and differential markers for each disease.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Professor dos Reis PP (PhD) for technical assistance.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 7; 27(45): 7813-7830

DOI: 10.3748/wjg.v27.i45.7813

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

ORIGINAL ARTICLE

Observational Study

Multimodality management of gallbladder cancer can lead to a better outcome: Experience from a tertiary care oncology centre in North India

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Author contributions: Singh S was the guarantor and designed the study; Goel S, Aggarwal A, and Iqbal A participated in the acquisition, analysis, and interpretation of the data, and drafted the initial manuscript; Talwar V, Mitra S, and Singh S revised the article critically for important intellectual content.

Institutional review board statement: The local Institutional Review Board (IRB) approved the study on May 26, 2020 (No. RGCIRC/IRB-BHR/48/2020).

Informed consent statement: All patients provided informed consent prior to any intervention, chemotherapy, radiotherapy, or surgery.

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: No additional data are available.

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Abstract

BACKGROUND

Surgical resection is a treatment of choice for gallbladder cancer (GBC) patients but only 10% of patients have a resectable disease at presentation. Even after surgical resection, overall survival (OS) has been poor due to high rates of recurrence. Combination of surgery and systemic therapy can improve outcomes in this aggressive disease.

AIM

To summarize our single-center experience with multimodality management of resectable GBC patients.

METHODS

Data of all patients undergoing surgery for suspected GBC from January 2012 to December 2018 was retrieved from a prospectively maintained electronic database. Information extracted included demographics, operative and perioperative details, histopathology, neoadjuvant/adjuvant therapy, follow-up, and recurrence. To know the factors associated with recurrence and OS, univariate and multivariate analysis was done using log rank test and cox proportional hazard analysis for categorical and continuous variables, respectively. Multivariate analysis was done using multiple regression analysis.

RESULTS

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

Country/Territory of origin: India

Specialty type: Gastroenterology and hepatology

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

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

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Received: April 26, 2021
Peer-review started: April 26, 2021
First decision: June 13, 2021
Revised: June 26, 2021
Accepted: September 2, 2021
Article in press: September 2, 2021
Published online: December 7, 2021

P-Reviewer: Tashkandi E
S-Editor: Wu YXJ
L-Editor: Wang TQ
P-Editor: Wang LYT

Of 274 patients with GBC taken up for surgical resection, 172 (62.7%) were female and the median age was 56 years. On exploration, 102 patients were found to have a metastatic or unresectable disease (distant metastasis in 66 and locally unresectable in 34). Of 172 patients who finally underwent surgery, 93 (54%) underwent wedge resection followed by anatomical segment IVb/V resection in 66 (38.4%) and modified extended right hepatectomy in 12 (7%) patients. The postoperative mortality at 90 d was 4.6%. During a median follow-up period of 20 mo, 71 (41.2%) patients developed recurrence. Estimated 1-, 3-, and 5-years OS rates were 86.5%, 56%, and 43.5%, respectively. Estimated 1- and 3-year disease free survival (DFS) rates were 75% and 49.2%, respectively. On multivariate analysis, inferior OS was seen with pT3/T4 tumor (P = 0.0001), perineural invasion (P = 0.0096), and R+ resection (P = 0.0125). However, only pT3/T4 tumors were associated with a poor DFS (P < 0.0001).

CONCLUSION
Multimodality treatment significantly improves the 5-year survival rate of patients with GBC up to 43%. R+ resection, higher T stage, and perineural invasion adversely affect the outcome and should be considered for systemic therapy in addition to surgery to optimize the outcomes. Multimodality treatment of GBC has potential to improve the survival of GBC patients.

Key Words: Gallbladder cancer; Multimodality; Surgical resection; Adjuvant; Chemotherapy; Chemoradiotherapy

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Core Tip: Gallbladder cancer (GBC) is an aggressive malignancy with only 10% of cases amenable to resection at presentation and a dismal overall 5-year survival rate of 5%-13% after curative surgery. Recently, several experts have recommended that multimodality treatment, including neoadjuvant and adjuvant therapies, can improve survival. In this study, we share our experience with multimodality approach in GBC. Five-year overall survival was approaching 50%, and therefore we suggest that such approach can improve survival in this aggressive malignancy.

Citation: Goel S, Aggarwal A, Iqbal A, Talwar V, Mitra S, Singh S. Multimodality management of gallbladder cancer can lead to a better outcome: Experience from a tertiary care oncology centre in North India. *World J Gastroenterol* 2021; 27(45): 7813-7830
URL: https://www.wjgnet.com/1007-9327/full/v27/i45/7813.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i45.7813

INTRODUCTION

Gallbladder cancer (GBC) is the most common and most aggressive malignant disease of the biliary tract. A distinct geographical variability has been observed in the prevalence of GBC. Countries like India, Pakistan, Chile, Korea, and Japan have reported a higher prevalence as compared to the Western world. The highest incidence has been reported in regions like Delhi, India (21.5/100000), La Paz, Bolivia (15.5/100000), South Karachi, Pakistan (13.8/100000), and Quito, Ecuador (12.9/100000)[1,2].

Surgical resection is the treatment of choice but only 10% of patients have a resectable disease at presentation. Even after surgical resection, overall survival (OS) has been poor due to high rates of recurrence[2]. Recently, there has been an increased interest in multimodality treatment including both neoadjuvant and adjuvant therapy to improve outcomes. Although there are no randomized trials on the issue but improved outcomes have recently been reported using multimodality treatment. A recent expert consensus statement on GBC recommended that all patients with clinical T3–4 N+ disease should be considered for neoadjuvant chemotherapy (NACT) trials [3]. After curative resection, patients with T2 or higher and N+ disease should undergo adjuvant systemic chemotherapy or chemoradiotherapy (CRT). Adjuvant CRT should

be used in patients with positive margins after resection[3]. With advancements in surgical approach and systemic therapy, multimodality approach has a potential to obtain favorable outcomes in this aggressive disease[4].

We have adapted various aspects of the multimodality approach for GBC in the last decade. In this study, we aimed to analyze our outcomes for multimodality management of GBC.

MATERIALS AND METHODS

Patients

Institutional review board approval was taken for waiver of informed consent (RGCIRC/IRB-BHR/48/2020). All patients undergoing surgery for suspected gall bladder cancer from January 2012 to December 2018 were included. Data containing demographics, operative and perioperative details, histopathology, and neoadjuvant/adjuvant therapy was retrieved from a prospectively maintained electronic database. Follow-up data was collected from the database as well as telephonically.

Preoperative evaluation

All patients with suspicion of GBC were evaluated by contrast-enhanced computed tomography (CECT) of the abdomen and pelvis. CEA and CA19-9 were routinely measured in all cases. Since April 2015, all patients who had a resectable disease on CECT underwent an additional 18-FDG positron emission tomography (PET) scan to rule out distant metastasis as a part of the study to evaluate the role of PET scan in GBC[5]. All patients who presented with jaundice underwent magnetic resonance cholangiopancreatography to confirm the level of obstruction and biliary drainage procedure as indicated. Patients who underwent laparoscopic cholecystectomy outside for a benign disease and were found to have to have GBC on histopathology were defined as incidental GBC. They were evaluated similarly except that they were excluded from PET scan study due to possible high false positivity rate in view of ongoing inflammation at the postoperative site. Patients with locally advanced diseases were considered for NACT after discussion in multidisciplinary board. The following criteria were used to select patients for neoadjuvant therapy in primary GBC patients: (1) T4 lesion involving two or more adjacent organs or the hepatic hilum; (2) Extensive hepatic infiltration which required major liver resection (> 2 segments); (3) N2 disease (AJCC 7th); (4) Bulky regional nodes (> 3 cm in short axis); and (5) During waiting period after portal vein embolization.

The main aim for NACT was to select good tumor biology patients and improve R0 resection rate. Incidental cases were referred for NACT if they have a history of bile spillage in index surgery. Neoadjuvant CRT was not done in any patient.

The most commonly used regimen for NACT was gemcitabine (1000 mg/m^2 intravenously over 30-60 min) on days 1 and 8, and cisplatin (75 mg/m^2 intravenously over 2 h) on day 1, every 21 d. In case of renal compromise, carboplatin was used. After three cycles, patients were reassessed for response using PET-computed tomography (CT) and CECT of the abdomen and pelvis. Due to the retrospective nature of this study, the type and duration of chemotherapy were not controlled and were decided by the team of medical oncologists.

Data collection was done in concordance with ethical guidelines of Declaration of Helsinki. All patients provided informed consent prior to any intervention, chemotherapy, or surgery.

Surgical treatment

All patients underwent staging laparoscopy to rule out distant metastases. This was followed by exploratory laparotomy and inter-aortocaval (IAC) lymph node sampling for frozen section. Definitive procedure was generally abandoned if IAC nodes were positive for malignancy except for select cases. Resectable primary GBC underwent radical cholecystectomy which included *en bloc* resection of the gallbladder with a non-anatomical liver wedge (2 cm liver margin) or segment IVB/V resection with regional lymphadenectomy including retropancreatic lymph nodes (station 13) and common hepatic artery nodes (station 8) along with all the soft tissue around and in between hilar structures (station 12). In the initial period, the decision between non-anatomical wedge and segment IVB/V was taken by operative surgeon intraoperatively, but since 2014, all patients were part of a randomized controlled trial (RCT) comparing wedge resection and segment IVB/V resection for GBC (CTRI/2018/05/014324). Selected cases with extensive liver involvement or infiltration into right portal structures

underwent modified extended right hepatectomy (*en bloc* resection of the gallbladder along with segments V, VI, VII, VIII, and IVB) with regional lymphadenectomy. We did not perform hepato-pancreatoduodenectomy or vascular resections for GBC at our centre. Port sites were resected for all patients with incidental GBC before 2016, but it is not done routinely now. Common bile duct resection and adjacent organ (colon/stomach/duodenum) resections were performed only when necessary to achieve R0 status. All intraoperative and perioperative data was recorded. Postoperative complications were recorded and graded according to the Clavien-Dindo classification[6]. Histopathological data for all patients were retrieved and staging was done as per AJCC 8th classification[7].

All patients were discussed in multidisciplinary meetings for planning adjuvant therapy. Since January 2015, all T2/node positive GBC patients were included in an institutional RCT comparing adjuvant chemotherapy and CRT after radical cholecystectomy (R0 resection) [CTRI/2018/01/011296]. The patients randomized to chemotherapy were given single agent gemcitabine 1 gm/m^2 on days 1, 8, and 21 in each cycle for six cycles starting 3 wk after surgery. Chemo-radiation group received external beam radiation therapy (50.4 Gy, 1.8 Gy for 28 fractions). Radiation area included gallbladder fossa, tumor bed, and adjacent liver and regional nodes. Chemotherapy included injection of 5-FU 750 mg/m^2 on days 1-5 and on last days of radiotherapy in a concurrent fashion. All patients who received NACT completed a total of six cycles of perioperative chemotherapy. Patients with R1 resection received radiation therapy in addition to chemotherapy.

Follow-up

All patients were kept on regular follow-up, every 3 mo for first 2 years, and every 6 mo for next 3 years. At each visit, physical examination and tumor marker (CA19-9 and CEA) measurement were done. CECT of whole abdomen was done every 6 mo and those with suspicious or equivocal findings underwent PET-CT followed by histological confirmation of recurrence. All patients with recurrence were counselled for palliative therapy.

Statistical analysis

Demographic and preoperative data was given for all patients, including those who were found to have an unresectable/metastatic disease intraoperatively. But these patients were excluded from final analysis. Categorical variables are described using counts/percentages and the mean/median was used for continuous variables. OS and disease free survival (DFS) were calculated using Kaplan-Meier curves. OS was calculated from the date of diagnosis to death or last follow-up and DFS was calculated from the date of surgery to recurrence of disease. To know the factors associated with recurrence and OS, univariate and multivariate analysis was done using log rank test and cox proportional hazard analysis for categorical and continuous variables, respectively. Multivariate analysis was done using multiple regression analysis. The statistical review of the study was performed by a biomedical statistician.

RESULTS

Patient characteristics

From January 2012 to December 2018, a total of 298 patients were taken up for surgery for a suspected GB malignancy. Out of these, 22 patients were found to have benign disease on final histopathology and 2 had neuroendocrine tumors of the gallbladder, so they were excluded from final analysis (Figure 1). Among 274 patients with a confirmed histopathological diagnosis of GBC, 172 (62.7%) were female and the median age was 56 (range, 28-80) years. The most common presenting symptom was abdominal pain (80.7%), followed by jaundice (8.1%), non-specific symptoms (5.5%), dyspepsia, weight loss, loss of appetite, and fever. Ninety-six (35%) patients had incidental presentation and the median time interval between cholecystectomy and radical surgery was 30 (range, 11-175) d. Cholelithiasis was seen in 173 (63.1%) cases. Although CEA and CA19-9 levels were not available in some patients, CEA was raised in 57/174 (32.8%) and CA19-9 was raised in 94/209 (45%) cases.

Neoadjuvant therapy

Twenty-seven percent (75/274) of all patients received NACT. Out of the 75 patients,

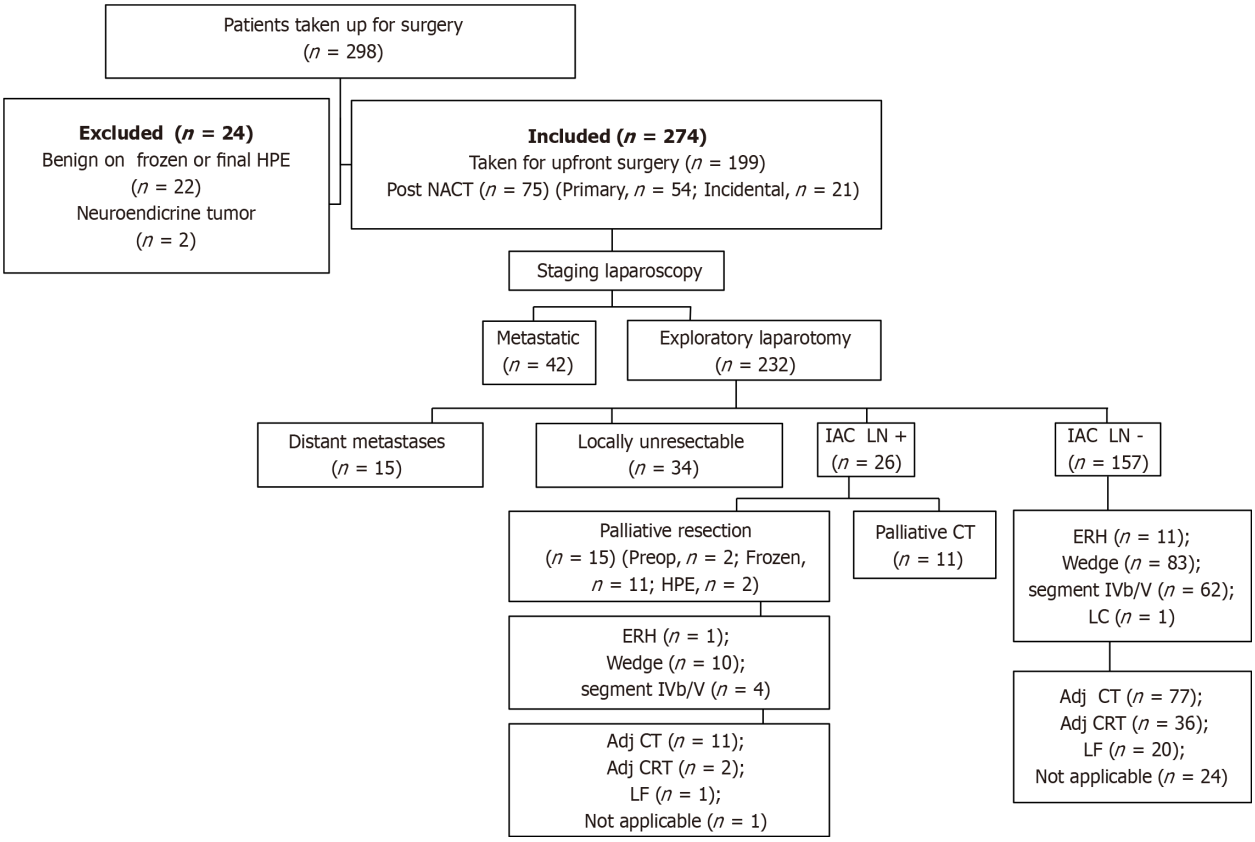


Figure 1 Details of suspected gallbladder cancer patients taken up for surgical exploration. NACT: Neoadjuvant chemotherapy; IAC LN: Inter-aortocaval lymph node; CT: Chemotherapy; LC: Laparoscopic cholecystectomy; LF: Lost to follow-up; CRT: Chemoradiotherapy; HPE: Histopathological examination; ERH: Extended right hepatectomy.

21 had incidental presentation and the rest 54 were non-incidental. Fifty-seven percent (43/75) of patients who received NACT could undergo curative resection and the rest 43% (32/75) were found to have an either metastatic or locally unresectable disease on exploration. Of 43 patients who successfully underwent surgery, 29 received gemcitabine with cisplatin, 12 received gemcitabine with carboplatin, and 2 received gemcitabine only. After NACT, 37 patients underwent radical cholecystectomy (22 had wedge liver resection, and 15 underwent anatomical segment IVb/V resection) and 6 had modified extended right hepatectomy.

Surgery

On exploration, 102 (staging laparoscopy, 42; laparotomy, 60) patients were found to have a metastatic or unresectable disease. Distant metastasis was seen in 66 patients (peritoneum, 40; liver, 15; IAC nodes, 11) and 34 had a locally unresectable disease on exploration. Two patients who were planned for major hepatectomy were found to have liver cirrhosis and surgery was abandoned. Of 172 patients who finally underwent surgical resection, 93 (54%) underwent wedge resection followed by anatomical segment IVb/V resection in 66 (38.4%) and modified extended right hepatectomy in 12 (7%) patients. One patient underwent laparoscopic cholecystectomy but was found to have T1a disease on final histopathology. Adjacent organ resection was done in 66 patients (CBD, 31; colon, 11; stomach/duodenum, 13; and multiple organs, 11). Median blood loss was 200 (range, 50-2000) mL and median duration of surgery was 270 (range, 120-540) min.

Morbidity and mortality

The postoperative mortality at 90 d was 4.6% (8/172), and the most common cause of death was bile leak and subsequent sepsis (n = 3) followed by postoperative liver failure (n = 2), acute myocardial infarction (n = 2), and ARDS (n = 1). Overall morbidity rate was 30.8% (53/172) but clinically significant complications (Clavien-Dindo grade III or more) were seen in only 12.2% (21/172) of cases. Median hospital stay was 9 (range, 3-54) d.

Histopathology

Histopathological details are described in Table 1. The most common histological diagnosis was adenocarcinoma seen in 160/172 (93%) cases. All patients were staged according to the AJCC 8th TNM classification. The majority of patients had a T2/T3 (83%) disease and 55/172 (32%) had a node positive disease. Median number of lymph nodes resected was 9 (range, 1-25). On final staging, the maximum number of patients had a stage III disease (III, 73; II, 45; IV, 33; I, 21).

Adjuvant therapy

Excluding the patients who had a stage I (n = 21) disease on final histopathology, 151 patients were eligible for adjuvant therapy. Approximately 86% (126/147) of patients received adjuvant therapy. Out of these, 88 received chemotherapy only and 38 received CRT. Ninety-seven percent of patients in the radiotherapy group (37/38) and 90.9% (80/88) patients in the chemotherapy group completed the intended treatment. Overall, 117 out of 126 (92.8%) patients completed the adjuvant therapy.

Follow-up and survival

During a median follow-up period of 20 mo, 71 (41.2%) patients developed recurrence. In the majority of them, recurrence was seen at a distant site (47/71, 66.2%) followed by loco-regional failure in 18/71 (25.4%) and at multiple sites in 6 (8.4%). The most common site of distant metastases was the peritoneum (n = 22) followed by the liver (n = 15), distant nodes (n = 9), and lung (n = 1). Median DFS and OS were not reached in our study. However, median OS for stage III and stage IV patients was 27.1 mo and 19.6 mo, respectively. Median DFS for stage III and stage IV patients was 24 mo and 13 mo, respectively. Estimated 1-, 3-, and 5-year OS rates were 86.5%, 56%, and 43.5%, respectively. Estimated 1- and 3-year DFS rates were 75% and 49.2%, respectively. Stagewise OS and DFS are shown in Figure 2. On log rank test, they correlated significantly.

Factors affecting survival

On univariate analysis, inferior OS and DFS were associated with upfront presentation (non-incidental), positive resection margin, lymph node involvement, higher T stage (T3 or T4), and lymphovascular and perineural invasion (PNI) (Table 2). Neoadjuvant therapy was given in advanced cases, hence the cohort was associated with a poor outcome. However, on multivariate analysis, inferior OS was seen with pT3/T4 tumour (P = 0.0001), PNI (P = 0.0096), and R+ resection (P = 0.0125). On multivariate analysis, only pT3/T4 tumors were associated with a poor DFS (P < 0.0001). Also, association of R+ resection with early recurrence was approaching the level of significance (P = 0.0513).

Impact of adjuvant therapy on overall outcome

In our study, 147 patients were advised to receive adjuvant therapy, out of which 117 patients completed the adjuvant therapy (adjuvant group) whereas 30 patients did not take/complete adjuvant therapy (non-adjuvant group). These two groups were comparable in baseline characteristics except for a higher incidence of post-cholecystectomy GBC in the adjuvant group (Table 3).

Estimated median OS for the adjuvant group and non-adjuvant group was 49.9 mo and 28.5 mo, respectively; however, the difference was not significant (P = 0.21). Estimated median DFS was 30.6 mo and 17.7 mo for the adjuvant and non-adjuvant group, respectively (P = 0.14) (Figure 3).

DISCUSSION

According to GLOBOCAN 2018 data, GBC accounts for 1.2% of all cancer diagnoses worldwide with a median survival of less than a year in advanced cases[8]. It is an aggressive malignancy with usually late presentation with an overall estimated 5-year survival rate of 5%-13%[9-11]. Radical surgery is the mainstay of treatment but survival with surgery alone is dismal in locally advanced cases[10].

Presentation is usually a decade late in Western patients as compared to those in our series[12], which can be attributed to endemicity of GBC in Indian subcontinent which has higher composition of younger population. It is diagnosed either incidentally (where cholecystectomy is performed for benign conditions) or mostly in advanced stage where patients present with cachexia with or without jaundice.

Table 1 Histopathological and perioperative details of resectable gallbladder patients (*n* = 172)

Patient characteristic	*n* (%)
Type of surgery	
Wedge resection	93 (54)
Anatomical segment IVb/V resection	66 (38.4)
Modified extended right hepatectomy	12 (7)
Lap cholecystectomy	1 (0.6)
Histology	
Adenocarcinoma	160 (93)
Adenosquamous	9 (5.2)
Carcinosarcoma	2 (1.2)
Squamous	1 (0.6)
Histological grade	
Well differentiated	33 (19.2)
Moderately differentiated	116 (67.4)
Poorly differentiated	23 (13.4)
pT stage	
T1	25 (14.5)
T2	70 (40.7)
T3	73 (42.4)
T4	4 (2.4)
pN stage	
N0	117 (68)
N+	55 (32)
LVI positive	54 (31.4)
PNI positive	56 (32.5)
IAC positive	15 (8.7)
R0/R1 resection	
R0	161 (93.6)
R1	11 (6.4) [liver (*n* = 4), cystic duct (*n* = 4), bile duct (*n* = 3)]
Final stage (AJCC 8th)	
I	21 (12.2)
II	45 (26.2)
III	73 (42.4)
IV	33 (19.2)
Postoperative morbidity	
Overall	53 (30.8)
Clavien-Dindo grade III & above	21 (12.2)
Bile leak	14 (8.1)
Clinically significant	5 (2.9)
90 d mortality	8 (4.6)
Adjuvant therapy	117/147 (79.6)
Chemotherapy only	80

Chemoradiotherapy	37
Advised but not taken/incomplete	30
Not indicated	25

AJCC: American Joint Committee on Cancer; IAC: Inter-aortocaval lymphnode; LVI: Lymphovascular invasion; PNI: Perineural invasion.

Table 2 Association between patient and disease characteristics with outcomes

	Overall survival			Disease free survival		
Factor	**HR**	**95%CI**	***P* value**	**HR**	**95%CI**	***P* value**
Sex						
Female	0.92	[0.56-1.51]	0.76	0.98	[0.61-1.6]	0.96
Male						
Jaundice						
Yes	0.9	[0.37-2.16]	0.81	1.19	[0.48-2.93]	0.67
No						
Incidental						
Yes	0.24	[0.07-0.81]	0.001	0.54	[0.34-0.86]	0.01
No						
Neoadjuvant therapy						
Yes	2.2	[1.27-3.81]	< 0.001	2.91	[1.64-5.16]	< 0.001
No						
Resection						
R+	4.08	[1.22-13.64]	< 0.001	4.13	[1.22-13.9]	< 0.001
R0						
Lymph node status						
Positive	1.91	[1.13-3.25]	0.006	2.44	[1.4-4.17]	0.001
Negative						
T stage						
T1a-T2						
T3-T4	5.01	[3.06-8.18]	< 0.001	4.21	[2.55-6.94]	< 0.001
LVI						
Yes	2.08	[1.22-3.5]	0.001	2.12	[1.23-3.64]	0.001
No						
PNI						
Yes	3.06	[1.75-5.37]	< 0.001	2.54	[1.45-4.45]	< 0.001
No						
Poorly differentiated						
Yes						
No	1.75	[0.81-3.7]	0.07	1.43	[0.66-3.08]	0.28

HR: Hazard ratio; CI: Confidence interval; LVI: Lymphovascular invasion; PNI: Perineural invasion.

Cholelithiasis has been associated with GBC in several studies with a prevalence of stones in approximately 70%-88% of cases of GBC[13,14]. Our study showed the absence of gallstones in approximate one-third of cases, which might be explained by

Table 3 Clinicopathological characteristics of patients who received adjuvant therapy *vs* those who did not

Patient characteristic	Adjuvant group (*n* = 117), *n* (%)	Non-adjuvant Group (*n* = 30), *n* (%)	*P* value
Age, yr (mean)	54.5	60	0.01
Sex (M:F)	47:70	10:20	0.63
Incidental GBC	55 (47)	7 (23.3)	0.03
Neoadjuvant therapy	33 (28.2)	7 (23.3)	0.76
Type of surgery			0.71
Wedge resection	62 (53)	16 (53.3)	
Anatomical segment IVb/V resection	48 (41)	11 (36.7)	
Modified extended right hepatectomy	7 (6)	3(10)	
Histology			0.91
Adenocarcinoma	108 (92.3)	28 (93.3)	
Adenosquamous	7(6)	2 (6.7)	
Carcinosarcoma	1	0	
Squamous	1	0	
Histological grade			0.12
Well differentiated	21 (18)	2 (6.7)	
Moderately differentiated	82 (70.1)	21 (70)	
Poorly differentiated	14 (11.9)	7 (23.3)	
pT stage			0.56
T1	6 (5.1)	2 (6.7)	
T2	57 (48.7)	11 (36.7)	
T3	52 (44.4)	17 (56.6)	
T4	2 (1.7)	0	
pN stage			0.47
N0	77 (65.8)	17 (56.7)	
N+	40 (34.2)	13 (43.3)	
LVI positive	37 (31.6)	13 (43.3)	0.32
PNI positive	40 (34.2)	12 (40)	0.7
R0/R1resection			0.84
R0	109 (93.2)	27 (90)	
R1	8 (6.8)	3 (10)	
Final stage (AJCC 8th)			0.79
I	3 (2.6)	1 (3.3)	
II	36 (30.8)	8 (26.7)	
III	56 (47.9)	13 (43.3)	
IV	22 (18.1)	8 (26.7)	

AJCC: American Joint Committee on Cancer; GBC: Gallbladder cancer; LVI: Lymphovascular invasion; PNI: Perineural invasion.

environmental and genetic predisposition of the study population to GBC.

Factors affecting survival

Incidental detection of GBC after cholecystectomy usually confers a favorable prognosis as the malignancy is usually detected in early stage[15,16]. Non-incidental cases are more likely to have advanced T stage, high-grade tumors, lymphovascular

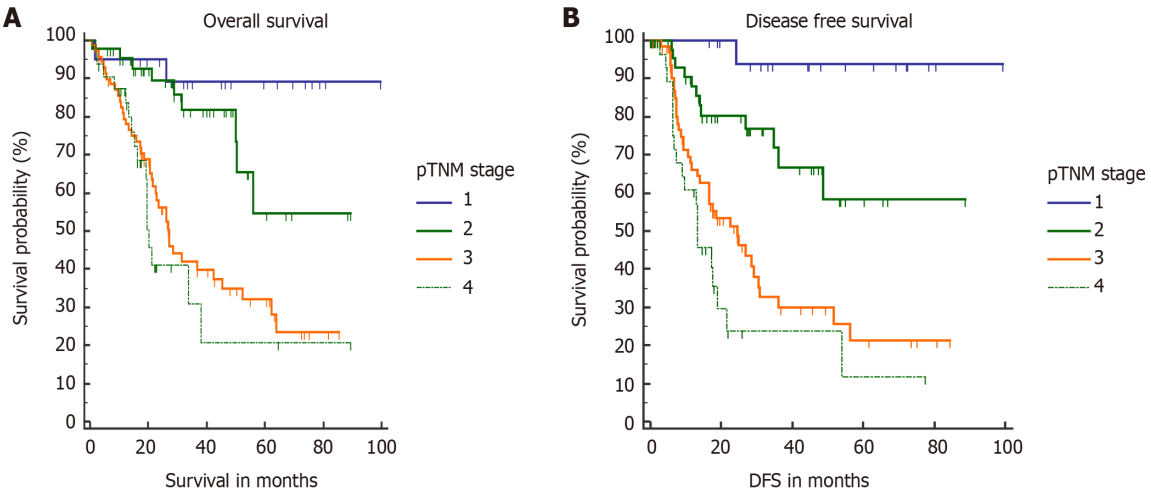


Figure 2 Stagewise overall survival and disease free survival. A: Stagewise overall survival; B: Stagewise disease free survival. DFS: Disease free survival.

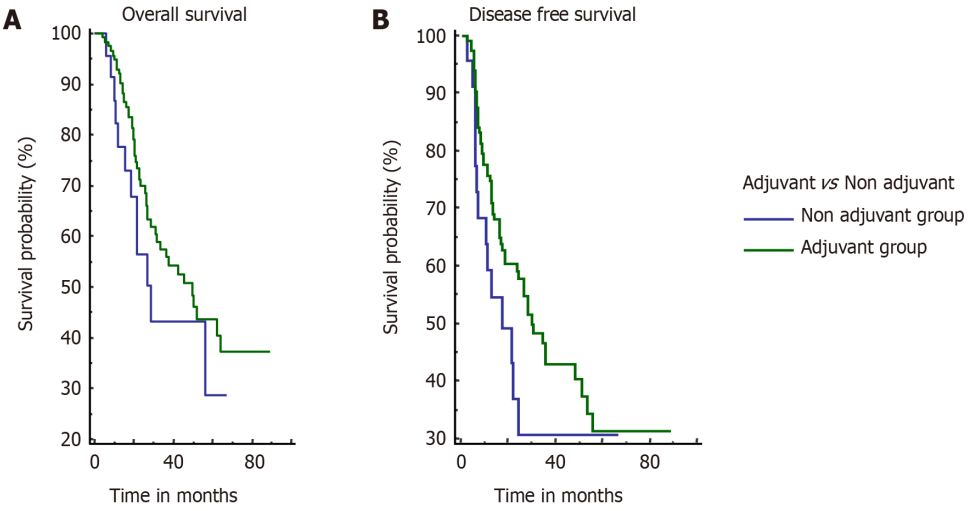


Figure 3 Comparison of overall survival and disease free survival of patients who received adjuvant therapy *vs* those who did not. A: Overall survival; B: Disease free survival.

invasion, positive lymph nodes, and R2 resection[16]. Optimal timing of completion of radical cholecystectomy is still debatable. Early surgery may lead to higher morbidity due to recent inflammation and adhesions and is also associated with a higher rate of unresectability due to breach of tumor and dissemination with seeding of tumor cells in the peritoneal cavity during index surgery[17]. Recently, a multi-institutional study showed a better survival when re-resection was performed between 4-8 wk from the index surgery although the retrospective and observational nature of the study casts apprehension over its universal application[18]. However, it is pertinent to give importance to bile spillage during index surgery, residual disease, and tumor biology rather than relying solely on the time interval[10,19]. In the present study, median time interval between index and redo surgery was 4 wk. Surgery was usually delayed with administration of NACT if there was any evidence of bile spillage. Future outcome also correlates well with the presence of residual disease on final exploration. Risk factors for finding residual disease include T3 tumors, PNI, and lymphovascular invasion[20]. Even half of the patients with incidental T1b/T2 GBC have residual disease on re-exploration and subsequently have a poor outcome[21]. However, higher T stage and poorly differentiated tumors have shown a high probability of residual disease at redo surgery[22]. In our study, 39.7% (29/73) of patients with incidental GBC were found to have residual disease at re-exploration. Incidental cases were found to have a significantly better survival on univariate analysis but not on multivariate analysis. This might be due to a smaller sample size of incidental cases in view of its lesser prevalence as compared to Western studies (Table 4). Also the

Table 4 Comparison of the present study with other studies on multimodality management of gallbladder cancer

Parameter	Our study	Patkar *et al*[4], 2018	Creasy *et al*[15], 2019
Sample size	274	400	437
Patients who underwent complete resection	172	320	255
Unresectable	102	80	182
Major liver resection, (%)	Yes (7)	No	Yes (24.3)
Incidental GBC, (%)	35	40	60.7
R1 resection, *n* (%)	11/172 (6.4)	10/320 (3.1)	15/255 (5.9)
Neoadjuvant in resectable group, *n* (%)	43/172 (25)	83/320 (25.9)	16/255 (6.3)
Final stage III/IV, *n* (%)	106/172 (61.6)	232/400 (58)	306/437 (70)
LN positivity, *n* (%)	56/172 (32)	98/320 (30.62)	NA
Residual disease in incidental cases, *n* (%)	29/73 (39.7)	68/160 (42.5)	172/276 (62.3)
Adjuvant therapy, *n* (%)	117/147 (79.6)	206/320 (64.4)	78/255 (30.7)
Recurrence, *n* (%)	71/172 (41.2)	98/320 (30.6)	NA
Dying of disease, *n* (%)	69/172 (40.1)	45/320 (14)	149/255 (58.4)
Estimated 3 yr OS, (%)	56	64	NA
Estimated 5 yr OS, (%)	43.5	NA	43 (only survivors)
Estimated 3 yr DFS, (%)	49.2	49	36

DFS: Disease free survival; GBC: Gallbladder cancer; LN: Lymph node; NA: Not available; OS: Overall survival.

number of truly incidental GBC (pT1) was much higher in Western studies as compared to our series (pT1 = 16.4%)[23].

Curative surgery with R0 resection improves the survival of GBC patients. The tendency of GBC to have early systemic dissemination often rules out radical surgery. A recently published study from our centre showed that routine application of 18-FDG PET changed management in approximately one-fourth of all resectable primary GBC patients and in one-third of locally advanced cases due to detection of unsuspected distant metastasis[5]. Similarly, routine application of staging laparoscopy before surgical exploration prevented non-therapeutic laparotomy in 23% of overall GBC patients with higher yield in locally advanced cases[24]. We universally applied staging laparoscopy in GBC patients before proceeding with curative surgery. It prevented laparotomy in 15.3% (42/274) of cases and helped in not only preventing surgical morbidity but also leading to quick commencement of palliative treatment. Staging laparoscopy is now routinely recommended prior to laparotomy for all suspected or proven GBC cases[3].

For non–metastatic GBC, standard surgical treatment is radical cholecystectomy which includes non-anatomical wedge or segment IVb/V resection with locoregional lymphadenectomy. Adjacent organ resection or major hepatectomy may be necessary to achieve negative margins. R0 resection was one of the major factors that significantly affected OS survival in our series. R1 resection was associated with a higher risk of death (hazard ratio [HR] = 4.08, 95%CI: 1.22-13.64, $P < 0.001$) and recurrence (HR = 4.13, 95%CI: 1.22-13.9, $P < 0.001$). All the patients with positive microscopic margin had a stage III or IV disease. Median OS in patients with R1 resection was significantly poor (19.6 *vs* 56.1 mo) (Figure 4). Patkar *et al*[4] also showed an inferior survival after R1 resection (17 *vs* 71 mo). It seems logical to give neoadjuvant treatment to avoid R1 resection in cases where tumor is close to resection margins on imaging, which is mostly in stages III and IV disease.

T stage is an important determinant of final outcome of GBC patients[25,26]. Increasing T stage is also associated with a higher probability of lymph nodal involvement and PNI[4,27]. Higher T stage (pT3/T4) was the only factor which negatively impacted both OS and DFS in our study. Median OS in pT3/T4 tumors was 21.5 mo (Figure 5).

PNI is acknowledged as a poor pathological factor with inferior outcome[26,28]. PNI is more frequently found in proximal tumors (tumors located in GB neck and

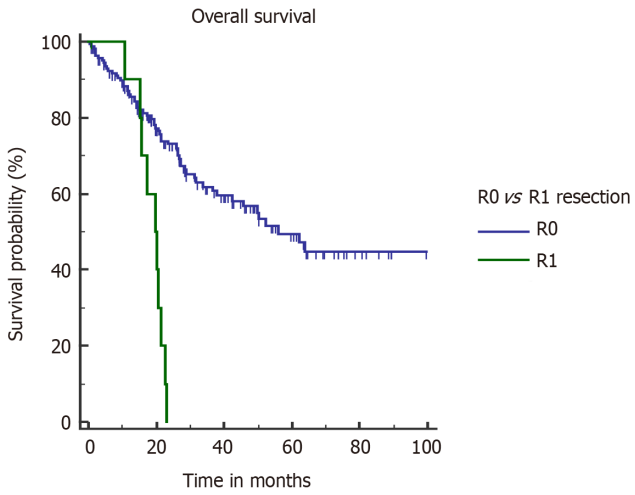


Figure 4 Survival curves of patients with R0 resection compared to R1 resection cases.

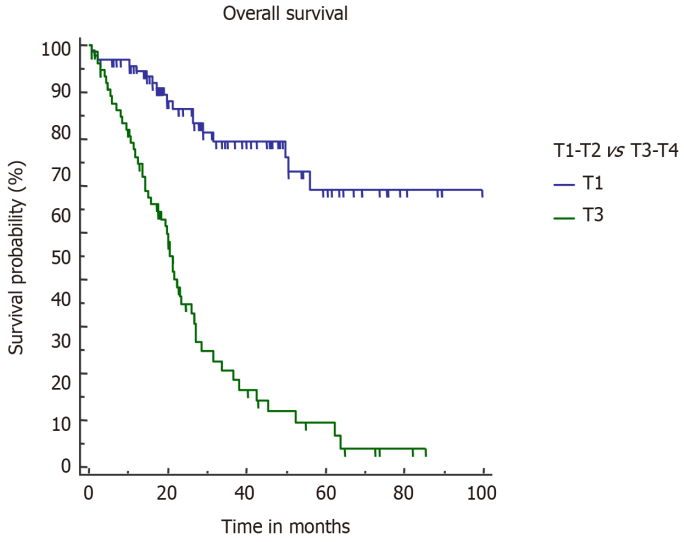


Figure 5 Survival curves of patients with pT1/T2 tumors compared to those with pT3/T4 tumors.

cystic duct) and with higher T stage[27]. PNI positive patients are shown to have significantly lower OS and DFS[26-28]. In our study, on multivariate analysis, PNI adversely affected OS (median OS, 21.3 mo) in PNI positive patients (Figure 6). Median OS was not reached in the PNI negative cohort. None of the patients with stage I disease was found to have PNI positivity, which correlates with the results of a recent study[27]. However, almost half of combined stage III/IV patients had PNI (48/106).

In past, various studies have reported about the adverse impact of node positivity on survival[4,15,29]. From the AJCC 8th edition, N classification of GBC was modified with more emphasis laid on the number rather than the location of involved nodes. Suspicious or confirmed involvement of lymph nodes is also one of the indications for neoadjuvant therapy[30]. In our study, 32% of operated patients had pathological involvement of lymph nodes but it did not affect survival or recurrence on multivariate analysis. LN sampling was adequate, with a median LN harvest of 9. Seventy-three percent of node positive patients completed intended adjuvant therapy. This might explain partly why lymph node positivity did not affect survival and recurrence in the present study.

Multimodality treatment

Chemotherapy is used as an adjunct to surgery in several settings of GBC: (1) As adjuvant therapy after surgical resection, with or without radiation to minimize recurrence; and (2) As neoadjuvant therapy in locally advanced GBC to downstage

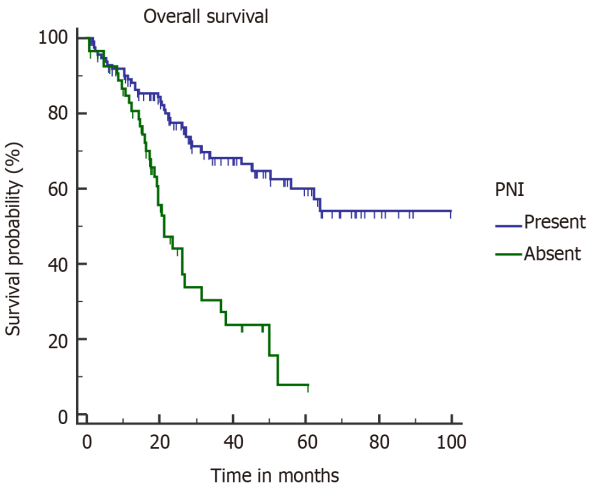


Figure 6 Survival curves of patients with perineural invasion compared to those without. PNI: Perineural invasion.

disease and select good biology tumors for surgery. Due to the rarity of GBC in the West, the data is often clubbed with other biliary malignancies, which leads to heterogeneity of data and hampers their applicability to GBC. Recently published studies from high volume centres have highlighted the need of multimodality management of GBC patients for further improvement in outcomes (Table 4).

Neoadjuvant chemotherapy

Neoadjuvant therapy for GBC is still not standardized in terms of indications, regimen, and duration. Institutions follow their own protocols based on the local data in the absence of randomized trials. The most suitable cases for implementation of NACT in GBC would be incidental GBC patients with residual mass on imaging or evidence of bile spillage during index surgery or locally advanced GBC where R0 resection is not feasible. Locally advanced GBC usually refers to T3 tumors with extensive liver involvement, T4 tumors, or those with any T stage and nodal involvement on imaging.

No randomized control trial has been conducted till date to test the efficacy of neoadjuvant therapy in GBC. A recent systematic analysis reviewed eight studies, out of which five were from India and only two were prospective studies. This calls attention to the paucity of the literature on neoadjuvant therapy for GBC[31]. The median OS for locally advanced cases that undergo curative resection following neoadjuvant therapy is found to be significantly better than that of patients who did not have surgery following neoadjuvant therapy[30,31]. In one of the largest studies, on a retrospective review of 160 patients, Chaudhari *et al*[30] reported a response rate of 52% with surgery feasible in 41% of cases. In another study from the same centre, 74% of patients who received neoadjuvant therapy could undergo R0 resection[4]. In a study from the West, Creasy *et al*[15] showed a median survival of 50 mo in locally advanced GBC patients who underwent surgery after preoperative gemcitabine based chemotherapy. In our study, the neoadjuvant therapy cohort had a poor survival due to the advanced nature of the disease in this subclass. However, 57% of patients with locally advanced disease initially could undergo surgery after NACT. Improvement in chemotherapeutic drug regimen with possible addition of targeted therapy might further improve resectability rate in future.

Adjuvant chemotherapy

Even after R0 resection, 30%-70% of patients develop recurrence over the time[4,15,32]. On analysis, 41% (71/172) of our patients developed recurrence after surgery, out of which 2/3 relapsed at distant sites. Higher rate of distant relapse in spite of R0 resection emphasizes on the need of inclusion of novel systemic therapies for further improvement in outcome and survival.

In contrast to neoadjuvant therapy, adjuvant therapy has been tested in the RCT setting with mixed results. In a meta-analysis by Ma *et al*[33], patients with positive lymph nodes, R1 resection, and non-stage I, benefited most from administration of adjuvant chemotherapy. Recently, several studies have highlighted various chemotherapy drug combinations with promising results after surgery. In the ABC-02 trial, 410 patients with advanced or metastatic biliary malignancy (36% cases were

GBC) were randomized to receive gemcitabine + cisplatin or cisplatin alone. The results demonstrated significant improvements in OS (11.7 *vs* 8.1 mo, $P < 0.001$) with the combination regimen[34]. Another French study (PRODIGE-12/ACCORD-18) evaluated 196 patients with biliary malignancy after surgical resection, out of which only 20% of patients had GBC. The trial randomized patients to receive gemcitabine + oxaliplatin or observation alone. The study found no survival benefit in the chemotherapy group. The study was criticized for including a lower proportion of high-risk patients (R1 resection and node positive patients) who can derive maximum benefit from adjuvant therapy[35].

More recently, in a study from UK (BILCAP trial), patients with biliary malignancies were randomized to receive either adjuvant capecitabine or observation alone after surgery. A total of 447 patients were included in the study, out of which only 18% were GBC cases. This study clearly demonstrated the benefit of adjuvant therapy in improving the OS and decreasing the recurrence rate during the first 2 years after surgery. However, in this study, there were issues with quality of surgery performed as 54% of cases had positive microscopic margins and also 38% had node positive disease which is a subclass that derives maximum benefit from adjuvant therapy[36].

Adjuvant chemoradiotherapy

In view of a 25%-68% rate of recurrence in loco-regional basin, researchers have been advocating administration of adjuvant CRT[4,37]. In a study from the United States (SWOG0809 trial), 79 patients with biliary tract cancer were analyzed after receiving adjuvant capecitabine and gemcitabine followed by radiotherapy and concurrent capecitabine. GBC comprised 32% of the study population. The local recurrence at 2 years was 11% with a median OS of 35 mo. In spite of the lack of a control group, this study provided clinicians with a well-supported regimen[38]. However, Fareed *et al* [39] found no survival benefit with adjuvant chemoradiation in resected GBC patients. In a recent multi-institutional analysis, resected GBC patients with high-risk features such as T3/T4 tumor, lymph node positivity, and R1 resection were found to derive maximum benefit after adjuvant therapy[40]. In present times, the data is still insufficient to conclusively advocate adjuvant chemotherapy over chemoradiation in node negative R0 resected patients. However, adjuvant chemoradiation is unanimously considered to be the treatment of choice in patients with R1/2 resection margins[3].

In the absence of standard clinical guidelines, in the current study, all patients with T stage ≥ 2 and/or positive lymph node were advised to receive adjuvant therapy. Three–fourth of all our patients received adjuvant therapy. Estimated 5-year OS rate was 43.5%, which is comparable to that observed in the MSKCC study[15] (Table 4). Historically, the 5-year OS rate after aggressive resection for GBC was 16%[41]. Even after all the advancements in surgical technique and perioperative care, the median survival for patients with stage I-III disease was 12.9 mo and 5.8 mo for those presenting with stage IV disease in the absence of multimodality treatment at MSKCC in 2008 with improvement in survival after increase in administration of systemic therapy[15,42]. Our study showed a better median survival for stage III and IV cases with multimodality treatment (27 mo and 20 mo, respectively). When comparing early stage disease (stages I and II) with locally advanced stage GBC (stages III and IV), the former had a significantly better survival (73.1 *vs* 41.4 mo, respectively, $P < 0.0001$), which emphasizes on the need for better chemotherapeutic regimen as well as uniform application of systemic therapy in the adjuvant setting.

Our study is one of the largest studies worldwide reporting improved outcomes following multimodality treatment in surgically resected patients. In wake of the scarcity of data on multimodality management of GBC, our study highlights the feasibility of better outcomes with proper utilization of systemic therapy with surgery to obtain optimum results. Correlation between specific chemotherapy regimens and survival is beyond the scope of this study due to its retrospective nature. Despite inherent limitations with potential biases, our study stresses on the urgent need for conducting randomized trials to form consensus on tackling an aggressive disease like GBC. In future, addition of genomic profiling-guided targeted therapy may potentially improve the survival and personalize the therapy of GBC patients.

CONCLUSION

GBC is an aggressive malignancy which warrants equally aggressive measures to provide patients with a meaningful survival. With addition of systemic therapy to

curative surgery, the 5-year survival rate in our study was 43%. R+ resection, higher T stage, and PNI adversely affected the outcome. Patients with higher stage (III/IV), nodal involvement, and high-risk features should be considered for systemic therapy in addition to surgery to optimize the outcomes. Multimodality treatment of GBC has a potential to improve the survival of these patients.

ARTICLE HIGHLIGHTS

Research background

Gallbladder cancer (GBC) is an aggressive biliary tract cancer with only 10% of cases amenable to resection at presentation with a dismal overall 5-year survival of less than 15% after surgery. Even after surgical resection, overall survival (OS) has been poor due to high rates of recurrence. With advancements in surgical approach and systemic therapy, multimodality approach has a potential to obtain favorable outcomes in this aggressive disease; however, there is a paucity of data in the literature for its uniform application.

Research motivation

In the management of patients with GBC, adoption of a multimodality approach should be considered.

Research objectives

The research purpose was to share our experience and give an overview on multimodality management of GBC patients.

Research methods

All the data of patients undergoing surgery for suspected GBC from January 2012 to December 2018 was retrieved from a prospectively maintained electronic database and analyzed.

Research results

Multimodality treatment significantly improved the 5-year survival of patient with GBC. Microscopically positive resection margin, higher T stage, and perineural invasion adversely affected the outcome.

Research conclusions

Gallbladder cancer has a favorable survival when treated with multimodality approach. Patients with high-risk features may particularly benefit from this approach

Research perspectives

Multimodality treatment of GBC has a potential to improve the survival of GBC patients.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Jaipuria J, Consultant (Surgical Oncology), for his valuable inputs.

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10.1002/jso.21141]

World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 7; 27(45): 7831-7843

DOI: 10.3748/wjg.v27.i45.7831

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

ORIGINAL ARTICLE

Observational Study

In-hospital mortality of hepatorenal syndrome in the United States: Nationwide inpatient sample

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of data, revising the article, final approval; Cheungpasitporn W performed the overall supervision and final approval.

Institutional review board statement: The Mayo Clinic Institutional Review Board approved this study (IRB number 21-007353 and date of approval; July 27, 2021).

Informed consent statement: The Mayo Clinic institutional review board approved this study and exempted the need for informed consent because the data in NIS database was publicly available and de-identified.

Conflict-of-interest statement: The authors deny any conflict of interest.

Data sharing statement: We conducted a retrospective cohort study of hospital admissions for HRS from 2005 to 2014 in the National Inpatient Sample (NIS) database (publicly available and de-identified).

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

Country/Territory of origin: United States

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

Open-Access: This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external

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Abstract

BACKGROUND

Hepatorenal syndrome (HRS) is a life-threatening condition among patients with advanced liver disease. Data trends specific to hospital mortality and hospital admission resource utilization for HRS remain limited.

AIM

To assess the temporal trend in mortality and identify the predictors for mortality among hospital admissions for HRS in the United States.

METHODS

We used the National Inpatient Sample database to identify an unweighted sample of 4938 hospital admissions for HRS from 2005 to 2014 (weighted sample of 23973 admissions). The primary outcomes were temporal trends in mortality as well as predictors for hospital mortality. We estimated odds ratios from multi-level mixed effect logistic regression to identify patient characteristics and treatments associated with hospital mortality.

RESULTS

Overall hospital mortality was 32%. Hospital mortality decreased from 44% in 2005 to 24% in 2014 ($P < 0.001$), while there was an increase in the rate of liver transplantation ($P = 0.02$), renal replacement therapy ($P < 0.001$), length of hospital stay ($P < 0.001$), and hospitalization cost ($P < 0.001$). On multivariable analysis, older age, alcohol use, coagulopathy, neurological disorder, and need for mechanical ventilation predicted higher hospital mortality, whereas liver transplantation, transjugular intrahepatic portosystemic shunt, and abdominal paracentesis were associated with lower hospital mortality.

CONCLUSION

Although there was an increase in resource utilizations, hospital mortality among patients admitted for HRS significantly improved. Several predictors for hospital mortality were identified.

Key Words: Hepatorenal syndrome; Liver transplantation; Mortality; Nationwide; Big data; Hospitalization; Outcomes; Predictors

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Core Tip: In this study, we utilized the national inpatient sample database to assess the temporal trend in mortality and identify predictors for mortality among hospital admissions for hepatorenal syndrome in the United States. We demonstrated that the overall hospital mortality was 32%. Hospital mortality decreased from 44% in 2005 to 24% in 2014. There was an increase in the rate of liver transplantation, renal replacement therapy, length of hospital stay, and hospitalization cost.

Citation: Kaewput W, Thongprayoon C, Dumancas CY, Kanduri SR, Kovvuru K, Kaewput C, Pattharanitima P, Petnak T, Lertjitbanjong P, Boonpheng B, Wijarnpreecha K, Zabala Genovez JL, Vallabhajosyula S, Jadlowiec CC, Qureshi F, Cheungpasitporn W. In-hospital mortality of hepatorenal syndrome in the United States: Nationwide inpatient sample. *World J Gastroenterol* 2021; 27(45): 7831-7843
URL: https://www.wjgnet.com/1007-9327/full/v27/i45/7831.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i45.7831

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Received: September 4, 2021
Peer-review started: September 4, 2021
First decision: October 16, 2021
Revised: October 24, 2021
Accepted: November 25, 2021
Article in press: November 25, 2021
Published online: December 7, 2021

P-Reviewer: Kowalewski G, Kulkarni AV, Singh SA, Zaghloul MS
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

INTRODUCTION

Hepatorenal syndrome (HRS) is a serious complication of cirrhosis with an incidence as high as 32% among patients with advanced liver disease[1-7]. Previous studies have consistently demonstrated high morbidity, mortality, and resource utilizations[1,8-17]. Several factors have been associated with poor outcomes, including high model for end stage liver disease (MELD) score[18], degree of acute kidney injury (AKI)[11,19], extrahepatic organ failure[20], and sepsis[18,21].

In recent decades, there have been significant advances in knowledge, treatment, and optimal management of patients with HRS[1,7-17,22-24]. While terlipressin, a synthetic vasopressin analog with predominant vasopressin 1A receptor effect[25], has been used to treat HRS in many Asian and European countries, it is currently not yet available in the United States for the treatment of HRS[1,26]. Thus, currently available treatment options for HRS in the United States include albumin volume expansion, octreotide with or without midodrine, and intravenous cardiovascular medications like vasopressin and norepinephrine[1]. Nevertheless, there have been improvements in the overall care for patients with HRS, including liver transplantation and renal replacement therapy. In addition, several studies have suggested the use of transjugular intrahepatic portosystemic shunt (TIPS) for patients with HRS[27-29]. However, data specific to HRS, hospital mortality trends, and hospital admission resource utilization remain limited.

In this study, we aimed to evaluate patient characteristics, in-hospital treatments, mortality, and resource utilization during hospital admissions for HRS in the United States. We also assessed the temporal trend in mortality and identified the predictors for mortality.

MATERIALS AND METHODS

Study population

We conducted a retrospective cohort study of hospital admissions for HRS from 2005 to 2014 in the national inpatient sample (NIS) database. The detail of the NIS database was previously described[30]. We identified hospital admission with a primary discharge diagnosis using the international classification of disease-9 (ICD-9) diagnosis code of 572.4. The Mayo Clinic institutional review board approved this study (IRB number 21-007353 and date of approval; July 27, 2021) and exempted the need for informed consent because the data in NIS database was publicly available and de-identified.

Data collection

We abstracted patient and hospital characteristics, procedures, outcomes, and resource utilization from the database (Supplementary Table 1). Patient characteristics included age, sex, race, etiology of liver disease, medical comorbidity based on Elixhauser index [31], and admission day. Hospital characteristics included hospital size, ownership, location, teaching status, and region. Procedures included renal replacement therapy, liver transplantation, TIPS, abdominal paracentesis, and mechanical ventilation. Outcomes included hospital mortality, resource utilization, including length of hospital stay, and hospitalization cost. Since this study used data over 10 different calendar years, we adjusted hospitalization costs for inflation using the consumer price index and converted them to 2014 United States dollar equivalents.

Statistical analysis

The NIS database contains hospitalization data from a stratified sample of 20% of hospitals in the United States. As such, we used discharge weight provided by the Healthcare Cost and Utilization (HCUP) to estimate the total number of hospital admissions for HRS. We used descriptive statistics to summarize patient and hospital characteristics, procedures, outcomes, and resource use of HRS admission. We fitted logistic regression model for hospital mortality and liver transplantation, and standard least square linear regression for length of hospital stay, and hospitalization cost, using calendar years as the independent variable to assess the annual trend from 2005 through 2014. We estimated adjusted odds ratio (OR) for hospital mortality from multivariable multi-level mixed effect logistic regression, employing hospital identification number as random effect with patients-level characteristics clustered within hospital-level characteristics. We performed all statistical analyses using STATA, version 15 (StataCorp LP, College Station, TX, United States).

RESULTS

Patient characteristics, in-hospital treatments, outcomes, and resource use in hospital admission for HRS

There were 4938 hospital admissions with HRS as the primary diagnosis in the unweighted sample and 23973 admissions in the weighted sample. Table 1 shows patient and hospital characteristics of hospital admissions for HRS. The mean age was 58.8 ± 12.3 years, and the majority of patients were males (63%). Alcohol-related liver disease (46%) and viral hepatitis (25%) were the most common liver disease etiologies. Most patients were admitted to large urban teaching hospitals. Of those patients admitted for HRS, 21% received renal replacement therapy and 2% underwent liver transplant during their hospitalization. During this 10-year period, there was a 32% mortality observed for HRS admissions. The mean length of hospital stay was 8.8 d and the mean hospitalization cost was 73731 United States dollars.

Trends in hospital mortality, liver transplantation, length of stay, hospitalization cost in hospital admission for HRS

Table 2 showed the annual trend in hospital mortality, liver transplantation, length of hospital stay, and hospitalization cost in HRS admissions from 2005 to 2014.

There was a decreasing trend in hospital mortality from 44% in 2005 to 24% in 2014 among hospital admissions for HRS in the United States (OR: 0.92, 95%CI: 0.90-0.94 *per* year; $P < 0.001$) (Figure 1A).

Meanwhile, there was an increase in the rate of liver transplantation (OR: 1.11, 95%CI: 1.02-1.20 *per* year; $P = 0.02$) (Figure 1B) and renal replacement therapy (OR: 1.05, 95%CI: 1.02-1.08 *per* year; $P < 0.001$) (Figure 1C) performed in hospitalization for HRS.

There was an increasing trend in mean length of hospital stay (coefficient estimate 0.2 d *per* year; $P < 0.001$) (Figure 1D) and hospitalization cost (coefficient estimate 5778 United States dollars *per* year; $P < 0.001$) (Figure 1E) among hospitalization for HRS during 10-year period from 2005 to 2014.

Predictors for hospital mortality

In multivariable analysis (Table 3), older age (OR: 1.45 for 40-59 years, 1.77 for 60-79 years, 2.12 for ≥ 80 years, compared to 18-39 years; all $P < 0.001$), alcohol use (OR: 1.35; $P < 0.001$), coagulopathy (OR: 1.15; $P = 0.001$), and presence of a neurological disorder (OR: 1.38; $P < 0.001$) predicted higher hospital mortality.

Need for mechanical ventilation (OR: 9.24; $P < 0.001$) was associated with higher mortality, whereas liver transplantation (OR: 0.15; $P < 0.001$) and TIPS (OR: 0.23; $P < 0.001$), and abdominal paracentesis (OR: 0.48; $P < 0.001$) were associated with lower hospital mortality. Renal replacement therapy was not significantly associated with mortality risk.

DISCUSSION

In this study based on a large United States database of hospitalizations, the mortality rate for hospitalized patients with HRS decreased by approximately 50% during the 10-year study period. During the same period, there was a 2-fold increase in the incidence of HRS patients receiving a liver transplant and the incidence of in-hospital renal replacement therapy increased by 60%. Notably, there were also increase in length of hospital stay and a 2-fold increase in the estimated hospital cost, which is likely related to higher utilization of healthcare resources. This highlights the high economic burden of chronic liver disease in the United States[32,33].

The marked improvement in the in-hospital mortality rate for HRS is likely reflective of changes in both medical and surgical management during the study period. Our study shows that there was an apparent increase in the number of liver transplants and renal replacement therapy around 2007 to 2008. This trend coincided with overall changes in clinical practice over the preceding years[34]. Although the unique pathophysiology of HRS has long been recognized as a functional renal failure occurring as a result of advanced liver disease[35], its treatment, including the initiation of in-hospital dialysis, and the role for liver transplantation have significantly evolved[36]. Historically, the initiation of renal replacement therapy in patients with HRS was felt to be controversial and futile. Increasing experience with liver transplantation in the setting of HRS as well as improved access to continuous renal

Table 1 Patient characteristics, in-hospital treatments, outcomes, and resource use in hospital admission for hepatorenal syndrome (mean ± SD)

	Unweighted, *n* (%)	Unweighted % ± SE	Weighted, *n* (%)	Weighted % ± SE
Total, *n* (%)	4938		23973	
Sex				
Male	3130	63.39 ± 0.68	15183	63.33 ± 0.31
Female	1808	36.61 ± 0.68	8790	36.67 ± 0.31
Age (yr)		58.8 ± 12.3		58.8 ± 12.3
18-39	266	5.39 ± 0.32	1299	5.42 ± 0.15
40-59	2461	49.84 ± 0.71	11933	49.77 ± 0.32
60-79	1927	39.02 ± 0.69	9365	39.06 ± 0.31
≥ 80	284	5.75 ± 0.33	1376	5.74 ± 0.15
Race				
White	3098	72.23 ± 0.68	15050	72.12 ± 0.31
Black	421	9.81 ± 0.45	2055	9.85 ± 0.21
Hispanic	511	11.91 ± 0.49	2495	11.95 ± 0.22
Asian/Pacific islander	81	1.89 ± 0.21	395	1.89 ± 0.09
Native American	57	1.33 ± 0.17	280	1.34 ± 0.07
Other	121	2.82 ± 0.25	593	2.84 ± 0.11
Admission day				
Weekday	3955	80.09 ± 0.57	19223	80.18 ± 0.26
Weekend	983	19.91 ± 0.57	4751	19.82 ± 0.26
Liver disease etiology				
Alcoholic liver disease	2249	45.54 ± 0.71	10935	45.61 ± 0.32
Viral hepatitis	1218	24.66 ± 0.61	5915	24.67 ± 0.28
Comorbidities				
Diabetes Mellitus	1260	25.52 ± 0.62	6132	25.58 ± 0.28
Hypertension	1937	39.23 ± 0.69	9437	39.36 ± 0.32
Fluid/electrolyte disorders	3548	71.85 ± 0.64	17233	71.88 ± 0.29
Coagulopathy	2115	42.83 ± 0.70	10286	42.90 ± 0.32
Anemia	1937	39.23 ± 0.69	9422	39.30 ± 0.31
Weight loss	872	17.66 ± 0.54	4255	17.75 ± 0.25
Cancer	658	13.32 ± 0.48	3198	13.34 ± 0.22
Congestive heart failure	630	12.76 ± 0.47	3042	12.69 ± 0.21
Chronic pulmonary disease	613	12.41 ± 0.47	2973	12.40 ± 0.21
Obesity	456	9.23 ± 0.41	2218	9.25 ± 0.19
Neurological disorders	234	4.74 ± 0.30	1147	4.78 ± 0.14
Pulmonary circulation disorders	176	3.56 ± 0.26	847	3.53 ± 0.12
Valvular disease	164	3.32 ± 0.25	795	3.32 ± 0.12
Peripheral vascular disorders	119	2.41 ± 0.22	587	2.45 ± 0.10
Depression	413	8.36 ± 0.39	2002	8.35 ± 0.18
HIV/AIDS	36	0.73 ± 0.12	173	0.72 ± 0.05
Substance use				

Smoking	583	11.81 ± 0.46	2825	11.78 ± 0.21
Alcohol	1930	39.08 ± 0.69	9394	39.19 ± 0.31
Drug use	209	4.23 ± 0.29	1023	4.27 ± 0.13
Bed size				
Small	611	12.30 ± 0.50	2898	12.09 ± 0.21
Medium	1210	24.41 ± 0.66	5905	24.63 ± 0.28
Large	3117	63.28 ± 0.74	15171	63.28 ± 0.31
Location/Teaching status				
Rural	651	13.89 ± 0.53	3167	13.21 ± 0.22
Urban, non-teaching	1723	36.76 ± 0.74	8320	34.70 ± 0.31
Urban, teaching	2564	49.35 ± 0.77	12487	52.08 ± 0.32
Hospital region				
Northeast	984	20.24 ± 0.62	4817	20.09 ± 0.26
Midwest	1122	23.03 ± 0.65	5406	22.55 ± 0.27
South	1699	34.03 ± 0.73	8261	34.46 ± 0.31
West	1133	22.70 ± 0.64	5489	22.90 ± 0.27
Medical procedures/interventions				
Renal replacement therapy	1018	20.61 ± 0.58	4929	20.56 ± 0.26
Paracentesis	2226	45.08 ± 0.71	10843	45.23 ± 0.32
Mechanical ventilation	499	10.10 ± 0.43	2412	10.06 ± 0.19
TIPS	46	0.93 ± 0.14	218	0.91 ± 0.06
Liver transplantation	85	1.68 ± 0.18	404	1.68 ± 0.08
LTA	66	1.34 ± 0.16	321	1.34 ± 0.07
SLKT	19	0.38 ± 0.09	93	0.39 ± 0.04
Outcomes				
Mortality	1573	31.90 ± 0.66	7616	31.81 ± 0.30
Length of hospital stay (d)		8.8 ± 10.9		8.8 ± 11.0
Hospitalization cost (United States $)		735701 ± 135526		73731 ± 135876

SE: Standard error; HIV/AIDS: Human immunodeficiency virus/acquired immunodeficiency syndrome; TIPS: Transjugular intrahepatic portosystemic shunt; LTA: Liver transplant alone; SLKT: Simultaneous liver-kidney transplantation.

replacement have resulted in a change in practice and a decrease in mortality[37].

In 2007-2008, multiple randomized control trials on terlipressin were published and have influenced the medical management HRS as well as patient outcomes[9-11,14,15]. Studies have shown potential beneficial effects of terlipressin, a potent selective splanchnic and extrarenal vasoconstrictor, on kidney function among patients with HRS[10,38,39]. Additionally, non-response to vasoconstrictors can also predict HRS mortality[40,41]. Unfortunately, as of 2020, the FDA has not yet approved the use of terlipressin for HRS in the United States. Results from the phase 3 trial terlipressin did not show any significant survival benefit and its use was associated with adverse events, such as respiratory failure[42,43]. Although terlipressin is currently not yet available in the United States[1,26], the observed findings of decreasing mortality trends for HRS in the Unites States are likely due to improvements in healthcare, increased access and acceptance of chronic intermittent hemodialysis for patients with liver disease as well as increased acceptance of liver transplantation for patients with acute decompensation[44].

In addition to liver transplantation, our study interestingly showed that TIPS and abdominal paracentesis were associated with lower hospital mortality among patients with HRS. Possible mechanisms underlying reduced mortality among patients who received paracentesis were that those who had abdominal paracentesis received more

Table 2 The annual trend in hospital mortality, liver transplantation, renal replacement therapy, length of hospital stay, hospitalization cost in hepatorenal syndrome admission from 2005 to 2014 (mean ± SD)

Year	Unweighted sample[1]	Weighted sample[1]	Hospital mortality weighted % ± SE	Liver transplantation weighted % ± SE	Renal replacement therapy weighted % ± SE	Length of stay (d)	Hospital cost (United States $)
Total	4931	23941	31.8 ± 0.7	1.7 ± 0.2	20.6 ± 0.6	8.8 ± 11.0	73731 ± 135876
2005	312	1471	43.8 ± 2.8	0.7 ± 0.5	13.8 ± 2.0	8.2 ± 8.6	42857 ± 67978
2006	330	1551	40.8 ± 2.7	0.6 ± 0.4	13.6 ± 1.9	7.2 ± 7.6	41841 ± 67254
2007	287	1358	36.7 ± 2.9	1.1 ± 0.6	17.0 ± 2.2	8.0 ± 8.9	49879 ± 77833
2008	367	1737	37.1 ± 2.5	1.9 ± 0.7	21.7 ± 2.2	8.6 ± 9.4	65419 ± 109901
2009	486	2363	31.1 ± 2.1	2.2 ± 0.7	20.8 ± 1.8	8.9 ± 11.3	71737 ± 123006
2010	610	2973	31.1 ± 1.9	0.9 ± 0.4	21.3 ± 1.7	9.3 ± 13.7	69778 ± 106971
2011	628	2934	30.3 ± 1.9	1.9 ± 0.5	23.3 ± 1.7	8.6 ± 9.6	82917 ± 154746
2012	603	3015	31.2 ± 1.9	2.0 ± 0.6	21.9 ± 1.7	9.1 ± 13.5	74951 ± 113671
2013	622	3110	28.3 ± 1.8	2.7 ± 0.7	21.4 ± 1.6	9.4 ± 12.1	95671 ± 210352
2014	686	3430	24.1 ± 1.6	1.7 ± 0.5	22.4 ± 1.6	9.0 ± 9.0	90829 ± 149495
P value			< 0.001	0.02	< 0.001	< 0.001	< 0.001

[1]Sample of hepatorenal syndrome patients having complete data on mortality status.
SE: Standard error.

aggressive treatments such as albumin and vasopressors, TIPS, and liver transplantation than those who received palliative care. Furthermore, abdominal paracentesis may have led to the diagnosis and treatment for spontaneous bacterial peritonitis[45]. The use of TIPS in patients with HRS remains controversial, although there is increasing data suggesting there may be benefit[24,29]. According to current best practice recommendations, the presence of HRS is not an absolute contraindication for TIPS and the presence of other indications, such as ascites, should guide decision making[29]. Specific to this topic, there is a clear need for additional randomized controlled trials, however, in the interim, there are an increasing number of small studies demonstrating positive outcomes in select HRS patients receiving TIPS[24,46,47]. Since mortality in patients with HRS undergoing TIPS is driven mainly by poor liver function it may be possible that there was a population selection bias and these patients had initially better liver function resulting in better survival.

Our study also showed several risk factors associated with in-patient mortality for HRS. These factors include advanced age, history of alcohol use, coagulopathy and presence of a neurological disorder. It is well known that older age, coagulopathy, and neurological disorder are associated with poor outcomes in patients with HRS[11,18-21]. Hepatic encephalopathy is known to be associated with mortality[48], and thus this could be the underlying reason for association between neurological disorder and increased in-patient mortality for HRS. Although specific knowledge regarding the duration and timing of alcohol use prior to hospitalization is a limitation of this dataset, active alcohol use is a known decompensating event that can result in AKI and HRS. It is also possible that recent alcohol use prevented certain patients from being suitable for liver transplantation. In this foreseeable scenario, initiation of renal replacement therapy has increasingly been used as a bridge to liver transplant eligibility and liver compensation.

There are several limitations in our study. The NIS is a hospitalized database. Thus, we did not evaluate the long-term outcomes of HRS following hospitalization. Although our study showed a decreasing trend of in-hospital mortality rates, it should not be generalized to the overall survival of patients with HRS. Estimates of in-hospital mortality do not include deaths that occur after discharge. The database did not contain MELD score, which predicted mortality in HRS patients[48]. In addition, treatment of HRS was not assessed in this study[40,41]. Data on medications including midodrine, octreotide, vasopressor, albumin infusion were not available in the database. Thus, we could not assess the effects of these agents and the response to

Table 3 Clinical characteristics associated with in-hospital mortality

Characteristics	Univariable analysis		Multivariable analysis	
	Unadjusted OR (95%CI)	*P* value	Adjusted OR (95%CI)	*P* value
Female sex	0.96 (0.85-1.09)	0.52	0.91 (0.78-1.07)	0.27
Age (yr)				
18-39	1 (ref)	-	1 (ref)	-
40-59	1.21 (0.91-1.60)	0.19	1.45 (1.28-1.64)	< 0.001
60-79	1.24 (0.93-1.65)	0.14	1.77 (1.68-1.87)	< 0.001
≥ 80	1.68 (1.17-2.42)	0.005	2.12 (1.51-3.00)	< 0.001
Race				
White	1 (ref)	-	1 (ref)	-
Black	1.38 (1.11-1.71)	0.003	1.26 (0.91-1.75)	0.16
Hispanic	1.05 (0.86-1.29)	0.61	1.12 (0.78-1.61)	0.53
Asian/Pacific Islander	1.44 (0.91-2.27)	0.12	1.30 (0.98-1.73)	0.07
Native American	1.04 (0.59-1.84)	0.88	1.11 (0.86-1.43)	0.43
Other	0.88 (0.59-1.32)	0.55	0.93 (0.48-1.81)	0.84
Weekend admission	1.14 (0.98-1.32)	0.08	1.05 (0.82-1.34)	0.69
Liver disease etiology				
Alcohol-related	0.90 (0.80-1.01)	0.08	0.85 (0.71-1.01)	0.06
Viral hepatitis	0.98 (0.86-1.13)	0.83	1.00 (0.81-1.24)	1.00
Comorbidities				
Smoking	0.96 (0.79-1.16)	0.66	1.15 (0.83-1.60)	0.40
Alcohol use	0.98 (0.87-1.11)	0.79	1.35 (1.26-1.45)	< 0.001
Drug use	0.84 (0.62-1.14)	0.26	0.77 (0.55-1.08)	0.13
HIV/AIDS	1.02 (0.51-2.07)	0.95	0.81 (0.58-1.13)	0.22
Autoimmune arthritis	1.10 (0.64-1.91)	0.73	1.14 (0.54-2.41)	0.72
Congestive heart failure	1.05 (0.88-1.26)	0.59	0.99 (0.87-1.12)	0.84
Chronic pulmonary disease	1.00 (0.84-1.21)	0.96	0.95 (0.78-1.16)	0.63
Coagulopathy	1.01 (0.90-1.15)	0.82	1.15 (1.16-1.25)	0.001
Diabetes mellitus	0.78 (0.67-0.89)	< 0.001	0.87 (0.73-1.04)	0.12
Hypertension	0.76 (0.67-0.86)	< 0.001	0.83 (0.70-1.01)	0.06
Lymphoma	1.42 (0.68-2.96)	0.35	1.53 (0.42-5.60)	0.52
Fluid/electrolyte disorders	0.85 (0.74-0.97)	0.02	0.87 (0.75-1.01)	0.07
Cancer	1.34 (1.13-1.59)	0.001	1.40 (0.88-2.23)	0.15
Neurological disorders	1.29 (0.98-1.70)	0.07	1.38 (1.21-1.58)	< 0.001
Obesity	0.87 (0.70-1.07)	0.20	0.92 (0.62-1.38)	0.70
Peripheral vascular disorders	0.78 (0.51-1.18)	0.23	0.78 (0.42-1.46)	0.44
Psychoses	0.80 (0.55-1.16)	0.24	0.93 (0.78-1.12)	0.44
Pulmonary circulation disorders	0.75 (0.53-1.05)	0.10	0.68 (0.43-1.08)	0.11
Valvular disease	0.75 (0.53-1.07)	0.12	1.01 (0.64-1.60)	0.96
Weight loss	0.91 (0.78-1.07)	0.27	1.05 (0.97-1.13)	0.21
Medical procedure				
Renal replacement therapy	0.98 (0.85-1.14)	0.81	0.92 (0.68-1.25)	0.59

Liver transplantation	0.33 (0.23-0.46)	< 0.001	0.15 (0.11-0.21)	< 0.001
TIPS	0.40 (0.18-0.90)	0.03	0.23 (0.12-0.43)	< 0.001
Paracentesis	0.46 (0.41-0.53)	< 0.001	0.48 (0.43-0.53)	< 0.001
Mechanical ventilation	6.97 (5.66-8.59)	< 0.001	9.24 (7.90-10.81)	< 0.001

HIV/AIDS: Human immunodeficiency virus/acquired immunodeficiency syndrome; TIPS: Transjugular intrahepatic portosystemic shunt; OR: Odds ratio; 95%CI: 95% confidence interval.

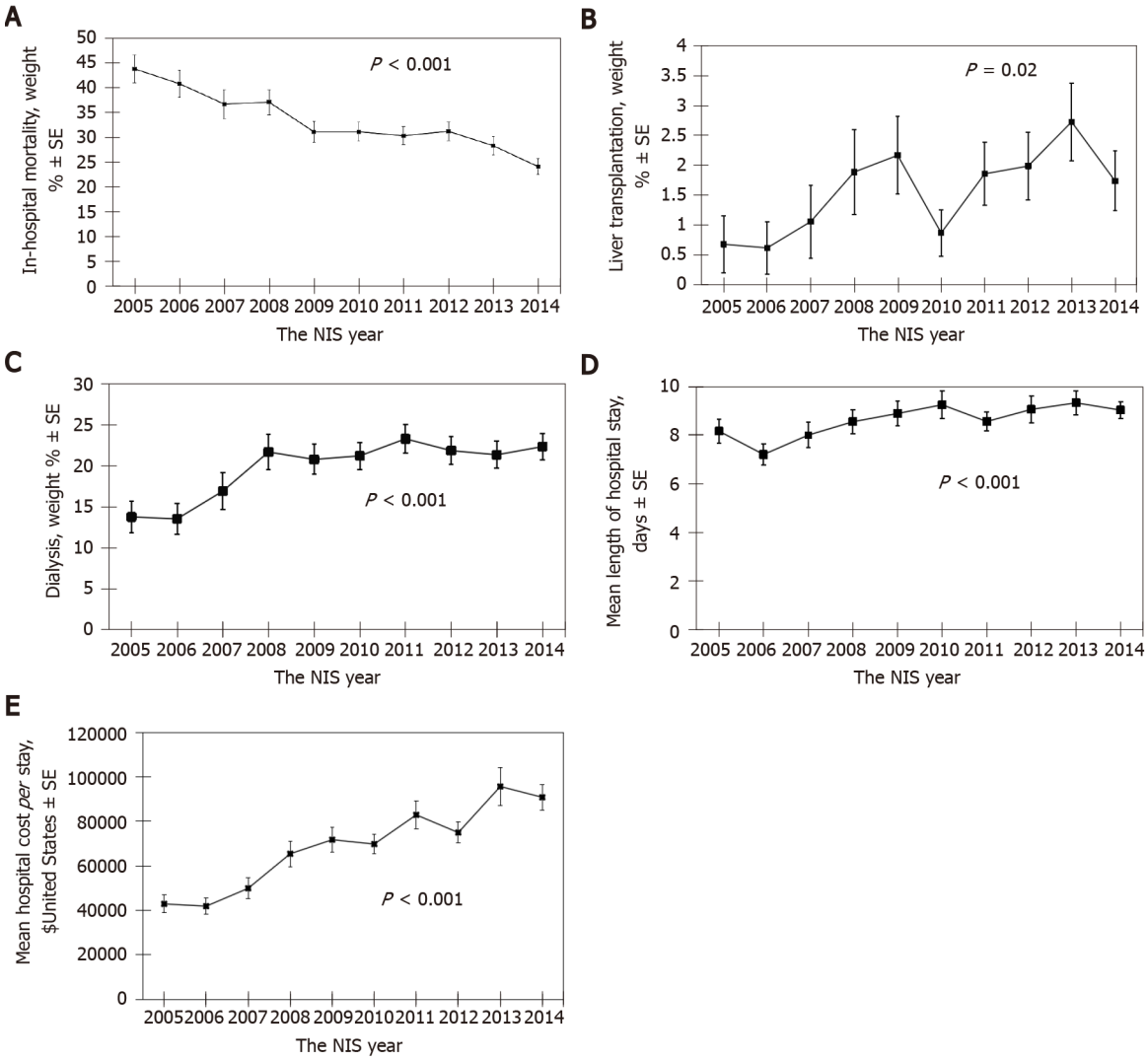


Figure 1 Data on admissions in the United States due to hepatorenal syndrome. A: Decreasing trend in hospital mortality among hospital admissions; B: Increase in the rate of liver transplantation among hospital admissions; C: Trend of renal replacement therapy among hospital admissions; D: Trend of mean length of hospital stay among hospital admissions; E: Trend of hospitalization cost among hospital admissions. NIS: National inpatient sample.

treatments on the outcomes of HRS. Lastly, HRS was identified by ICD-9 diagnosis code. Given definition of the HRS has changed over the years, these changes in definition may have affected the incidence of HRS in our study overtime.

CONCLUSION

In summary, our study showed a decreasing trend of in-hospital mortality rates in patients with HRS. These trends were likely related to advances in medicine, increased access and acceptance of renal replacement therapy, and increased utilization of liver transplantation which is the definitive treatment for HRS. Future studies are needed to

understand if these trends are impacted by other factors such as facility performance, patient care teams, health insurance reimbursement policies, or other factors.

ARTICLE HIGHLIGHTS

Research background

Hepatorenal syndrome (HRS) is a serious complication of cirrhosis, associated with high morbidity, mortality, and resource utilizations. In recent decades, there have been significant advances in knowledge, treatment and optimal management of patients with HRS.

Research motivation

There has been improvement in overall care for patients with HRS. Data on trends of hospital mortality and resource utilization in hospital admissions for HRS were limited.

Research objectives

We aimed to evaluate patient characteristics, in-hospital treatments, mortality, resource use among hospital admissions for HRS s in the United States. We also assessed the temporal trend in mortality and identified the predictors for mortality.

Research methods

We used the national inpatient sample database to identify unweighted sample of 4938 hospital admissions primarily for HRS from 2005 to 2014 (weighted sample of 23973 admissions). The primary outcome was the temporal trend in and predictors for hospital mortality. We estimated odds ratio from multi-level mixed effect logistic regression to identify patient characteristics and treatments associated with hospital mortality.

Research results

The overall hospital mortality was 32%. Hospital mortality decreased from 44% in 2005 to 24% in 2014 ($P < 0.001$), while there was an increase in the rate of liver transplantation ($P = 0.02$), renal replacement therapy ($P < 0.001$), length of hospital stay ($P < 0.001$), and hospitalization cost ($P < 0.001$). Multivariable analysis older age, alcohol abuse, coagulopathy, neurological disorder, and need for mechanical ventilation predicted higher hospital mortality, whereas liver transplantation, TIPs, and abdominal paracentesis were associated with lower hospital mortality.

Research conclusions

Although there was an increase in resource utilizations, hospital mortality among hospital admissions for HRS significantly improved.

Research perspectives

These trends were likely related to increased utilization of liver transplantation which is the definitive treatment for HRS. Future studies are needed to understand if these trends are impacted by other factors such as facility performance, patient care teams, health insurance reimbursement policies, or other factors.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 7; 27(45): 7844-7854

DOI: 10.3748/wjg.v27.i45.7844

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

CASE REPORT

Clinical presentation of gastric Burkitt lymphoma presenting with paraplegia and acute pancreatitis: A case report

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Informed consent statement: The patient and his legal guardian provided informed written consent during the treatment.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

CARE Checklist (2016) statement: The authors have read the CARE Checklist (2016), and the manuscript was prepared

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Abstract

BACKGROUND

The incidence of gastric Burkitt lymphoma (BL), presenting as paraplegia and acute pancreatitis, is extremely low. BL is a great masquerader that presents in varied forms and in atypical locations, and it is prone to misdiagnosis and missed diagnosis. The prognosis of BL remains poor because of the difficulty in early diagnosis and the limited advances in chemotherapy.

CASE SUMMARY

A 53-year-old man was referred to our hospital from the local county hospital due to abdominal pain for two weeks and weakness in the lower extremities for one day. Magnetic resonance imaging of the abdomen and lumbar spine showed a swollen pancreas and gallbladder, with peripancreatic exudation and liquid collection, indicating acute pancreatitis and acute cholecystitis. Additionally, we observed abnormally thickened lesions of the gastric wall, multiple enlarged retroperitoneal lymph nodes and a well-demarcated, posterolateral extradural mass lesion between T9 and T12, with extension through the spinal foramen and definite bony destruction, suggesting metastasis in gastric malignancy. Subsequent whole-body positron emission tomography/computed tomography examination showed multifocal malignant lesions in the stomach, pancreas, gallbladder, bone, bilateral supraclavicular fossa, anterior mediastinum, bilateral axillary and retroperitoneal lymph nodes. Gastroduodenal endoscopy revealed primary BL with massive involvement of the gastric body and duodenum. The patient refused chemotherapeutic treatment and died one week later due to upper gastrointestinal hemorrhage. Afterward, we reviewed the characteristics of 11

patients with BL involving the stomach, pancreas or spinal cord.

CONCLUSION
Clinicians should be aware that BL can be the potential cause of acute pancreatitis or a rapidly progressive spinal tumor with accompanying paraplegia. For gastric BL, gastroscopy biopsies and pathology are necessary for a definite diagnosis.

Key Words: Burkitt lymphoma; Paraplegia; Acute pancreatitis; Case report

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Core Tip: The incidence of Burkitt lymphoma (BL) is extremely low, and the clinical symptoms are atypical. The misdiagnosis rate is high, and the patient's prognosis is poor. The patient in this case was eventually diagnosed with BL involving the stomach, pancreas and vertebral column presenting with acute pancreatitis and neurological symptoms secondary to compression of the spinal cord. Chemotherapeutic treatment was refused by the patient, and he eventually died after one week due to upper gastrointestinal hemorrhage. This case reminds us that further transcriptomic and clinical studies are needed to explore desirable biomarkers for early BL. Eleven cases were reviewed with an emphasis on diagnostic criteria and treatment protocols. Clinicians need to raise awareness of BL and reduce misdiagnosis rates.

Citation: Lin Y, Pan YH, Li MK, Zong XD, Pan XM, Tan SY, Guo YW. Clinical presentation of gastric Burkitt lymphoma presenting with paraplegia and acute pancreatitis: A case report. *World J Gastroenterol* 2021; 27(45): 7844-7854
URL: https://www.wjgnet.com/1007-9327/full/v27/i45/7844.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i45.7844

INTRODUCTION

Burkitt lymphoma (BL) is a subgroup of high-grade non-Hodgkin's lymphoma (NHL) with an aggressive clinical course that was first described as a clinical entity in children in Central Africa by Denis Burkitt in 1958[1]. Clinically, patients with BL often present with solid tumors or large lymph nodes or symptoms similar to acute leukemia, and bone marrow invasion is present in more than 25% of cases[2]. BL has been classified into three subtypes according to the World Health Organization classification: Sporadic type, endemic type and immunodeficiency-associated type[3]. Endemic BL is most prevalent in children from equatorial Africa and New Guinea. Approximately 50% of endemic BL affects the jaw or kidneys. This endemic subtype could also occur in the distal ileum, cecum, greater omentum, ovaries and breasts. Nearly all cases are associated with Epstein-Barr virus[4]. Sporadic BL most commonly affects children[5] but represents less than 1% of NHL cases among adults[6]. Most sporadic BL occurs in the bowel, respiratory tract-associated lymphoid tissue and gut-associated lymphoid tissue. Immunodeficiency-associated BL is most frequently present in Human Immunodeficiency Virus-positive patients[4]. BL is highly sensitive to chemotherapy. Despite the long-term treatment-related sequelae of patients with BL treated with high-intensity chemotherapy regimens, patients who tolerate highly intensive combination chemotherapy regimens tend to have excellent oncologic outcomes. Currently, most treatment protocols for adult patients are based on pediatric clinical trials, and treatment-related toxicities remain a major barrier for those with advanced age. Hence, the overall prognosis for adult patients remains dismal[7]. Due to the rapid proliferation of BL, early diagnosis is essential for the effective treatment of BL. Until recently, BL was diagnosed mainly on the basis of clinical presentation, histopathological changes, morphology, immunophenotype and genotype. There have been few reports on adult patients with sporadic BL, especially adult patients with severe involvement of the stomach, pancreas and spinal cord. Herein, we report a case of gastric BL in an adult patient presenting with paraplegia and acute pancreatitis, along with a review of the literature.

according to the CARE Checklist (2016).

Supported by The Science and Technology Planning Project of Guangzhou City, No. 201803010018.

Country/Territory of origin: China

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): 0
Grade D (Fair): 0
Grade E (Poor): 0

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Received: August 9, 2021
Peer-review started: August 9, 2021
First decision: August 29, 2021
Revised: September 11, 2021
Accepted: November 24, 2021
Article in press: November 24, 2021
Published online: December 7, 2021

P-Reviewer: Dias E
S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

CASE PRESENTATION

Chief complaints

A 53-year-old male patient was admitted to the hospital with abdominal pain for two weeks and weakness in the lower extremities for one day.

History of present illness

This patient was admitted to the local hospital because of epigastric pain after alcohol consumption. He described the pain as intermittent, non-radiating and worsening with food consumption. The patient denied nausea, vomiting, constipation, fever or progressive weight loss. Based on abdominal pain, elevated levels of serum amylase, and findings of peripancreatic exudation and effusions by computed tomography (CT), the patient was diagnosed with acute pancreatitis. The patient was treated with antibiotics, proton pump inhibitors, fasting and short-term intravenous feeding and fluid therapy, and the abdominal pain was alleviated slightly. Unfortunately, on the 14th d of hospitalization, this patient developed a sudden onset of aconuresis and paraplegia. He was referred to our hospital for further examination.

History of past illness

The patient reported no remarkable history of past illness.

Personal and family history

There was no family history of malignant tumors.

Physical examination

The patient's vital signs were stable. No superficial lymphadenopathy was palpable. Regarding the pulmonary and cardiac examination, no obvious abnormality was observed. The abdomen was flat and soft. Physical examination revealed epigastric tenderness without rebound tenderness or Murphy's sign. No jaundice or palpable masses were observed. Neurologic examination revealed no abnormality in his cranial nerves. The muscle strength of the upper limbs was normal, while it was grade I in the lower limbs. Deep tendon reflexes in the affected limbs were diminished or absent. Bilateral Babinski signs were positive. Hypoesthesia beneath the T8 sensory dermatome was observed. Meningeal irritation signs were negative. He also showed bladder-urinary dysfunction.

Laboratory examinations

The auxiliary examination at admission showed that the white blood cell count was 14.68 × 10^9/L (normal range, 3.5 × 10^9/L - 9.5 × 10^9/L), RBC count was 3.95 × 10^9/L (normal range, 4.3 × 10^9/L - 5.8 × 10^9/L), HGB was 136.0 g/L (normal range, 130-175 g/L), PLT count was 324 × 10^9/L (normal range, 100 × 10^9/L-350 × 10^9/L), C-reactive protein was 34.64 mg/L (normal range, 0-6 mg/L), procalcitonin was 0.12 ng/mL (normal range, 0-0.05 ng/mL), serum amylase was 266 U/L (normal range, 0-125 U/L), lactic dehydrogenase (LDH) was 526 U/L (normal range, 71-231 U/L), and uric acid was 799 μmol/L (normal range, 71-231 μmol/L). Laboratory tests showed no abnormalities in liver function or electrolytes. His carbohydrate antigen 19-9 was 461.28 U/mL (normal range, 0-35 U/mL), and carbohydrate antigen 12-5 was 126.90 U/mL (normal range, 0-35 U/mL). Other tests revealed normal tumor marker levels, including carcino-embryonic antigen and alpha fetoprotein levels of 0.56 ng/mL (normal range, 0-5 ng/mL) and 2.6 ng/mL (normal range 0-8.1 ng/mL), respectively.

Imaging examinations

A CT scan at admission showed a swollen pancreas and gallbladder, with peripancreatic exudation and liquid collection, indicating a diagnosis of acute pancreatitis and acute cholecystitis. Magnetic resonance imaging (MRI) of the abdomen and lumbar spine at the 14th d after admission showed a swollen pancreas and gallbladder, with less peripancreatic exudation and liquid collection, indicating the remission of acute pancreatitis and acute cholecystitis. Additionally, MRI showed abnormally thickened lesions of the gastric wall, multiple enlarged retroperitoneal lymph nodes and a well-demarcated, posterolateral extradural mass lesion between T9 and T12, with extension through the spinal foramen and definite bony destruction (Figures 1 and 2). Whole-body positron emission tomography-CT (PET-CT) was then performed and showed multifocal malignant lesions in the stomach, pancreas, gallbladder, bone, bilateral supraclavicular fossa, anterior mediastinum, bilateral axillary and retroperitoneal

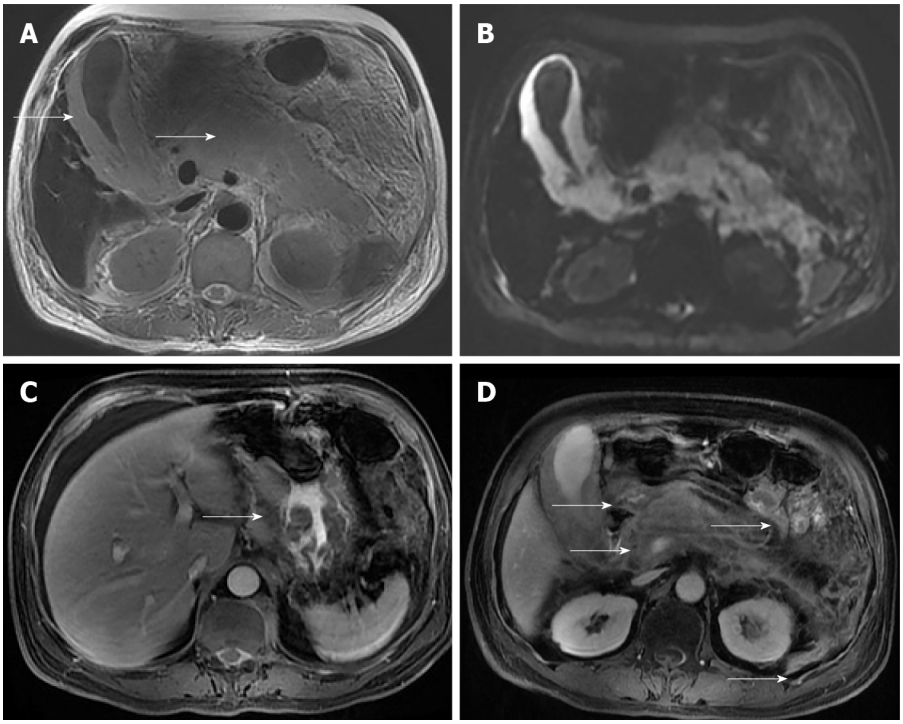


Figure 1 Magnetic resonance imaging of the abdomen at diagnosis. A: Axial T2-weighted magnetic resonance imaging (MRI) demonstrates homogeneous, hyperintense lesion in the whole pancreas and a markedly swollen gallbladder (arrows); B: Diffusion-weighted MRI shows abnormal hyperintensity in gall bladder wall and pancreas; C: Axial contrast-enhanced T1-weighted MRI shows the abnormal thickened lesions of the gastric wall (arrows), which display contrast enhancement in a × homogeneous fashion; D: Axial contrast-enhanced T1-weighted MRI shows the swollen gallbladder and multiple enlarged retroperitoneal lymph nodes (arrows), which display contrast enhancement in a homogeneous fashion.

lymph nodes (Figure 3), indicating multiple metastases of malignant tumors. Gastroduodenal endoscopy revealed massive involvement of the gastric body and duodenum with tumors (Figure 4). Histology and immunohistochemistry of gastric biopsies were suggestive of BL (Figure 5).

FINAL DIAGNOSIS

The histological findings, immunophenotype of the biopsies, and radiological findings were consistent with BL involving the stomach, pancreas and vertebral column. However, the primary lesion of BL is unclear. Because the patient had no symptoms of fever or weight loss, it was classified as group A. Due to the lack of bone marrow aspirate and trephine biopsy, we could not confirm the accuracy of the stage classification of BL in this case. Curiously, this patient presented with acute pancreatitis as the initial manifestation. One possible explanation for the presentation of acute pancreatitis is that the main pancreatic duct was obstructed by the substantial mass. Obstruction of the pancreatic orifice may impair the outflow of pancreatic juice and eventually induce pancreatitis.

TREATMENT

After admission to our department, this patient received short-term fasting, acid suppression, pancreatic enzyme suppression and fluid replacement for acute pancreatitis. Due to suspicion of necrotic pancreatitis, sulbactam sodium/cefoperazone sodium (3 g/d) was administered IV for one week.

Unfortunately, this patient developed sudden onset of aconuresis and paraplegia. According to the neurology consultation, acute myelitis was suspected. To inhibit the inflammatory response and block the antibodies, high doses of glucocorticoids and gamma globulin were applied for three days. Nevertheless, the efficacy of these treatments appeared poor. Concerning the high cost and potential side effects of these treatments, glucocorticoid and gamma globulin treatment was abandoned. Based on



Figure 2 Magnetic resonance imaging of the thoracic and lumbar vertebrae at diagnosis. A: Sagittal T2-weighted magnetic resonance imaging (MRI) shows epidural mass at the centrum and left posterolateral aspect of the spinal cord at the T9 to T12 levels, resulting in severe cord compression; B: Sagittal contrast-enhanced T1-weighted MRI shows the lesions displaying contrast enhancement in a heterogeneous fashion; C: Axial T2-weighted MRI shows that epidural mass involves the centrum and left posterolateral aspect of the spinal cord; D: Axial contrast-enhanced T1-weighted MRI shows the lesions displaying contrast enhancement in a heterogeneous fashion.

the indication for further imaging tests, this patient was diagnosed with gastric BL *via* endoscopic biopsy. Accordingly, a chemotherapy combination of cyclophosphamide, adriamycin, vincristine and prednisolone (CHOP) was recommended for the patient, but he refused the chemotherapeutic treatment.

OUTCOME AND FOLLOW-UP

One week after diagnosis and refusal of chemotherapy, the patient died of upper gastro-intestinal hemorrhage.

DISCUSSION

The incidence of BL is extremely low, and the clinical symptoms are atypical. Thus, we need to raise awareness of BL and reduce the misdiagnosis rates. BL was first described in 1958 by a British surgeon named Denis Burkitt as a sarcoma involving the jaw in African children with characteristic symptoms[1]. There has been some improvement in the understanding of its epidemiological diagnosis and treatment in the ensuing half century. In this article, we report the 11th case of BL involving the stomach, pancreas and spinal cord diagnosed based on the radiological findings and immunophenotype of the biopsies. The clinical features of 10 previous cases of BL

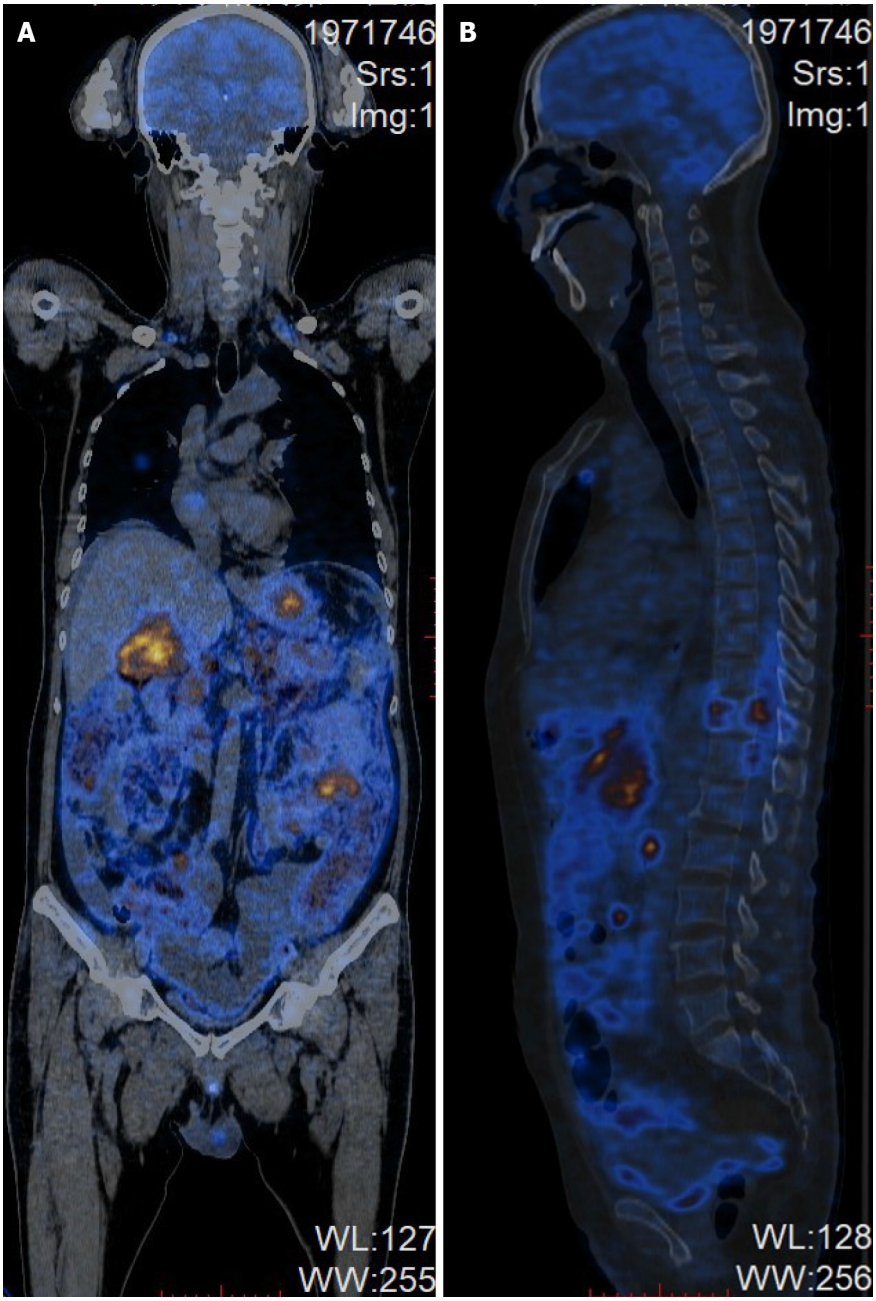


Figure 3 Positron emission tomography-computed tomography of the whole body at diagnosis. A: Coronal images; B: Sagittal images.

involving the stomach, pancreas or spinal cord are summarized in Table 1[8-17].

Among the previous cases, nine cases were reported in foreign countries, while only one patient came from China. From our review of the literature, a clear male predominance (70%) can be established. The ages of the patients range from 9 to 69 years, with a median age of 23 years. The initial symptoms, including abdominal distension, abdominal pain, lumbago, weakness in the lower extremities, fulminant hematemesis and progressive weight loss, are atypical. Regarding the detailed treatment protocols, seven patients received chemotherapy. Only one patient received palliative radiation treatment due to severe spinal cord involvement. Among the four patients who underwent surgical intervention, one patient underwent surgery for intraspinal decompression and mass separation, and the other three patients underwent distal or total gastrectomy. The outcome and follow-up of BL were reported in a total of eight cases. Regrettably, only a 9-year-old patient remained in clinical remission with completed chemotherapy, and no treatment-related sequelae 4 years were observed from initial diagnosis. Severe complications, including gastric perforation, sepsis and bacteremia, are always derived from intensive chemotherapy. Due to lymphoma recurrence or severe complications associated with chemotherapy, the other seven patients died within 6 mo of diagnosis.

Table 1 Clinical features of 10 previous cases of Burkitt lymphoma involving stomach, pancreas or spinal cord

Author	Age	Gender	Initial symptom	Affecting area	Biopsy area	Immunohistochemical studies	EB	HIV	Treatment	Prognosis
Kim *et al*[8]	69	Female	Low back pain radiating down to the right leg	Spinal cord at the L2 to L4 levels, intestine, live, bone and left supraclavicular lymph node	A posterolateral extradural mass lesion between L2 and L3	CD20 (+), CD79a (+), BCL-6 (+), CD10 (+), BCL-2 (-)	+	NA	NA	NA
Seo *et al*[9]	40	Male	Progressive pain and weakness in lower extremities	Spinal cord at the T2 to T4 levels, liver	An intraspinal extramedullary mass from T2 to T4, liver	CD20 (+), CD45RO (-)	NA	+	Chemotherapy and radiation therapy with HAART after surgery for intraspinal decompression and mass separation. Radiation	Died by massive pulmonary thromboembolism at 13 wk postoperatively
Chieng *et al*[10]	9	Male	Progressive pallor, peripheral oedema and respiratory distress	Stomach	Gastric body mass	CD20 (+), CD10 (+) and CD43 (+)	NA	NA	Induction chemotherapy with COP. Further chemotherapy included two courses of COPADAM followed by two courses of CYM and double intrathecal chemotherapy of methotrexate and hydrocortisone	Remains in clinical remission with complete resolution of the protein-losing enteropathy and no treatment related sequelae 4 yr from initial diagnosis
Bolandparvaz *et al*[11]	21	Male	Abdominal pain	Stomach	A huge mass in greater curvature of the stomach	NA	NA	NA	Total gastrectomy and roux-en-y esophagojejunostomy, chemotherapy was given for the patient 1 wk later without any other complication	NA
Gurzu *et al*[12]	60	Female	Fulminant hematemesis, recurring melena, epigastric pain, inappetence, and weight loss	Stomach	A huge mass in the antrum and posterior wall of the gastric body	CD20 (+), CD79a (+), BCL-6 (+), CD10 (+), Ki-67 (100%+), CD3 (-), CD5 (-), CD23 (-), TdT (-), bcl-2 (-), and Cyclin D1 (-)	-	NA	Distal gastrectomy	Died ten days after surgical intervention
Krugmann *et al*[13]	28	Male	Hematemesis and increasing abdominal pain	Stomach	A huge mass in the middle third of the stomach	CD20 (+), CD10 (+), BCL-6 (+), Ki-67 (95%+), CD3 (-), CD5 (-), CD23 (-), Cyclin D1 (-), BCL-2 (-) and TdT (-)	-	NA	Billroth-II surgical resection	Died due to lymphoma recurrence four months after onset
Liao *et al*[14]	26	Male	Fulminant hematemesis, abdominal pain	Stomach	A mass in the body and antrum of the stomach	CD20 (+), CD10 (+), BCL-6 (+), MUM-1 (-), CD30 (-)	NA	NA	Induction chemotherapy with two courses of R-ECHOP. Further chemotherapy included two courses of R-hyper CVAD followed by five courses of intrathecal prophylactic injection of chemotherapy drugs	Lymphoma recurrence six months after onset
Sağlam *et al*[15]	20	Male	Weight loss, back pain, mandible numbness, night sweats, and poor exercise tolerance	The body of the pancreas	A mass in the body of the pancreas	NA	NA	NA	Doxorubicin based combination chemotherapy	Died from sepsis during the second month of chemotherapy
Nistala *et al*[16]	21	Male	Jaundice, increasing swelling in	The head of the pancreas, cystic duct,	The first and second parts of	CD20 (+), CD10 (+), BCL-6 (+), CD5 (-), Mib-1 (99%+)	NA	NA	Two cycles of CHOP followed by hyper CVAD regimen as	NA

			the epigastric region	portal vein and hepatic artery, duodenum	duodenum				definitive therapy	
Konjeti *et al* [17]	68	Female	Belching, abdominal bloating and weight loss	The head of the pancreas, central hepatic duct and portal vein	The pancreatic head mass	CD20 (+), CD10 (+), C-myc (+), BCL-6 (+), CD3 (-), TdT (-), BCL-2 (-), Ki-67 (> 90%+)	NA	NA	Two cycles of chemotherapy regimen consisting of etoposide, prednisone, vincristine (Oncovin), and doxorubicin hydrochloride (Hydroxydaunorubicin hydrochloride)	Die due to the sepsis and bacteremia

EB: Epstein-Barr virus; HIV: Human Immunodeficiency Virus; HAAART: Highly active antiretroviral therapy; COP: Cyclophosphamide, vincristine and prednisolone; COPADAM: Cyclophosphamide, vincristine, prednisone, cytarabine, doxorubicin and methotrexate; CYM: Cytarabine and methotrexate; R-ECHOP: Rituximab, etoposide, cyclophosphamide, doxorubicin, vincristine and prednisone; CVAD: Cyclophosphamide, vincristine, doxorubicin, dexamethasone; CHOP: Cyclophosphamide, doxorubicin, vincristine and prednisolone; NA: Not available.

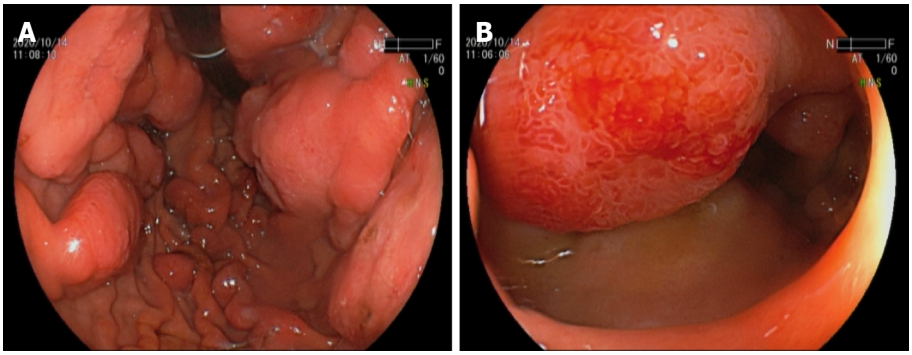


Figure 4 Gastric endoscopy. A: Multiple large (2 to 3 cm in diameter) raised ulcerated tumors involving both the greater and smaller curvatures of the gastric body; B: Numerous smaller tumors involving the anterior wall of the duodenal bulb and the second part of duodenum.

Three variants of BL have been described worldwide: Endemic, sporadic, and immunodeficiency-associated. Among the three subtypes, sporadic BL is regarded as the most common type[18]. The clinical features of BL are variable. In endemic BL, patients tend to present with jaw and other facial diseases. Cases of sporadic BL with an intraperitoneal mass as the initial manifestations are more common. Additionally, the clinical course of sporadic BL is usually aggressive, with frequent extranodal and central nervous system (CNS) involvement and an overall poor prognosis. BL with extranodal involvement usually occurs in the gastrointestinal tract (50%) and head and neck (25%). According to the statistics, CNS involvement is recognized in 13%-17% of all cases of BL[19]. This kind of cancer cell proliferates rather rapidly, with a doubling time of approximately 24 h, and the Ki-67 proliferation index tends to be 90%-100%. Clinically, a blood test usually reveals markedly elevated LDH and uric acid levels in the early stages, indicating a high tumor burden[20]. Herein, we report a case of gastric BL in an adult patient presenting with paraplegia and acute pancreatitis. Similarly, the auxiliary examination in this case also showed markedly elevated LDH and uric acid levels at admission. During hospitalization, this patient developed acute compression of the spinal cord. Abdominal CT at admission revealed no apparent abnormal findings except for the indication of acute pancreatitis. Unexpectedly, MRI of the abdomen and lumbar spine at the 14th d after admission indicated multisite metastasis in gastric malignancy, including in the pancreas, bone, bilateral supraclavicular fossa, anterior mediastinum, bilateral axillary and retroperitoneal lymph nodes. Finally, gastroduodenal endoscopy revealed massive involvement of the gastric body and duodenum with BL. Dawson's criteria are used to label primary gastrointestinal lymphoma, including absence of peripheral lymphadenopathy at the time of presentation, lack of mediastinal lymph node enlargement, normal total and differential white blood cell count, predominance of bowel lesion at the time of laparotomy with only lymph nodes obviously affected in the immediate vicinity and no lymphoma involved in the liver and spleen[21]. In this case, the patient had leukocytosis and multiple enlarged retroperitoneal lymph nodes and therefore did not fulfil the criteria. Hence, it was not a case of primary gastric lymphoma and the primary lesion of BL is unclear. This case reminds us that malignant tumors can originate from hematopoietic

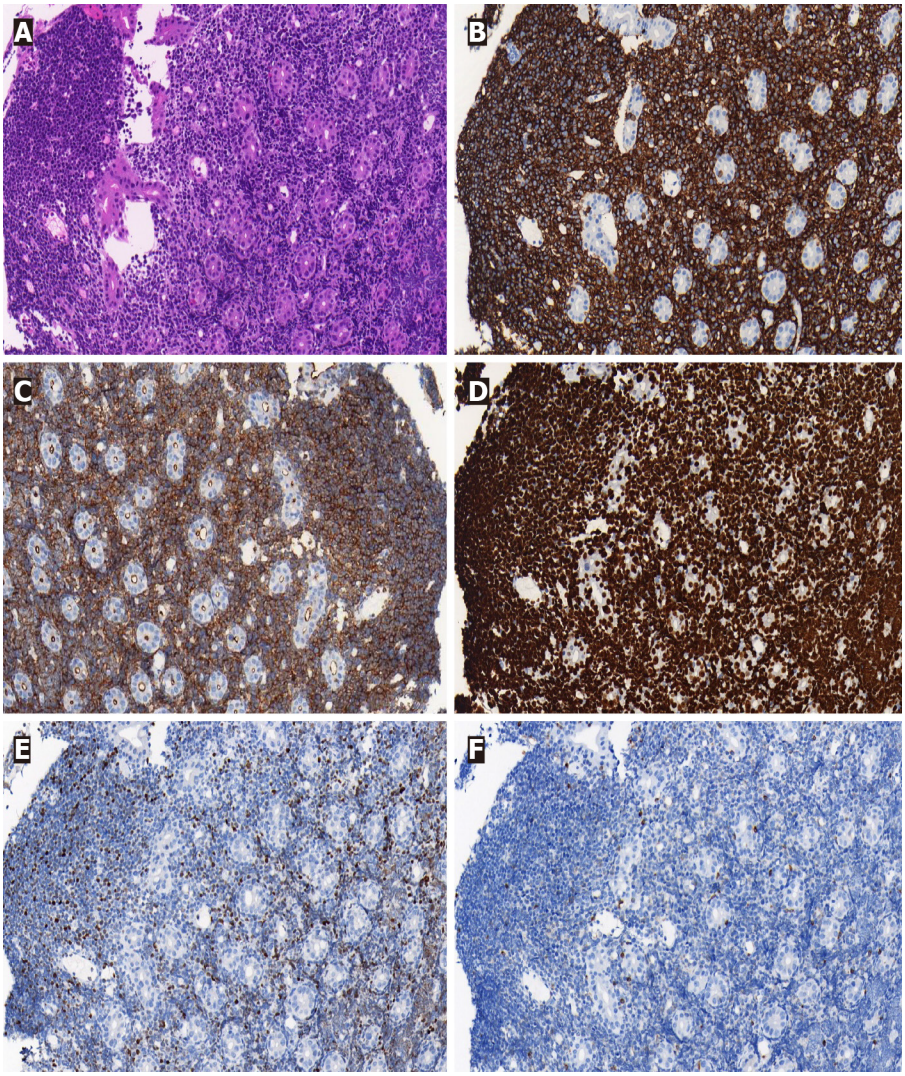


Figure 5 Histology and immunohistochemistry of gastric biopsies (× 200). A: Haematoxylin and eosin staining showed a characteristic "starry sky" appearance; B: Immunohistochemical staining was positive for CD20; C: Immunohistochemical staining was positive for CD10; D: Immunohistochemical staining was positive for Ki-67 (> 90% +); E: Immunohistochemical staining was positive for BCL-6; F: Immunohistochemical staining was negative for BCL-2.

malignancies, especially BL, and that this needs to be taken into consideration when there is abnormally rapid progression of the disease and when there are numerous affected areas or markedly elevated indicators of tumor burden.

Regarding the imaging evaluation of BL, CT scanning and three-dimensional reconstruction are more useful for accurately displaying bone destruction. When spinal cord involvement is suspected for clinical reasons, the preferred choice is MRI since it outperforms CT in depicting associated soft tissues. Additionally, diffusion-weighted MRI is a favorable diagnostic tool in oncologic imaging since it can reflect cellularity and proliferative activity in most malignancies. It is acknowledged that most malignancies are characterized by sustained proliferation, contributing to a high cellular density. More specifically, on diffusion-weighted MRI, BL demonstrates a markedly high signal intensity due to the relative restriction of water associated with high cellular density[22,23]. Since repeated serial imaging is essential for evaluating disease progression, MRI is also superior to CT due to its lack of ionizing radiation. For superior staging and assessment of the treatment response, PET/CT is a better choice since it can evaluate the functional status of abnormally hypermetabolic tissues throughout the whole body[24].

Histologically, the tumor cells of BL are medium-sized with an abundant, basophilic cytoplasm and display the typical "starry sky" pattern. The tumor cells are positive for BCL-6, CD19, CD20, CD22, CD10 and CD79a but negative for CD3, CD5, CD23 and TdT[25]. BL is characterized by the t (8; 14) (q24; q32) translocation of the c-myc and IgH genes, resulting in IgH-myc fusion, which can be detected by molecular analysis *via* fluorescence in situ hybridization. In our case, the tumor cells were negative for

creatine kinase and CD3, indicating that the tumor was not derived from the epithelium or T-cells. Additionally, the tumor cells were positive for CD20, CD79a, CD10, and BCL-6, suggesting germinal center-derived B cells. Combined with the high Ki67 index, the diagnosis of BL can be established.

Systemic chemotherapy is the preferred choice for the treatment of BL. Additionally, conventional radiotherapy, surgery, or a combination of both are recommended as the standard treatment unless severe compression of vital organs by lymphoma is observed[26]. Currently, most treatment protocols for adults are based on pediatric clinical trials. At present, most classical chemotherapy regimens show good efficacy and safety in children and relatively young patients. However, the prognosis of adult patients is poor due to their low response rate and severe treatment-related toxicity. Chemotherapy regimens, including CHOP, hyper-cyclophosphamide, vincristine, epirubicin, dexamethasone, etoposide, prednisone, vincristine, cyclophosphamide, epirubicin and cyclophosphamide, epirubicin, doxorubicin, vincristine, high-dose methotrexate/isophosphamide, cytarabine and etoposide, are still the backbone of therapeutic strategies for BL. Rituximab is an anti-CD20 chimeric antibody that acts by depleting CD20-positive B lymphocytes[27]. It has been reported that common chemotherapy regimens combined with rituximab can significantly improve the 3-year overall survival rate of BL patients (83% *vs* 70%)[28]. Treatment with prophylactic intrathecal methotrexate or cytarabine can lower the incidence of CNS relapse. Hence, it is regarded as a part of the first-line treatment option for BL[29,30]. In this case, the patient refused the chemotherapeutic treatment and died of upper gastrointestinal hemorrhage one week after diagnosis.

CONCLUSION

The incidence of BL is extremely low, and the clinical symptoms are atypical, contributing to the high misdiagnosis rate and poor prognosis. Clinically, malignant tumors originating in hematopoietic malignancies, especially BL, need to be taken into consideration if there is abnormally rapid progression of the disease and if there are numerous affected areas or markedly elevated indicators of tumor burden. CT or MRI could be an option for the detection of BL, while PET/CT is essential for the staging of BL. Histological assessment is indispensable for a definite diagnosis. Regarding the treatment of BL, chemotherapy is the preferred choice. Prophylactic intrathecal methotrexate or cytarabine is also recommended to lower the incidence of CNS relapse.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 7; 27(45): 7855-7858

DOI: 10.3748/wjg.v27.i45.7855

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

LETTER TO THE EDITOR

SARS-CoV-2 infection in people with pre-existing liver disease: Further research is warranted

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Author contributions: Verma HK and Bhaskar LVKS wrote and revised the letter.

Conflict-of-interest statement: The authors declare no conflict of interest for this article.

Country/Territory of origin: Italy

Specialty type: Gastroenterology and hepatology

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

Peer-review report's scientific quality classification

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

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Abstract

Patients with severe liver disease who have been infected with severe acute respiratory syndrome coronavirus-2 (coronavirus disease 2019) frequently develop acute respiratory distress syndrome and multiple organ failure, with a high mortality rate, as a result of the hyper-proinflammatory state known as the cytokine storm. Clinicians must recognize cytokine storms earlier to avoid intensive care admission and multi-organ damage, a critical life-threatening condition with prognostic and therapeutic implications

Key Words: Cytokine storm; Liver disease; Angiotensin-converting enzyme 2; Therapeutics; Inflammatory markers

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Core Tip: Understanding the hepatic consequences of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection and its molecular mechanism has greatly evolved. Evidence suggests that coronavirus disease 2019 fatalities are primarily due to cytokine storm and abnormal immune function. Throughout the infection, interleukin-6, nuclear factor kappa B, and tumor necrosis factor-alpha are inflammatory cytokines released by SARS-CoV-2-infected macrophages and monocytes that cause acute liver injury. Anti-viral treatment with anti-inflammatory receptors, such as monoclonal antibodies, can be used to reduce the morbidity and mortality associated with SARS-CoV-2 infection.

Citation: Verma HK, Bhaskar L. SARS-CoV-2 infection in people with pre-existing liver disease: Further research is warranted. *World J Gastroenterol* 2021; 27(45): 7855-7858

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Received: May 4, 2021
Peer-review started: May 4, 2021
First decision: June 12, 2021
Revised: June 13, 2021
Accepted: September 8, 2021
Article in press: September 8, 2021
Published online: December 7, 2021

P-Reviewer: Kashyap MK, Pham TTT
S-Editor: Fan JR
L-Editor: Wang TQ
P-Editor: Wang LYT

URL: https://www.wjgnet.com/1007-9327/full/v27/i45/7855.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i45.7855

TO THE EDITOR

Recently we have seen a paper entitled "Impact of cytokine storm and systemic inflammation on liver impairment patients infected by SARS-CoV-2: Prospective therapeutic challenges" contributed by Ali *et al*[1] in your well-regarded journal "*World J Gastroenterology*"[1]. Regarding this paper, we would like to draw your attention to several valuable and interesting aspects. The current scenario is that the second wave of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) [coronavirus disease 2019 (COVID-19)] pandemic is much more aggressive, with many more cases reported in various countries. As of April 2021, nearly 2.5 million deaths worldwide have been attributed to COVID-19. Based on the geographical distribution of the COVID-19 pandemic, it was found that in areas with a higher frequency, such as China, the rate of SARS-CoV-2 infected patients with liver impairment is also higher [2]. Most hospitalized COVID-19 patients have elevated liver biomarkers, primarily aminotransferase and bilirubin, which cause multi-organ failure[3,4]. This review paper by Ali *et al*[1] shows great public health interest. In their article, the authors elegantly described the impact of SARS-CoV-2 on hepatic impairment conditions. Besides, they focused on several current studies that indicated the role of the hyperinflammatory state that is known as "cytokine storm" concerning the angiotensin-converting enzyme 2 (ACE2) receptor as the main factor for the high rate of SARS-CoV-2 spreading and mortality and its putative therapies[5].

The SARS-CoV-2 directly enters the host cell through surface receptors and binds to ACE2[6]. ACE2 expression has been reported in different normal human organs, including the liver, where its expression is significantly low compared to the duodenum, kidney, and small intestine[7]. Accumulating evidence indicated the hepatic sharing of ACE2 after virus entry into the host cell. The underlying mechanisms of liver injury in COVID-19 patients are currently indistinguishable. However, human liver single-cell RNA-seq data indicated the co-expression of ACE2 and transmembrane serine protease 2 in liver progenitor cells, suggesting that the liver is the target of coronavirus disease[8].

Further, there is a 59.7% increase in ACE2 expression in cholangiocytes compared to 2.6% in hepatocytes, indicating that SARS-CoV-2 may directly bind to the ACE2 receptor, and the liver may be a good host for SARS-CoV-2[9,10]. Histological analysis of liver biopsies of COVID-19 patients revealed moderate microvascular steatosis, mild lobular, portal activity, and T cell overexpression, showing that the liver injury could have been caused by either SARS-CoV-2 infection or treatment[3,11]. A hospital-based study in China revealed elevated levels of proinflammatory cytokines, chemokines, and growth factors in COVID-19 patients compared to healthy adults[12,13]. Further, the patients with severe COVID-19 show hepatic dysfunction or liver disorders, including chronic liver disease, hepatitis viruses (types B, C, D, and E), hepatotropic virus infection, non-alcoholic fatty liver disease (NAFLD), and non-alcoholic steatohepatitis with elevated platelet, neutrophil, and lymphocyte counts, resulting in the worst outcomes from acute respiratory distress syndrome[14,15].

There is no consensus among researchers regarding liver damage in COVID-19 patients; some studies proposed the immediate cytopathic effect of the virus on hepatocytes or the biliary epithelium *via* ACE receptors[16,17]. Others postulated inflammatory and immune-mediated liver failure in patients with multiple organ damage[18]. However, hepatic inflammation involving cytokine activation was well-documented. A case study of COVID-19 patients demonstrated that the C-reactive protein (CRP) of 20 mg/L and a lymphocyte count of 1.1 109/L were independent risk factors for liver injury[19]. Kupffer cell activation is indeed a common finding in the liver of SARS-CoV-2 infected patients. Further, the altered macrophage polarization in SARS-CoV-2-infected patients with NAFLD suggests that SARS-CoV-2 has mechanisms to divert macrophage polarization in their preferred direction and increase the synthesis of inflammatory cytokines[18].

Regardless of the precise definition, the combinations of clinical manifestation and inflammatory markers (such as elevated plasma levels of CRP, lactate dehydrogenase, interleukin (IL)-6, IL-1, tumour necrosis factor-alpha (TNF)-α, and ferritin) could be used to define the "cytokine storm syndrome" in COVID-19 patients[20-22]. Besides this, treatment with anti-IL-6 receptor monoclonal antibodies (sarilumab and

tocilizumab), anti-IL-6 monoclonal antibodies (siltuximab), IL-1 inhibitors (Anakinra, Rilonacept, and Canakinumab), and TNF-α inhibitors (adalimumab, etanercept, and infliximab) showed promising results against SARS-CoV-2-induced cytokine storm[23-25]. In addition, corticosteroids that are known to alter the nuclear factor kappa B pathway central to the cytokine storm were used to manage the severe SARS and Middle East respiratory syndrome patients[26]. As a cytokine storm is a critical life-threatening condition and has prognostic and therapeutic implications, the clinicians must recognize cytokine storms earlier to avoid intensive care admission and multi-organ damage.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 December 7; 27(45): 7859-7861

DOI: 10.3748/wjg.v27.i45.7859

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

LETTER TO THE EDITOR

Therapeutic potentials of fasudil in liver fibrosis

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Author contributions: Xu PF designed the framework and supervised the preparation; both authors have wrote the letter, prepared the figures, read and approved the final letter.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Country/Territory of origin: United States

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

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Abstract

Fasudil has the potential to prevent liver fibrosis by activating natural killer cells and inhibiting the proliferation of hepatic stellate cells. Fasudil may be a promising clinical therapeutic drug for the prevention and treatment of liver fibrosis.

Key Words: Fasudil; Liver fibrosis; Natural killer cell; Hepatic stellate cell; Clinical therapeutic drug

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Core Tip: This letter to the editor is to supplement the ongoing discussion on the therapeutic potentials of Fasudil in the treatment of hepatic fibrosis. Fasudil is potential for the treatment of liver fibrosis through activating natural killer cells and inhibiting the proliferation of hepatic stellate cells. Fasudil, a vasodilator used in clinical treatment of cerebral vasospasm, exhibits the protective and therapeutic effect on liver fibrosis.

Citation: Xi Y, Xu PF. Therapeutic potentials of fasudil in liver fibrosis. *World J Gastroenterol* 2021; 27(45): 7859-7861

URL: https://www.wjgnet.com/1007-9327/full/v27/i45/7859.htm

DOI: https://dx.doi.org/10.3748/wjg.v27.i45.7859

TO THE EDITOR

We read with great interest the recent basic study by Han *et al*[1], that reported Fasudil, a potent RhoA/ROCK inhibitor and vasodilator, prevents and treats liver fibrosis and liver injury. They found Fasudil alleviates thioacetamide (TAA)-induced liver fibrosis in mice. The anti-fibrotic phenotypic exhibition of Fasudil is impressive.

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Received: July 4, 2021
Peer-review started: July 4, 2021
First decision: August 8, 2021
Revised: August 11, 2021
Accepted: November 24, 2021
Article in press: November 24, 2021
Published online: December 7, 2021

P-Reviewer: Zhang J
S-Editor: Wang LL
L-Editor: A
P-Editor: Wang LL

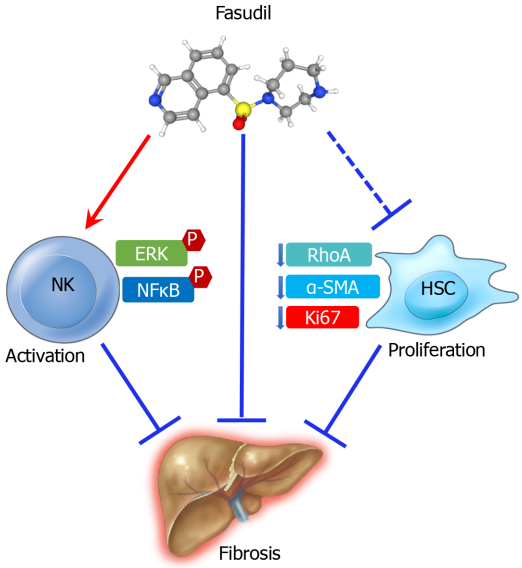


Figure 1 Schematic illustration depicting that fasudi prevents and treats liver fibrosis by activating natural killer cells and inhibiting hepatic stellate cells proliferation. NK: Natural killer; HSCs: Hepatic stellate cells.

Hepatic fibrosis is the formation of scar tissue in response to chronic liver damage, such as chronic hepatitis and hepatic steatosis[2]. Currently, there is no pharmacotherapy available approved by Food and Drug Administration (FDA) in the treatment of liver fibrosis[3]. Fasudil has been approved in Japan and China for the prevention of artery tightening and ischemia caused by cerebral vasospasm and pulmonary hypertension[4]. Due to its safety and efficacy, Fasudil might be a promising clinic agent for the prevention and treatment of liver fibrosis.

Hepatic stellate cells (HSCs) and natural killer (NK) cells play key roles in the pathogenesis of liver fibrosis. They isolated NK cells from mice treated with vehicle, TAA, or TAA and Fasudil and treated the NK-92 cells with different concentrations of Fasudil. These results showed that Fasudil robustly promotes NK cell activation. When discussing the effect of Fasudil on HSCs, they used human stellate cell line LX2 cells and observed that Fasudil directly induces apoptosis and inhibits the proliferation of LX2 cells. LX2 cell is indeed a model for the study of HSC activation. But to investigate HSCs activation, the model of primary HSCs subjected to culture activation and **L**X2 cells subjected to the stimulation of the potent profibrogenic cytokine transforming growth factor-beta 1 (TGF-β1) and then treated with the drugs under study are more widely accepted. Here, the authors proposed that Fasudil inhibited liver fibrosis by blocking HSCs activation by directly using the LX2 cells treated with Fasudil, which is far-fetched and hard to interpret. As primary HSCs are activated by prolonged culture, HSCs isolated from human or mouse livers and treated with the studied drug may be a more comprehensive approach to evaluate HSC activation.

Other studies also showed that Fasudil has anti-fibrotic phenotypic exhibition in rat models of hepatic fibrosis, such as Fasudil alleviated hepatic fibrosis in type 1 and 2 diabetic rats and carbon tetrachloride (CCl_4)-induced rat liver injury[5-7]. Combined with these studies, we proposed that Fasudil is potential for the treatment of liver fibrosis through multitargeted effects, as outlined in Figure 1. Taken together, Fasudi is a promising medication for the prevention and treatment of liver fibrosis.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 7; 27(45): 7862-7865

DOI: 10.3748/wjg.v27.i45.7862

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

LETTER TO THE EDITOR

Diagnostic biomarkers for pancreatic cancer: An update

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Author contributions: Yang M and Zhang CY collected data, wrote, finalized the letter, and contributed equally.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Country/Territory of origin: United States

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

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Abstract

Pancreatic ductal adenocarcinoma accounts for the primary type of pancreatic cancer (PC) with a 5-year survival rate of only about 10% in the United States. Early diagnosis will improve chances for curative treatment. To date, a broadly used serum marker for PC diagnosis is carbohydrate antigen 19-9, which is the only approved biomarker currently by the United States Food and Drug Administration. However, it has low specificity; therefore, development of novel biomarkers is urgently needed. Clinical trials are ongoing to evaluate candidate biomarkers for PC diagnosis, and the use of a multi-biomarker panel with current PC diagnostic biomarkers appears promising.

Key Words: Pancreatic ductal adenocarcinoma; Diagnosis; Biomarkers; Panel; Clinical trials

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Core Tip: The development of ideal diagnostic biomarkers for pancreatic cancer (PC) is critically important for early diagnosis, large-scale screening, monitoring of therapeutic response, prediction of risk, and prognosis. So far, the only approved serum marker for PC diagnosis is carbohydrate antigen 19-9 (CA 19-9) in the United States; although, many potential biomarkers have been investigated. However, CA 19-9 has low sensitivity; hence, new solutions are needed. Herein, we summarize some of the ongoing clinical trials that aim to investigate the application of biomarkers in PC diagnosis.

Citation: Yang M, Zhang CY. Diagnostic biomarkers for pancreatic cancer: An update. *World J Gastroenterol* 2021; 27(45): 7862-7865

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Received: July 20, 2021
Peer-review started: July 20, 2021
First decision: August 6, 2021
Revised: August 10, 2021
Accepted: November 24, 2021
Article in press: November 24, 2021
Published online: December 7, 2021

P-Reviewer: Imai Y, Jin ZD, Ling Q, Yu F
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

URL: https://www.wjgnet.com/1007-9327/full/v27/i45/7862.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i45.7862

TO THE EDITOR

We read with great interest a review paper recently published by O'Neill and Stoita [1], reviewing diagnostic biomarkers currently applied in pancreatic cancer (PC). The biomarkers are from serum, urinary, pancreatic, salivary, biliary, and fecal sources and comprise many different types of molecules. For example, serum biomarkers include proteins of glycolipids, growth factors, cytokines, chemokines, adhesion molecules, non-coding RNAs (long non-coding RNAs and microRNAs), and liquid biopsy (exosomes, circulating tumor DNA or ctDNA, and circulating tumor cells or CTCs)[1].

Moreover, we agree with the authors' suggestion that early diagnosis of PC improves chances for curative treatment. PC comprises two main subtypes, including the more common exocrine cancers and less common endocrine cancers. Pancreatic ductal adenocarcinoma (PDAC) accounts for the primary type of PC, consisting of around 95% in exocrine cancers and about 90% in all PCs. The 5-year survival rate of PC is relatively low and was only 10% for all patients with PC in the United States from 2010 to 2016[2]. To date, the only approved serum marker for PC diagnosis is carbohydrate antigen 19-9 (CA 19-9) in the United States, even though it has low specificity[3]. However, CA 19-9 is a non-PC-specific marker, shown to increase in colorectal, liver, lung, and ovarian cancers, as well as desmoplastic fibroblastoma[4,5]. Because of the low specificity of CA 19-9, a multi-marker panel that combines some of the currently investigated biomarkers (with CA 19-9) can be used to improve the specificity and sensitivity of PC diagnosis. For example, a multi-biomarker panel with enzyme-linked immunosorbent assay using three potential biomarkers, leucine-rich alpha-2-glycoprotein 1, transthyretin, and CA 19-9, improved the diagnosis of PDAC in normal pancreas and benign pancreatic disease and other tumors[6]. Although a multi-biomarker panel provides a better approach for early PC diagnosis, some limitations, including cost, the requirement for large sample volumes, good technique and analytical performance, and practical feasibility, may impact their broad application[3,7,8].

In addition, many of the biomarkers discussed in the abovementioned paper, including extracellular matrix-associated proteins such as matrix metalloproteinase and tissue inhibitor of metalloproteinase 1, profibrotic factors such as transforming growth factor-beta, growth factors such as vascular endothelial growth factor, cell-cell interacting protein such as intercellular adhesion molecule 1, and microRNAs such as mi-R21, are not specific markers implicated in many other cancers and diseases[9-12]. Furthermore, germline mutations in genes such as cyclin-dependent kinase inhibitor 2A, tumor protein p53, serine/threonine kinase ATM, MutL homolog 1, and breast cancer 1 and 2 have been significantly associated with PC[13]. The authors also mentioned genetic factors associated with PC, such as *KRAS* in ctDNA and *KRAS* mutation in CTCs. Therefore, genetic mutation or inherited factors may be a predisposing factor for PC and should be considered during the diagnosis.

Finally, this letter summarizes the actively recruiting and completed clinical trials to evaluate diagnostic methods or biomarkers for PC (Table 1). The data were collected from the website https://clinicaltrials.gov (accessed on July 18, 2021) using the keywords biomarkers and PC. Overall, the specificity and sensitivity of PC diagnosis can be increased by using multiple marker panels in combination with CA 19-9 or with novel screened biomarkers. In addition, accuracy, cost-effectiveness, and ease of application together will ensure the broad application of any new diagnostic method.

Table 1 Clinical trials for pancreatic cancer with representative diagnostic biomarkers

Trial number	Biomarkers	Status	Year to complete	Results/Trial titles
NCT03311776	HA and PRO-C3	Completed	2035	Serum HA and PRO-C3 were prognostic for overall survival in patients with PC[14]
NCT04241367	ctDNA	Recruiting	2025	Verification of predictive biomarkers for pancreatic cancer treatment using multicenter liquid biopsy
NCT04143152	sTRA and CA 19-9	Recruiting	2023	Two biomarker panels with sTRA and CA 19-9 improved sensitivity and accuracy, compared to using only CA19-9 [15]
NCT03404661	Methylated DNA markers	Recruiting	2023	Optical and biochemical biomarkers in early pancreatic cancer significance: a prospective study
NCT04584996	CircRNAs	Recruiting	2023	Circular and non-coding RNAs as clinically useful biomarkers in pancreaticobiliary cancers
NCT04636788	Circulating exosomal small RNAs	Recruiting	2022	Diagnostic and prognostic values of EUS-FNA specimens and circulating exosomal small RNA in patients with pancreatic cancer
NCT03536793	Urinary tissue factor and Endo180	Recruiting	2022	Study of uTF and Endo180 as markers of early malignancy in cystic pancreatic lesions
NCT04549064	AREG	Recruiting	2021	Identification of AREG for the detection of pancreatic cancer by the biosensor
NCT03817866	Chromogranin A	Recruiting	2021	To validate the performance of Brahms Chromogranin A II Kryptor assay to monitor the course of disease in patients with well-defined gastroentero-pancreatic neuroendocrine tumors
NCT03214991	DNA	Unknown	2021	Circulating tumor DNA as a prognostic marker in patients with pancreatic cancer
NCT01664169	VEGF-A and VEGF-R2	Completed	2018	Validation of circulating biomarkers using the immunological multiparameter chip technology (IMPACT) platform on plasma specimens collected on CALGB 80303
NCT02974764	Circulating tumor cells	Completed	2018	Alterations in circulating tumor cells predicted the progression of pancreatic ductal adenocarcinoma, treatment response, and clinical outcomes[16]
NCT00674973	AREG, EGF, sHER2, TGF-α	Completed	2015	Exploratory analyses suggested that high AREG might predict progression-free survival in patients with pancreatic cancer treated with erlotinib[17]
NCT01675258	Four messenger RNA biomarkers (*KRAS*, *MBD3L2*, *ACRV1*, and *DPM1*) in salivary samples	Completed	2013	The logistic regression model using four biomarkers yielded an area under the curve value of 0.971 (cutoff 0.433) to detect resectable pancreatic cancer with 90.0% sensitivity and 95.0% specificity[18]
NCT00899158	Caspase-3 and pAkt in muscle, and urinary 3-MH	Completed	2008	Role of caspase-3, phosphatidylinositol-3 kinase, and 3-methylhistidine in the pathophysiology of skeletal muscle loss in weight-losing pancreas cancer patients

ACRV1: Acrosomal vesicle protein 1; AREG: Amphiregulin; DPM1: Dolichyl-phosphate mannosyltransferase subunit 1; EGF: Epidermal growth factor; circRNAs: Circular RNAs; ctDNA: Circulating tumor DNA; HA: Hyaluronan; MBD3L2: Methyl-CpG binding domain protein 3 like 2; pAkt: Phosphorylated Akt; PRO-C3: Propeptide of type III collagen; sHER2: Soluble human epidermal growth factor receptor 2; sTRA: Sialylated tumor-related antigen; TGF-α: Transforming growth factor-alpha; 3-MH: 3-methylhistidine.

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Help Desk: https://www.f6publishing.com/helpdesk
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ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2021 December 14; 27(46): 7866-8039



Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

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CORRECTION

8031 Erratum: Author's research-fund support notation correction. Mass forming chronic pancreatitis mimicking pancreatic cystic neoplasm: A case report *World J Gastroenterology* 2018; Jan 14; 24 (2): (297-302)

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LETTER TO THE EDITOR

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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: *Hua-Ge Yu*; **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, Subrata Ghosh

EDITORIAL BOARD MEMBERS
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PUBLICATION DATE
December 14, 2021

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World Journal of Gastroenterology

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World J Gastroenterol 2021 December 14; 27(46): 7866-7893

DOI: 10.3748/wjg.v27.i46.7866

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

FRONTIER

Imaging of the chemotherapy-induced hepatic damage: Yellow liver, blue liver, and pseudocirrhosis

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Author contributions: Calistri L, Rastrelli V and Colagrande S contributed equally to this work, in terms of conceptualization, writing the original draft, reviewing and editing; Maraghelli D, Vidali S, Pietragalla M contributed in figures and references research; and all the authors have read and approved the final manuscript.

Conflict-of-interest statement: There are no known conflicts of interest associated with this publication and there has been no financial support for this work that could have influenced its outcome.

Country/Territory of origin: Italy

Specialty type: Radiology, Nuclear Medicine and Medical Imaging

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

Peer-review model: Single blind

Peer-review report's scientific

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Abstract

The liver is the major drug-metabolizing and drug-detoxifying organ. Many drugs can cause liver damage through various mechanisms; however, the liver response to injury includes a relatively narrow spectrum of alterations that, regardless of the cause, are represented by phlogosis, oxidative stress and necrosis. The combination of these alterations mainly results in three radiological findings: vascular alterations, structural changes and metabolic function reduction. Chemotherapy has changed in recent decades in terms of the drugs, protocols and duration, allowing patients a longer life expectancy. As a consequence, we are currently observing an increase in chemotherapy-associated liver injury patterns once considered unusual. Recognizing this form of damage in an early stage is crucial for reconsidering the therapy regimen and thus avoiding severe complications. In this frontier article, we analyze the role of imaging in detecting some of these pathological patterns, such as pseudocirrhosis, "yellow liver" due to chemotherapy-associated steatosis-steatohepatitis, and "blue liver", including sinusoidal obstruction syndrome, veno-occlusive disease and peliosis.

Key Words: Hepatic damage; Yellow liver; Chemotherapy-associated steatohepatitis; Blue liver; Sinusoidal obstruction syndrome; Veno-occlusive disease; Peliosis; Pseudocirrhosis

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Core Tip: Chemotherapy-induced hepatic damage represents an increasingly frequent condition observed in oncology patients: recent pharmacological innovations and specific and longer therapies have led to longer life expectancy and, inevitably, to an

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

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Received: April 19, 2021
Peer-review started: April 19, 2021
First decision: June 3, 2021
Revised: June 15, 2021
Accepted: November 29, 2021
Article in press: November 29, 2021
Published online: December 14, 2021

P-Reviewer: Chen LW, Pan W
S-Editor: Wang JL
L-Editor: A
P-Editor: Wang JL

increase in systemic side effects and organ damage, primarily in the liver because of its detoxifying function. Even for experienced radiologists, the assessment of radiological patterns associated with liver injury derived from chemotherapy can sometimes be challenging. Our aim is to summarize useful ways to recognize, understand and monitor the evolution of these forms of hepatic damage to support clinicians in decision making.

Citation: Calistri L, Rastrelli V, Nardi C, Maraghelli D, Vidali S, Pietragalla M, Colagrande S. Imaging of the chemotherapy-induced hepatic damage: Yellow liver, blue liver, and pseudocirrhosis. *World J Gastroenterol* 2021; 27(46): 7866-7893
URL: https://www.wjgnet.com/1007-9327/full/v27/i46/7866.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i46.7866

INTRODUCTION

The liver plays key roles in the metabolism and detoxification of many commonly used drugs, predisposing hepatocytes to xenobiotic- and toxin-induced injury[1]. Chemotherapy has recently evolved, from the use of cytotoxic drugs to new biological drugs acting on specific molecules critical for cell growth, differentiation, and nutrient supply. The advent of these new treatments, as well as the frequent use of multidrug regimens and the longer duration of systemic therapies due to longer survival, have increased the potential for liver parenchymal damage, collectively referred to as chemotherapy-associated liver injury (CALI)[2].

The first case of CALI was reported in the early 1950s in reference to clinical and laboratory signs of hepatic fibrosis presented by 5 children with acute leukemia during folic acid antagonist treatment[3]. Recently, efforts to identify imaging features and standardize the management of CALI have been made[4,5]. However, with the advent of newer molecular targeted oncological therapies and, more recently, immune checkpoint inhibitors, the evaluation and treatment of liver toxicity associated with these drugs are still evolving[6-9].

Regardless of the cause of injury, CALI can manifest as nonspecific symptoms and signs of abdominal discomfort, evidence of hepatomegaly, and/or elevated liver function tests, often representing a diagnostic dilemma for the oncologist, as the same symptoms and signs of liver injury may also be unrelated to chemotherapy[4,10].

Moreover, many chemotherapy-induced hepatic parenchymal effects can impair the detection of liver metastases. As patients with metastatic cancer increasingly undergo chemotherapy with curative intent, it is mandatory that radiologists understand the pathophysiology of these therapy-induced liver changes and become familiar with their imaging features[11]. Finally, the early recognition of certain adverse reactions is essential for cancer patients to prevent dangerous complications such as acute hepatitis, liver cirrhosis, and even liver failure. Often, patients can be managed with supportive therapies, and the liver toxicity may resolve after discontinuation of chemotherapy.

After a summary of the main forms of liver damage, including drug-induced liver injury (DILI), we analyze the role of imaging in detecting certain pathological patterns of CALI that may appear during oncologic follow-up, such as so-called "yellow liver", "blue liver" and "pseudocirrhosis".

HEPATIC DAMAGE

Various forms of hepatocyte injury are known: infectious (viral hepatitis), autoimmune hepatitis, toxicity/drug-induced injury, metabolic injury (nonalcoholic fatty liver disease), and intracellular depositions (hemochromatosis, alpha-1-antitrypsin, Wilson disease, and metabolic diseases such as glycogen storage disorders)[12]. Other types of damage involve biliary stasis-induced injury ("long standing obstruction of the bile duct"), injury to the hepatic artery that affects circulation, and damage from physical/chemical agents[13-16]. Vascular alterations, inflammation and oxidative stress represent the pathogenesis of various forms of liver damage: in liver ischemia–reperfusion injury (during the liver transplantation process), the damage

involves all parenchymal cells (hepatocytes, endothelial cells and cholangiocytes)[17]. The lack of substrates and oxygen during the ischemic phase of injury results in the mitochondrial production of reactive oxygen species (ROS); in the reperfusion phase, the availability of oxygen further accentuates the oxidative stress, increasing damage to the donor liver; moreover, there is a concomitant release of inflammatory cytokines and an influx of inflammatory cells that amplify tissue injury[18]. Considering a more general definition of damage as a reversible or irreversible modification of cellular and/or tissue function in response to a stressful stimulus, the liver response to injury includes a relatively narrow spectrum of morphologic changes, and accordingly, there are only a few pathologic patterns that can be recognized microscopically[19].

Therefore, dividing liver injury patterns by the cell type being destroyed, regardless of the cause, we can categorize injuries into cell-indiscriminate (most frequently in response to mechanical injury, ischemia, and liver resection), cholestatic (typically in response to mechanical and presumed autoimmune biliary injury), and hepatocyte-associated injuries[20]. Hepatocyte-related injury includes cell death (apoptosis, necrosis, necroptosis, and autophagy) and degenerative and/or intracellular accumulation (*i.e.*, ballooning degeneration, steatosis and iron or copper accumulation)[20,21].

Clinical data and animal models suggest that hepatocyte death is the key trigger of liver disease progression, manifested by the subsequent development of inflammation due to an influx of acute or chronic inflammatory cells involving the lobular parenchyma (diffuse inflammation), foci inside lobules or limited to the portal tracts (focal inflammation). If the damage is severe enough, and if the blood flow is adequate, then hepatic regeneration can restore a functional liver mass. If the damage is chronic, liver fibrosis/cirrhosis may develop[19,22].

LIVER BLOOD FLOW

Knowing the peculiarities of hepatic vascularity helps with understanding the imaging features of liver damage. Hepatic feeding is guaranteed for a 70%-75% by portal flow, with a low oxygen content and high metabolite content, and for a 20%-25% by arterial flow with a high oxygen content and a low metabolite content. The two systems are interconnected through transvasal, transsinusoidal and peribiliary communications that allow the arterial supply to compensate for any small reduction in portal inflow, according to a mechanism regulated by humoral mediators (adenosine, histamine, vasopressin, and prostacyclin) and by the autonomic nervous system, activated by hepatocyte demand for oxygen and metabolites[13,23]. This condition appears on computed tomography (CT)/magnetic resonance (MR) images as hyperdensity/hyperintensity of the involved parenchyma during the arterial phase, also called transient hepatic parenchymal enhancement (THPE)[24]. Depending on the level of the obstacle and the predominance of the shunt involved, different THPE, either localized or diffuse, can be seen on images. There are three diffuse types: (1) If the obstacle diffusely compromises the intralobular vein or the structures downstream (*e.g.*, in Budd-Chiari or right-sided heart failure), the prevailing plexus is the trans-sinusoidal plexus, and the resulting THPE is of the "mosaic" type (Figure 1A); (2) If the obstacle is at the level of the portal axis or upstream from the intralobular vein (as happens in portal thrombosis or cirrhosis, respectively), the prevailing shunt is peribiliary, and the resulting THPE is of the "central-peripheral" type (Figure 1B); and (3) In contrast, if the peribiliary plexus is blocked, as occurs in bile duct dilatation or sometimes in cholangitis, arterialization is "peribiliary"[24] (Figure 1C).

In addition to the major vascular systems, a third type of vasculature contributes no more than 2%–3% of hepatic blood flow, establishing communication between the systemic venous system and the portal system, and it includes capsular veins, Sappey's paraumbilical veins, epiploic and hilar veins, suspensory ligament and diaphragmatic veins, and accessory cystic veins[24]. These components may act according to the pressure gradient through an anomalous blood supply or drainage from vessels to certain areas of the parenchyma, mainly located in segments I–IV. Normally, the third inflow is "afferent" to the liver, but its flow direction can be reversed. Therefore, during portal hypertension or under other stress conditions, the intraportal pressure becomes higher than that of the systemic veins, and the "third" hepatic system becomes efferent, allowing a preferential outflow that can cause a localized reduction in portal inflow, sometimes resulting in a compensatory "arterial buffer response" and correlated sequelae[24]. The same diversion of the third inflow explains both the development of shunting systems in cirrhotic liver and the appearance of pseudonodular lesions in noncirrhotic liver, especially after che-

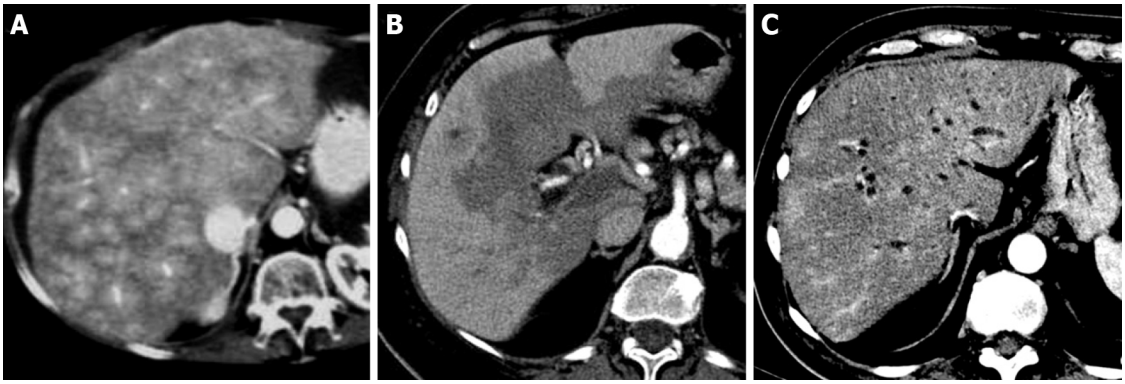


Figure 1 Transient hepatic parenchymal enhancement. A: Contrast-enhanced computed tomography axial scan in the arterial phase shows a "mosaic" pattern of enhancement in patients with Budd-Chiari syndrome; B: "Central-peripheral" pattern in patients with portal thrombosis; C: "Peribiliary" pattern in patients with cholangitis.

motherapy, which can damage minor portal vessels and facilitate blood inflow through the third inflow system[24].

In general, if the cause of arterial rebound persists, hepatocyte can be injured as in normal conditions they need low oxygen tension and high nutrient levels typical of those supplied by portal inflow. Therefore, persistent hemodynamic changes can determine focal metabolic alterations that result in focal sparing in fatty liver or nodular fat accumulation in normal liver, which are typically found in the subdiaphragmatic aspect of the right lobe, the posterior aspect of the left lobe, the periportal aspect of segment IV, around the falciform ligament and around the gallbladder bed [25,26] (Figure 2). Finally, if the obstacle remains, then the insufficient blood supply to much of the liver leads to metabolic infarction, fibrosis and atrophy of the liver (especially in segments V, VI and VII, where the third inflow is lacking or poorly represented) (Figure 3), along with compensatory nodular regenerative hyperplasia (NRH) and large regenerative nodules in areas of hepatic parenchyma that maintain an adequate portal and arterial blood supply (especially the left lobe and segment VIII, with higher third inflow)[24,27-29].

DILI

DILI is a current hot topic, as seen by the increasing number of publications in recent years[30-32]. It is a challenging clinical problem with respect to both diagnosis and management, with an estimated incidence of 14 to 19 cases per 100000 persons[30]. Iproniazid, cinchophen, and sulfonamides were the first prototypical hepatotoxins to be identified[31,33]. By the mid-1980s, close to 1000 drugs were linked to hepatic injury[34]. Clinically, DILI ranges from asymptomatic hypertrasaminasemia and hepatitis to acute or fulminant hepatic failure[35]. Although severe DILI is rare, drugs have become the overall leading cause of acute liver failure in the United States and other Western countries[31]: acetaminophen (paracetamol) is the responsible drug in 40%-50% of these cases, with another 11%-12% of cases caused by herbal compounds and dietary supplements, equaling the frequency of cases due to acute viral hepatitis [36,37].

On the basis of liver function tests, DILI may be defined as predominantly hepatic, distinguished by disproportionate elevations in serum aminotransferases compared with the level of alkaline phosphatase, or cholestasis, distinguished by inverted, disproportionate and mixed patterns[36].

Considering the histopathology, DILI is particularly complex. The United States DILI Network recognizes 18 distinct categories of DILI: acute and chronic hepatitis, acute and chronic cholestasis, cholestasis-hepatitis, granulomatous, macro- and microvesicular steatosis, steatohepatitis, zonal and nonzonal necrosis, vascular injury, hepatocellular alterations, nodular regenerative hyperplasia, mixed or unclassified injury, minimal nonspecific changes, absolutely normal, and massive necrosis[38].

Currently, DILI is classified as either idiosyncratic (injury unpredictable, not dose-dependent, and caused by agents that have little or no intrinsic liver toxicity) or direct (injury predictable, dose-dependent, and caused by agents that are intrinsically toxic to the liver), but indirect injury is now accepted as a third type (caused by the action of

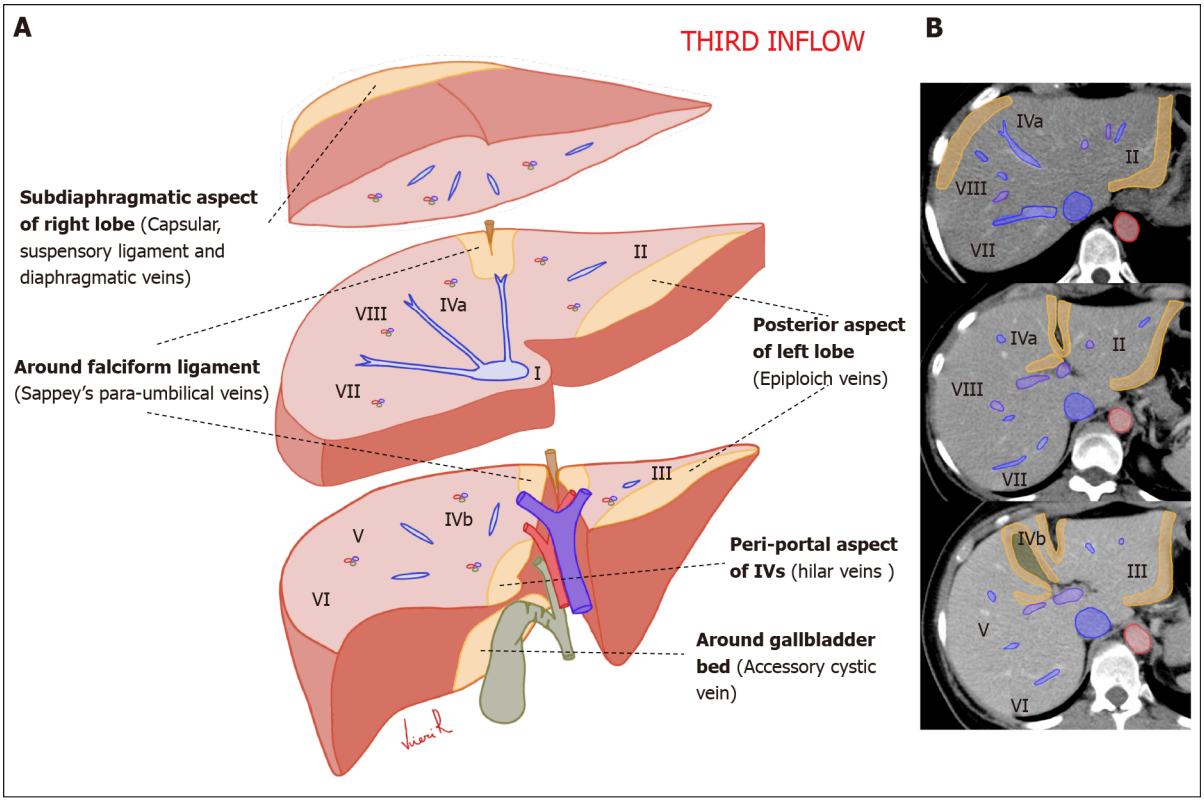


Figure 2 Schematic representation of the anatomical sites of the liver "Third inflow" in hepatic sections. Yellow areas show the typical sites of focal sparing in fatty liver or nodular fat accumulation in the normal liver. A: Volumetric representation; B: Computed tomography axial scan.

the drug not by its toxic or idiosyncratic properties, such as the induction of immune-mediated hepatitis or the worsening of pre-existing hepatitis or fatty liver disease)[30].

The most common forms of DILI involve idiosyncratic hepatotoxicity, including acute and chronic hepatitis (most often associated with isoniazid, nitrofurantoin, and diclofenac and with methyldopa, minocycline, and statins, respectively), acute and chronic cholestasis (correlated with estrogens, androgenic steroids and flurixidine), and mixed hepatitis-cholestasis (due to amoxicillin-clavulanate and fluoroquinolones) patterns[30,32,39].

Many antineoplastic agents can cause acute hepatic necrosis due to direct hepatoxicity, as well as sinusoidal obstructive syndrome (SOS) (myeloablative agents, alkylating agents and monoclonal antibody-cytotoxic conjugates such as gemtuzumab and ozogamicin) or NRH (azathioprine, mercaptopurine and thioguanine)[30,32,39].

Finally, an increasing form of indirect injury is immune-mediated liver injury due to various immunomodulatory agents, tumor necrosis factor antagonists, and, most important, antineoplastic checkpoint inhibitors[40-42]. There are several reports of the reactivation of both hepatitis B and hepatitis C in patients treated with agents such as rituximab, cyclophosphamide, doxorubicin, vincristine, or prednisolone for lymphoma [2,43].

CALI

Among the various forms of DILI, CALI is often reported in the literature, mainly in association with patients with colorectal liver metastases[44-46]. CALI appears to be regimen-specific, generally including two main types of liver injury, vascular changes and fatty changes, which are primarily associated with the development of ROS that lead to cellular damage and activate apoptosis pathways[5]. The prevalence of CALI increases with the duration of chemotherapy, and currently, no convincing data on the reversibility of CALI are available[46].

Various studies support the important clinical impact of CALI. Karoui *et al*[47] demonstrated that CALIs increased the risk of postoperative liver failure by 11%, with others such as Vauthey *et al*[48] demonstrating increased 90-d postoperative mortality in patients with steatohepatitis (14.7% *vs* 1.6%). Nevertheless, it remains unclear

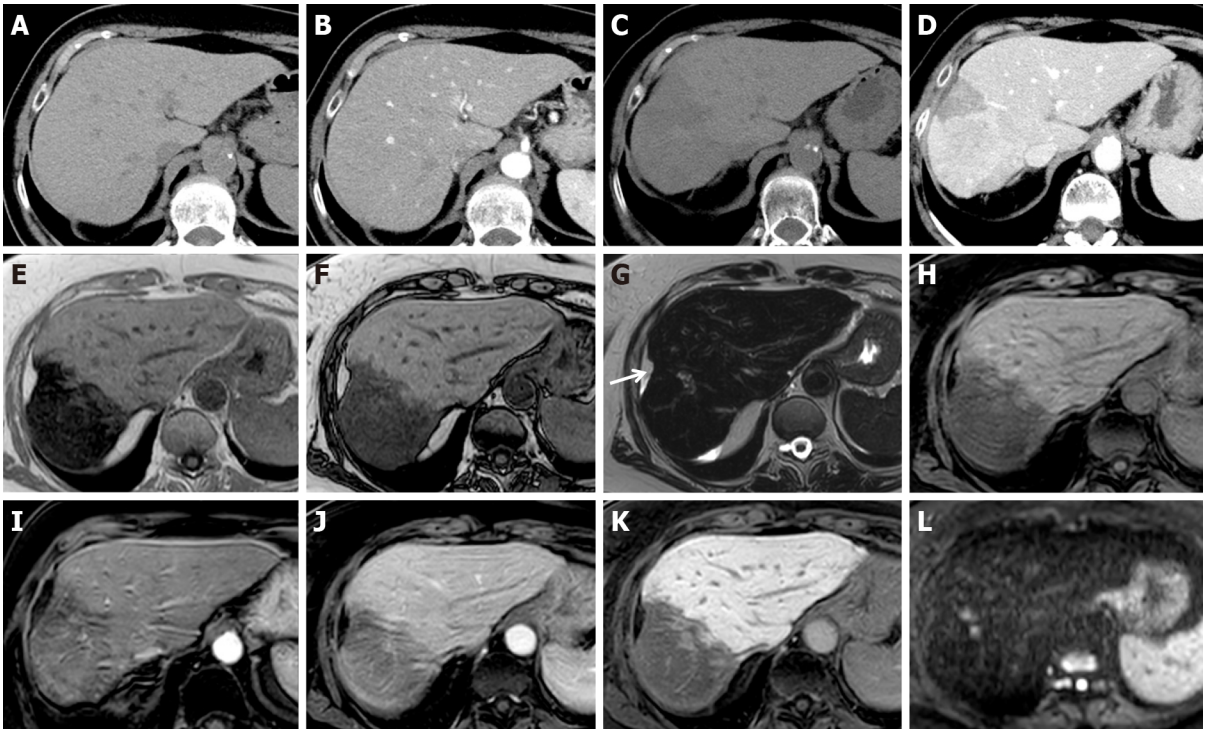


Figure 3 Liver metabolic infarction areas in patients with breast cancer. A, B: Unenhanced (A) and arterial phase (B) computed tomography (CT) axial scan before therapy show normal liver; C, D: Unenhanced (C) and late arterial phase (D) CT axial scans after 3 mo of therapy show early steatotic changes of the parenchyma and inhomogeneous enhancement in segments VII-VIII; E-L: Magnetic resonance after 6 mo of therapy shows progression of parenchymal involution and atrophy in gradient echo (GE) T1w in-phase (E), GE T1w out-of-phase (F), fat sat GE 3D T1w unenhanced (H), arterial (I), portal (J) and hepatobiliary phase (K). Capsular retraction is seen (white arrow). On T2w (G), and high b-value diffusion-weighted (L) images, no signal alteration was detectable.

whether CALI influences survival. Although Tamandl *et al*[49] reported lower survival in patients with SOS, other studies demonstrated that SOS is associated with a lesser degree of regression of liver metastases, and this regression, not SOS, impacts prognosis[50,51]. Controversial data have also been reported for steatosis[50,52]. Moreover, postoperative morbidity and mortality due to liver failure are often related to inadequate function of the residual liver[53]. Therefore, the improved detection of CALI during the preoperative assessment of the future liver remnant is an important clinical issue.

More frequently, oxaliplatin treatment is associated with SOS, which occurs in 19%–52% of patients and is linked to an increased occurrence of NRH[54]. Irinotecan-based treatments are related to the appearance of steatohepatitis, with a rate of 20.2%, and its effects are exacerbated by baseline obesity and/or metabolic syndrome[51,55]. Furthermore, the development of steatosis was observed in 30 to 47% of patients who received 5-fluorouracil therapy, which remains a cornerstone of modern chemotherapy[10,56].

More unusual forms of CALI include pseudocirrhosis, which is mostly observed in patients undergoing chemotherapy for breast cancer, and chemotherapy-induced sclerosing cholangitis (CISC)[57]. CISC is a form of secondary sclerosing cholangitis, occasionally resulting from ischemic injury to the bile ducts associated with hepatic artery infusion with fluoropyrimidines (incidence of 8%-55%). Since biliary endothelial cells, in contrast to hepatocytes, derive their vascular supply almost exclusively from the branches of hepatic arteries[58], arterial occlusion may cause bile duct ischemia and fibrosis without parenchymal infarction. The main finding was segmental or diffuse narrowing of the cystic, biliary-shared, left and right hepatic ducts, with sparing of the common intrapancreatic bile duct, which is usually supplied by branches of the gastroduodenal artery[58]. Reports of CISC triggered by systemic chemotherapy (taxanes, bevacizumab, paclitaxel, or cisplatin) are even more rare[59,60]. Although CISC should be considered rare, it is clinically important, requiring frequent endoscopic intervention to maintain biliary drainage[58].

Finally, over the past two decades, molecular targeted agents, including small-molecule protein kinase inhibitors, monoclonal antibodies, and immune checkpoint inhibitors, have become promising for use in the treatment of various malignant neoplasms (especially malignant melanoma, non-small cell lung cancer and renal cell

carcinoma)[61]. Protein kinase inhibitors are reported to induce a low-grade elevation in serum transaminases in approximately 30% of patients and high-grade elevation in 2% of patients[62]. Liver injury due to immune checkpoint inhibitors most often presents with a hepatocellular biochemical pattern, occurring in 2%-30% of patients, with increasing risk when multiple immune checkpoint inhibitors are administered and in patients who develop other immune-related adverse events, although severe cases remain very rare[63].

Overall, radiologists should know that chemotherapy frequently modifies not only the radiological appearances of liver tumors but also the imaging features of the non-tumor-bearing liver. Excluding CISC, due to its rarity, and acute hepatitis, due to its nonspecific imaging features (hepatosplenomegaly, collapsed gallbladder with wall thickening, decreased liver enhancement, ascites and widening of the periportal space due to edema)[2], we analyze the role of imaging in the identification of more typical features of CALI, namely, yellow liver, blue liver and pseudocirrhosis.

YELLOW LIVER

The term "yellow liver" refers to a macroscopic feature of the liver that can be observed upon histopathologic examination and is determined by a general increase in the parenchymal lipidic content, compared to physiological normal texture[5]. Different pathological conditions fall under the generic definition of yellow liver. Hepatic steatosis is identified by pathological deposition of lipid vesicles in hepatocytes, usually associated with metabolic syndrome, obesity, diabetes, insulin resistance or alcohol[64]. Hepatic steatosis must be differentiated by steatohepatitis, a more serious histologic complication where fat deposition is associated with an inflammatory response, consisting of ballooning of hepatocytes, lobular inflammation, hepatocyte degeneration and thus fibrosis of different grades, including liver cirrhosis [48]. CASH is a form of steatohepatitis that can sometimes develop as a consequence of therapies with chemotherapeutic agents (CTAs) that produce side effects critical for hepatocyte[65,66].

Among the forms of CALI, other than the aforementioned association with 5-fluorouracil-based treatment[57], steatosis is seen in 14.6% and 41.1% of patients with estrogen-receptor-positive breast cancer treated with tamoxifen and anastrozole, respectively[10,67]. Additionally, cases of steatosis in patients receiving pazopanib and bevacizumab, alone and in combination with paclitaxel, have been reported[6]. Similarly, an increased incidence of CASH in recent decades has been reported[56,68,69]. Although the true frequency of these pathologies is not easily determined[65,70], as mentioned above, the prevalence of CASH is increasing in patients undergoing treatment with 5-fluorouracil and irinotecan[71], especially when the drugs are coadministered[48]. Additionally, platinum derivatives (oxaliplatin), taxanes and methotrexate have been linked to this condition, and with a lower frequency, L-asparaginase, dactinomycin, mitomycin C and bleomycin sulfate[72,73].

Interestingly, steatosis and CASH occur not only during treatment for liver metastases but also in the course of systemic chemotherapy for nonmetastatic cancer [71]. Considering the pathogenesis of steatosis, as discussed above (see "Liver blood flow"), persistent hemodynamic changes determining focal metabolic alterations and the third hepatic inflow system are involved. The pathogenesis of CASH remains under discussion, and different mechanisms have been proposed. First, CTAs can be responsible for decreasing fatty acid oxidation, thus generating oxidative stress with hepatocyte dysfunction[74]. According to You *et al*[11], CTAs have been reported to produce abundant ROS, damaging not only cancer cells but also normal cells. This damage promotes both the deposition of lipid vesicles into hepatocytes and inflammation[65,75]. Robinson *et al*[57] described a "two hits" model, in which patients with underlying hepatic steatosis, undergo a "second hit" to the parenchyma, represented by chemotherapy-induced oxidative stress or mitochondrial dysfunction, creating the conditions of inflammation and giving rise to CASH. Finally, minor portal vessels can be increasingly susceptible to the direct damage caused by CTAs, facilitating the reversion of the "third inflow" system and triggering consequent arterial compensation. Therefore, the resultant inadequate perfusion, together with direct damage to hepatocytes, can contribute to the metabolic dysfunction critical for CASH development[24].

Fatty infiltration leading to yellow liver requires a relatively short development time (generally only a few weeks after the beginning of chemotherapy)[57]. Hepatic steatosis and steatohepatitis are typically asymptomatic even when liver function tests

are abnormal, making an early diagnosis a difficult goal[4]. When chemotherapy is withdrawn, there is often a regression of steatosis, suggesting that most of the changes caused by chemotherapy are at least partially reversible[6,57]. In other cases, especially when the diagnosis is delayed or under-evaluated, the parenchymal inflammatory response can lead to more serious and irreversible changes, including fibrosis and atrophy. Finally, the regenerative phenomenon of liver parenchyma, such as in NRH, is a possible compensatory response to injury, as long as adequate perfusion is maintained[28,56,76]. As expected, when steatosis is present prior to the therapy, liver is thought to be more susceptible to CALI due to its impaired regenerative capability and abnormal innate immunity[10]. Diffuse forms of hepatic steatosis and steatohepatitis can be obstacles to surgical planning, *e.g.*, not allowing large liver resection due to presence of metastases[69]. Patients with steatosis who undergo major hepatectomy have increased blood loss, more postoperative complications, and a longer stay in the intensive care unit than patients with healthy livers[19]. Finally, patients' postoperative morbidity and mortality can be increased since both steatosis and steatohepatitis impair liver function[48].

Imaging

Clinicians increasingly demand the quantification of liver fat to grade the level of hepatic damage, not only in living donors for liver transplantation and for patients who must undergo liver resections or bariatric surgery but also in patients receiving potentially hepatotoxic therapies. The ability of MR-based methods to detect and quantify steatosis has been investigated in the past 30 years, and substantial correlations between pathologically and radiologically determined fat fractions have been demonstrated[77-79].

However, most often, in daily practice, steatosis and steatohepatitis are evaluated qualitatively. From a radiological point of view, it is impossible to differentiate between these two forms of liver disease, as they can be distinguished only by histologic alterations[71]. The distribution of CASH, as well as hepatic steatosis, can vary from diffuse to focal infiltration. Ultrasound allows a subjective estimation of the degree of diffuse fatty infiltration using some features that include liver brightness and contrast between the liver and the kidney[80]. On an unenhanced CT scan, diffuse steatosis can be diagnosed when attenuation of the liver is at least 10 Hounsfield units less than that of the spleen, the hepatic-to-splenic attenuation ratio is less than 1, or the liver attenuation is less than 40 Hounsfield units (Figure 4A). In more severe cases, intrahepatic vessels may appear hyperdense relative to fat-containing liver tissue[81]. With MRI, chemical shift gradient-echo imaging with in-phase and out-of-phase acquisitions is the most widely used technique for the assessment of fatty liver (Figure 4E and F). These scans show signal intensity loss on out-of-phase images in comparison with in-phase images, whereas the application of chemical fat saturation sequences is less sensitive[82,83].

While diffuse forms of steatosis are not difficult to recognize, focal fat deposition or fatty sparing can sometimes mimic a hepatic mass or single and multimetastatic disease[84]. However, MRI often serves as a problem-solving tool because signal loss on out-of-phase T1-weighted images cannot be seen in metastasis[85]. Additionally, focal fatty deposition or sparing can be recognized by the characteristic location (in the anatomic sites of the third inflow system), geographic pattern rather than round or oval shapes, absence of a mass effect on the vasculature, poorly delineated margins, and contrast enhancement that is similar to or less than that of the normal liver parenchyma[9,86] (Figure 5). These features usually allow for the differentiation of focal or multifocal fat accumulations from hepatocellular carcinoma, hepatic adenoma, focal nodular hyperplasia (FNH), FNH-like nodules and hypervascular metastases that show mass effects, marked or heterogeneous enhancement, and sometimes necrotic and hemorrhagic areas; however, hepatocellular carcinomas, hepatic adenomas and, more rarely, FNH, may involve microscopic fat content[87]. Clinical manifestations and diffusion-weighted images can help in the more complex differentiation of ischemic or mucinous metastases, abscesses, lymphoma and hypovascular metastases[87-89] (Figure 4K). The differential diagnosis between large areas of focal steatosis with infiltrative hepatocellular carcinoma may be more difficult; irregular liver contours, mild mosaic pattern enhancement and the presence of portal vein thrombosis are suggestive of the latter[87]. Shape, location and MR chemical shift imaging allow us to distinguish periportal fat deposition from other periportal abnormalities (edema, inflammation, hemorrhage, and lymphatic dilatation) and focal sparing in the liver with diffuse steatosis mimicking hypervascular lesions (such as hemangiomas or arterioportal shunts)[87]. Interestingly, when steatosis develops during chemotherapy, in the presence of hepatic metastases, the parenchyma

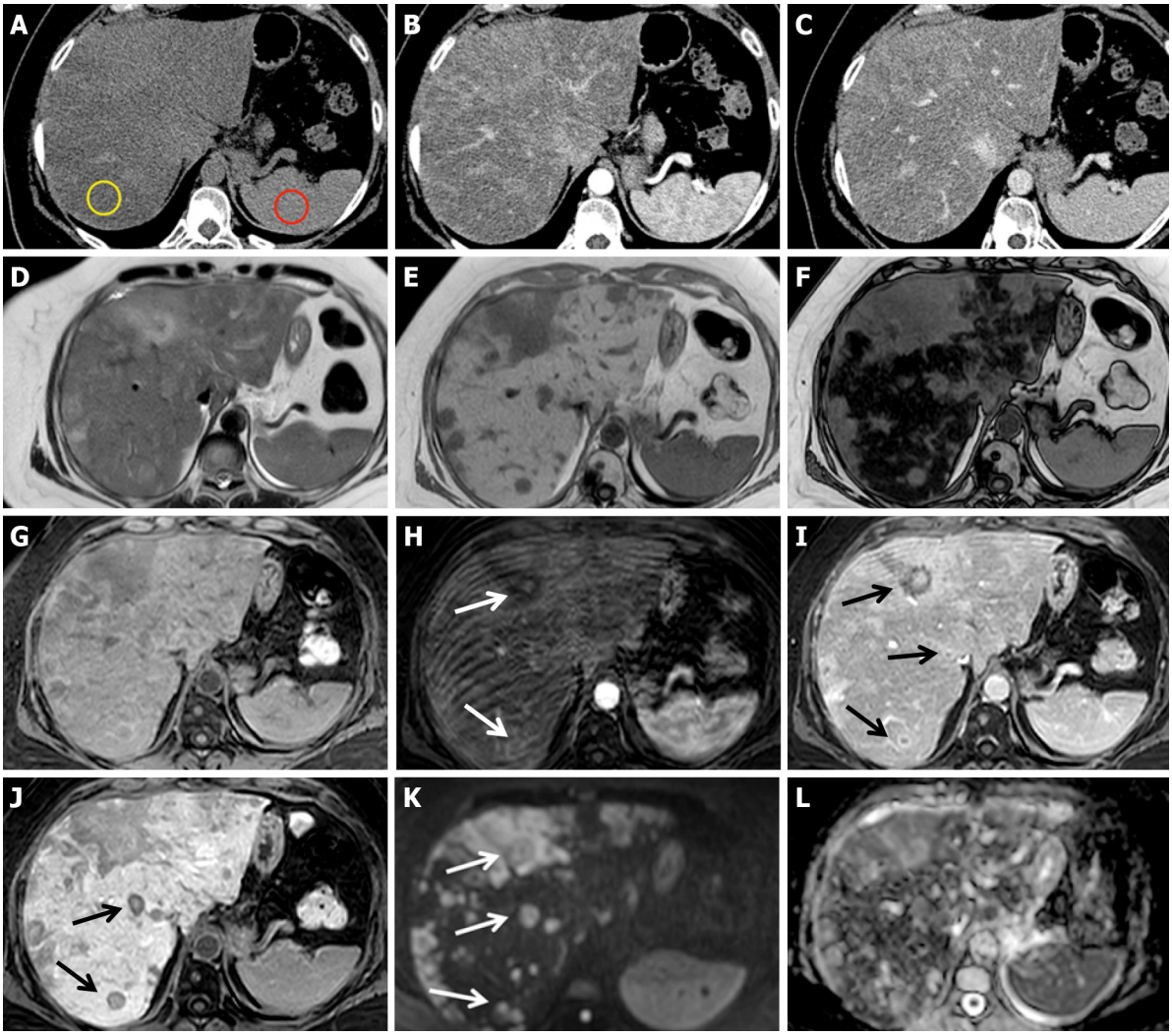


Figure 4 Chemotherapy-associated diffuse steatosis in patients with liver metastatic colorectal cancer 6 mo after the beginning of chemotherapy with 5-fluorouracil + folinic acid + irinotecan. A-C: Unenhanced computed tomography axial scan (A), arterial (B) and portal phase (C) show severe inhomogeneous hepatic steatosis. Liver attenuation in steatotic areas is 8 HU (yellow ROI), less than that of the spleen (43 HU, red ROI), and the hepatic/splenic attenuation ratio is << 1. No nodules are visible; D-L: On magnetic resonance performed during the following week, gradient echo (GE) T1w in-phase (E) and out-of-phase (F) images confirm severe hepatic steatosis. On unenhanced fat sat GE 3D T1w images (G), arterial (H), portal (I), hepatobiliary (J) phases and high b-value diffusion-weighted images (K), multiple metastases are evident, some of which are characterized by rim enhancement (arrows). The T2-weighted image (D) and apparent diffusion coefficient map (L) are also shown.

surrounding the lesion can be spared from steatosis[90]. Once again, this outcome may be attributed to a modification of parenchymal perfusion, and in particular, a reduction of portal inflow in the peritumoral area caused by direct compression of the adjacent parenchyma, the presence of tumor emboli in the portal vein branches[90] and/or the neoangiogenesis that accompanies tumoral growth, increasing arterial perfusion[91,92].

Morphological changes such as increased craniocaudal liver diameter and an increased caudate-to-right lobe ratio are more characteristic of steatohepatitis[93]. Finally, when CASH is diagnosed in advanced stages and, in particular, when it progresses toward cirrhosis, typical morphological changes of the latter can be observed[94].

BLUE LIVER

Blue liver refers to parenchymal venous congestion resulting from blockage of blood outflow, macroscopically characterized by an intraoperative subcapsular livid appearance and a similar "marble" bluish-red discoloration on the cut surface[95]. Budd-Chiari syndrome is a typical postsinusoidal form of blue liver. In this case, the physical obstacle to hepatic outflow, represented by stenosis or a thrombus, is located

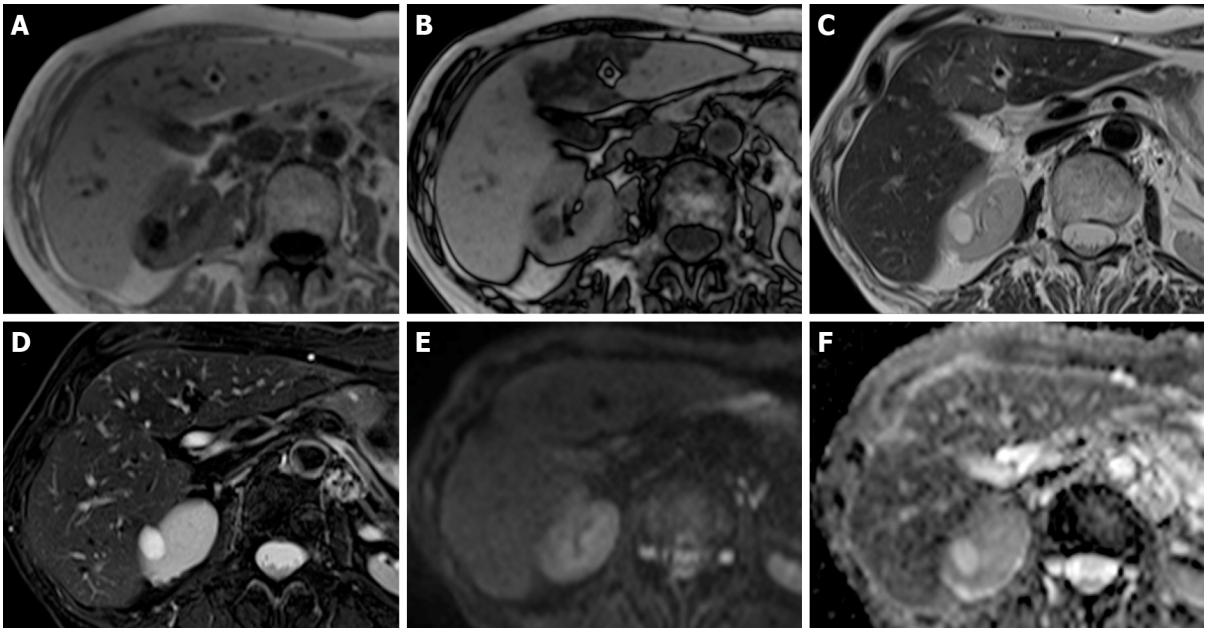


Figure 5 Chemotherapy-associated focal steatosis in patients with lung cancer 3 mo after the beginning of immunotherapy. A-F: On magnetic resonance, focal geographic fatty deposition, poorly delineated, is seen as signal hypointensity on gradient echo (GE) T1w out-of-phase (B) in the periportal aspect of segment IV and around the falciform ligament. On T2w images (C) it is weakly hyperintense. No signal alterations are seen on the GE T1w in-phase images (A), T2w fat saturation (D), diffusion-weighted (E) images, or apparent diffusion coefficient map (F).

in the hepatic veins or in the inferior vena cava, and therefore, considering the blood flow, after the hepatic sinusoids[24]. However, different causes of blue liver are possible. Other postsinusoidal forms include increased blood pressure in the right atrium (*e.g.*, congestive heart failure, constrictive pericarditis, and mitral stenosis)[96]. Moreover, injuries to the sinusoidal endothelium itself can cause a sinusoidal form of blue liver that is mainly known as SOS, but that includes a full spectrum of histologic features involving restrictive (nonthrombotic sinusoidal obstruction and perisinusoidal fibrosis), dilating (hepatic sinusoid dilation and peliosis) and regenerative (NRH) aspects of the disease[97].

Considering CALI, sinusoidal endothelial injury can be defined as nontumor-bearing hepatic parenchymal damage resulting from chemotherapy itself, not associated with the presence or infiltration of hepatic metastasis[11,98]. Therefore, the injury primarily originates at the level of the sinusoidal endothelium, eventually extending distally to the centrilobular veins or proximally to the portal branches. Its pathogenesis remains under discussion, but some consistent data have been reported. Namely, oxaliplatin induces depolymerization of F-actin in sinusoidal endothelial cells and activates matrix metallopeptidase (MMP-9 and MMP-2)[57,99,100]. This activation results in cytokine production, which induces sinusoidal endothelial damage and swelling[101]. Depletion of antioxidants such as glutathione and nitric oxide from endothelial cells can increase the damage[102]. Therefore, floating red blood cells enter the space of Disse through the gaps formed in the sinusoidal endothelium (the basis of peliosis), and collagen fibers are deposited in the extravascular space (perisinusoidal fibrosis). A combination of these factors, in addition to clogging of necrotic sinusoidal endothelial cells in sinusoids, results in sinusoidal narrowing, thus causing obstruction and increased pressure in the sinusoids. When sinusoidal outflow blockage is sustained, sinusoids upstream of the obstruction undergo dilation and disruption, resulting in pseudocystic blood-filled lacunae typical of peliosis. Concurrently, the damage from altered hepatic blood flow induces the regeneration of residual hepatocytes to replace the parenchymal damaged cells, giving rise to NRH[103].

As a consequence, the histology of sinusoidal injury involved in blue liver is heterogeneous. In the early phase, vascular alterations are predominant, including sinusoidal dilatation and congestion, perisinusoidal hemorrhage and peliosis. With the progression of the damage, fibrosis of different grades (which can be localized in the perisinusoidal space around the centrilobular vein or portal vein) is dominant, and hepatocyte disruption and NRH are evident. Although primarily originating from impaired hepatic perfusion, blue liver is characterized by parenchymal nodularity without fibrous septa, with a benign aspect similar to that of FNH, resulting from the

regeneration of hepatocytes replacing the parenchymal damaged cells[57]. Oxaliplatin is a well-known drug implicated in the development of NRH, vascular injury, such as sinusoidal ballooning, microvascular injury, and long-term fibrosis. Furthermore, paclitaxel, capecitabine, doxorubicin, and trastuzumab are also known to be causative CTAs[104].

In cases of severe progression, the chronic presentation of blue liver can be similar to that observed in cirrhosis[105]. However, in the advanced stage of chemotherapy-induced sinusoidal injury, fibrosis develops primarily between the centrilobular veins; in contrast, in primary liver disease, such as cirrhosis, bridging fibrosis, promoted by inflammation, usually develops between portal spaces. Therefore, the histologic pattern that occurs in certain forms of blue liver has been variably defined as "cardiac cirrhosis", "reversed lobulation" or "centrilobular cirrhosis"[96].

Veno-occlusive disease and SOS

Veno-occlusive disease (VOD) is a well-established condition historically associated with myelosuppressive therapy in hematologic malignancies, and it is characterized by the obliteration of small hepatic venules and centrilobular fibrosis without macroscopic signs of obstruction[97]. This pathologic alteration was first reported in 1920 by Willmot *et al*[106] and was caused by lethal intoxication by pyrrolizidine alkaloids, which are present in certain herbal remedies. The association between chemotherapy and VOD became clear in the 1950s[107]. Finally, in 1999, when DeLeve *et al*[98] recognized that the disease originated primarily in the hepatic sinusoid and did not necessarily involve the centrilobular vein, the disease was renamed SOS. These two forms therefore indicate a non-thrombotic obstruction of hepatic sinusoids with (VOD) or without (SOS) involvement of the centrilobular veins, whereas large hepatic veins remain patent[98].

More often, these pathological conditions diffusely involve the nontumor-bearing hepatic parenchyma. However, rare cases of focal SOS have been reported. The true incidence of sinusoidal focal injury remains unknown, but radiologists should be aware of it since, similar to focal steatosis, it can mimic hepatic metastasis[11,108].

Several CTAs are critical for the sinusoidal type of CALI. In particular, cyclophosphamide has been associated with the development of a rapidly progressive form of VOD[109]. Even 5-fluorouracil, mercaptopurine, dacarbazine, and vincristine have been associated with it[110], with an onset of damage ranging from 1 to 3 wk after initiation of therapy[4].

However, oxaliplatin, more than other CTAs, seems to be particularly involved in the development of SOS. According to Rubbia-Brandt *et al*[103], 51%–79% of patients who underwent oxaliplatin-based therapy developed SOS, compared with only 21%–30% of the patients who received different regimens[75,111]. Nonetheless, the incidence of sinusoidal injury is significantly higher in patients who receive more than 6 cycles of chemotherapy, while adding the anti-vascular endothelial growth factor antibody bevacizumab seems to have a protective effect[112].

Most patients with diffuse SOS are asymptomatic, in contrast to patients with VOD [97]. Clinical presentation in the acute phase may include abdominal pain and ascites. In the subacute setting, patients present with recurrent ascites and hepatosplenomegaly resulting from portal hypertension, which can be confirmed with a transjugular biopsy (pressure gradient > 10 mmHg), while the chronic presentation is similar to that of cirrhosis. Hematic tests may show nonspecifically increased bilirubin and hepatic enzyme levels[105].

Concerning prognosis, diffuse forms of SOS are associated with poor outcomes and a higher complication rate after major hepatectomy. Especially since the liver parenchyma tends to become soft and brittle, patients who undergo hepatectomy show an increased risk of perioperative morbidity (approximately 30%)[97,99] and postoperative complications[113]. Indeed, the presence of SOS is associated with a reduced liver functional reserve[108].

Imaging

Since the disease was renamed, imaging findings of VOD have been limited to case reports[114], whereas data regarding SOS are more consistent.

Concerning oxaliplatin-based chemotherapy, the radiological features reach a maximal severity approximately 4 mo after the beginning of therapy, and they show radiologic remission approximately 3 mo after discontinuation[115]. The cessation of chemotherapy is often followed by a reduction in these abnormalities, suggesting that SOS, at least for mild-to-moderate forms, both diffuse and focal, is potentially reversible[108].

Morphological alterations such as hepatosplenomegaly, periportal edema, edematous wall thickening of the gallbladder and ascites can be seen[116]. With the detection of ascites, it is important to confirm the diagnosis of SOS and distinguish it from the malignant ascites associated with peritoneal spread or metastasis[117]. US may show a decrease or reversal of blood flow in the portal veins, but its usefulness is debated[105,108]. Contrast-enhanced CT imaging features include arterial-portal heterogeneous parenchymal enhancement, characterized by a "mosaic pattern" or diffuse linear hypoattenuation lesions resulting from hepatic congestion, which tend to be homogenous in the late phase. These alterations are predominantly located in the peripheral area (67.1%) and right hepatic lobe (62.4%), with an irregular distribution of abnormal areas intermingled with intact lobules[11,118]. A reduced caliber of suprahepatic veins has also been reported[4]. Eventually, complications of portal hypertension, such as the presence of periesophageal varices, form[116].

Similarly, MR images show a heterogeneous reticular or linear pattern in the nontumor-bearing parenchyma characterized by hypointensity on T1-weighted images and hyperintensity on T2-weighted images. Using liver-specific contrast agent (CA), reticular hypointensity of background liver tissue on hepatobiliary phase images with a high prevalence in peripheral areas of the liver is highly specific for SOS, occurring in 69% of cases[11,119]. This radiological finding is probably due to reduced uptake of liver-specific CA resulting from dysfunctional damaged hepatocytes and reduced portal flow[119] (Figure 6I, J and K). In focal SOS, the presence of ill-defined margins, especially on hepatobiliary phase images, and the absence of restricted diffusion on diffusion-weighted images, can help differentiate focal hepatic toxicity from metastasis[118].

Severe forms of SOS can also progress after discontinuation of therapy, leading to the appearance of regenerative phenomena including cirrhotic alterations[10]. In addition, in oxaliplatin-treated patients, portal hypertension and histological changes in NRH can arise during long-term therapy with 6-thioguanine for acute lymphatic leukemia[120]. The nodularity of NRH is usually microscopic and thus is not detectable on images. Larger nodules can show hyper/hypointensity on T1/T2-weighted images and increased vascularity (hyperintensity in the arterial phase, followed by iso- or slight hyperintensity in the portal and equilibrium phases), but their benign nature can be confirmed by their normal uptake of liver-specific MR CAs [57].

Finally, FNH-like lesions have been described to occur in these patients many years after the discontinuation of chemotherapy[108] (Figure 7).

Hepatic sinusoid dilatation and peliosis hepatis

Hepatic sinusoid dilatation (HSD) is a rare hepatic vascular lesion characterized by diffuse dilatation of hepatic capillaries with or without venous outflow obstruction. Causes of HSD with hepatic outflow obstruction include Budd Chiari syndrome, pericardial disease or right heart failure, and sinusoidal occlusion secondary to endothelial sinusoidal damage itself, as in SOS. It can be classified according to the affected zone of the hepatic lobule as centrilobular, periportal, or irregular[108]. On the other hand, forms of HSD without venous outflow obstruction are caused by extrahepatic acute inflammatory conditions (pyelonephritis, cholecystitis, pneumonia, pancreatitis, and inflammatory bowel diseases), use of oral contraceptives (still debated as a possible cause) and chronic conditions, such as congenital or idiopathic vascular diseases, neoplasms with or without secondary liver involvement, inflammatory or infectious diseases, the use of hormones and drugs.

HSD can be distinguished from peliosis, since the latter shows evidence of rupture of the reticulin fibers that support hepatocytes and sinusoids[121]. More precisely, peliosis hepatis is characterized by multiple blood-filled cystic lesions at the level of the sinusoids, with dimensions ranging from 1 mm to several centimeters, randomly distributed throughout the lobule, with loss of the endothelium[120]. Peliosis hepatitis was first described in 1861 on the basis of the Greek word "pelios", meaning "reddish" or "bluish", referring to the parenchymal color[122,123]. It is often a primary idiopathic condition (Figure 8). However, different etiologies have been proposed for secondary forms, including toxins (arsenic and polyvinyl chloride) and certain drugs, such as steroids, oral contraceptives, tamoxifen, 6-thioguanine, 6-mercaptopurine and methotrexate. Chronic wasting diseases have been proposed as another possible causes (diabetes mellitus, malignancy, acquired immunodeficiency syndrome, pregnancy, and infectious diseases, such as tuberculosis, leprosy, Bartonella and adenovirus)[105,120,124].

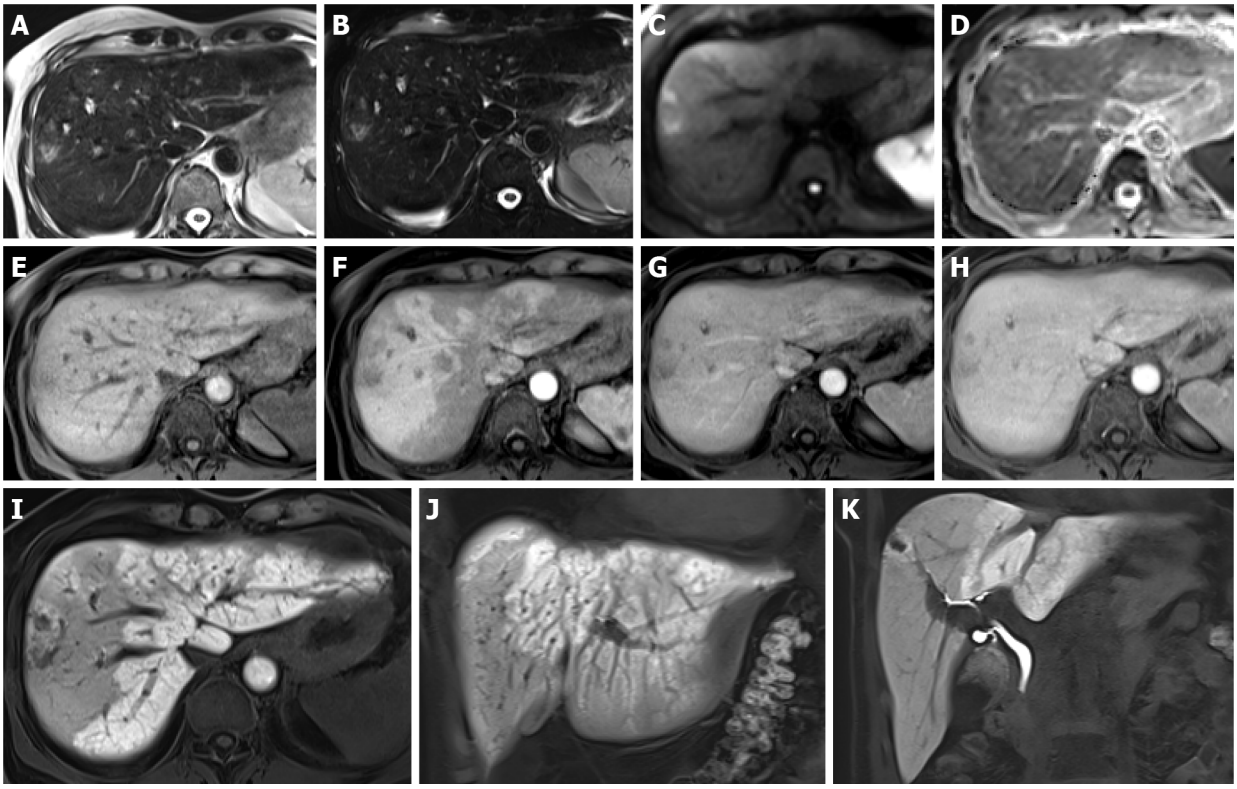


Figure 6 Association between sinusoidal obstructive syndrome and peliosis in patients without a history of hepatopathy, with lung cancer treated with 3 cycles of cisplatin and etoposide and 12 subsequent cycles of immunotherapy. A-K: After 1 year of therapy, magnetic resonance was performed for abdominal pain and an increase in liver enzymes. Hyperintense nodules on T2w (A), T2w fat sat (B) and high b-value diffusion-weighted images (C) are seen. On the apparent diffusion coefficient map (D), they are hypointense. On dynamic imaging, weak enhancement is seen (E: Unenhanced image; F, G, H: Arterial, portal and equilibrium phases, respectively). In the hepatobiliary phase, they appear predominantly hypointense (I-K). A transcutaneous biopsy was performed, resulting in peliosis nodules. Mosaic pattern enhancement of the liver parenchyma in the arterial phase (F) and reticular aspects in the hepatobiliary phase (I-J) were consistent with sinusoidal obstructive syndrome.

Its pathogenesis is not completely clear, except for SOS-dependent peliosis, and regardless of the cause, sinusoidal damage has been generally proposed as being critical to outflow blockage and dilation of the sinusoids/central vein of the hepatic lobule[120,125]. Moreover, hepatocellular necrosis may represent another possible mechanism with the subsequent formation of blood-filled lacunae[126,127]. Two different histological forms of peliosis can be identified: the parenchymal type, usually associated with hemorrhagic parenchymal necrosis and characterized by a lack of endothelial lining within the blood-filled lacunae, and the phlebectatic type, with a dilated central vein, showing an endothelial lining within the cystic spaces[128]. However, these seem to represent different temporal phases of the same condition, with the endothelial lining in the blood-filled lacunae continuously being disrupted and rapidly reconstituted[129].

The distribution of the lesions can vary considerably, from focal areas of peliosis within the liver parenchyma to widespread forms occupying most of the liver parenchyma[130,131]. Peliosis hepatis is usually an asymptomatic condition and therefore is often incidentally diagnosed. However, patients can present with hepatomegaly, portal hypertension, hepatic failure and ascites. Severe abdominal pain is a possible complication associated with minor trauma, resulting in hepatic rupture and hemoperitoneum[132]. The evolution of peliosis is variable and unpredictable. Peliosis sometimes worsens in terms of extension, thus remaining asymptomatic[133,134]. In the presence of underlying conditions such as HCV-related cirrhosis, it can promote the risk of liver failure[130,134]. In some cases, especially in young patients, this alteration can cause compression and stenosis of the vena cava[135]. However, regression is also possible once the etiologic agent causing secondary peliosis is identified and treated[130,134], and idiopathic forms can undergo spontaneous regression[136].

Imaging

On contrast-enhanced CT and MR images, HSD is associated with the typical features

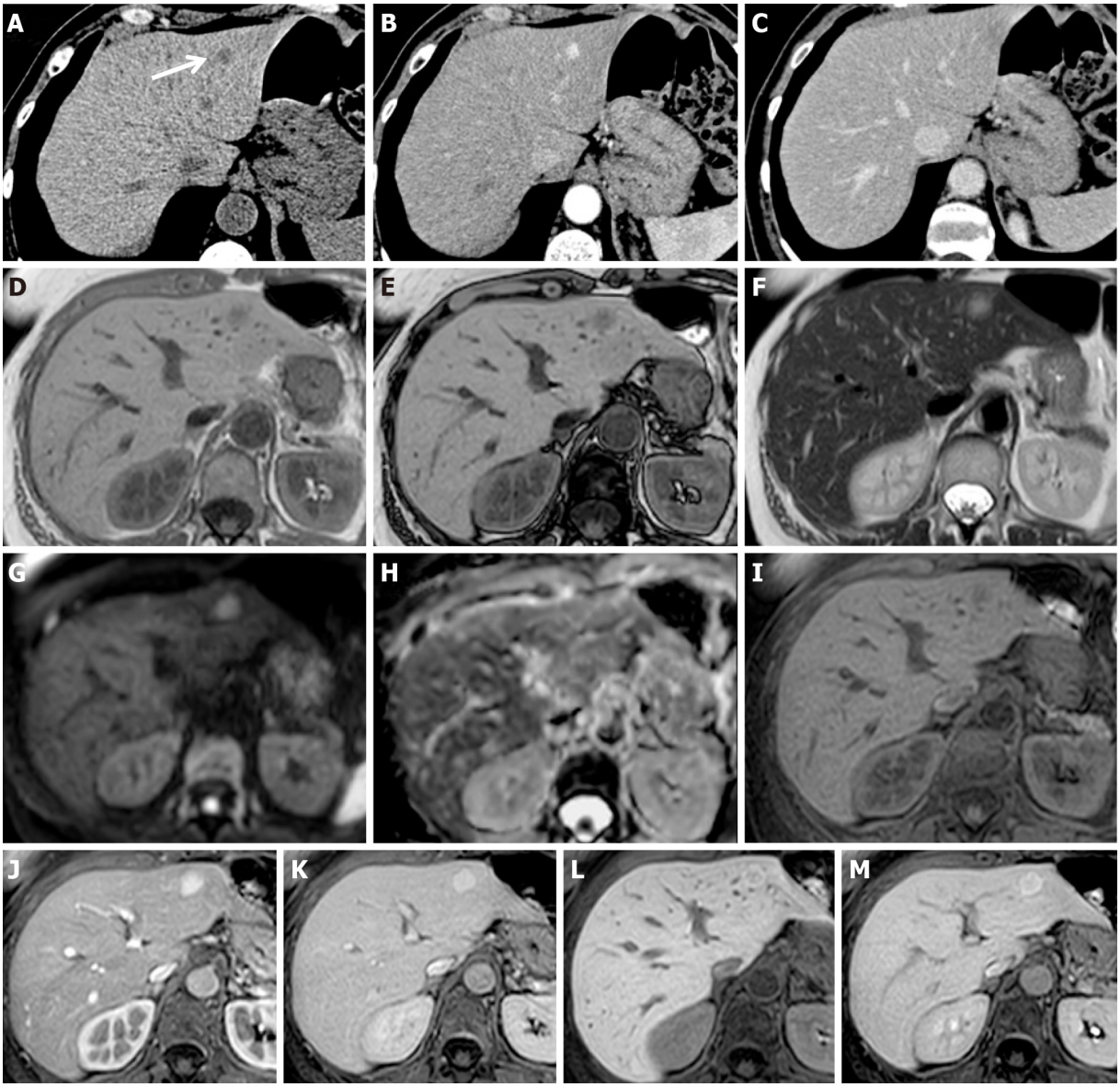


Figure 7 Focal nodular hyperplasia-like nodules in patients with colorectal cancer treated with surgery and adjuvant chemotherapy. A-C: Six months after adjuvant chemotherapy discontinuation, contrast-enhanced computed tomography (CT) showed a newly appeared nodule in segment II (white arrow), hypodense on unenhanced scan (A), with contrast enhancement on arterial phase (B) without washout in portal phase (C); D-M: Magnetic resonance performed 3 mo after CT showed a volumetric increase in the nodule, characterized by signal hypointensity in gradient echo T1w in-phase (D) and out-of-phase (E) and weak hyperintensity in T2w images (F), without diffusivity restriction on diffusion-weighted imaging (G-H). After liver-specific contrast agent administration, it presented homogeneous wash-in on the arterial phase (J) compared to the unenhanced image (I), no wash-out (K), and weak central signal hypointensity on equilibrium (L) and hepatobiliary (M) phases. A transcutaneous biopsy was performed, resulting in focal nodular hyperplasia-like nodules.

described for SOS, with mosaic pattern enhancement in the arterial-portal phase and reticular hypointense appearance in the MR hepatobiliary phase. On T2-weighted images, the affected areas may show slightly increased and heterogeneous signal intensity[137] (Figure 9).

The imaging features of peliosis depend on its extension, pathologic type and stage of blood components. In a few cases, the number or size of the peliotic lesions can increase in a short period and disseminate throughout the liver, resembling the progression of liver carcinoma or metastases[138].

On US, peliotic lesions appear homogeneous and hyperechoic, associated with pseudocyst formation, which may correspond to venous lacunae in the parenchyma [124], whereas in fatty liver, they will appear as hypoechoic lesions. In addition, when hemorrhage is present, US shows heterogeneously hypoechoic lesions. Unenhanced CT generally shows hypodense lesions, eventually associated with hyperdense foci, secondary to hemorrhage or calcifications (Figure 10). In dynamic phases, the pattern is variable; usually, in the arterial phase, the lesions show vessel-like enhancement at the center (target sign), with centrifugal enhancement during the venous phase;

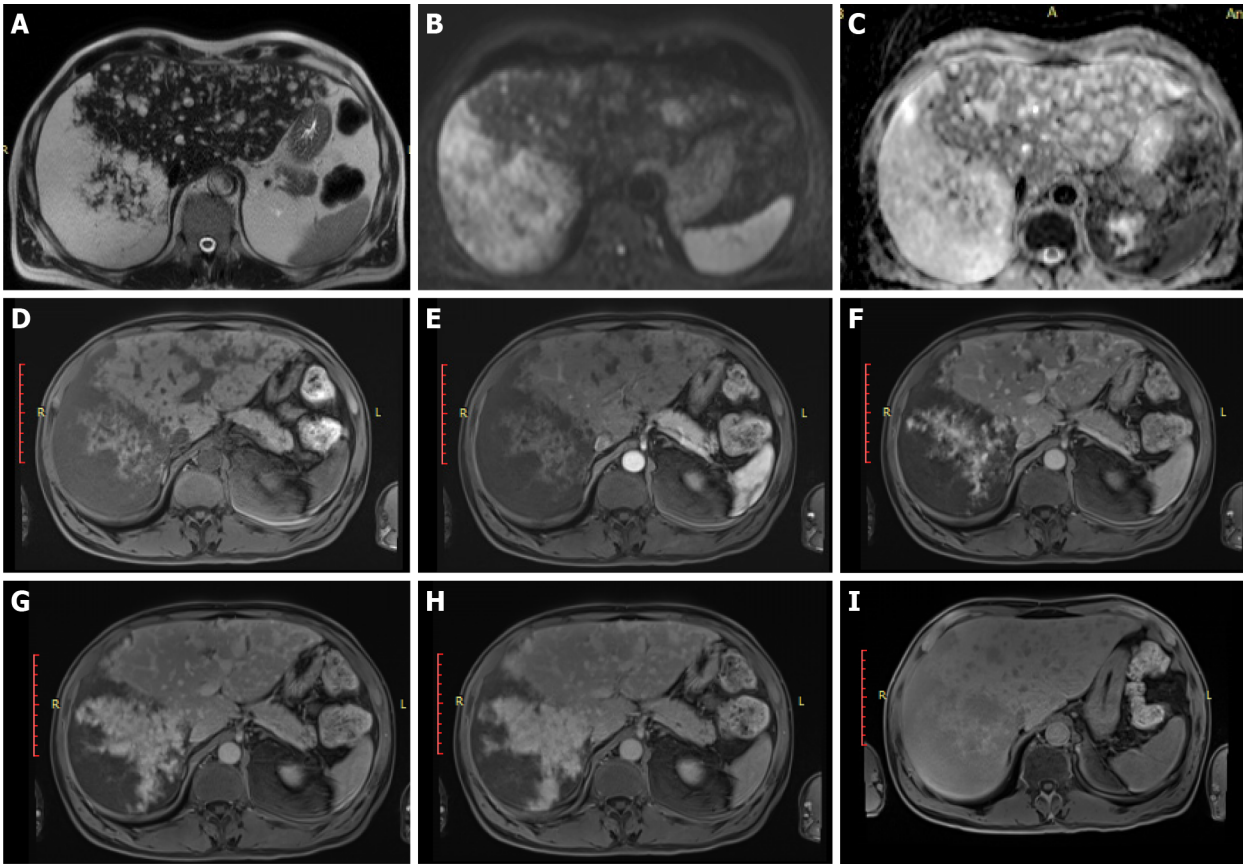


Figure 8 Primary idiopathic diffuse peliosis in patients without a cancer history. A-C: On magnetic resonance T2w images (A) and on diffusion-weighted imaging (B: High b value; C: Apparent diffusion coefficient map), numerous hemangioma-like lesions are visible, the largest in segments VII-VIII; D-I: After interstitial contrast agent administration, progressive centrifugal enhancement of the lesions was observed (D: Fat sat gradient echo 3D T1w unenhanced image; E, F, G: Arterial, portal and equilibrium phases; H-I: 5 and 30 min after contrast agent administration).

however, a centripetal enhancement pattern is also possible, which may be confused with that of a hemangioma[139]. Lesions tend to acquire diffuse homogeneous enhancement in the delayed phase[120,128]. In the presence of thrombosed cavities, these latter may show no enhancement. MR examination is the gold standard for radiologic diagnosis, presenting high specificity and sensitivity in the detection of the features of peliosis[134,136]. In MRI, on T2-weighted sequences, peliotic lesions are usually hyperintense compared to liver parenchyma with foci with a higher signal, which is likely attributable to hemorrhagic necrosis. On T1-weighted sequences, the lesions are hypointense, although isointense and hyperintense foci have also been described, depending on the age and the status of the blood components[120,140]. Exophytic extension of the peliotic nodules and fluid-fluid level, probably due to old and new blood products in the lesions, are rarely reported[140]. The dynamic behavior after CA administration is similar to that seen on CT scan, typically centrifugal, and more rarely centripetal[120]. In the hepatobiliary phase, a "branching" appearance caused by the direct demonstration of the vascular component within the lesion has been reported[134]. Although peliosis hepatis is a benign condition, the apparent diffusion coefficient values are lower than those of a normal-appearing liver, probably due to its content, including thrombi and hemorrhaged areas[140] (Figure 6). If the clinical and radiological findings are suggestive of peliosis, percutaneous liver biopsy should be avoided because of the significant risk of severe bleeding[141].

A summary of the liver mosaic appearance enhancement in blue liver and a classification of hepatic peliosis types are shown in Figure 11 and Figure 12, respectively.

PSEUDOCIRRHOSIS

Pseudocirrhosis is a pathological condition characterized by morphological changes of the liver mimicking macronodular cirrhosis without histopathological confirmation [104,142]. The ''pseudo'' prefix can also lead to confusion, indicating a more benign

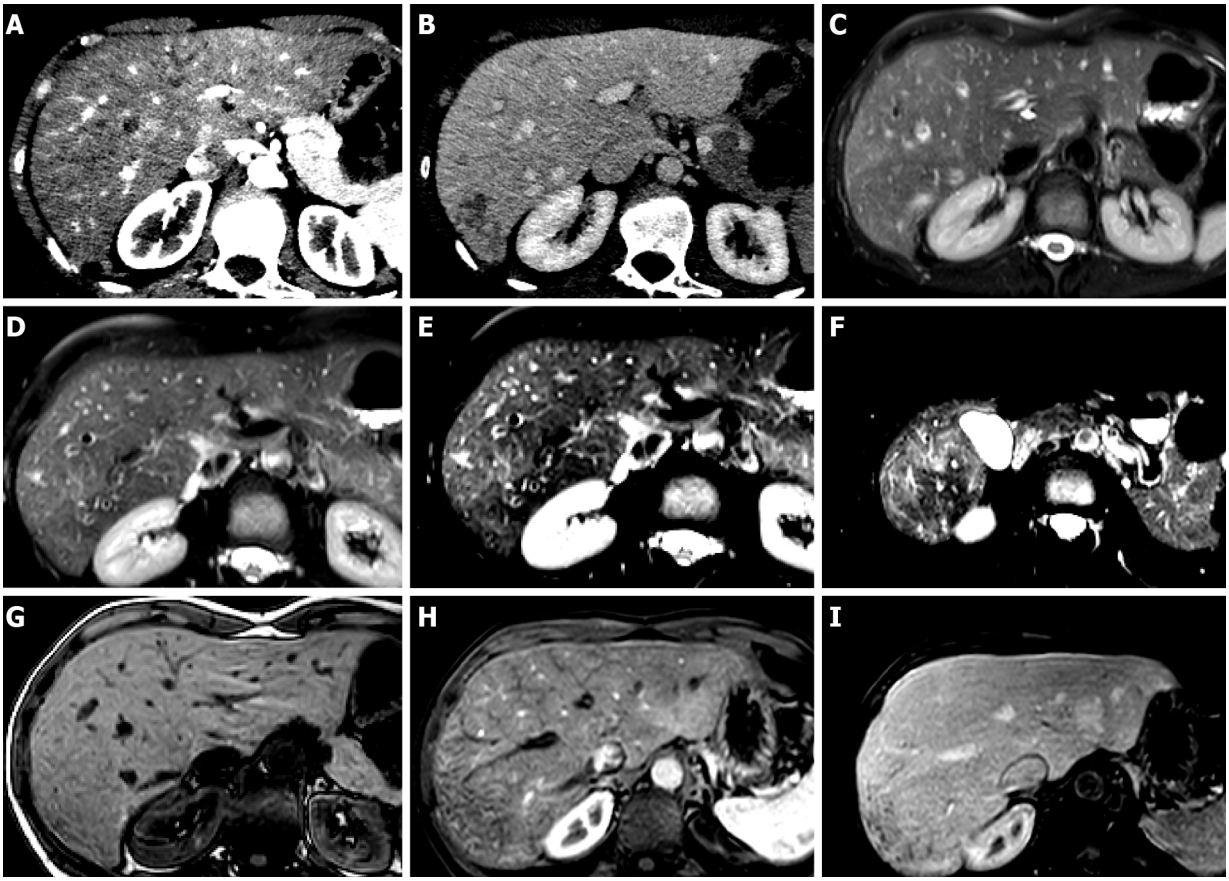


Figure 9 Hepatic sinusoid dilatation in patients with breast cancer during hormone therapy. A, B: Arterial (A) and portal (B) computed tomography axial scans show a liver mosaic pattern of arterial enhancement, with reticular aspects on the subcapsular parenchyma of segment VII in the portal phase; C-F: Magnetic resonance (MR) T2w images show mild signal hyperintensity on different liver sections and different echo times; G-I: MR unenhanced (G), arterial (H) and portal phases (I) confirm the mosaic pattern mostly subcapsular of the liver parenchyma.

condition than cirrhosis; indeed, even for patients who are asymptomatic and pseudocirrhosisis identified only incidentally during oncological follow-up, most patients can develop serious systemic complications, sometimes life-threatening, including portal hypertension, ascites and splenomegaly[143]. Abdominal distension, ascites and splenomegaly are the most common initial presentations in patients. Therefore, early recognition is important.

Breast cancer liver metastasis treated with chemotherapy is the most commonly reported cause of pseudocirrhosis[143,144]. However, it has also been linked to other metastatic diseases, including gastroenteric (pancreatic, esophageal, and colon), small-cell lung and thyroid cancers[144,145]. Vuppalanchi *et al*[146] estimated a prevalence of up to 50% in patients with metastatic breast cancer. Qayyum *et al*[142] said that approximately 75% of patients with liver metastatic breast cancer receiving chemotherapy demonstrated various degrees of hepatic contour abnormalities, from limited retraction to diffuse nodularity, and that approximately 9% of these patients developed portal hypertension. Morphological changes were seen after a median follow-up interval of 15 mo[142]. Indeed, the real prevalence of pseudocirrhosis has not yet been defined[147]. Interestingly, it is often observed in patients with a major morphologic response to chemotherapy[145]. Among the various CTAs, most cases of pseudocirrhotic changes are described after patients receive regimens including gemcitabine, 5-flurouracil, oxaliplatin[2] and trastuzumab[4,104]. More recently, Vuppalanchi *et al*[146] described two cases of pseudocirrhosis in patients after they had received the latest target therapy.

The pathophysiology of postchemotherapy pseudocirrhosis is still unknown, but it is proposed to be multifactorial and represent a mechanism of both cancer regression as a response of hepatic metastasis to CTAs and a consequence of the hepatotoxic effect of the treatment itself and cancer progression, with fibrosis surrounding the infiltrating hepatic tumor[147,148]. Tumor shrinkage in response to chemotherapy causes hepatic capsular retraction and scar formation around metastatic lesions, thus resulting in macronodular cirrhosis[149,150]. The regenerative response of hepatocytes

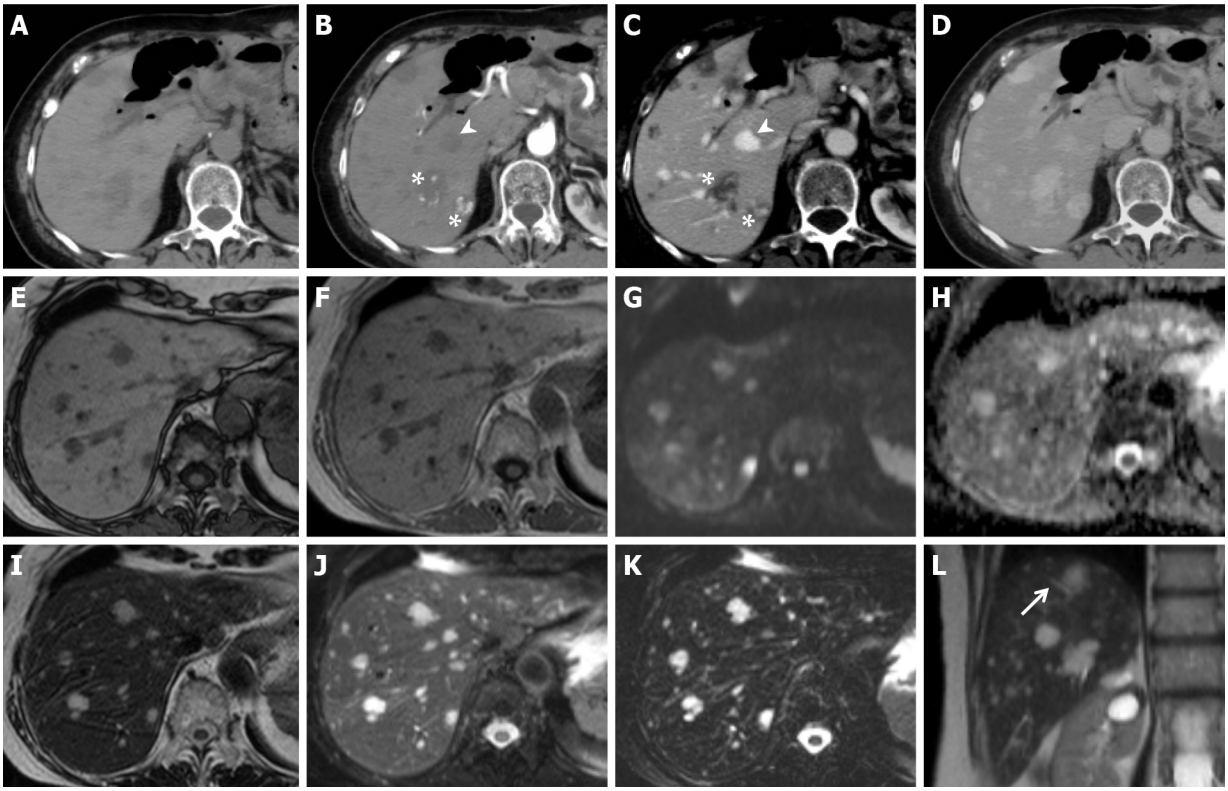


Figure 10 Secondary idiopathic multiple peliotic lesions in patients with a history of 6-mercaptopurine treatment for leukemia. A-D: Contrast-enhanced computed tomography shows multiple lesions, hypodense on unenhanced scan (A) with dystrophic calcifications and hyperdense foci, probably secondary to hemorrhage. On dynamic imaging (B, axial arterial phase; C, axial portal phase), the lesions present centripetal (arrowhead) or centrifugal (asterisk) globular contrast enhancement without signs of washout. In the delayed phase (D), they appear isodense compared with the hepatic parenchyma; E-L: Magnetic resonance confirming the presence of hypointense lesions on T1w images (E-F) and hyperintense lesions on T2w images (I, J and L, arrow), which maintain high signal in long echoes echo time 320 ms (K). No signs of altered diffusion (G-H) or mass effects are shown. These characteristics were consistent with multiple peliotic lesions.

to ischemia following chemotherapy-induced injury has been proposed as another mechanism; in this case, the development of NRH is thought to be critical to compression of the surrounding parenchyma, resulting in atrophy[151]. Finally, sinusoidal obstruction may contribute to pseudocirrhosis[146,149]. This effect may be secondary to both chemotherapy-induced sinusoidal damage and mechanical compression resulting from metastases, leading to rebound arterialization and portal flow reduction, which helps to explain the atrophy of the parenchyma and the cirrhotic appearance of the liver[152]. Interestingly, the mechanism is quite similar to that proposed by Breen for hepatic changes during cirrhotic progression[153]. A general rule of progression is proposed as follows: less portal inflow, an arterial phenomenon, metabolic infarction and fatty changes, fibrosis and atrophy[24,154]. Importantly, in this setting, in contrast to liver cirrhosis, histologic examination is consistent with NRH without bridging fibrosis[151]. In chemotherapy-naïve patients, however, pseudocirrhosis seems to occur only rarely[155] (Figure 13). This second type of pseudocirrhosis is linked to cancer progression and may be related to tumor size, with extensive fibrosis corresponding to a desmoplastic reaction surrounding the infiltrating tumors[155,156]. The pressure generated by fibrosis determines parenchymal portal flow lessening, with a consequent arterial reaction[24]. Therefore, in chemotherapy-naïve patients, the pathogenesis may also be similar to that after chemotherapy. Histologic examination of this second setting of pseudocirrhosis shows extensive fibrosis resulting from a desmoplastic reaction determined by the infiltrating lesion[149].

Imaging

The diagnosis of pseudocirrhosis is radiological and is defined by features typical of cirrhosis[104,142]. Because it progresses rapidly compared with 'true' liver cirrhosis, it can be easy to detect serial changes in liver morphology on imaging studies.

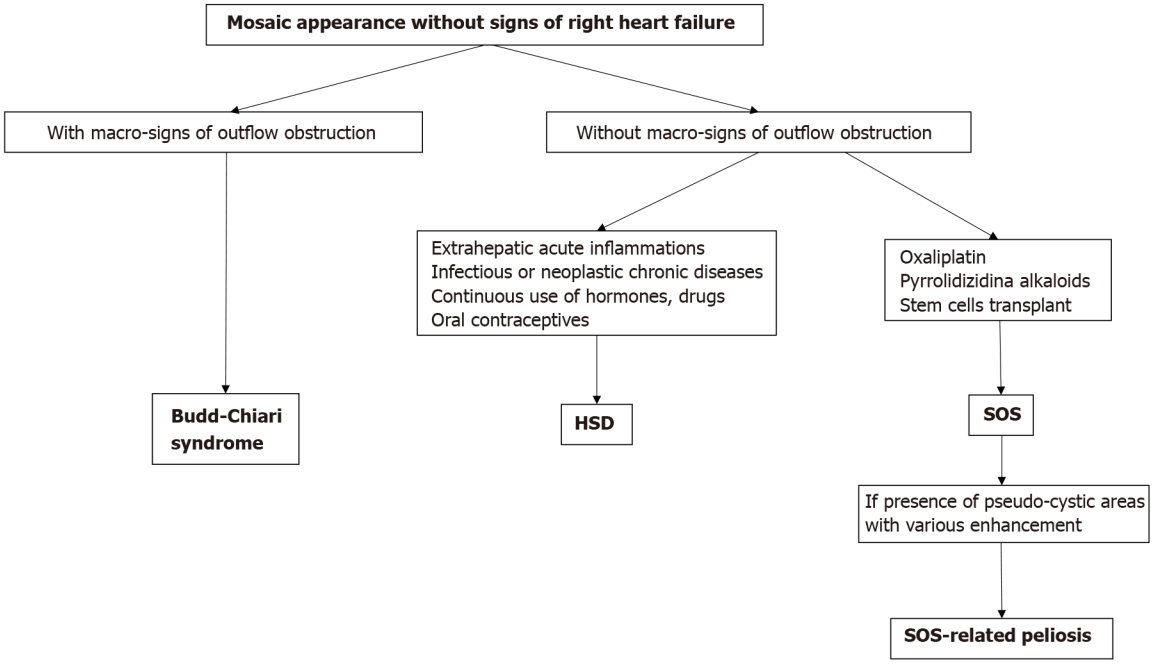


Figure 11 Diagram of different forms of mosaic pattern enhancement in blue liver syndrome. HSD: Hepatic sinusoid dilatation; SOS: Sinusoidal obstruction syndrome.

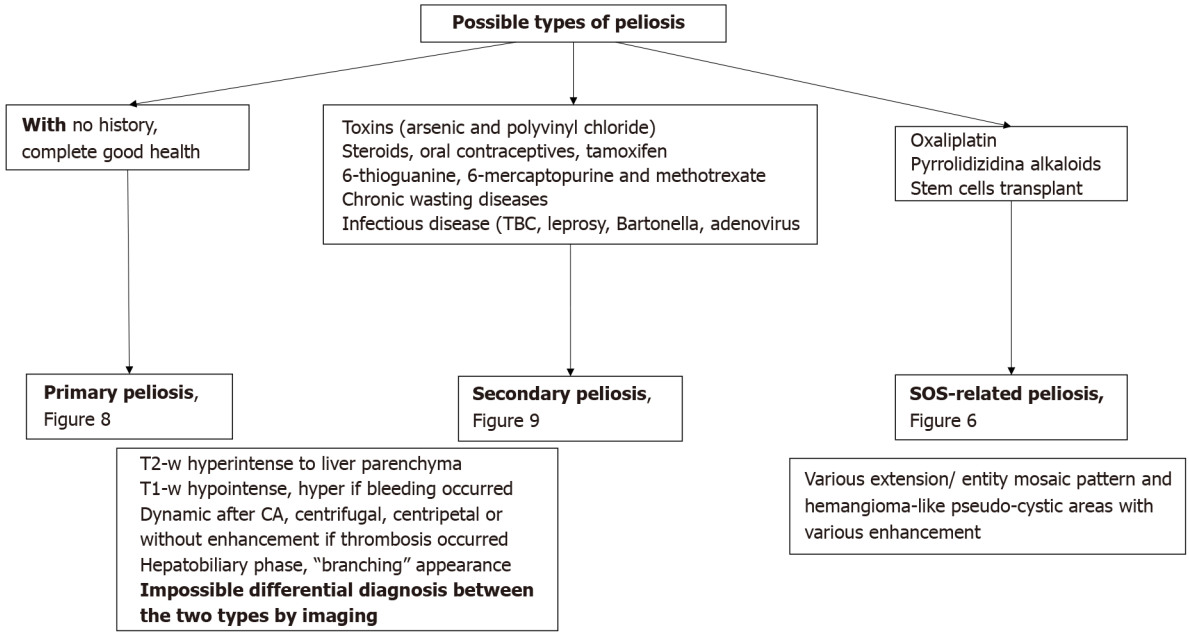


Figure 12 Diagram showing the classification of hepatic peliosis. SOS: Sinusoidal obstruction syndrome; CA: Contrast agent.

On CT or MR, hepatomegaly and diffuse fatty changes of the liver parenchyma were initially seen, with smooth hepatic surfaces and metastases that focally bulge out. These are followed by a reduction in the hepatic volume along with capsular retraction [104] (Figure 14). With time, fibrosis becomes prominent, confluent low-attenuation nodularity with irregular enhancement can be seen, and parenchymal atrophy of the right lobe associated with relative hypertrophy of the caudate and left lobe becomes more evident[104]. Moreover, other findings complicating cirrhotic changes include signs of portal hypertension such as splenomegaly, ascites and portosystemic varices [143]. Liver-specific gadolinium-enhanced MR can confirm the same morphological alterations, allowing for more accuracy in the characterization of any metastases. These lesions may appear as several focal lesions with high signal intensity on T2-weighted images and low signal intensity on T1-weighted images, with rim

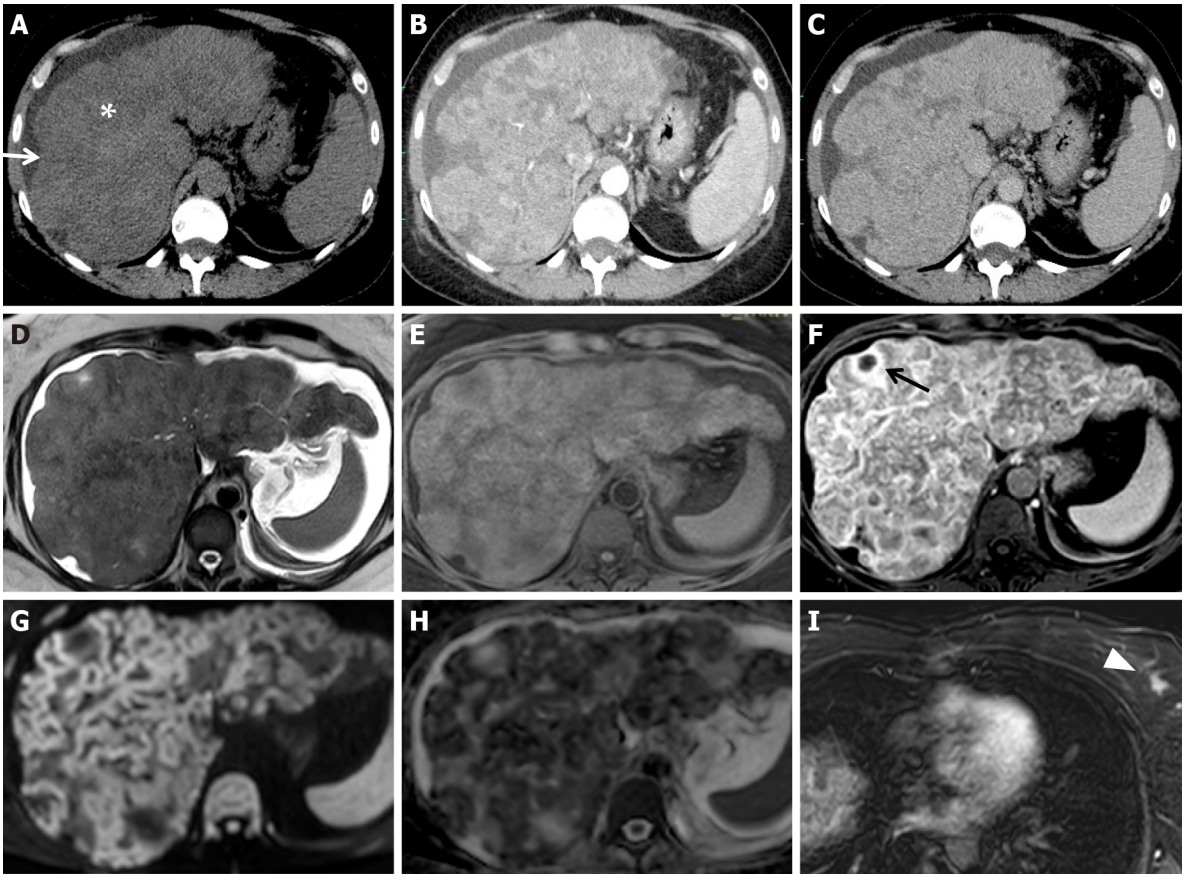


Figure 13 Pathologically proven pseudocirrhosis due to a small breast cancer in a chemotherapy "naïve patient", having received no chemotherapy. A-C: On unenhanced (A) computed tomography (CT) axial scans, a lobulated liver contour with retraction of the capsular surface (white arrow), low-attenuation parenchymal areas, and ascites (white asterisk) are seen. On arterial (B) and portal (C) CT axial scans, architectural disorder and heterogeneous contrast enhancement are detectable; D-I: On magnetic resonance, the presence of ascites is confirmed on T2w images (D). Profound structural and architectural changes due to the presence of coarse nodules separated by areas of fibrosis in an unenhanced fat sat gradient echo 3D T1w image (E) and a contrast-enhanced phase T1w image at equilibrium (F) are visible; various confluent nodules with irregular hyperintense rims on high b-value diffusion-weighted images (G) and low signal intensity in apparent diffusion coefficient map value (H) were observed. A small necrotic area inside a nodule is indicated in F (black arrow). One small left breast cancer nodule (white arrowhead) on a contrast-enhanced T1w image is visible in the arterial phase (I).

enhancement after CA administration[157] (Figure 15). Tumor markers do not increase during the period of pseudocirrhosis, indicating that progression of metastasis is unlikely[104]. Furthermore, nonspecific radiological findings may lead to a misinterpretation of the cancer response[147,158]. In addition, noncirrhotic causes of diffuse liver surface nodularity vary, and the clinical presentations are quite similar. In some of these causes, such as chronic Budd-Chiari syndrome, chronic portal vein thrombosis and pseudomyxoma peritonei, hepatic contour changes are easily distinguishable from cirrhosis because of their characteristic features. The latter shows coarse and lobulated contours, while nodularity associated with cirrhosis is typically relatively fine and diffuse. However, noncirrhotic causes of fine, diffuse nodularity are occasionally shown not only in pseudocirrhosis but also in hepatic failure and sarcoidosis[143]. Fulminant hepatic failure can present with diffuse surface nodularity due to a combination of alternating foci of confluent regenerative nodules and necrosis[159]. Sarcoidosis of the liver is rarely observable on imaging because noncaseating granulomas are usually microscopic. However, it can sometimes be visible as diffuse granular heterogeneity with or without fine nodularity of the hepatic surface[160,161].

Moreover, once pseudocirrhosis has been properly assessed, careful monitoring and appropriate management of complications are necessary to avoid progression toward life-threatening complications, such as hepatic failure, encephalopathy, and esophageal/gastric variceal bleeding, similar to those seen in classic severe cirrhosis [144,147]. Therapy should be modified and sometimes interrupted[154] because imaging features of pseudocirrhosis have been shown to completely resolve in some patients[154,158].

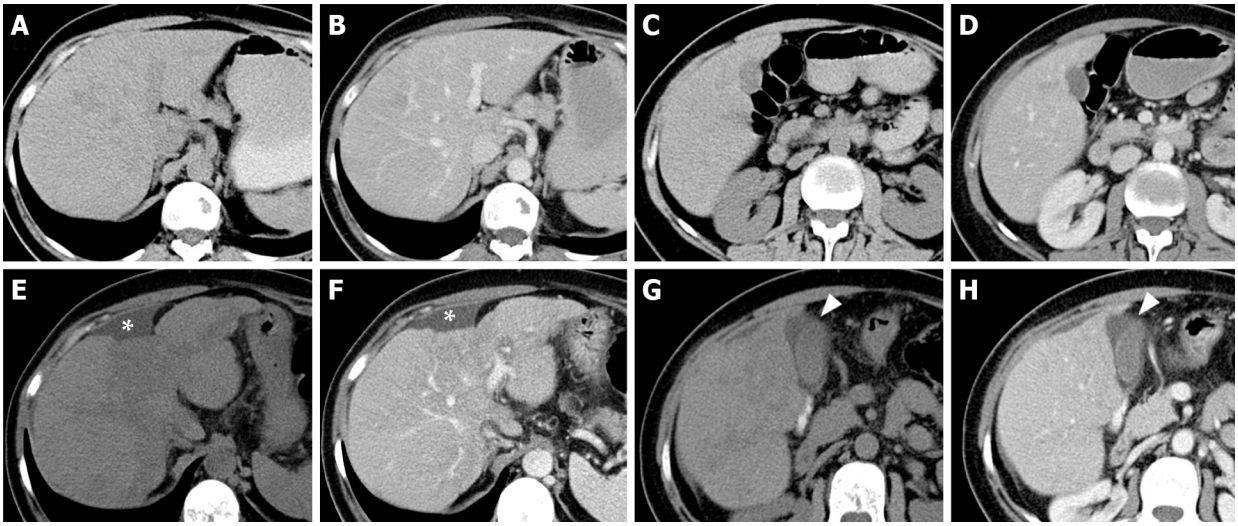


Figure 14 Early features of pseudocirrhosis in patients with metastatic breast cancer treated with gemcitabine for 12 mo. A-D: On axial unenhanced (A, C) and contrast-enhanced portal (B, D) computed tomography (CT) scan images, executed prior chemotherapy, the liver presents a regular volume, morphology and a smooth surface. No signs of ascites are present; E-H: On CT exam after chemotherapy (12 mo) at the same levels, in the same phases, fatty changes of the liver parenchyma, reduction of the hepatic volume with relative hypertrophy of the left lobe, irregular margins and capsular retraction corresponding to the IV segment (asterisk) were detectable. Peri-hepatic and pericholecystic effusion occurred (arrowhead).

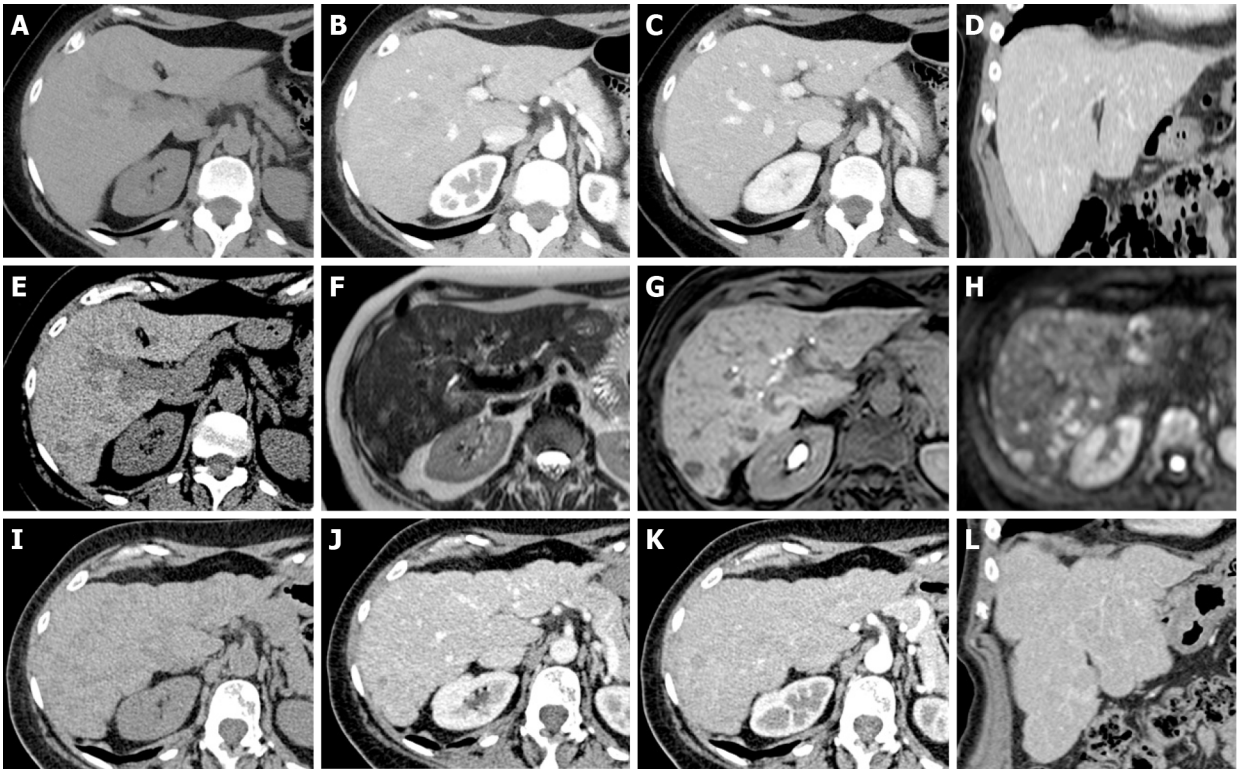


Figure 15 Pseudocirrhosis in patients with breast cancer treated with surgery and 6 mo of chemotherapy (capecitabine and monoclonal antibodies). A-D: Unenhanced (A: Axial) and contrast-enhanced computed tomography (CT) (B: Axial arterial phase; C: Axial portal phase; D: Coronal portal phase) was performed at staging. The liver shows regular volume, morphology and a smooth surface. No focal lesions were found; thus, no chemotherapy was undertaken; E: At the 1-year follow-up, unenhanced CT demonstrated the appearance of a hypodense focal lesion (arrowhead); F-H: A complete magnetic resonance study with liver-specific contrast agent confirmed the presence of new focal lesions consistent with metastases. Mild hyperintensity in the T2w sequence (F), clear hypointensity in the fat sat gradient echo 3D T1w hepatobiliary phase (G) and high signal in diffusion-weighted images (H) are shown. Chemotherapy was started. I-L: A 6-mo follow-up unenhanced (I: Axial) and contrast-enhanced CT (J: Axial arterial phase; K: Axial portal phase; L: Coronal portal phase) shows typical signs of liver pseudocirrhotic changes: parenchymal volume reduction, irregular macrocyclic margins, right lobe atrophy and caudate lobe hypertrophy.

CONCLUSION

In conclusion, many drugs can cause liver damage through various mechanisms in

oncologic patients. As a consequence of the longer life expectancy of these patients, chemotherapy-associated liver injury is becoming increasingly frequent. Radiologists need to be aware of and know the imaging patterns of chemotherapy injury, supporting clinicians in therapeutic decisions and thus preventing severe complications for patients.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 December 14; 27(46): 7894-7908

DOI: 10.3748/wjg.v27.i46.7894

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

FRONTIER

Hepatic pseudolesions caused by alterations in intrahepatic hemodynamics

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Author contributions: Kobayashi S designed and wrote the manuscript, and collected relevant data and approve the final manuscript.

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

Country/Territory of origin: Japan

Specialty type: Anatomy and morphology

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

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Abstract

Hepatic pseudolesion may occur in contrast-enhanced computed tomography and magnetic resonance imaging due to the unique haemodynamic characteristics of the liver. The concept of hepatic arterial buffer response (HABR) has become mainstream for the understanding of the mechanism of the reciprocal effect between hepatic arterial and portal venous flow. And HABR is thought to be significantly related to the occurrence of the abnormal imaging findings on arterial phase of contrast enhanced images, such as hepatic arterial-portal vein shunt and transient hepatic attenuation difference, which mimic hypervascular tumor and may cause clinical problems. Third inflow to the liver also cause hepatic pseudolesion, and some of the cases may show histopathologic change such as focal hyperplasia, focal fatty liver, and focal sparing of fatty liver, and called pseudotumor. To understand these phenomena might be valuable for interpreting the liver imaging findings.

Key Words: Pseudolesion; Focal sparing of fatty liver; Computed tomography; Hepatic blood flow; Hepatic hemodynamics; Hyperplastic change

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Core Tip: Understanding the characteristics of hepatic blood flow and the pathophysiology of pseudolesions caused by alterations in intrahepatic hemodynamics is important for diagnostic imaging of liver lesions. The concept of hepatic arterial buffer response, a unique mechanism for regulating hepatic blood flow, might be essential for elucidating the pathogenesis of hepatic arterial-portal vein shunting and transient hepatic attenuation difference on dynamic contrast-enhanced imaging of the liver. In addition, some pseudolesions are associated with histopathologic changes such as focal hyperplasia, focal fatty liver, and focal sparing of fatty liver. Understanding these

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Received: April 19, 2021
Peer-review started: April 19, 2021
First decision: June 23, 2021
Revised: June 23, 2021
Accepted: November 25, 2021
Article in press: November 25, 2021
Published online: December 14, 2021

P-Reviewer: Chen Q
S-Editor: Gong ZM
L-Editor: A
P-Editor: Gong ZM



phenomena may aid in interpreting liver imaging findings.

Citation: Kobayashi S. Hepatic pseudolesions caused by alterations in intrahepatic hemodynamics. *World J Gastroenterol* 2021; 27(46): 7894-7908
URL: https://www.wjgnet.com/1007-9327/full/v27/i46/7894.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i46.7894

INTRODUCTION

Hepatocellular carcinoma (HCC) is among the leading causes of cancer-related death, and colorectal carcinoma is prone to induce hepatic metastasis. Thus, there is a growing need to develop diagnostic imaging techniques that can properly identify localized malignancies in the liver.

Ultrasound is widely available, making it helpful for screening patients with hepatic mass lesions. However, given its lack of objectivity, computed tomography (CT) and magnetic resonance (MR) imaging are mainly utilized for closer examination of potentially malignant lesions.

The liver is an organ with a unique blood supply involving two types of inflow vessels: the hepatic artery and portal vein. The differential diagnosis of hepatic masses is made using imaging findings observed in dynamic contrast-enhanced studies, including hepatic arterial phase, portal venous phase, and equilibrium phase images. However, unique hemodynamic characteristics of the liver may lead to the occurrence of pseudolesions on contrast-enhanced images[1].

Radiologically, a pseudolesion is defined as a focal mass-like finding observed only on diagnostic imaging, without any actual histopathological abnormality[1]. Hepatic pseudolesions represent an important imaging challenge because they sometimes present findings similar to those of hepatic malignancy. In addition, some pseudolesions may cause focal parenchymal changes due to localized impairments in blood flow compared to the surrounding hepatic parenchyma, and such pseudolesions are referred to as pseudotumors[2].

Given their importance in the diagnostic imaging of liver lesions, we first introduce the characteristics of hepatic blood flow, following which we describe the mechanisms by which alterations in intrahepatic hemodynamics can lead to pseudolesions. Finally, we review hepatic parenchymal changes that occur in the region containing the intrahepatic hemodynamic abnormality.

OVERVIEW OF HEPATIC BLOOD FLOW

The liver is the largest parenchymal organ in the abdomen. It differs from other abdominal parenchymal organs in that there are two types of inflow vessels: the hepatic artery and the portal vein. Total hepatic blood flow is approximately 800-1200 mL/min (approximately 100 mL/min per 100 g liver wet weight). The portal vein supplies 75%-80% of the hepatic blood flow, while the hepatic artery supplies the remaining percentage. Hepatic blood volume is approximately 25-30 mL/100 g liver weight, representing roughly 10%-15% of the total blood volume. The average pressure in the hepatic artery is almost the same as aortic pressure; in contrast, portal vein pressure is approximately 6-10 mmHg in humans, while hepatic venous pressure is approximately 2-4 mmHg[3].

The portal vein collects blood from the splenic, gastric, superior mesenteric, and inferior mesenteric veins and flows into the liver through the hepatic hilum. Portal blood is mostly composed of blood from the gastrointestinal tract, and portal blood flow varies greatly depending on the feeding state. That is, portal blood flow increases after ingestion and decreases during fasting.

Hepatic arterial blood is rich in oxygen, and the peripheral hepatic arterial branches — either directly or after forming a capillary plexus around the bile duct and nourishing the bile duct — flow into sinusoids to supply oxygen to the hepatocytes and other structures.

The ratio of portal to hepatic arterial blood inflow to the liver is approximately 3:1, and the oxygen supply is mainly bestowed by hepatic arteries. Researchers have assumed that there is a complementary interaction between portal blood flow and hepatic arterial blood flow, meaning that hepatic arterial blood flow increases when portal blood flow decreases and that an increase in portal blood flow compensates for a decrease in hepatic arterial blood flow[2].

Several mutual routes of communication connect the hepatic artery and portal vein within the liver, including the trans-sinusoidal route, tumor thrombus-induced transvasal route, transtumoral shunt, transplexal route (peribiliary route), and arterio-portal fistula[4,5]. Among these connecting routes, the trans-sinusoidal route may represent the main complementary interaction between portal and hepatic arterial blood flow.

Recent studies have proposed the concept of hepatic arterial buffer response (HABR) for understanding the mechanism underlying the reciprocal effect between hepatic arterial and portal venous flow. As portal blood flow increases or decreases depending on the activity of the gastrointestinal tract, the liver has no control over portal blood flow. Therefore, when portal blood flow is reduced, hepatic arterial blood flow is controlled to maintain hepatic blood flow (*i.e.*, the oxygen supply to the liver) [6].

To elaborate, the space of Mall, which surrounds the terminal branches of the portal vein and hepatic artery before they drain into the hepatic sinusoid, constantly secretes adenosine, a vasoactive substance that serves to dilate the hepatic artery. When the normal portal blood flow is abundant, adenosine in the space of Mall is washed away by the influence of portal blood flow and does not dilate the hepatic arteries. However, when portal blood flow decreases, adenosine remains in the space of Mall, dilates the hepatic artery, and increases hepatic arterial flow to compensate for the decrease in portal blood flow to maintain hepatic sinusoidal blood flow. This is called the adenosine wash-out theory[7-9]. This mechanism of hepatic artery dilatation takes place in the hepatic arteriole, the distal part of the intrahepatic hepatic artery within the portal tract. HABR is thought to be significantly related to abnormal imaging findings observed on contrast-enhanced arterial phase images, such as hepatic arterial-portal vein shunting (AP shunting) and transient hepatic attenuation difference (THAD), as described below (Figure 1).

DEFINITIONS OF AND IMAGING FINDINGS ASSOCIATED WITH AP SHUNTING AND THAD

Choi *et al*[4] defined AP shunting as an organic or functional communication between the hepatic arterial branch and the portal venous system, resulting in the redistribution of arterial flow into a focal region of the portal venous flow. When blood flow through the portal vein is diminished or absent, the hepatic artery takes over perfusion of the liver through the AP shunt[4]. When portal vein obstruction occurs, increased hepatic arterial blood flow occurs mainly through the peribiliary plexus[10]. Namely, AP shunting is a consequence of the HABR mechanism.

On dynamic contrast-enhanced images, AP shunting is associated with (1) early enhancement of peripheral portal vein branches before the central portal vein is enhanced; and (2) THAD[4] (Figure 1).

THAD refers to transient, peripheral, wedge-shaped hepatic parenchymal enhancement (usually with a straight margin) that occurs during the hepatic arterial phase of contrast-enhanced imaging[10]. This phenomenon arises because increased arterial flow compensates for decreased portal venous flow and because the inflow of contrast material from a high-pressure arterial blood system into a low-pressure portal branch opacifies the focal area of the liver, while contrast material in the adjacent parenchyma is diluted by the unenhanced portal venous flow[4]. On portal venous phase images, the involved site returns to normal or nearly normal attenuation. Normal vessels pass through the area of THAD, and this finding can aid in differentiating THAD from hypervascular liver tumors such as HCC on contrast-enhanced imaging.

CAUSES OF LOCALIZED INTRAHEPATIC HEMODYNAMIC ALTERATIONS

The ratio of portal blood flow to hepatic artery blood flow is usually considered to be

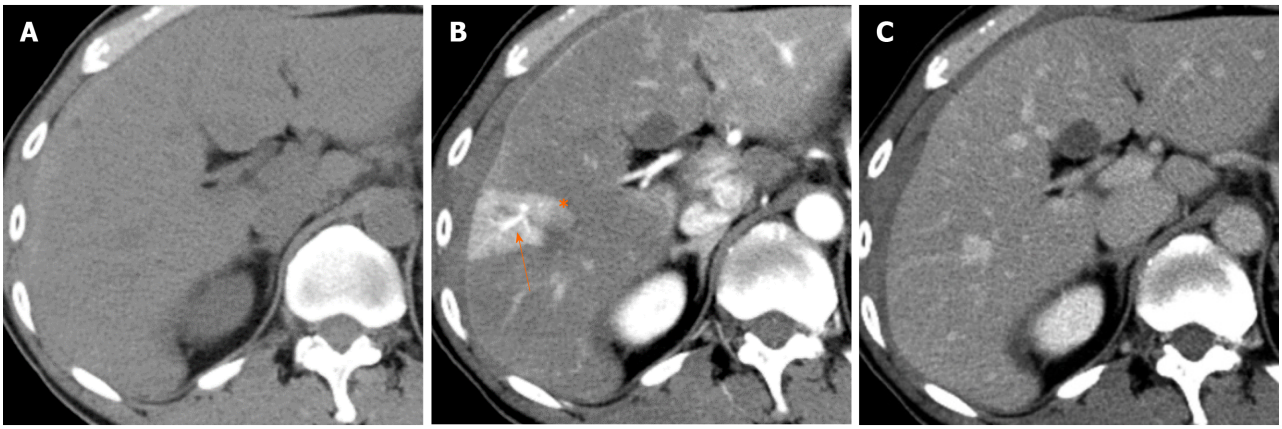


Figure 1 Arterial-portal vein shunt of 40th male. A: Pre contrast enhanced computed tomography (CT) of the liver shows no definite focal liver mass in segment V of the right lobe. B: On arterial phase contrast enhanced CT image, wedge shaped focal enhanced area is observed in peripheral part of segment V of the liver (*). Well opacified portal vein branch is observed within the focal enhanced area (arrow). C: On equilibrium phase of contrast enhanced CT, there are no attenuation differences in the peripheral part of segment V of the liver. Therefore, focal enhanced area observed in arterial phase contrast enhanced CT image is diagnosed as transient hepatic attenuation difference.

approximately 3:1 within the liver. Local disruption of this ratio leads to focal changes in blood flow on contrast-enhanced CT and MR images. The causes of intrahepatic hemodynamic changes include increased or decreased hepatic arterial blood flow, increased or decreased portal vein blood flow, and decreased hepatic venous blood flow. Anatomical variations can also cause intrahepatic hemodynamic changes.

Increased or decreased flow in regular liver vessels

Causes of increased hepatic arterial blood flow include HABR due to decreased portal blood flow and the presence of congenital or acquired shunt pathways [*e.g.*, hepatic AP shunts), hepatic arterial–hepatic venous shunts (AV shunts)], hereditary hemorrhagic telangiectasia (Figure 2), hepatic trauma, and others[11].

Physiological causes of increased portal blood flow include diurnal variations that occur with food intake, while pathological causes include "small-for-size grafts" at liver transplantation.

Causes of reduced portal blood flow include extrahepatic portal obstruction (Figure 3), portal vein thrombosis, portal vein tumor thrombus (Figure 4), agenesis of the portal vein (congenital porto-systemic shunt), patent ductus venosus, and porto-sinusoidal vascular disease (formerly known as idiopathic portal hypertension). In addition, blood flow from the portal vein to hepatic sinusoids is reduced in patients with liver cirrhosis exhibiting porto-systemic shunting.

Causes of decreased hepatic venous blood flow include Budd-Chiari syndrome (BCS) (Figure 5), sinusoidal obstruction syndrome (SOS) (Figure 6), hepatic vein thrombosis, and hepatic vein tumor thrombus. Secondary to these lesions, the liver exhibits a state of hepatic congestion. Increased hepatic venous pressure leading to decreased hepatic venous blood flow may also occur in patients with congestive heart failure and those who have undergone the Fontan procedure.

Localized portal hypoperfusion and the associated focal increases in hepatic arterial blood flow due to HABR are associated with THAD and the presence of hypervascularized pseudolesions on contrast-enhanced images due to increased inflow of contrast medium into the sinusoids during the arterial phase[12,13] (Figures 1-4). In contrast, when hepatic venous blood flow is decreased due to obstruction of hepatic venous outflow, blood drainage from the sinusoids toward the inferior vena cava is stagnant, and the sinusoidal pressure increases. This results in a decrease in the inflow of low-pressure portal blood into the sinusoids and an increase in the inflow of hepatic arterial blood, which causes reticular heterogeneous staining on arterial and portal phase contrast-enhanced images (Figures 5 and 6). This type of contrast-enhanced imaging finding is usually observed in patients with congestive liver, which occurs secondary to congestive heart failure, BCS, and SOS. Thus, when arterial phase contrast-enhanced images show THAD or reticular staining of the liver parenchyma, the presence of intrahepatic hemodynamic abnormalities can be inferred.

In addition to focal alterations in intrahepatic hemodynamics, anatomical variations in the portal venous system and other characteristic anatomical features of the vessel surrounding the liver can cause focal hemodynamic changes in several specific



Figure 2 Hereditary hemorrhagic telangiectasia of 70th male. On arterial phase contrast enhanced computed tomography of the liver, there are multiple pathy attenuated areas throughout the liver. Which are multiple transient hepatic attenuation difference caused by multiple arterial-portal venous shunts in hereditary hemorrhagic telangiectasia.

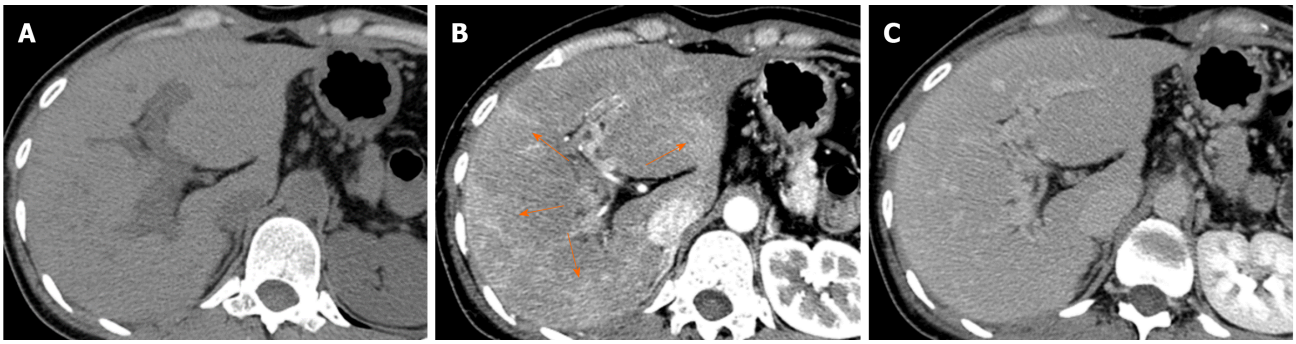


Figure 3 Extrahepatic portal obstruction of 30th female. A: Pre contrast enhanced computed tomography (CT) of the liver shows no definite focal liver lesion. B: On arterial phase contrast enhanced CT of the liver, there are multiple pathy attenuated areas in the subcapsular peripheral portion of the liver (arrow). C: On equilibrium phase contrast enhanced CT of the liver, there are no attenuation differences in the peripheral part of the liver.



Figure 4 Portal vein tumor thrombus of gastric cancer in 70th male. A: Pre contrast enhanced computed tomography (CT) of the liver shows no definite focal liver lesion. Wall thickening on the lesser curvature side of the stomach caused by gastric cancer is observed (*). B: On arterial phase contrast enhanced CT image, focal segmental enhanced area is observed in the right lobe of the liver (*). There is hypovascular tumor thrombus within the right portal vein (arrow). C: On equilibrium phase contrast enhanced CT of the liver, there are no attenuation differences in the liver.

portions of the liver[14].

Third inflow

The parabiliary venous system, epigastric–paraumbilical venous system, and cholecystic vein directly enter the liver independently of the portal venous system. These vessels are called the "third inflow," referring to the third hepatofugal flow after the hepatic arterial and portal vein systems[15].



Figure 5 Acute Budd-Chiari syndrome of 20th female. A: Pre contrast enhanced computed tomography (CT) of the liver shows no definite focal liver lesion in the liver. B: On portal phase contrast enhanced CT image, Irregular reticular hypo-enhancement is observed in the liver, which is caused by congestive change induced by hepatic outflow obstruction of Budd-Chiari syndrome. C: On equilibrium phase contrast enhanced CT of the liver, although intrahepatic parenchymal attenuation differences of the liver in the left lobe of the liver have disappeared, minimal attenuation differences are still observed in the right lobe of the liver.

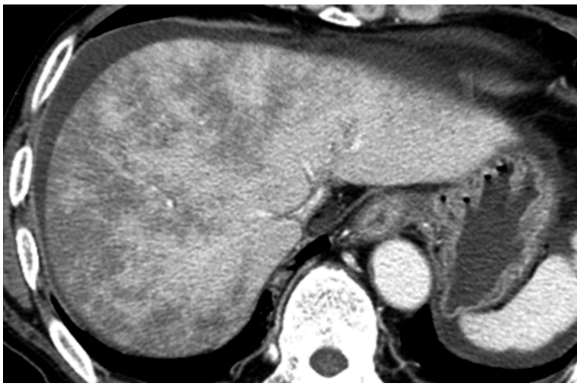


Figure 6 Sinusoidal obstruction syndrome after umbilical cord blood transplantation to acute myelocytic leukemia in 60th male. Portal phase image of contrast enhanced computed tomography shows irregular reticular hypodensity which are caused by hepatic congestion caused by sinusoidal portal flow disturbance.

Parabiliary venous system

The parabiliary system, termed the pancreatico-pyloro-duodenal vein, collects venous blood from the pancreatic head, stomach, and duodenum and usually joins the main portal vein outside the liver before flowing into the liver. However, in some cases, the pancreatico-pyloro-duodenal vein does not connect to the main portal vein before it enters the liver, instead directly entering the liver and perfusing the hepatic sinusoids at the posterior aspect of segment IV without fusion to the main portal vein. As this venous system includes the right gastric vein, this anatomical variation is sometimes referred to as "aberrant right gastric vein"[17]. The incident of aberrant right gastric vein varies from 1.5% to 49%[16-18], while the incidence of aberrant left gastric vein varies from 0.8% to 4%[18,19].

Although most aberrant gastric veins enter the liver and perfuse the hepatic sinusoids at the posterior aspect of segment IV (Figure 7), some may enter the liver and perfuse the liver parenchyma at the posterior edge of segment II or III[20] (Figure 8).

Epigastric–paraumbilical venous system

The paraumbilical vein is divided into the vein of Burow, superior vein of Sappey, and inferior vein of Sappey[21]. Among them, under conditions of portal hypertension, the inferior vein of Sappey is often dilated and forms a porto-systemic collateral pathway connected with the portal system in the anterolateral part of segment IV adjacent to the falciform ligament and epigastric veins.

When superior vena cava obstruction occurs, hyperenhancement of segment IV (*i.e.*, quadrate lobe hot-spot sign) can be observed on contrast-enhanced CT/MR images[22] (Figure 9). This phenomenon is the result of the inferior vein of Sappey acting as the hepatopetal collateral route.

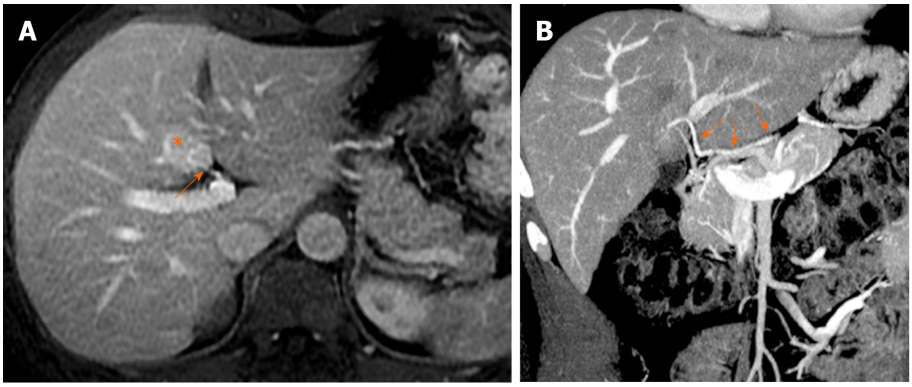


Figure 7 Hypervascular pseudolesion observed in posterior aspect of segment IV (50th male). A: On portal phase image of contrast enhanced magnetic resonance imaging with Gadolinium based contrast agent, focal hyper-attenuation area is observed in posterior aspect of segment IV of the liver (*). Tiny vascular branch is directly entering the area at hepatic hilum, which is an aberrant right gastric vein directly entering to the liver (arrow). B: On coronal reconstruction of portal phase image of contrast enhanced computed tomography, aberrant right gastric vein directly enters the posterior aspect of segment IV of the liver without fusion to the main portal vein (arrows).

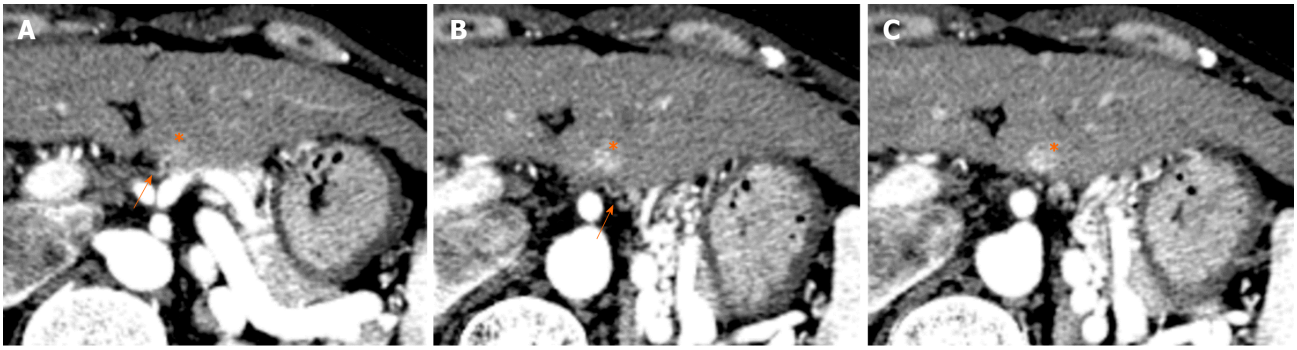


Figure 8 Hypervascular pseudolesion observed in posterior aspect of segment II (40th female). A-C: On arterial phase images of contrast enhanced computed tomography, tiny focal hyper-attenuation area is observed in posterior aspect of segment II of the liver (*). Tiny vascular branch is directly entering the area from outside of the liver, which is an aberrant left gastric vein directly entering to the liver (arrows).



Figure 9 Hypovascular pseudolesion in the drainage area of the vein of Sappey (70th female). On arterial phase contrast enhanced computed tomography (CT) image, focal hypoattenuation area is observed in anterior portion of segment IV of the liver adjacent to the falciform ligament, which is not detected on both pre-contrast CT and equilibrium phase contrast enhanced CT (images are not shown). This is hypovascular pseudolesion in the drainage area of the vein of Sappey.

Cholecystic veins

The blood supply and drainage of the gallbladder are also related to the occurrence of focal hepatic hemodynamic changes. Arterial supply of the gallbladder is provided by the hepatic artery (mainly the right hepatic artery[23]), and the cholecystic vein drains into the liver sinusoids surrounding the gallbladder usually after being connected to the peripheral branch of the intrahepatic portal vein. In detail, cholecystic venous

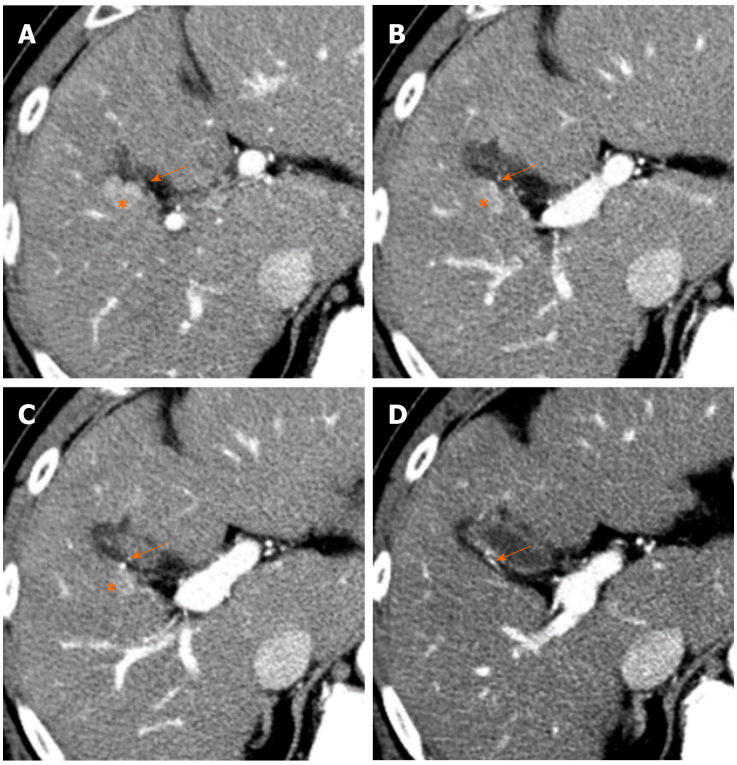


Figure 10 Hypervascular pseudolesion in cholecystic venous drainage area (50th male). A-D: On sequential images of arterial phase contrast enhanced computed tomography, round hyper-attenuation area is observed in segment V of the liver adjacent to the gallbladder (*). Tiny enhanced vessel is directly entered to the enhanced liver area from the gallbladder wall (arrows), which is cholecystic venous drainage to the liver and hypervascular pseudolesion in cholecystic venous drainage area.

blood most frequently enters the peripheral portal branches of liver segments V (96%) and IV (93%)[23]. As the cholecystic venous blood originates from the hepatic artery, the concentration of nutrients and humoral factors such as hormones, which flow into the hepatic sinusoids of the cholecystic venous drainage area, differs from that in other hepatic sinusoids into which the portal venous blood flows. Such differences in the composition of influx blood between the cholecystic venous drainage area and the rest of the liver can cause focal differences in contrast-enhanced imaging and histopathological findings (Figure 10).

FOCAL PARENCHYMAL CHANGES IN THE LIVER DUE TO HEMODYNAMIC ALTERATIONS

Focal alterations in intrahepatic hemodynamics may not only present as pseudolesions on contrast-enhanced images but may also result in histological changes of the liver parenchyma at the site of the blood flow change. There are three major patterns of such histological changes in the liver parenchyma.

Focal hyperplasia

Focal hyperplasia of the liver is observed in patients with cirrhotic liver and may be related to the presence of anomalous portal flow such as aberrant gastric venous drainage. Matsui *et al*[24] reported that in patients with cirrhotic liver with aberrant gastric venous drainage, 22%-50% of cases are associated with focal hyperplastic changes at the posterior aspect of segment IV, where the aberrant gastric venous drainage is present[24] (Figure 11). Focal hyperplastic changes such as anomalous portal venous drainage in the caudate lobe have also been reported in cases of cirrhotic liver, with the authors surmising that the etiology of such hyperplastic changes is intimately related to the anomalous portal flow[25,26]. Similarly, focal hyperplasia with anomalous portal flow in the caudate lobe has also been reported in a patient without cirrhosis[27].

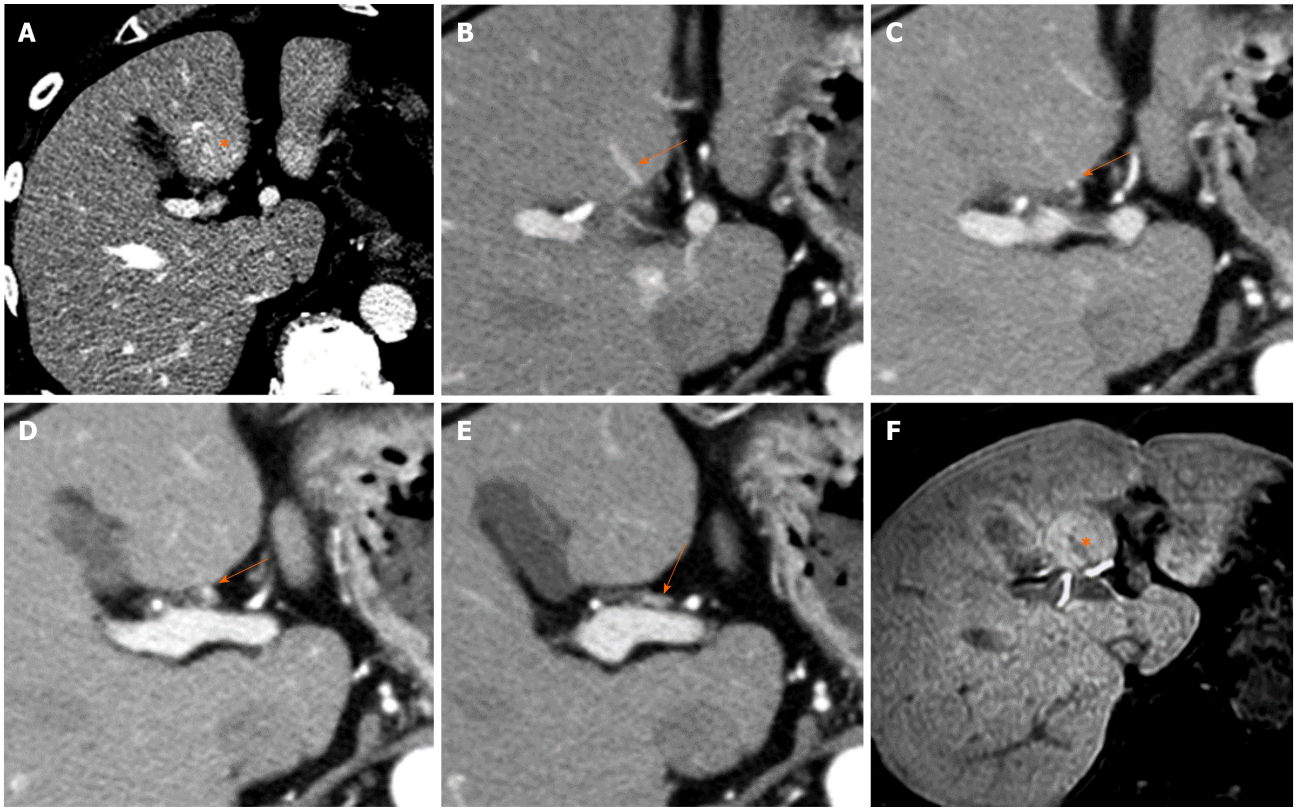


Figure 11 Focal hyperplastic change in posterior aspect of segment IV (70th male). A: On the portal phase contrast enhanced computed tomography (CT) image, focal hyper-attenuation area is observed in the posterior aspect of segment IV of the liver (*), which is not detected on both pre-contrast CT and equilibrium phase contrast enhanced CT (images are not shown). This is hypervascular pseudolesion observed in the area of aberrant right gastric venous drainage to the liver. B-E: On sequential images of arterial phase contrast enhanced CT, aberrant right gastric vein directly entering to the posterior aspect of segment IV of the liver is well opacified (arrows). F: On hepatobiliary phase of Gd-EOB-DTPA enhanced magnetic resonance imaging, slightly hyper-intensity area is observed in the same place of focal hyper-attenuation area observed in the portal phase contrast enhanced CT image (*), which represents focal hyperplasetic change of the liver in aberrant right gastric venous drainage area in the posterior aspect of segment IV.

Researchers have examined the etiology of liver hyperplasia occurring after hepatectomy or portal vein embolization. At present, it is believed that blood flow, shear stress, and adenosine are involved in the development of hyperplasia in the liver [28]. Studies on liver regeneration after hepatectomy or portal vein embolization have also suggested that hyperplasia and atrophy of the liver occur when the liver is unable to compensate for changes in blood flow caused by surgery or portal vein embolization. Several studies support the hypothesis that after portal vein embolization, portal blood flow increases in the unembolized liver lobe, which causes acute portal hypertension. This change leads to increased shear stress and nitric oxide production, which in turn triggers liver regeneration[29-31]. In contrast, decreased intrahepatic shear stress is thought to induce liver atrophy[31]. This mechanism is thought to maintain the ratio between liver mass and blood flow, most likely to ensure maintenance of adequate clearance function[31].

In small-for-size syndrome after liver transplantation, HABR reduces hepatic arterial flow due to excessive portal flow, which leads to decreased oxygen delivery to the liver parenchyma. The lack of adequate oxygen for liver regeneration increases the risk of liver failure. However, normalization of portal pressure and portal blood flow is believed to improve liver regeneration[7]. Thus, the degree of hepatic blood flow, especially portal blood flow, plays a major role in liver regeneration and atrophy.

Ethanol consumption is also involved in liver regeneration and atrophy. Gluud *et al* [32] observed that the frequency of hyperplastic nodules decreased with higher ethanol intake in patients with cirrhosis, indicating that ethanol consumption may inhibit liver regeneration[32]. Histopathologically, ethanol intake causes damage to the hepatic veins, especially perivenular fibrosis[33]. Impaired hepatic veins lead to decreased outflow of sinusoidal blood, resulting in stasis of blood flow and hepatic congestion, thereby inducing liver atrophy.

Focal hyperplastic changes are observed in the aberrant right gastric venous drainage area in patients with alcoholic cirrhotic liver[24]. One possible explanation

for this is the difference in blood ethanol concentrations of the main portal and aberrant right gastric vein. In humans, 20% of ingested ethanol is absorbed through the stomach, while 80% is absorbed through the small intestine[34]. This means that the ethanol concentration in the venous blood of the small intestine is higher than that in the venous blood of the stomach. Normally, both the venous blood from the stomach and small intestine join and form the main portal vein before flowing into the liver, so there is no difference in ethanol concentration in the portal venous blood within the liver. However, in the presence of aberrant right gastric venous drainage, hepatic venous injury is less likely to occur at the drainage area because the ethanol concentration is lower in the gastric vein. In contrast, in the area perfused by the portal flow, hepatic venous injury is more likely to occur as the blood flow of the main portal vein collects the venous flow of the small intestine, which contains higher ethanol concentration.

As a result, most of the liver areas that receive blood flow from the main portal vein exhibit congestion due to hepatic vein injury, while the area supplied by the aberrant right gastric venous drainage exhibits less extensive hepatic vein injury and less severe congestion than the rest of the liver. Hepatic atrophy occurs in areas with severe hepatic congestion, while areas with less hepatic congestion are relatively hyperplastic. This may be why focal hyperplasia occurs in the aberrant right gastric venous drainage area in patients with alcoholic cirrhosis.

Similarly, hepatic venous injury is less likely to occur at the cholecystic venous drainage area surrounding the gallbladder in segments IV and V of the liver due to the relatively low amount of ethanol in the venous blood compared to the rest of the liver. Therefore, focal hyperplastic changes may also occur in this area in patients with alcoholic cirrhosis (Figure 12).

The contribution of hepatic venous drainage to liver deformity and atrophy has also been noted in patients with conditions other than alcoholic liver disease. Ozaki *et al*[35] demonstrated that, because the diameter of the middle hepatic vein is small and venous blood pressure is high compared to that in the other hepatic veins, the middle hepatic venous drainage area tends to exhibit congestive changes. Such changes lead to selective atrophy of the middle hepatic venous drainage area (*e.g.*, segment IV) as well as relative hyperplastic changes in the right and left hepatic venous drainage area [35].

Other factors that may contribute to focal hyperplastic changes in the third inflow area include different concentrations of bile acid in the main portal vein and the third inflow. Previous studies have reported that enterohepatic circulation is involved in liver regeneration[36], and that decrease in bile acid return to the liver triggers hepatocyte proliferation[37]. Because bile acids circulate in the gut–liver axis, the concentration of bile acids in the third inflow is lower than that in the main portal venous flow. Therefore, differences in the concentration of bile acids in the inflow may contribute to the development of focal hyperplasia of the liver parenchyma in the aberrant venous drainage area[38-40].

Localized hepatocellular hyperplastic changes in the normal liver that mimic liver neoplasms on imaging are referred to as focal nodular hyperplasia (FNH). Researchers have proposed that the pathogenesis of FNH is related to a disturbance of sinusoidal blood outflow[41-43] or to the presence of abnormal anomalous vessels[44]. These studies indicate that localized alterations in intrahepatic hemodynamics play a major role in the pathogenesis of FNH.

In summary, the presence of intrahepatic hemodynamic alterations is essential for the development of focal hyperplastic changes in the liver. Such changes are also influenced by concomitant factors such as differences in the blood concentrations of nutrients, ethanol, hormones, and so on at the site. Further research is required to elucidate the mechanism underlying the development of focal hyperplastic changes in the liver.

Focal fat deposition

Focal fat deposition in the liver is occasionally observed in the anteromedial portion of segment IV (adjacent to the falciform ligament)[45] and in the posterior aspect of segment IV[46] (Figures 13 and 14). Focal fat deposition at the posterior aspect of segment IV is related to the presence of aberrant right gastric venous drainage[47], while that at the anteromedial portion adjacent to the falciform ligament is related to the presence of inferior vein of Sappey drainage[47,48].

Vilgrain *et al*[49] suggest that differences in the concentration of insulin in the blood entering the liver contribute to focal fat deposition in the liver. As an aberrant right gastric vein may collect venous blood from the head of the pancreas and flow into the posterior aspect of segment IV, the concentration of insulin may in turn be higher in

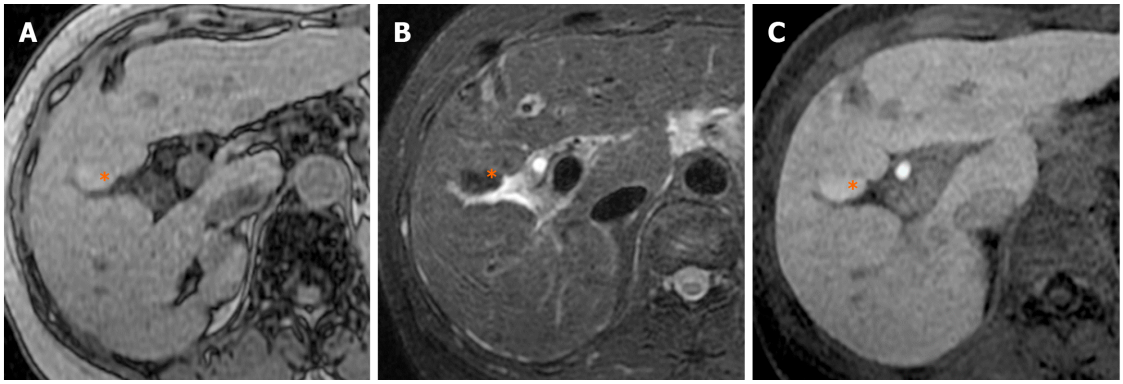


Figure 12 Focal hyperplastic change in cholecystic venous drainage area (50th male). A: On T1 weighted opposed-phase magnetic resonance (MR) image, focal hyperintense lesion is observed at segment IV of the liver adjacent to the gallbladder (*). B: The focal lesion shows hypointensity on fat suppressed T2 weighted MR image (*). C: The focal lesion shows hyperintensity on hepatobiliary phase of Gd-EOB-DTPA enhanced magnetic resonance imaging (*). These findings observed at segment IV of the liver adjacent to the gallbladder represent focal hyperplastic change of the liver in cholecystic venous drainage area.

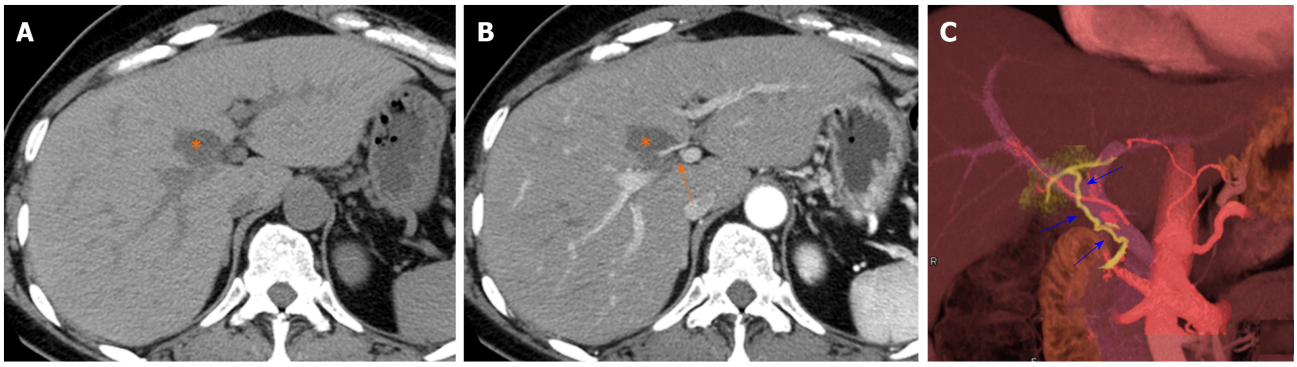


Figure 13 Focal fat deposition in posterior aspect of segment IV (50th female). A: On pre-contrast enhanced computed tomography (CT), focal hypodense lesion is observed at the posterior aspect of segment IV of the liver (*). B: On arterial phase contrast enhanced CT image, the lesion shows hypodense (*) and an enhanced vascular branch is directly entering the area at hepatic hilum (arrow). C: On three-dimensional reconstructed CT image with contrast enhancement, an aberrant right gastric vein directly drains into the posterior aspect of segment IV of the liver without connecting the main portal vein is observed (arrows).

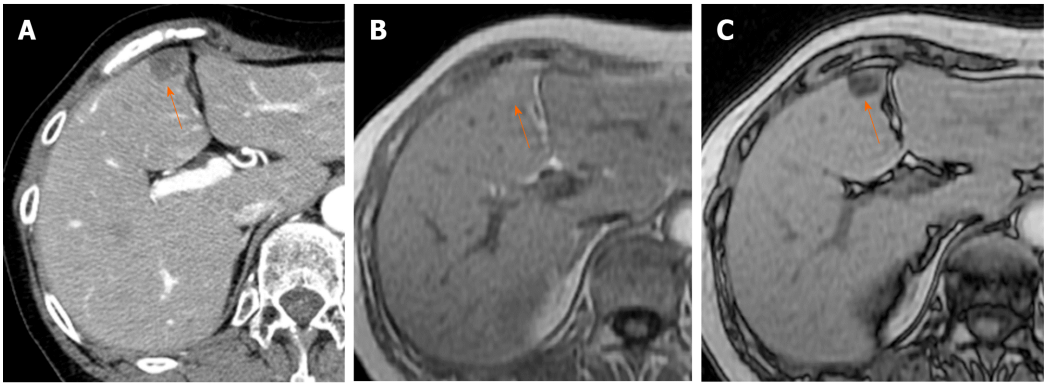


Figure 14 Focal fat deposition in the drainage area of the vein of Sappey (60th female). A: On arterial phase contrast enhanced computed tomography image, focal hypoattenuation area is observed in anterior portion of segment IV of the liver adjacent to the falciform ligament (arrow). B and C: On T1 weighted in-phase and opposed-phase image of the liver, the lesion shows hyperintense on in-phase (B, arrow) and shows hypointense on opposed-phase (C, arrow), which represent focal fat deposition of the liver at the drainage area of inferior vein of Sappey.

the inflow area of the aberrant right gastric vein than in other areas. This may lead to focal fat deposition in the posterior aspect of segment IV, where aberrant right gastric venous drainage is present.

Focal fat deposition is also observed in the hepatic parenchyma surrounding the metastasis of pancreas islet cell tumors, which produce insulin. The etiology of focal fat deposition in such cases may be the same as that described above[49,50].

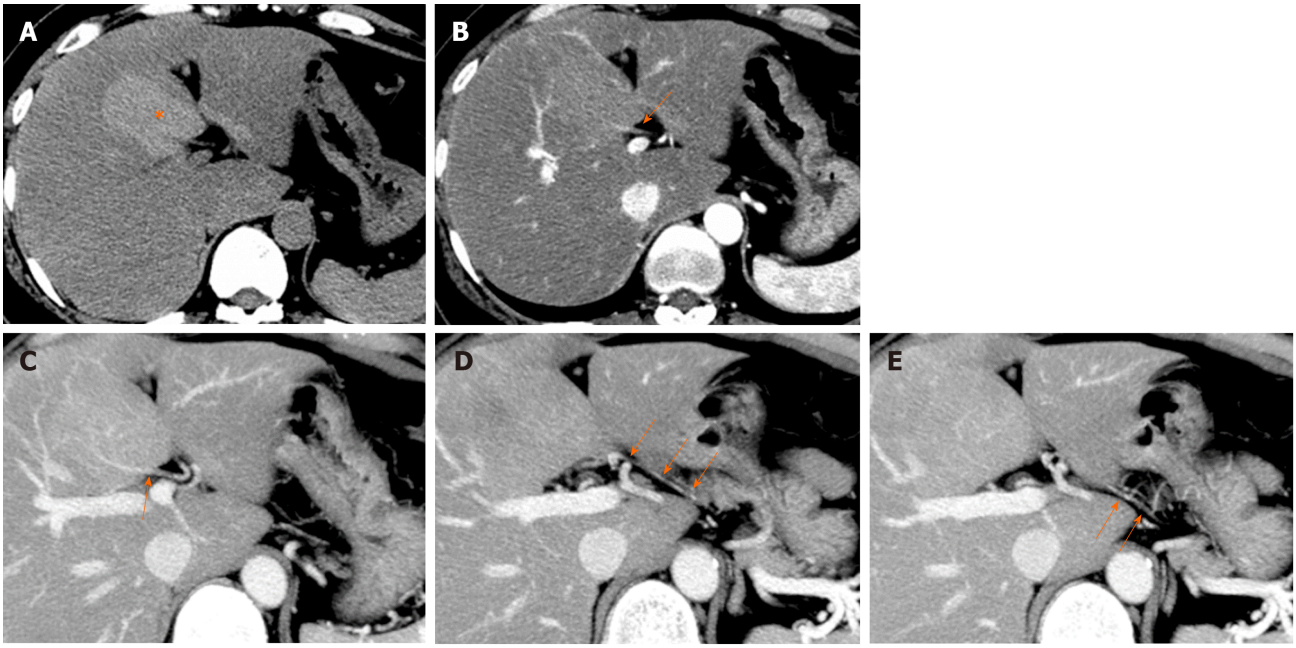


Figure 15 Focal spared area of fatty liver in posterior aspect of segment IV (40th female). A: On pre-contrast enhanced computed tomography (CT), a focal hyperdense lesion compared to the background liver parenchyma is observed in posterior aspect of segment IV of the liver (*). Background liver shows decreased density and suggestive of fatty liver and hyperdese area is diagnosed as focal sparing of fatty liver. B: On arterial phase contrast enhanced CT image, an enhanced vascular branch is directly entering the area at hepatic hilum (arrow). C-E: On sequential images of arterial phase contrast enhanced CT, aberrant right gastric vein directly entering to the posterior aspect of segment IV of the liver is well opacified (arrows). These findings represent focal spared area of the fatty liver in aberrant right gastric venous drainage area of the liver at the posterior aspect of segment IV of the liver.

Focal sparing of fatty liver

Focal sparing of fatty liver refers to the presence of focal areas exhibiting a relative decrease in the degree of fat deposition in cases of fatty liver. This type of focal sparing represents the opposite of focal fat deposition in terms of steatotic liver changes and is intimately related to alterations in intrahepatic hemodynamics. Focal sparing of fatty liver is sometimes observed in the posterior aspect of segment IV (Figure 15) and in the liver parenchyma surrounding the gallbladder in segments IV and V.

Matsui *et al*[51] reported a strong correlation between the focally spared area at the posterior edge of segment IV in fatty liver and aberrant gastric venous drainage directed to segment IV.

Fatty liver is an abnormality of the liver caused by overnutrition. However, when aberrant right gastric venous flow with a low level of nutrients compared to the main portal vein enters the posterior aspect of segment IV, focal sparing of fatty liver is assumed to occur in the third inflow area. Vilgrain *et al*[49] reported that if the insulin concentration of the aberrant gastric venous flow is low in patients with fatty liver, the aberrant venous drainage area will exhibit less fat deposition and focal sparing on liver imaging.

The blood supply of the gallbladder is provided by the cholecystic artery originating from the hepatic arterial branches, in which the blood contains enough oxygen but contains fewer nutrients than the portal venous blood. The cholecystic vein drains into the liver parenchyma surrounding the gallbladder. The venous flow that perfuses the liver area surrounding the gallbladder contains less nutrients than other areas of the liver supplied by the portal vein. For this reason, focal sparing of fatty liver occurs in the liver parenchyma surrounding the gallbladder in segments IV and V. This hypothesis is further supported by the finding that the incidence of focal sparing of fatty liver is significantly lower in patients who have undergone cholecystectomy than in those with intact gallbladders[52].

CONCLUSION

In the present review, we discussed the characteristics of hepatic blood flow and pathophysiology of pseudolesions that can occur due to alterations in intrahepatic hemodynamics. Understanding HABR, a unique mechanism for regulating hepatic

blood flow, might be essential for elucidating the pathogenesis of AP shunting and THAD on dynamic contrast-enhanced imaging of the liver. In addition, some pseudolesions are associated with histopathologic changes such as focal hyperplasia, focal fatty liver, and focal sparing of fatty liver. Understanding these phenomena may aid in interpreting liver imaging findings.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 14; 27(46): 7909-7924

DOI: 10.3748/wjg.v27.i46.7909

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

REVIEW

Enteric nervous system and inflammatory bowel diseases: Correlated impacts and therapeutic approaches through the P2X7 receptor

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Author contributions: Magalhães HIR was responsible for the literature review and analysis and wrote the review; Castelucci P performed the critical interpretation and revised the manuscript for intellectual content.

Conflict-of-interest statement: The authors declare no conflicts of interest for this article.

Supported by the São Paulo Research (FAPESP, Brazil), No. 2014/25927-2 and No. 2018/07862-1; and the National Council for Scientific and Technological Development (CNPq, Brazil).

Country/Territory of origin: Brazil

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): A

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Abstract

The enteric nervous system (ENS) consists of thousands of small ganglia arranged in the submucosal and myenteric plexuses, which can be negatively affected by Crohn's disease and ulcerative colitis - inflammatory bowel diseases (IBDs). IBDs are complex and multifactorial disorders characterized by chronic and recurrent inflammation of the intestine, and the symptoms of IBDs may include abdominal pain, diarrhea, rectal bleeding, and weight loss. The P2X7 receptor has become a promising therapeutic target for IBDs, especially owing to its wide expression and, in the case of other purinergic receptors, in both human and model animal enteric cells. However, little is known about the actual involvement between the activation of the P2X7 receptor and the cascade of subsequent events and how all these activities associated with chemical signals interfere with the functionality of the affected or treated intestine. In this review, an integrated view is provided, correlating the structural organization of the ENS and the effects of IBDs, focusing on cellular constituents and how therapeutic approaches through the P2X7 receptor can assist in both protection from damage and tissue preservation.

Key Words: Chemical coding; Enteric nervous system; Gastroenterology; Inflammatory bowel diseases; P2X7 receptor; Purinergic signaling

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Core Tip: This review summarizes the impacts caused by inflammatory bowel diseases

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

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Received: July 10, 2021
Peer-review started: July 10, 2021
First decision: August 19, 2021
Revised: August 19, 2021
Accepted: November 25, 2021
Article in press: November 25, 2021
Published online: December 14, 2021

P-Reviewer: Xu B
S-Editor: Wang LL
L-Editor: A
P-Editor: Wang LL

on enteric nervous system cells and brings together the findings of the most recent literature on therapeutic approaches through the P2X7 receptor. Despite the great advancement of knowledge in the field, data on the mechanisms and effects of neuronal loss during colitis are still scarce. Furthermore, clinical trials that would make the use of P2X7 receptor antagonists in human patients feasible are lacking. In the laboratory, the results of animal models reinforce that the P2X7 receptor may be an important future target for the treatment of intestinal disorders.

Citation: Magalhães HIR, Castelucci P. Enteric nervous system and inflammatory bowel diseases: Correlated impacts and therapeutic approaches through the P2X7 receptor. *World J Gastroenterol* 2021; 27(46): 7909-7924
URL: https://www.wjgnet.com/1007-9327/full/v27/i46/7909.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i46.7909

INTRODUCTION

The gastrointestinal (GI) tract is a set of organs responsible for performing several complex functions that are essential for an individual's survival, including mainly food transportation, the digestion and absorption of nutrients, and the secretion of water, electrolytes, and mucus[1]. In the GI tract, there is an extensive intrinsic nervous system responsible for the control and coordination of local motility, the movement of fluids through the mucous epithelium, changes in blood flow, and interactions with the immune system[2]. Sometimes, this influence continues even if there is complete separation of the GI tract from the central nervous system (CNS)[2,3].

The enteric nervous system (ENS) is composed of thousands of small ganglia interconnected by their neural fibers and is arranged in two plexuses. The myenteric plexus is located between the fibers of the muscular layer throughout the GI tract, and the submucosal plexus is located in the submucosal layer of the small and large intestines[2,4,5]. Thus, the ENS shares many synaptic and ultrastructural characteristics of the neuronal interrelationship of the GI tract and the CNS[6], with many similarities demonstrated between them, which are reflected in neurological diseases [7]. Enteric innervation has been widely studied, and when preserved and functionally active, enteric innervation is considered equally essential to life as CNS innervation[8].

The study of the ENS has progressed from a healthy context to several pathological models, identifying neuroplastic changes that possibly contribute to modifying intestinal and perception functions in GI disorders[9]. It has been found that purinergic neurotransmission also plays a fundamental role in preserving the internal balance of these organs[10], interacting directly with motor and secretory functions[11] by the expression of several of its receptors on neurons located in the ENS[12]. In addition, the purinergic signaling pathway has also been widely recognized as a fundamental component in the course of inflammation during intestinal diseases[10,13,14].

In this context, the P2X7 receptor appeared to be one of the most correlated representatives in studies of infectious and inflammatory diseases[15]. The most striking differences in the P2X7 receptor in comparison to other purinergic receptors arise not only from its structural conformation but also from a sensitivity that is 10 to 100 times lower for its functional activation, suggesting it as a "danger" detector for tissue damage[16]. Therefore, a better understanding of the behavior of the P2X7 receptor and how it could be affected or modulated in some specific cases is sought, for example, in the treatment of Crohn's disease and ulcerative colitis - inflammatory bowel diseases (IBDs) that cause neuronal death in the ENS and compromise the functionality of the affected organs[17-19].

The great impact of Crohn's disease and ulcerative colitis is that both are capable of influencing all areas of patients' lives, from school and work to social and family life, affecting patients' productivity in each area[20]. In addition, when these conditions are poorly controlled, they can have negative effects on psychosocial well-being[21], increasing even the rates of anxiety and depression according to the severity of the conditions[22]. Worryingly, the occurrence of IBDs cases worldwide increased from 3.7 million to over 6.8 million between 1990 and 2017[23], which makes an individual approach with strong multidisciplinary care increasingly important, as this type of

approach could offer a higher quality of life even for individuals of different ages[20].

Thus, this review aimed to provide an integrated view of the structural organization of the ENS and the deleterious effects arising from IBDs, focusing on the cellular constituents and how therapeutic approaches through the P2X7 receptor can assist in both protection from damage and tissue preservation.

THE ENTERIC NERVOUS SYSTEM

The ENS, also known as the "second brain"[6,24], acts in an essential way in the motility of the esophagus, stomach, and small and large intestines[4,6], modulating the different contraction types of each organ[25]. In addition, the functions of endocrine and exocrine secretion, control of local blood flow, and regulation of inflammatory and immune processes are also related to ENS function[26].

The enteric neural circuit is organized as an interconnected network of enteric neurons and glial cells[4] throughout the entire GI tract and bile and pancreatic ducts [27]. The enteric neural circuit is arranged in two plexuses: the submucosal plexus, which in large mammals is present in two individualized levels (outer/inner) and is located in the outer connective tissue layer and the inner mucosal layer, and the myenteric plexus, located between the longitudinal and circular muscle layers[2,4,5, 28].

Within this complex innervation system, in humans, there are approximately 400 to 600 million neurons[5] grouped into several ganglia that connect[2] through the primary interganglionic tracts, which characterize the primary plexus[4,5]. The secondary and tertiary plexuses are also present in the myenteric plexus, represented by thinner filaments that are arranged parallel to the fibers of the circular musculature [29] and by even thinner filaments that branch among the constituents of the primary plexus[30]. This extensive neuronal network ends up projecting itself toward various effector structures, such as muscular and immune cells and blood vessels[27].

As proposed by Aleksandr S. Dogiel in 1899, the morphological classification of enteric neurons can be based on their conformation and dendritic distribution. Dogiel described type I cells as flattened, slightly elongated, with an angled or star-shaped contour, and, as remarkable characteristics, as having only one axon and four to 20 Lamellar dendrites that frequently extend at a short distance from the cell body[31].

Type II neurons have large round or oval cell bodies and eccentric nuclei[31], and the surface is grooved by bundles of neural fibers[32]. The main characteristic of type II neurons is the presence of several axonal processes that are emitted either directly from the cell body (multipolar neuron) or from a single initial process that branches into short subsidiary axons (pseudounipolar neurons)[4,33]. Such structures run toward the mucosa[34] and sometimes also provide collateral innervation to the submucosal ganglia[35].

Additionally, enteric neurons can also be identified as intrinsic primary afferent neurons (IPANs), interneurons, and motor neurons[4], classified into at least 18 subtypes and using more than 30 neurotransmitters in their synapses[28,30]. Of these neurotransmitters, acetylcholine (ACh) and nitric oxide (NO) stand out as the most abundant[27], as well as adenosine-5′-triphosphate (ATP)[26], vasoactive intestinal polypeptide (VIP), and substance P (SP)[36]. It is not rare that the same chemical compound stimulates neurons that perform distinct functions[26].

IPANs (classified as Dogiel type II) are recognized for responding to chemical stimuli, mucosal deformation and GI muscle tension, translating these signals into a neural impulse that will trigger a local motor reflex[37]. Altogether, IPANs represent approximately 14% and 30% of the neurons of the submucosal and myenteric plexuses, respectively. IPANs often project to form synapses with myenteric interneurons, motor neurons of the longitudinal and circular muscles[38], and with other IPANs[4].

The interneurons of the ENS (classified as Dogiel type I) are interposed with the IPANs and motor neurons[26], acting as mediators that are activated by the first neuron after a stimulus is received in the mucosa[27,39,40]. Thus, four neuronal types have been reported: one ascending (5%)[38], related to the pathways of the propulsive reflexes[41]; and three descending[38], related to local motility reflexes (5%), the conduction of the migratory myoelectric complex in the small intestine (4%), and secretomotor reflexes (2%)[4,30]. The interconnection of motor, secretory, and vasomotor pathways was suggested on the basis of the double projection of some of these neural fibers in both the submucosal and myenteric plexuses[38].

Motor neurons (classified as Dogiel type I) mark direct connections with muscle cells and, according to their neurotransmitter, can be classified as excitatory by

acetylcholine transferase (ChAT) labeling or as inhibitory by neuronal nitric oxide synthase (nNOS) labeling[4,5,36]. In addition, Furness *et al*[30] classified motor neurons as secretomotor/vasodilator neurons (60%), secretomotor neurons that are not vasodilators (29%), and neurons that innervate only enteroendocrine cells. On the basis of distribution analysis, it is already known that this neuronal class is also present in both enteric plexuses[2].

In summary, neurons of the submucosal plexus innervate the mucosal epithelium and submucosal arterioles to control and maintain water and electrolyte balance, luminal secretion and vascular tone, whereas the myenteric plexus promotes motor innervation of both layers of the muscle region[5], controlling the reflex pathways of the motor complex[42]. However, it is worth noting that the former is present only in the small and large intestines, whereas the latter is found continuously from the initial esophageal region to the internal anal sphincter[4].

The great difference in ENS innervation is that because the enteric ganglia possess all the necessary components to generate and complete a complex reflex circuit (IPANs, interneurons, and motor neurons)[28,43], the ENS has the capacity to regulate GI functions even in the absence of extrinsic neural connections[43]. Therefore, several authors have confirmed that ENS action can occur independently of the CNS[4,24,26,36,44,45], even though the latter often initiates or modulates some of the actions of the ENS[18,24,26].

However, according to Furness[5] and Furness *et al*[2], this autonomy does not actually occur. There are dependencies through interactions between local enteric reflexes, reflexes that pass through sympathetic ganglia, and reflexes that pass in return to the CNS[2,5]. Conveniently, these connections can be classified as vagal and thoracolumbar spinal, being represented by pre-enteric neurons that terminate inside the enteric ganglia, controlling and modifying the activities of neurons present there, or even by direct innervation of effector regions, *e.g.*, the striated skeletal muscles of the esophagus and the sphincters of the GI tract[2].

All this structural and functional complexity characterizes the ENS as the largest and most varied division of the peripheral nervous system[46], leading initially John N. Langley[47] to recognize the ENS no longer as a distribution of parasympathetic postganglia but rather as a distinct segment of the autonomic nervous system that, due to its prominence, should stand alongside the sympathetic and parasympathetic divisions.

INFLAMMATORY BOWEL DISEASES AND THEIR IMPACTS ON THE ENTERIC NERVOUS SYSTEM

IBDs, classically subdivided into Crohn's disease and ulcerative colitis[48,49], are complex and multifactorial disorders characterized by chronic and recurrent inflammation of the intestine[50,51]. Usually, debilitating[48], these disorders reach their peak onset in patients between the ages of 15 and 30 years[52], who, on a purely individual basis, may alternate between periods of symptomatic flares and clinical remission[49].

Although the etiology of IBDs is not yet fully understood[53,54], a growing body of evidence has suggested that the occurrence of IBDs is related to genetic predispositions[55,56] and aberrant immune responses in the face of various environmental triggers[56,57], including antigens from the gut microbiota[56,58,59], poor dietary habits, and high antibiotic consumption in childhood and adolescence[57,60]. Worryingly, an increase in both the incidence and prevalence of IBDs has been reported worldwide[23,52,61,62], but this increase is even more pronounced in newly industrialized countries with more westernized societies[63,64].

Commonly, the symptoms of IBDs may include abdominal pain, diarrhea, rectal bleeding, and weight loss. Ulcerative colitis primarily affects the rectum and is limited to the superficial part of the large intestine mucosa[48], and Crohn's disease is manifested by transmural lesions that may extend from the mouth to the anus, promoting possibly irreversible damage[65]. Sometimes the appearance of and gradual increase in intestinal ulcers associated with cumulative destructive effects can cause stenosis, fistulas, and colorectal cancer[66-68]. Therefore, it is clear that IBDs have an expressive influence on the quality of daily life in these patients[20,21].

In this sense, several efforts are being made to more closely mimic these diseases in the laboratory through the use of animal models, either to understand the relationship between their pathophysiological components or to identify the mechanisms and drugs that mitigate the symptomatology[69]. For this, two main substances have been

used quite satisfactorily for colitis induction: dextran sulfate sodium (DSS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS). DSS is a soluble polysaccharide supplied in drinking water and chemically interferes with gut mucosal barrier integrity, allowing the dissemination of luminal antigens into underlying tissue. TNBS is a reagent administered rectally in combination with ethanol that disrupts the mucosal barrier, allowing TNBS to induce colitis by haptenating colonic proteins, causing them to become preferential targets for immune cells. In both cases, the onset of acute or chronic lesions is dependent on the concentration and/or the frequency of the administration of each substance[69-73].

Specific to the ENS, reports have pointed out that intestinal inflammation can cause functional and structural changes in neurons[74-76] and necrosis, apoptosis and degeneration in enteric ganglia[17,18,77]. In fact, different authors have already demonstrated important variations in the cell number and neuronal profile of inflamed areas when compared to healthy tissues (Tables 1 and 2). In addition, damage to intestinal innervation during the inflammatory course may cause organ functional losses through modifications in motility patterns, increased excitability with changes in synaptic transmission in neural microcircuits, inadequate secretory responses of the epithelium to incoming stimuli[18,78], and enteric cell death from dependence on multiple caspases[19,79,80]. Despite this, little is still known about the mechanisms behind the loss of enteric innervation linked to IBDs[76].

In view of the therapeutic management of IBDs, the introduction of anti-TNF agents has positively marked this path[81-83], especially as they favor the healing of the mucosal layer with increases in its growth with stimulation[84], and as they demonstrate a greater safety of use when compared to conventional protocols[81,82]. In this same context, the P2X7 receptor is also emerging as a very important medical target for the prevention and treatment of these disorders[10], possibly in a similar way to the above, since its continuous activation may worsen the local inflammatory response[85,86]. However, little is known about the real involvement between the activation of this purinergic receptor and the subsequent cascade of events and how all these activities associated with chemical signaling interfere with the functionality of the affected or treated intestine.

THE PURINERGIC RECEPTORS

ATP is the central nucleotide of body metabolism[87], one of the most abundant molecules in living cells[88], and despite being recognized as an energy substrate[87], ATP also acts systemically in conjunction with adenosine and adenosine diphosphate (ADP). As an example, ATP presents actions in the control of vascular tone and remodeling[89,90] and in growth, differentiation[91], and cell communication[87,88,92,93].

Initially recognized for its fundamental role in several intracellular biochemical processes, the function of ATP as a neurotransmitter was greatly questioned when proposed by Geoffrey Burnstock in 1972[94]. In any case, the discovery of purinergic neurons - as they were named in reference to their relation with purine nucleotides[95] - answered the questions generated about the existence of neurons that are neither cholinergic nor adrenergic[36], and a high level of evidence has been reached on purinergic neurons in the scope of physiological and pathophysiological scientific research[92].

According to Burnstock[96], the presence of purinergic receptors was implicit in the hypothesis of this class of neurotransmission, and these receptors were classified into two types: P1 by the use of adenosine and P2 by the use of ATP and ADP. However, only in 1985 was it proposed on pharmacological grounds that this second type could be further subdivided into two other larger families[97]: P2Y, coupled with G-protein; and P2X, coupled with ion channel-dependent ligands[98]. Four subforms are currently recognized for P1 receptors (A1, A2a, A2b, and A3)[99], eight for P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14)[100], and seven for P2X receptors (P2X1-7)[101,102], making it plausible that purinergic receptors are the most abundant in mammalian tissues[103], found even in cells of neural origin[13,93,103-107].

In the ENS, the presence of purinergic receptors has been widely recognized in enteric neurons and glial cells of humans and other animal species[13,14,108]. In the guinea pig, the P2Y1 receptor has already been identified in the submucosal plexus of the ileum[109], and the P2Y2, P2Y6, P2Y12, P2X2, and P2X3 receptors have been identified in the submucosal and myenteric plexuses of the stomach, jejunum, ileum, and distal colon[110-114].

Table 1 Specific variations in cell number and neuronal profile area according to the respective chemical code observed in the submucosal plexus of the enteric nervous system

Ref.	Species	Colitis	Time	Submucosal plexus	
				Cellular chemical code (change in density compared to healthy tissues, %)	Cellular chemical code (change in profile area compared to healthy tissues, %)
Schneider *et al* [169]	Human	Crohn's disease	6.1±6,3 years	ChAT, nNOS, SP, and NSE (similar to); VIP (> 16%-CT)	N/A
Sigalet *et al*[170]	Rat	TNBS-50% ethanol	5 d	PGP9.5 (<)[1]; VIP (<)[1]; S100 β (<)[1]	N/A
da Silva *et al*[130]	Rat	TNBS-30% ethanol	24 h	P2X7 (< 21%-CT; < 13%-sham) ; Calret (< 11.7%-CT; < 8%-sham); Calb (< 34%-CT; < 30%-sham); HuC/D (< 33.4%-CT; < 28%sham); S100β (< 44.2%-CT; < 33%-sham)	Calbindin (< 25%-CT/sham)

[1]Count change without percentage information.

TNBS: 2,4,6-trinitrobenzenesulfonic acid; N/A: Not applicable; (<): Cell count/area decreased; (>): Cell count/area increased; ir: immunoreactive; CT: Control group; Sham: Sham group; P2X7: P2X7 receptor; ChAT: Acetylcholine transferase enzyme-ir; nNOS: Neuronal nitric oxide synthase enzyme-ir; Calret: Calretinin-ir; Calb: Calbindin-ir; SP: Substance P-ir; VIP: Vasoactive intestinal polypeptide-ir; HuC/D and PGP9. 5: Pan neuronal-ir; NSE: Neuron-specific enolase-ir; S100β: Protein β for calcium S100-ir labeling.

In mouse studies, P2X2, P2X3, and P2X5 receptors were identified in the submucosal and myenteric plexuses of the stomach, jejunum, ileum, and colon[11,115-117]. In rats, P2X2 and P2X3 receptors have been demonstrated in the submucosal and myenteric plexuses from the stomach to the large intestine and rectum[118-123], and P2X6 receptors have been demonstrated in the submucosal plexuses of the jejunum, ileum, and proximal and distal colon and in the myenteric plexuses of the stomach, ileum, and proximal and distal colon[124].

Specifically, the P2X7 receptor has also been visualized in the submucosal and myenteric plexuses of the colon of humans[19] and in the submucosal plexus of the ileum and the myenteric plexus of the stomach and small and large intestines of guinea pigs[125]. In mice, the presence of the P2X7 receptor was identified in the myenteric plexus of the colon[19] and in rats in the submucosal and myenteric plexuses of the esophagus, stomach, jejunum, ileum, large intestine, and distal colon [121,126-133]. Similar to the other purinergic receptors, the P2X7 receptor also presents a wide range of distributions in relation to enteric neurons with different chemical codes that integrate the ENS (Table 3).

The P2X7 receptor

The P2X7 receptor is a trimeric complex that typically contains 595 amino acids (594 in guinea pigs)[134,135]. The P2X7 receptor consists of two transmembrane domains (TM1 and TM2) linked by a large extracellular loop and by two intracellular domains known as the N-terminus and C-terminus[134,136]. The loop acts as a site for transition metal binding and assists in the activation of this receptor *via* ATP[136], allowing the channel formed by TM1 and TM2[86,135,137] to regulate the passage of calcium, sodium, and potassium[13,93,138]. The domains inside the cell modulate the functions and determine the kinetics of the depolarization and expansion of this channel[139]. It is worth noting that in the P2X7 receptor, the intracellular C-terminus is significantly longer than that in the other P2X receptors[134,136].

As another striking feature, the P2X7 receptor also demands higher concentrations of extracellular ATP for its activation than other purinergic receptors do[101], and this is a possible tissue "danger" sensor[101,140]. In response to inflammation[14,128], trauma or injury[91,141], the elevation of ATP causes a prolonged stimulus that induces the transition of the ion channel to a nonselective membrane pore[101,142,143], making the cell permeable to molecules up to 900 daltons[94,101,142,143]. In association, massive calcium influx[144] can contribute to cell death[85,137,145], with subsequent release of greater amounts of ATP[146-148].

Thus, in addition to its already recognized role in neurotransmission[141], the P2X7 receptor is also closely related to most diseases of the body[140], acting in multiple inflammatory processes[85,99149,150], immune responses[10,85,86,99,149,151], metabolism and cell proliferation[149]. The P2X7 receptor may also be responsible for

Table 2 Specific variations in cell number and neuronal profile area according to the respective chemical code observed in the myenteric plexus of the enteric nervous system

Ref.	Species	Colitis	Time	Myenteric plexus	
				Cellular chemical code (change in density compared to healthy tissues, %)	Cellular chemical code (change in profile area compared to healthy tissues, %)
Boyer *et al*[79]	Mice	DNBS-50% ethanol	0.5 - 120 h	HuC/D (< 42%-CT)	N/A
Linden *et al*[17]	Guinea pig	TNBS-30% ethanol	2 - 12 h;1-56 d	HuC/D 12 and 24 h (< 15%-CT); HuC/D 6 and 56 d (< 20%-CT); ChAT, nNOS, calret and NeuN 6 d (=); VIP 6 (>)[1] and 56 d (No differences)	N/A
Sarnelli *et al*[171]	Rat	TNBS-50% ethanol	7 d	HuC/D (< 20%-CT)	N/A
Gulbransen *et al* [19]	Mice	DNBS-50% ethanol	48 h	HuC/D (< 32%-CT)	N/A
Linden[77]	Guinea pig	TNBS-30% ethanol	24 h	HuC/D (< approximately 20%-25%-CT)	N/A
Da Silva *et al*[129]	Rat	TNBS-30% ethanol	24 h	P2X7 (< 11%-CT); ChAT (< 34.9%-CT); nNOS (< 42.3%-CT; < 18%-sham); Calret (< 60.6%-CT; < 15%-sham); Calbindin (< 22.9%-CT); HuC/D (< 33.3%-CT; < 16%-sham); S100β (< 29.2%-CT; < 23%-sham)	nNOS (< 6.6%-CT/sham); ChAT (< 21.2%-CT/sham); Calbindin (>19%-CT); Calretinin (< 2%-sham)
Souza *et al*[133],[2]	Rat	TNBS-30% ethanol	24 h	P2X7 (< 10.6%-sham; < 20.4%-BBG); ChAT (< 34%-sham; < 13.9%-BBG); nNOS (< 22.9%-sham; < 22.2%-BBG); HuC/D (< 15.4%-sham; < 19.5%-BBG); GFAP (< 14.4%-sham; < 17.7%-BBG)	nNOS (< 12%-sham; < 8%-BBG); ChAT and HuC/D (No differences)

[1]Change in count without percentage information.

[2]Data from ileum after colitis. DNBS: Dinitrobenzene sulfonic acid; TNBS: 2,4,6-trinitrobenzenesulfonic acid; N/A: Not applicable; (=): Similarity of cell count; (<): Cell count/area decrease; (>): Cell count/area increase; ir: Immunoreactive; CT: Control group; Sham: Sham group; BBG: Brilliant Blue G-treated animals group; P2X7: P2X7 receptor; ChAT: Acetylcholine transferase enzyme-ir; nNOS: Neuronal nitric oxide synthase enzyme-ir; Calret: Calretinin-ir; VIP: Vasoactive intestinal polypeptide-ir; HuC/D: Neuronal pan-ir; NeuN: Neuronal nuclear antigen-ir; GFAP: Glial fibrillary acid protein-ir; S100β: Protein β for calcium S100-ir labeling.

triggering the stimulation of necrosis and apoptosis after neurological injuries[85,152,153].

Most of the studies involving the ENS have demonstrated a decrease in the number of cells that are immunoreactive to the P2X7 receptor in the submucosal and myenteric plexus following ischemia/reperfusion in the ilea of rats [127,131,132] and intestinal inflammation in rats[128-130,133], mice, and humans[19]. Moreover, the alteration of these same neurons was observed in the ENS of the large intestine of rats subjected to undernourishment protein and renutrition[121].

Antonioli *et al*[128] also observed a higher intensity of immunofluorescence labeling of these cells in the myenteric ganglia of the distal colon of rats with experimentally induced colitis. These findings may reflect higher activation of the P2X7 receptor in the epithelium and lamina propria of the colon in response to inflammation[154] and in human patients with Crohn's disease or ulcerative colitis[155]. Moreover, it has already been shown that the P2X7 receptor also acts in regulating the activation of NF-κB[148,154] and in the release of proinflammatory cytokines (IL-1β, IL-6, IL-18, and TNF)[148,154,156]. In addition, higher colocalization rates between the P2X7 receptor and dendritic cells, T cells, and macrophages in the epithelium and lamina propria of the inflamed colon in humans have also been reported[155].

Thus, it is highlighted that the P2X7 receptor can promote the occurrence and progression of IBDs, altering the local biological behavior[10] and acting as a key factor in the pathogenesis of ulcerative colitis and Crohn's disease[19,154,157], sometimes even being responsible for neuronal loss[19,158]. Soon, effective pharmacological blockade of this receptor will emerge as a new target in the treatment of inflammatory conditions[99].

Table 3 Specific distribution of the P2X7 receptor in relation to cells with different chemical code that integrate the enteric nervous system

Ref.	Species	Tissue	Chemical cellular code (p2x7 receptor expression, %)	
			Submucosal plexus	Myenteric plexus
Hu *et al*[125]	Guinea pig	Ileum	ChAT, calret, NPY and SP	nNos, calret, calb, NPY, SP, and HuC/D
Hu *et al*[125]	Guinea pig	Stomach and intestines	N/A	nNos, calret, calb, NPY, SP, and HuC/D
Vanderwinden *et al*[126]	Rat	Stomach, jejunum, and colon	S100β	S100β
Gulbransen *et al*[19]	Human and mice	Colon	+	+
Girotti *et al*[121]	Rat	Large intestine	P2X7 in 100% of ChAT, calret, and calb; ChAT (22.5%), calret (35%) and calb (12.7%)	P2X7 in 100% of ChAT, nNOS, calret, and calb; ChAT (12.7%), nNOS (35.7%), calret (17.6%) and calb (8.3%)
Palombit *et al*[127]	Rat	Ileum	N/A	P2X7 in 100% of ChAT, nNOS, calret, and calb; ChAT (42.2%), nNOS (24.5%), calret (33.5%), and calb (10.7%)
Antonioli *et al*[128]	Rat	Distal colon	N/A	P2X7 in 100% of HuC/D
Da Silva *et al*[129]	Rat	Distal colon	N/A	P2X7 in 100% of ChAT, nNOS, calret, calb, and S100β
Da Silva *et al*[130]	Rat	Distal colon	P2X7 in 100% of calret, calb, HuC/D, and S100β	N/A

(+): P2X7 receptor positivity without cellular chemical code information; N/A: Not applicable; ir: Immunoreactive; ChAT: Acetylcholine transferase enzyme-ir; nNOS: Neuronal nitric oxide synthase enzyme-ir; Calret: Calretinin-ir; Calb: Calbindin-ir; NPY: Neuropeptide Y-ir; SP: Substance P-ir; HuC/D: Neuronal pan-ir; NF200: Neurofilament 200-ir; GFAP: Glial fibrillary acid protein-ir; S100β: Protein β for calcium S100-ir labeling.

THERAPEUTIC APPROACHES TO THE TREATMENT OF INFLAMMATORY BOWEL DISEASES THROUGH THE P2X7 RECEPTOR

Positive results from the use of P2X7 receptor antagonists have already been demonstrated in the treatment of ischiatic nerve lesions in mice[159], in brain infarction by middle cerebral artery occlusion in rats[160], and in ileal ischemia and reperfusion in rats[131]. During experimentally induced colitis, intraperitoneal application of Brilliant Blue G (BBG) significantly reduced weight loss in rats, the score of mucosal lesions observed through colonoscopy, the macro- and microscopic degrees of inflammation, the number of inflammatory cells, and the deposition of collagen fibers in this organ. Lower levels of P2X7 receptor expression in the epithelium and lamina propria and lower levels of cell apoptosis in the distal colon epithelium were also demonstrated by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. In addition, there was a stabilization of low concentrations of TNF-α, IL-1β and NF-κB, elementary members of this inflammatory process[154]. BBG was also effective in protecting intestinal regions distant from the inflammatory focus, as in the case of ileum in relation to distal colitis[56].

Moreover, in the context of colitis, various P2X7 receptor antagonists also slowed disease progression and reduced NF-κB activation, Caspase-1 expression, and concentrations of TNF and IL-1β in the mouse intestine[148]. Microscopic changes[148,154], changes in colonoscopy examination findings[154] and the loss of tight junctions due to inflammatory-cytokine-induced damage were also ameliorated[161]. In knockout (KO) mice, there was also an increase in specimen weight and reductions in histological lesions[155], with a greater preservation of the epithelial barrier, compared to wild-type (WT) animals[162]. Basically, there was no development of this disease in P2X7 receptor KO animals after the induction of inflammation[155,162].

Although all these therapeutic advances are exceptionally remarkable, only Eser *et al*[163] evaluated the use of some P2X7 receptor antagonists in humans with IBDs - a phase IIa study conducted specifically with patients in moderate to severe stages of Crohn's disease. According to the authors, the drug AZD9056 was well tolerated, and although it did not alter the concentrations of C-reactive protein or fecal calprotectin when compared to placebo, it caused a significant improvement in the Crohn's Disease

Activity Index (CDAI) and showing favorable effects on the remission of the disease and marked reduction in abdominal pain during the treatment period[163].

Taken together, this information reinforces the characterization of the P2X7 receptor as a promising target for the treatment of intestinal inflammatory conditions[14,127-133,148,154,155], especially in view of not only its wide expression in macrophages[10,164,165], mast cells[166] and T cells[10,162] but also its their strong involvement in the activation of caspases[167] and the release and regulation of transcription factors and pro-inflammatory cytokines[168,165].

CONCLUSION

It is concluded that IBDs are capable of aggressively and negatively affecting the cellular constituents of the ENS, and further studies are required in this area since knowledge in this area can still be considered, in a certain way, scarce. Studies of structural losses and/or structural deregulations in the enteric plexus may answer numerous questions about intestinal functionality, and therefore, the performance of these studies is of fundamental importance. Thus, it is also clear that the therapeutic approaches carried out through the P2X7 receptor have contributed to the advancement of this knowledge, but unfortunately: (1) We cannot fail to highlight that clinical trials with human patients are still lacking; (2) A better elucidation of the chemical signaling and functional regulation of immune cells upon the activation of this receptor is required; and (3) More quantitative studies on the structural components of the ENS involved in colitis and in its treatment are also required.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 14; 27(46): 7925-7942

DOI: 10.3748/wjg.v27.i46.7925

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

MINIREVIEWS

COVID-19 and gut immunomodulation

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Author contributions: Roy K and Agarwal S contributed equally; Roy K, Agarwal S, Banerjee R, Paul MK, and Purbey PK contributed to the draft preparation of the article; Paul MK and Purbey PK conceptualized and edited the article.

Conflict-of-interest statement: No Conflict-of-interest.

Country/Territory of origin: United States

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

Open-Access: This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external

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Abstract

The disease coronavirus disease 2019 (COVID-19) is a severe respiratory illness that has emerged as a devastating health problem worldwide. The disease outcome is heterogeneous, and severity is likely dependent on the immunity of infected individuals and comorbidities. Although symptoms of the disease are primarily associated with respiratory problems, additional infection or failure of other vital organs are being reported. Emerging reports suggest a quite common co-existence of gastrointestinal (GI) tract symptoms in addition to respiratory symptoms in many COVID-19 patients, and some patients show just the GI symptoms. The possible cause of the GI symptoms could be due to direct infection of the epithelial cells of the gut, which is supported by the fact that (1) The intestinal epithelium expresses a high level of angiotensin-converting enzyme-2 and transmembrane protease serine 2 protein that are required for the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) entry into the cells; (2) About half of the severe COVID-19 patients show viral RNA in their feces and various parts of the GI tract; and (3) SARS-CoV-2 can directly infect gut epithelial cells *in vitro* (gut epithelial cells and organoids) and *in vivo* (rhesus monkey). The GI tract seems to be a site of active innate and adaptive immune responses to SARS-CoV-2 as clinically, stool samples of COVID-19 patients possess proinflammatory cytokines (interleukin 8), calprotectin (neutrophils activity), and immunoglobulin A antibodies. In addition to direct immune activation by the virus,

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Received: February 18, 2021
Peer-review started: February 18, 2021
First decision: May 13, 2021
Revised: June 9, 2021
Accepted: November 29, 2021
Article in press: November 29, 2021
Published online: December 14, 2021

P-Reviewer: Atoum M, Fan Y, Fei C
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

impairment of GI epithelium integrity can evoke immune response under the influence of systemic cytokines, hypoxia, and changes in gut microbiota (dysbiosis) due to infection of the respiratory system, which is confirmed by the observation that not all of the GI symptomatic patients are viral RNA positive. This review comprehensively summarizes the possible GI immunomodulation by SARS-CoV-2 that could lead to GI symptoms, their association with disease severity, and potential therapeutic interventions.

Key Words: COVID-19; Gastrointestinal symptoms; Pathogenesis; Innate immune response; Adaptive immune response; Gut microbiota; Dysbiosis; Therapeutics; Probiotic; Pre-existing diseases

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Core Tip: Coronavirus disease 2019 (COVID-19) is a global pandemic. Many COVID-19 patients either present gastrointestinal (GI) symptoms in addition to respiratory symptoms or just GI symptoms. Syndrome coronavirus 2 (SARS-CoV-2) directly infects GI epithelial cells as they express significant levels of angiotensin-converting enzyme-2 and transmembrane protease serine 2 protein, required for SARS-CoV-2 entry. This article reviews gut infection and GI immunomodulation by SARS-CoV-2, leading to spectrum of GI symptoms and pathogenesis in COVID-19-patients. Special emphases are given on the innate and acquired immune responses in the GI tract due to intestinal and non-intestinal SARS-CoV-2 infection, COVID-19 severity in people with pre-existing intestinal diseases, role of gut microbiota, and possible therapeutic interventions are discussed.

Citation: Roy K, Agarwal S, Banerjee R, Paul MK, Purbey PK. COVID-19 and gut immunomodulation. *World J Gastroenterol* 2021; 27(46): 7925-7942
URL: https://www.wjgnet.com/1007-9327/full/v27/i46/7925.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i46.7925

INTRODUCTION

In December 2019, pneumonia cases with unrecognized etiology were reported in the Wuhan city of China, causing fever and acute respiratory distress. The causative agent is a novel coronavirus named syndrome coronavirus 2 (SARS-CoV-2), and the disease is referred to as coronavirus disease 2019 (COVID-19)[1,2]. SARS-CoV-2 belongs to the order "Nidovirales"; family of "Coronaviridae", and subfamily "Orthocoronavirinae" [3]. Coronaviruses are single-stranded RNA viruses, usually zoonotic but have regularly affected humans to cause a major health crisis[4,5]. COVID-19 rapidly spread like an epidemic in China, followed by worldwide transmission of the infection and, therefore, was declared a pandemic and global crisis by the World Health Organization (WHO) in March 2020. As of May 23, 2021, the WHO COVID-19 dashboard reported 166352007 confirmed cases of COVID-19, and 3449189 deaths worldwide, making this one of the worst pandemics in the 21st century. The high rate of human-to-human transmission, asymptomatic carriers, and the absence of therapeutic intervention led to the global pandemic.

The evolution of new variants of the virus has made the situation even worse. New SARS-CoV-2 strains are emerging like B.1.351 was detected in South Africa, B.1.207 in Nigeria, while strain B.1.1.7 was identified in the United Kingdom in December 2020 and is highly infectious. The new strains like B.1.1.7 strain harbor several mutations, especially in the S protein, including the N501Y (asparagine to tyrosine substitution), 69/70 deletion. P681H and enhances the virus-angiotensin-converting enzyme-2 (ACE-2) binding efficacy, thereby making the variants highly contagious. B.1.617.1, B.1.617.2, and B.1.617.3 are the three subtypes of the Indian variant reported in October 2020 is highly infectious and causing fresh waves of infection in many countries around the world. Three important mutations in the sequence coding for the viral spike protein co-occur in variant B.1.617.1: L452R, E484Q, and P681R. B.1.617.2 is also linked to the L452R, T478K, and P681R mutations[6]. Indications suggest that

these variants can trigger severe disease conditions or higher fatality rates. The complete impact of these mutations is not yet understood and is still being researched, but comprehensive genomic strain surveillance is needed to better understand the strain-specific infection, pathogenesis, epidemiological and therapeutic aspects. Several potential therapeutic and prophylactic interventions are under investigation or have undergone randomized controlled trials. Great strides have been made in vaccine development, and COVID-19 vaccines are now approved for mass use in several countries. Raising hopes for curbing the COVID-19 crisis and WHO's guidelines on wearing a mask, social distancing, and sanitization needs to be strictly followed to bend down the infection curve.

Though COVID-19 mainly causes respiratory illness, many patients experience gastrointestinal (GI) symptoms, including nausea, vomiting, belly pain, appetite loss, and diarrhea. GI symptoms are often associated with the presence of CoV2 RNA in many patients' stool (feces) samples[7]. Though the mechanism of lung infection is widely studied, there is a dearth of information regarding the enteric phase of SARS-CoV-2, especially the immune contexture and response. The gut microbiome is considered to play a key role in regulating the impact of SARS-CoV-2, and significant alterations in the microbiota profiles are reported in COVID-19 patients. The role of the gut-lung axis and the severe respiratory distress associated with gut imbalance is also very relevant[8]. COVID researchers have reported a disturbance of the gut microbiota and its association with lung and gut infections, which can cause hindrance in the gut-lung axis. Recent data suggest that GI symptoms might be a warning sign of a more serious condition with poor prognosis. Because of the GI infection and COVID severity, the present paper deals with a complete review of the COVID-19-associated gut-infection, pathogenesis, innate and acquired immune responses, gut microbiota, and possible therapeutic intervention. Figure 1, is a schematic showing SARS-CoV-2 infection and activation of cell death-associated release of damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), associated inflammation and host intracellular immune response.

COVID-19 PATHOGENESIS AND INFECTION PROCESS

Human-to-human transmission of COVID-19 can occur when the infectious respiratory droplets of patients are transmitted as droplets or aerosol that finally gets deposited into the nasal, oral, and conjunctival mucosa of an uninfected human being. SARS-CoV-2 prominently attacks the lungs and infects other organs such as the gut, heart, blood vessels, kidney, cortex, and central nervous system[9]. SARS-CoV-2 infects host cells when the viral spike (S) protein binds to the cell surface receptor ACE2. Thereby, ACE2 is the crucial cellular receptor for the entry of SARS-CoV-2[10]. Two functional domains are found in the S protein: A receptor-binding domain and a second domain with S1/S2 cleavage site containing multiple arginine residues that must be cleaved by cell proteases for cellular entry. The furin-mediated pre-cleavage of the S1/S2 site leads to further activation of viral fusion to the cells by transmembrane protease serine 2 protein (TMPRSS2)[11,12]. ACE2 receptors and TMPRSS2 are expressed in various human cells susceptible to viral infection, including epithelial cells in the lungs, small intestine, and colon, tubular cells of the kidney, neuronal and glial cells in the brain, enterocytes, vascular endothelial cells, smooth muscle cells and cardiomyocytes[13]. Viruses are shed in the feces long after the resolution of the pulmonary symptoms, making the fecal-oral route of SARS-CoV-2 transmission a possibility. Single-cell transcriptomics data suggest that the GI epithelium, especially the enterocytes lining of the ileum and colon, shows a higher frequency of coexpression of both the ACE2 and TMPRSS2 and therefore, are conducive for SARS-CoV-2 interaction and infection, which may explain the GI pathogenesis[14,15]. The viral entry is associated with the release of proinflammatory cytokines, immune cell infiltration, and overall immune activation leading to inflammation. The infection-associated GI-specific symptoms include anorexia, watery diarrhea, nausea and vomiting, and associated abdominal pain[14] (Figures 1 and 2).

COVID-19 AND GI SYMPTOMS

Similar to lung infection, the GI-infection by SARS-CoV-2 triggers an antiviral immune response characterized by the release of interferon (IFN), cytokines, and chemokines in the infected cells. Figure 2 presents a brief overview of the GI infection routes and

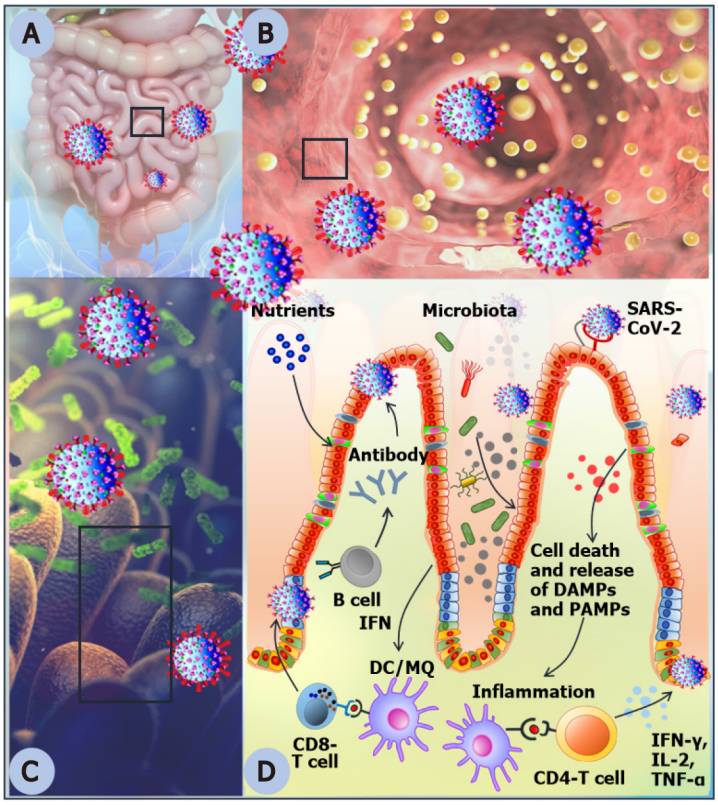


Figure 1 COVID-19 and gut Immunomodulation. A: Model is showing gut infection; B: Zoomed in area of the gut; C: Zoomed in representation of an area showing the intestinal crypts; D: Zoomed in C, showing a histological representation of intestinal crypts. The intestinal epithelium is folded and organized into crypts and villus. Villus is the finger-like projections gutting out towards the lumen of the intestine (red cells). The crypts base (shown in yellow and green cells) houses the intestinal stem cells, while the blue cells comprise the transit-amplifying cells. SARS-CoV-2 activates angiotensin-converting enzyme 2 receptors, and epithelial cell death-associated release of damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). DAMPs and PAMPs are considered a danger signal by immune cells, especially the dendritic cells, macrophages, and innate immune cells. This damage recognition is associated with proinflammatory cytokine production (like Interferon, tumor necrosis factor-α), followed by immune infiltration and virus-specific B and T cell response. CD8+ T cells undergo clonal expansion and kill the infected cells and launch an antiviral attack. B cells differentiation to plasma cells can lead to antiviral antibody production and subsequent neutralization of SARS-CoV-2[10,14]. Some images (Free Stock Media) are downloaded from Canva.com using subscription. IFN: Interferon; TNF-α: Tumor necrosis factor-α; DAMP: Damage-associated molecular patterns; PAMP: Pathogen-associated molecular patterns; DC: Dendritic cells; MQ: Macrophages.

symptoms. These inflammatory mediators promote infiltration of neutrophils, macrophages, and T cells to the site of infection, resulting in enteric inflammation that may lead to diarrhea and other GI symptoms[10]. Studies have shown that the elevated fecal levels of calprotectin (a marker protein expressed mainly by neutrophils) in patients with COVID-19 adds to the growing evidence that SARS-CoV-2 infection triggers an inflammatory response in the intestine. Calprotectin concentrations were found to be significantly higher in COVID-19 patients who had suffered from diarrhea along with elevated serum interleukin (IL)-6 levels[16]. An alternate mechanism implicated in GI symptoms in COVID-19 patients is oxygen deprivation [17]. Hypoxia is one of the major clinical symptoms in COVID-19 patients known to influence intestinal homeostasis, including microbiota composition and immune function. It is shown that oxygen deprivation (exacerbated hypoxia) can contribute to GI disorders and inflammatory disease severity[18].

The tissues that are targeted by SARS-CoV-2 go through an early phase of infection where a high viral load induces intestinal symptoms such as vomiting and diarrhea associated with COVID-19 during the initial phase in some patients. Thus, diarrhea should also generate awareness of a possible SARS-CoV-2 infection and should be investigated to reach an early diagnosis of COVID-19 to slow down its transmission instead of waiting for the respiratory symptoms to develop.

The first results linking GI symptoms with COVID-19 were obtained from a study conducted in COVID-19 confirmed patients in Wuhan, China[19]. In this study, 204 patients with COVID-19 who presented at three hospitals were analyzed. Although most patients presented with respiratory symptoms, many patients also presented with GI -specific symptoms. It is possible that GI symptoms associated with COVID-19 could be underreported due to the focus on fatal respiratory symptoms. However, a

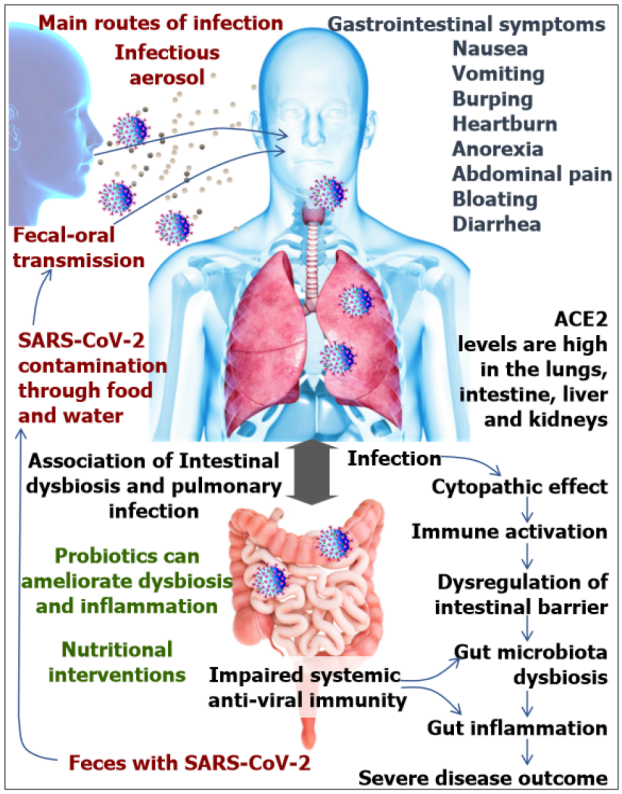


Figure 2 Illustrative model showing gastrointestinal infection routes and symptoms. The figure shows the main routes of infection. Infectious respiratory droplets or aerosols deposited on the nasal, oral, or conjunctiva may lead to person-to-person spread. SARS-CoV-2 is detected in feces of infected patients may infect by fecal-oral transmission. The right upper section of the figure also discusses the significant gastrointestinal symptoms associated with COVID-19 infection. The receptors (angiotensin-converting enzyme 2 and TMPRSS2) of SARS-CoV-2 are detected on various organs, especially the lungs, intestine, liver and kidneys. The right lower section describes the infection process leading to intestinal symptoms. After SARS-CoV-2 infection, cytopathic effect occurs due to infection and associated immune activation leading to compromised intestinal barrier function, microbial dysbiosis, and severe symptoms. Many studies have established the link between healthy intestinal flora and the gut-lung axis. COVID-19 severely induces the intestinal microbiota dysbiosis and affects the gut-lung axis, especially the immune response. Probiotics and appropriate nutritional supplements can help protect from SARS-CoV-2 associated symptoms[10,14]. Some images (Free Stock Media) are downloaded from Canva.com using subscription. ACE2: Angiotensin-converting enzyme 2.

study by Pan *et al*[20] reported that patients without GI symptoms were more likely to recover and be discharged than those with GI symptoms (60% *vs* 34%). This data indicates that GI symptoms like diarrhea may be associated with a worse outcome requiring respiratory assistance and intensive care admission. It was also found that patients with COVID-19, especially those with digestive symptoms, remained for a long time from the onset of symptoms to hospital admission with an average time of 9 d compared to patients with only respiratory symptoms who had an average admission time of 7.3 d[19]. This may indicate that those with digestive symptoms waited longer to be diagnosed in the hospital, as they were unsuspected of being SARS-CoV-2 positive in the absence of respiratory symptoms[21]. Besides, prolonged hospital stay could also be due to treatment time needed to resolve multiple symptoms in patients with GI and respiratory infections.

Wang *et al*[22] analyzed the biodistribution of SARS-CoV-2 in different tissues of patients with confirmed COVID-19[22]. In this study, SARS-CoV-2 was detected in multiple tissue specimens collected from 205 COVID-19 patients. Bronchoalveolar lavage fluid specimens showed the highest positive rates (14 of 15; 93%). However, the virus was also detected in feces suggesting that the infectious virions are secreted from the virus-infected GI cells. The virus has also been detected in GI histological samples and by endoscopy[23]. Therefore, the fecal-oral transmission could be a possible route for the viral spread. To further investigate the presence of SARS-CoV-2 in feces, Xiao *et al*[24] examined the viral RNA in feces from 73 patients with SARS-CoV-2 during their hospitalizations. Out of the 73 hospitalized patients infected with SARS-CoV-2, 39 (53.42%) tested positive for SARS-CoV-2 RNA in their stool. The study also found that 17 (23.29%) patients continued to have positive stool results after showing negative outcomes in the respiratory samples. Overall, these data suggest viral GI infection and a potential fecal-oral transmission that can last even after viral clearance in the respiratory tract, and also advocates implementing testing of the virus in feces by real-

time reverse transcription polymerase chain reaction for disease monitoring and surveillance.

IMMUNOMODULATION IN GI TRACT DUE TO INTESTINAL AND NON-INTESTINAL INFECTIONS

Clinical data suggest that co-infection of GI tract along with respiratory tract are quite prevalent[25]. Xiao *et al*[24] has reported the presence of replicating viruses in the epithelium of the GI tract[24], and the *in vitro* models of cell and organoid culture of human intestinal epithelial cells (hIECs) support efficient SARS-CoV-2 infection, replication and production of infectious de novo virus particles[25]. Intestinal viral load seems to show a stronger association with the severity of respiratory and GI symptoms in COVID-19 patients[26]. Recently, in a non-human primate (rhesus monkey) model of SARS-CoV-2 infection, *in vivo* infection of GI tract triggered reduced proliferation and increased apoptosis of intestinal epithelial and goblet cells along with intestinal inflammation by macrophages has been reported by performing immunohistochemistry for proliferation (Ki67), apoptosis (cleaved caspase 3), and recruited macrophages (CD68+), and multiplex cytokine assay of GI tract tissues[27]. These reports support immune modulation in the GI tract due to direct infection of GI tract cells by the virus or due to changes in the GI tract integrity and microbiota under the influence of systemic cytokines and hypoxic conditions or a combination of all. GI tract is a site of active immune reaction to generate tolerant immunity against various commensal pathogens and an effective immunity to fight the pathogenic infectious agents, such as bacteria, viruses, parasites, *etc.* Direct or indirect modulation in the GI tract's immune activation during SARS-CoV-2 infection seems a reason for observed GI symptoms in COVID-19 patients.

Innate immune response to SARS-CoV-2

The initial protection against pathogens is established by innate immunity. Although more studies are needed, it is reasonably convincing that intestinal epithelium gets infected and is associated with some sort of GI symptoms. Virally infected cells can recognize the virus and virus-associated molecular patterns to elicit initial innate immune pathways to release cytokines and chemokines to recruit body's innate-immune cells such as neutrophils, macrophages, *etc.* to the infected area of the gut, which further augments the inflammation in order to restrict the viral replication. This inflammatory response also promotes antigen processing and presentation to establish the adaptive immune response. However, some of the inflammatory cytokines are known to increase permeability of the intestinal lumen to the commensal microbes and may contribute to the onset of the GI-symptoms. The possible host immune responses during COVID-19 infection is discussed in this review.

Innate immune response mechanism to SARS-CoV-2: As explained in previous sections, it is evident that SARS-CoV-2 can infect various tissues of GI-tract followed by intestinal cell death, macrophage recruitment and release of various pro inflammatory cytokines to compromise the intestinal barrier[27]. Therefore, it is likely that SARS-CoV-2 infection of intestinal epithelial cells (IEcs) would trigger a coordinated innate immune response due to the recognition of SARS-CoV-2 associated molecular patterns (PAMPs), similar to that reported in the lung's epithelial cells[25,28]. The initial cytokine released by the infected cells can further recruit immune cells (neutrophils, macrophages, lymphocytes *etc.*), in the gut microenvironment to amplify the inflammatory response by recognition of PAMS and cytokines by their specialized receptors to restrict the virus propagation[29,30]. Notably, this early innate immune response is essential to facilitate the emergence of a more specific adaptive immune response by lymphocytes. The nature, timing and strength of innate and adaptive immune responses have been reported to be determining factors for the COVID-19 patient's symptoms[31]. Several components of inflammation exist but we have limited knowledge on the nature of inflammatory pathways triggered in the GI-tract by SARS-CoV-2.

One of the important components of inflammation is IFN response that includes large number of genes exerting antiviral effect. Recent report in a monkey model has shown many proinflammatory cytokines in the GI tract but they show no clear evidence on the IFN-I response genes and thus further omics studies may shed some lights in this regard[27]. It is apparent that asymptomatic and mild/moderate symptomatic patients develop a compelling early innate immune response to

successful viral clearance. While, patients with severe symptoms (especially the elderly and those with pre-existing health conditions) exhibit a dysfunctional early innate immune response against SARS-CoV-2 to allow the dissemination of infection leading to life-threatening complications[32,33]. In general, inadequate early innate immune response and failure to generate enough antiviral IFNs allow immune evasion, viral propagation, the spread of infection and subsequently cell death, and the release of PAMPs and DAMPs to cause cytokine storm. However, there is no strong correlation between viral load and severity of the disease highlighting the role of genetic or physiological state of the individual in developing the severe symptoms. Currently, we have little knowledge about the contribution of GI tract infection and inflammation towards cytokine storm and organ damage, which needs further exploration in the clinical and experimental setup. However, in the rhesus monkey model, it is evident that infection of GI-tract can contribute to systemic inflammation and inflammation to lungs[27].

IEcs and goblet cells undergo apoptosis[27]; however, other form of inflammatory cell death could be operational, which needs to be investigated in a preclinical and clinical setup as various types of cell death can occur due to the activation of innate immune recognition of PAMPs and DAMPs. The inflammatory cell death includes Pyroptosis, Apoptosis and Necroptosis, also termed as Panoptosis[30]. Pyroptosis is an inflammasome or Gasdermin mediated phenomena that involve caspase1, 4, and 5 activations (in humans) and gasdermin mediated pore formation and release of Il1b and IL-18. Recent data suggested a role of SARS-CoV-2 infection induced pyroptosis in peripheral blood mononuclear cells through NLRP3 (NLR family pyrin domain-containing 3) inflammasome activation, cleavage of caspase-1, and secretion of IL-1β and IL-18[34]. Necroptosis is a mixed-lineage kinase domain-like pseudokinase (MLKL)-mediated inflammatory cell death, during which oligomerized MLKL is translocated to form channels in the plasma membrane, which has been documented in SARS-CoV-2 infection[35]. Karki *et al*[35] have shown that a combination of just tumor necrosis factor (TNF)-α and IFN-γ can exert significant cell death in bone marrow-derived macrophages and their blockage can abrogate the cell death and severe symptoms in COVID-19 situation. We guess, possibly similar kind of cell death also operates in GI-tract as TNF-α and IFN-γ are induced in SARS-CoV-2 infected GI-tract[35]. Here, as part of an innate immune response, we elaborate on the evidence of IFN (IFN-I and IFN-III) and proinflammatory cytokines production in the context of human GI tract cells that may have consequences towards GI symptoms.

Induction of IFN and cytokines in the cells of the GI tract upon SARS-CoV-2 infection: To successfully combat and generate immune memory against SARS-CoV-2, the host' cells must generate an early innate immune response that includes the production of antiviral IFN and proinflammatory cytokines soon upon viral detection [36]. The severity of COVID-19 disease has been correlated with a defective or lower level of systemic IFN production but an elevated level of proinflammatory cytokines [37-39]. Since, infection of GI-tract contribute to the systemic cytokine pool[27], a detailed transcriptomic profile of GI-tract in non-human primate model can reveal some clues in future. In the *in vitro* models, similar to lung epithelial cells, the IEcs and intestinal organoids induce both type-I (IFN-I) and type-III IFN (IFN-III)[25,26,40]. Interestingly, SARS-CoV-2 induces stronger IFN-stimulated genes than SARS-CoV in the intestinal organoids, which seems similar to that observed in lung epithelial cells [26]. Analysis of feces of COVID-19 patients has revealed a significant association of elevated proinflammatory cytokine (IL-8) and lower level of anti-inflammatory cytokine (IL-10) in the COVID-19 patients as compared to healthy people, which indicate an inflammation/immune response in the intestine[26]. Post infection, expression of cytokines is evident in the time course analysis of 23 cytokines in the GI-tract of SARS-CoV-2 infected rhesus monkey[27]. Current studies analyzing the IEcs response upon SARS-CoV-2 infection have little data on IFN and ISG at later time points (24 h or longer). Also, analysis of cytokines in gut biopsy samples from various disease category may be useful. Future studies can be carried out to investigate other proinflammatory cytokines profiles that are usually observed in other viruses or bacterial infections. A comparative study would be necessary to dissect the molecular differences in response between IEcs and lung's epithelial cells. Whether intestinal inflammation contributes to the systemic cytokine pool (which seems convincing in rhesus monkey model), caused various types of cell death in intestinal epithelium and resident immune cells would be important aspects to explore.

Adaptive immune response to SARS-CoV-2 in the GI tract

The adaptive immune response mediated by B and T lymphocytes is usually

pathogen-specific and develops slowly relative to the innate immune response. B cells and T cells in the intestine are continually interacting with a vast amount of antigen-derived from diet and commensal microbes and maintain immune homeostasis. The interaction of gut-associated antigen and lymphocytes primarily happens in the gut-associated lymphoid tissues, including the Peyer's patches, isolated lymphoid follicles, and gut-draining mesenteric lymph nodes, leading to maturation and differentiation of lymphocytes[41]. We will discuss the potential adaptive immune response to SARS-CoV-2 infection.

Lymphopenia in COVID-19

Non-severe COVID patients show a near-normal number of circulating lymphocytes, while severe patients show a reduced number of circulating lymphocytes, a condition known as lymphopenia[42,43]. Whereas, a detailed analysis of lymphocyte subsets shows a significant reduction in T cells and NK cells, but without any alteration in B cell number in severe patients[22,42,43]. COVID-19 patients with pre-existing metabolic disease, like diabetes, show a higher proportion of severe infection[44], and lymphopenia has been linked to the severity of Crohn's disease[45]. Lymphopenia in severe COVID-19 patients may result from the synergistic effect of inflammation and metabolic disorder. The cellular mechanism of lymphopenia could be due to the following reasons and beyond. First, inhibition of lymphocyte proliferation in severe disease. Pre-existing metabolic disease, such as diabetes, enhances the propensity of the severity of COVID-19[46], and metabolic molecules, such as elevated blood lactic acid levels, may inhibit lymphocyte proliferation[47]. Second, higher lymphocyte death in severe disease. The potential mechanism of lymphocyte death could be due to metabolic disorder, inflammation, damage of lymphatic organs, and direct infection of lymphocytes[47]. Third, reduced lymphocyte production by skewed hematopoietic lineage cell fate decision. Metabolic disease (ulcerative colitis) and inflammation can skew hematopoietic fate decision towards the myelopoiesis with a concomitant decrease in lymphopoiesis[22,47,48]. Forth, infiltration of lymphocytes at the site of infection.

B cell and antibody-mediated immunity in COVID-19

In general, the intestine offers a model example of the diversity of antibody-secreting cells (ASCs) and comprises at least three subpopulations in humans[49]. Binding of antigen to antigen-specific B cells generates activated B cells that differentiates into ASCs with the help of T helper cells. It has been shown that 70% of non-severe COVID-19 patients have high and persistent SARS-CoV-2 neutralizing immunoglobulin (Ig)G in the sera after their recovery[50]. Antibody isotype analysis shows SARS-CoV-2 specific serum IgA and IgM in non-severe patients[51,52]. A longitudinal study in recovered patients showed that IgG antibodies are relatively stable up to 105 d post symptom onset while IgA and IgM antibodies rapidly decay[52]. Interestingly, an anti-SARS-CoV-2 antibody found in the mucosal fluid (saliva, nasal fluid, and tear fluid) and COVID-19 recovered patients have anti-SARS-CoV-2 specific IgG and IgA in saliva, indicating that antibody in the GI tract could be as crucial as antibodies in the serum for protective immunity[52-54]. ASCs in the intestine is a significant source of IgA producing cells in human. This indicates that the GI tract plays an essential role in generating anti-SARS-CoV-2 antibodies and protective immunity against SARS-CoV-2. The antibody can protect SARS-CoV-2 infection possibly by the following; antibody-mediated neutralization of the pathogen, phagocytosis of infected cells, and antibody-dependent cellular cytotoxicity. Patient-generated SARS-CoV-2-specific antibody can neutralize the virus SARS-CoV-2 in *ex-vivo* condition[53,55]. Furthermore, a mounting adaptive response in GI is supported by the prevalent presence of IgA in the stool of severe SARS-CoV-2 patients[26]. However, the importance and potency of antibody-mediated neutralization of SARS-CoV-2 *in vivo* require future studies in the model systems.

Longitudinal studies have shown that IgG and IgA levels to SARS-CoV-2 were significantly elevated as the disease progresses[52,56,57]. Anti-SARS-CoV-2 IgG was detected within the first week after onset of the symptoms in approximately 40% of patients. Within 15 d (late stage of infection), antibody levels increased by almost 100% of patients[57]. In general, severe patients showed a significantly higher IgG and IgA level compared to non-severe patients at late stages of infection. Surprisingly, the antibody level increases as the disease worsen in the severe group; on the contrary, the disease cured (patients recovered) in the non-severe group[51,56,57]. A few studies have shown that the severity of the disease positively correlated with an increased amount of IgG against S-protein and N-protein, especially in elderly patients[58].

Similarly, a very high level of anti-SARS-CoV-2 IgA correlated with severe acute respiratory distress syndrome[51]. Recent studies have shown that antibody levels (especially those of IgG and IgA) and B cell repertoire are highly dependent on the nature of microbiota in the gut[59,60]. Similarly, B cells and antibodies in the gut could affect the composition of the microbiota. B cell knock-out mice (a proxy for antibody deficiency) and AID deficiency mice (don't have secretory IgA in the gut) have reduced microbial diversity and alter the composition of gut microbiota[61]. Therefore, antibodies and microbiota have a feedback loop to maintain a healthy immune response. The current understanding is that people with dysbiosis (imbalance microbiota) have a prevalence of COVID-19[62]. Alternatively, it could be possible that high IgA levels in the severe patients who recovered have altered the composition of the microbiota and may have a long-term health effect. Figure 3 schematically represents the development of COVID-19 progress and its relationship to changes in the gut flora and disease progression.

T cell and cellular immunity in COVID-19

Cellular immunity is mediated by T cells, and microbiota profoundly affects T cell activation and differentiation, as observed in B cells. Dysbiosis can prompt multiple immune disorders mediated by T cells[63]. T cells have numerous subsets ($CD4^+$ T cells, $CD8^+$ T cells, and Treg) and distinct biological functions. $CD4^+$ T cells primarily regulate the function of other immune cells, $CD8^+$ T (cytotoxic T cells) cells can produce granzyme, and perforin results in the elimination of virus-infected cell, and regulatory T cell (Treg), which can restrain other activated T cells' function[64,65]. COVID-19 recovered patients have SARS-CoV-2 reactive IFN $^+$ T cells and granzyme B producing $CD8^+$ T cells[66,67]. The correlation of IFN $^+$ T cells and granzyme B producing $CD8^+$ T cells in recovered patients may indicate activated T cells mediated elimination of the virus-infected cells[54]. Interestingly, it has been observed that asymptomatic or mild SARS-CoV-2 infected patients have SARS-CoV-2 specific $CD4^+$ T cells, which could be due to cross-reactive $CD4^+$ T cell recognition between the common cold and SARS-CoV-2 coronaviruses[66,67].

The T cell functions are dysregulated in many severe patients[67,68]. The source of dysregulated T cell functions in severe disease could be due to the following reasons. First, it has been shown that both severe and non-severe patients have a comparable proportion of activated T cells suggesting functionality of activated T cells may be restrained by other immune cells, such as Treg, in non-severe patients[32,42]. In line with this, Qin *et al*[42] showed that severe patients have fewer Treg (specifically, induced Treg). Several studies have shown that GI tract dysbiosis can alter Treg/$CD4^+$ T cell axis and may have a pathogenic outcome[69]. The generation of fewer Treg in severe patients can be a synergistic effect of inflammation and mucosal microbiota imbalance. Second, T cells are exhausted in severe patients than non-severe COVID-19 patients[67,68]. Dysbiosis can promote T cells exhaustion[70]. So, it could be possible that T cell exhaustion in severe patients is a combined effect of hyper inflammation and imbalanced GI microbiota. However, we can't exclude other possibilities (such as bystander T cells) of dysregulated T cells in severe disease.

COVID-19 IN PEOPLE WITH PRE-EXISTING INTESTINAL DISEASES

Patients with chronic GI conditions may be at an increased risk of severe COVID-related illness, therefore management of these patients becomes important. Although the primary source of transmission for SARS-CoV-2 is respiratory droplets, there is increasing evidence supporting the possibility of a fecal-oral route of transmission. Patients with active ulcerative colitis and Crohn's disease have a greater tissue concentration of ACE2, increasing the possibility of an infection[71]. Additionally, the level of serine protease TMPRSS2, is about ten times higher in patients with inflammatory bowel disease (IBD) than in healthy subjects, suggesting an increased risk of infection in these patients[72]. Brenner *et al*[73] created the Surveillance Epidemiology of Coronavirus Under Research Exclusion for IBD (SECURE-IBD) database to identify potential IBD-associated COVID-19 risk factors. Out of 525 patients with IBD and COVID-19, severe infection (defined as intensive care unit admission, ventilator use, or death) was reported in seven percent of patients. Potential risk factors in these patients include increasing age, ≥ 2 comorbidities like diabetes mellitus, chronic inflammatory disease, and systemic glucocorticoids use, but not with anti-TNF therapy[73]. Using anti-TNF antibodies has been shown to reduce inflammatory cell death during experimental COVID-19 situation[35].

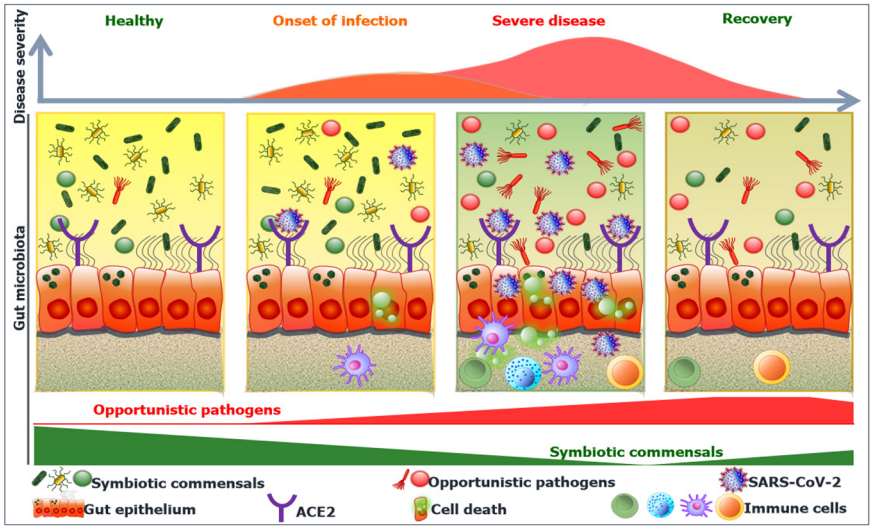


Figure 3 Schematic diagram showing COVID-19 disease progression and correlation with alterations with gut microbiota. The progression of gut microbiome alteration and its association with clinical symptoms and gut dysbiosis is evident. Cartoon inspired by[10,62,85]. ACE2: Angiotensin-converting enzyme 2.

The clinical presentation of several GI diseases (*e.g.*, Crohn's disease, ulcerative colitis) can mimic COVID-19 infection. Examples include diseases that manifest with diarrhea, nausea, vomiting, and/or anorexia. In a study by Mao *et al*[74], preliminary data have suggested that the prevalence of COVID-19 is not higher in IBD patients as compared to the general population[74]. Other studies have suggested that patients with IBD in remission are not at higher risk for SARS-CoV-2 virus infection and that such patients should continue maintenance therapy to sustain remission[74-76]. Digestive complications related to IBD relapse could be confused with symptoms of COVID infection and may skew the data for COVID symptoms in IBD patients. Discontinuing maintenance therapy for IBD has been associated with disease relapse and may lead to an increase of adverse outcomes such as hospitalizations, surgeries, and/or glucocorticoid therapy like prednisone that may increase the risk for severe COVID-19[76].

Patients with a flare of Crohn's disease/ulcerative colitis or active IBD, in the absence of COVID-19, may benefit from anti-inflammatory or biologic therapy to induce remission. Mild IBD therapeutic options include oral budesonide, aminosalicylates, and topical (rectal) therapy. While, the usual options for treating moderately to severely active IBD include biologic therapies (*e.g.*, anti-TNF agents, anti-integrin agents, and anti-interleukin agents)[76] are still viable. However, if systemic glucocorticoids are deemed necessary, the lowest dose of glucocorticoid with an appropriate clinical response is used for a short duration before transitioning to another therapy that is glucocorticoid-sparing[76]. Management of a patient hospitalized with severe ulcerative colitis in the absence of COVID-19 may include treatment with a glucocorticoid (like methylprednisolone) and in unresponsive cases medical therapy may be escalated to infliximab[76]. Surgery is an alternative option for patients who do not improve with medical therapy. Additionally, in the COVID-19 era, the initial use of infliximab at a dose of 5 mg/kg rather than glucocorticoid therapy is a reasonable approach.

IBD patients with known or suspected COVID-19 should have individualized medication regimen adjustments in order to balance the risk of disease flare[77]. The goal is to reduce immunosuppression during active viral infection to lower the risk of COVID-19-related complications (*e.g.*, pneumonia). Patients with suspected or confirmed COVID-19 infection can be treated with Budesonide, Aminosalicylates, including sulfasalazine, topical rectal therapy (*e.g.*, topical glucocorticoid), and antibiotics. However, Glucocorticoids require dose adjustment based on the severity of COVID-19 infection and Immunomodulators like thiotropines, methotrexate; Tofacitinib (Janus kinase inhibitor); biologic agents like anti-TNF agents, ustekinumab, or vedolizumab are held or delayed in patients with active symptoms of COVID-19 until symptoms resolve[78,79].

However, the association of comorbidities, and their effect on the prognosis of COVID-19 needs to be further evaluated. In a recent study, 18 (1%) of 1590 COVID-19

cases had a history of cancer of which three had a history of colorectal cancer, one each of colonic tubular adenocarcinoma, rectal carcinoma, and colorectal carcinoma. It was also noted that patients with a history of cancer and positive SARS-CoV-2 virus were observed to have a higher risk of severe events[80]. Several strategies have been proposed, such as delaying of adjuvant chemotherapy or elective surgery on a patient-by-patient basis, stronger personal protection provisions, and more intensive surveillance or treatment[80].

In a cross-sectional survey of 86602 individuals, 53130 reported prior abdominal pain, acid reflux, heartburn, and regurgitation with 6.4 percent COVID positivity. Proton pump inhibitors (PPI) users were shown to be considerably more likely than non-users to report a positive COVID-19 test result, with a dose-dependent increase in the likelihood of a positive test result, and further studies are required to ascertain the link. PPI increase the risk of enteric infections due to PPI-induced hypochlorhydria. The usage of Histamine-2 receptor antagonist was not associated with an increase in risk[81].

GUT MICROBE AND COVID-19

The gut microbiota, which includes approximately 10^{14} resident bacteria, archaea, virus, and fungi, regulates not only the metabolism and host immunity but also the overall health. The gut and the lung microbiota seem to bi-directionally modulate each other and maintain a healthy gut-lung axis and is reported to be altered in COVID-19 patients and other diseases[82]. Lung infections can also significantly change the composition of gut microbiota, a process collectively termed as "gut microbial dysbiosis." Viral respiratory infections are also known to induce inappetence and significantly impact the gut microbiota[7]. Severe pulmonary SARS-CoV-2 infection is associated with a hyperactive immune reaction and "cytokine storm". Inflammatory mediators cause significant lung cytopathy and hyper-permeability, leading to a viral transfer to the gut *via* circulation or some other unknown mechanisms. The inflammatory mediators also damage the intestinal barrier leading to the leakage of intestinal microorganisms and associated metabolites into the main bloodstream that may further the inflammation and GI symptoms.

Moreover, microorganism-associated molecular patterns and PAMPs are recognized by host immune mediators and evoke a strong detrimental immunological reaction in organs, including the lungs and intestine. This vicious cycle of chronic immune activation leads to tissue inflammation and damage. Giron *et al*[83], 2020 study the role of COVID-19-associated lung injury, systemic inflammation, and disruption of the gut's barrier functions, resulting in the enhanced vulnerability of microbial products [83]. Thus, COVID-19 affects the gut lung axis and induces microbial dysbiosis.

There is a dearth of information regarding the direct *vs* the indirect effect of SARS-CoV-2 on gut microbiota. Zuo *et al*[84], 2020 analyzed fecal microbiome from COVID-19 patients using shotgun metagenomic sequencing technology and detected higher opportunistic pathogens (including, *Clostridium hathewayi*, *Actinomyces viscosus*, and *Bacteriodes nordii*) and a concomitant decrease in beneficial commensals (including, *Faecalibacterium prausnitzii*, *Lachnospiraceae bacterium 5_1_63FAA*, *Eubacterium rectale*, *Ruminococcus obeum*, and *Dorea formicigenerans*[84]. Interestingly all patients in this study cohort did not present GI symptoms. Data regarding the use of probiotics and nutritional intervention can further confirm the hypothesis that SARS-CoV-2 associated disease severity may be dictated by the patient's microbiota status. Probiotics are live microbes, when consumed, can provide gut health. Several studies have shown that the administration of probiotics in COVID patients can ameliorate gut dysbiosis and improve host immune response[85,86]. In the absence of specific data, further investigation regarding the particular role of probiotics and supplements, microbial type or nutritional component needs investigation in larger SARS-CoV-2 infected patient cohorts[87].

Elderly people (> 60 years) are associated with severe symptoms and higher mortality rates. The link between aging and progressive alteration of detrimental gut microbiota is well worked out[88]. An increasing number of reports suggest that a strong relationship exists between the gut microbiome and SARS-CoV-2 infection severity. Therefore, the heightened risk of aged patients may be associated with microbial dysbiosis, leaky gut, inflammation, and a dysfunctional gut-lung axis in addition to pre-existing conditions. A major question that has not been addressed is why certain developed countries have significantly higher mortality rates as compared to some underdeveloped or developing nations. Amongst many possibilities, the role

of lung and gut microbiome and resulting interference with systemic immunity may also help explain the global disparities in COVID-19 associated disease severity and death[89]. To have a comprehensive idea investigation with a larger data set is warranted.

THERAPEUTICS OPTIONS FOR COVID-19

Though infection prevention, control strategies, and preventive treatment are the mainstay of the current management of COVID-19. Some glimmer of hope has arrived in the form of COVID-19 vaccines' approval for emergency use by many nations, but currently, no safe and effective treatment exists. The possible list of emerging therapeutics for the treatment of COVID-19 is steadily expanding and evolving, and that too in a short period of time. The United States Food and Drug Administration (FDA) has approved emergency use authorizations (EUAs) for a few medications and therapies and several others are under clinical trials[90]. This review will concentrate on the strategies especially aimed at prophylactic and therapeutic modulating the host immune system. Pre-infection immunoprophylaxis depends on the immune activation of the host immune system before infection and disease initiation. At the same time, therapeutic intervention strategies are focused to repair the immune systems during the duration of the illness, post-infection. Several therapeutic targets including IFN-I, TNF, JAK/STAT, IL-1, IL-6, GMCSF, convalescent plasma, and complements, are under investigation. A comprehensive list of all prophylactic and therapeutic molecules undergoing clinical trials is available online (https://www.who.int/ictrp/en/; clinicaltrials.gov).

Approved therapeutics for COVID-19

Several antiviral molecules are undergoing clinical trials, and Remdesevir has been approved by United States FDA for therapeutic management of COVID-19 patients [91]. Remdesevir (Veklury), being a nucleoside analog, prevents viral replication by inhibiting the viral RNA-dependent RNA polymerase (RdRp) activity, shortened recovery time, and reduced mortality rates. Eli Lilly and Company has also received EUA for a combinatorial use of Baricitinib (Olumiant; an inhibitor of JAK kinase) with Remdesevir in patients requiring supplemental oxygen.

The antibody cocktail of Casirivimab and Imdevimab by Regeneron Pharmaceuticals, Inc. has also obtained the EUA by the United States FDA for the treatment of mild to moderate COVID-19. Casirivimab and Imdevimab are monoclonal antibodies against spike protein of SARS-CoV-2, are supposed to neutralize the viral entry. Eli Lilly COVID-19 neutralizing antibody bamlanivimab (LY-CoV555) is also directed against the spike protein and has received EUA for non-hospitalized adults. Convalescent plasma (CP) is a passive immune therapy approach, where COVID-19 recovered patient can donate plasma rich in SARS-CoV-2-specific neutralizing antibodies to persons at high risk of contracting COVID-19[92]. United States FDA has provided a EUA for the use of CP as a treatment option for COVID-19 patients.

The potential vaccine can change the course of the COVID-19 pandemic. Researchers have used several technological vaccine development platforms, including nucleic acid-based (DNA and RNA), virus mimicking particle subunit vaccine, peptide vaccines, attenuated virus-based vaccines. mRNA vaccines developed by Pfizer-BioNTech and Moderna have received EUA from the United States FDA, bringing a big sigh of relief. mRNA vaccines prompt the cell to express the viral spike protein, which elicits a strong immune reaction against the infecting SARS-CoV-2 virus. The Oxford-AstraZeneca's COVID-19 vaccine has also received authorization in many countries. The Oxford-AstraZeneca vaccine uses the gene for the coronavirus S protein (double-stranded DNA) packed in an adenovirus. Other vaccine producers like Johnson & Johnson/Janssen Pharmaceuticals and Gam-COVID-Vac (Sputnik V) developed by the Gamaleya Research Institute of Epidemiology and Microbiology are also effective and are being used for mass vaccination in many countries. Bharat Biotech and the Indian Council of Medical Research collaborated to create Covaxin (codenamed BBV152), an inactivated virus-based COVID-19 vaccine. CoronaVac (inactivated vaccine) is produced by Sinovac is also used to vaccinate to fight against COVID-19. The new evolving strains with mutations in the S protein create a possibility of decreased susceptibility to monoclonal antibodies, vaccines and therapeutic agents. Scientists are investigating these mutations to help explain how quickly they can be spread and if vaccines will be effective.

Under trial therapeutics for COVID-19

Several strategies to target the uncontrolled host immune system have been attempted and are currently in clinical trials (clinicaltrials.gov). TNF-α, a proinflammatory cytokine, exhibits a positive correlation with advances in disease stages. Preliminary clinical data suggest the effectiveness of anti-TNF-α in reducing the cytokine storm as well as tissue inflammation. TNF-α-blockers, both small molecule and antibodies (Adalimumab and Otilimab), are currently under trial[93]. Patients with IBD with COVID respond better to anti-TNF-α blockers than alternative agents[94]. Dysregulated early IFN-I response may eventually lead to COVID complications, and an early IFN-Iα/β treatment with broad antiviral response can ameliorate disease progression [95]. IL-6 is associated with 'cytokine storm', and inhibition of IL-6 using a monoclonal antibody (Tocilizumab, Sarilumab) is under clinical trial and can be a potential treatment option. Initial clinical studies in China and a case study in France suggested a rapid favorable outcome on the therapeutic value of the anti-IL-6 receptor antibody [96]. More investigation is warranted as IL-6 is also reported to prevent enterocyte cell death after injury and help proliferation and repair[97]. Sanofi's KEVZARA Phase III trial investigating the efficacy of an anti-IL-6 receptor antibody in severe and critically ill patients did not yield promising results. Hence, further investigation is warranted to understand better the therapeutic advantage of inhibiting IL-6 signaling in COVID patients. Other than current therapeutics, patients suffering from systemic inflammation and IBD and associated diarrhea may benefit from the potential use of pro and pre-biotics[91,98].

CONCLUSION

SARS-CoV-2 has spread exponentially as a pandemic throughout the world. Scientists and researchers all over the world are working tirelessly to develop potential coronavirus treatment options. The United States FDA has recently granted EAU for several therapeutic modalities for targeting COVID-19. Pfizer and Moderna are producing United States FDA approved vaccines in millions of doses for the prophylactic use in COVID-19 patients. In this review, we have attempted to describe the link between COVID-19 associated GI infection, immune responses, and disease outcomes. SARS-CoV-2 primarily causes severe respiratory symptoms but also affects the GI system in many patients. The role of SARS-CoV-2 in gut infection, route of infection, relation with disease severity, localized *vs* systemic immune reaction, altered microbiota, dysbiosis, and the mechanism underlying pre-existing conditions and therapy. Many queries remain unexplored, especially in the context of GI infection, and need further investigation. The bidirectional gut-lung axis has been implicated in the homeostasis of the immune system. GI inflammation and dysbiosis may contribute to systemic inflammation and affect lung and other organs' health, and may be associated with severe COVID consequences. The vice versa may also be confirmed and the underlying mechanism that pathologically upsets the gut-lung communications during COVID-19 infection is not clearly understood. The role of probiotics in enhancing the immune system and the attenuation of dysbiosis may be a promising approach for reducing the GI-symptoms and preventing the COVID-19 severity. The emergence of new strains like B.1.207, B.1.351, B.1.1.7, B.1.617.1, B.1.617.2, and B.1.617.3 can impact GI significantly and therefore strain surveillance is important and its role in gut infection also needs to be studied. Hence the use of bioinformatics, mutational analysis, structural modeling to better understand the spike-ACE2 interaction, and the use of organoid and non-human primate models to study the viral infection process and therapeutic screening are key in the fight against COVID-19.

ACKNOWLEDGEMENTS

Manash K Paul acknowledges Steven Dubinett, Brigitte Gomperts, and Volker Hartenstein from UCLA for providing constant support and mentoring.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 14; 27(46): 7943-7955

DOI: 10.3748/wjg.v27.i46.7943

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

MINIREVIEWS

Transmembrane serine protease 2 and angiotensin-converting enzyme 2 anti-inflammatory receptors for COVID-19/inflammatory bowel diseases treatment

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Author contributions: Lashgari NA, Roudsari NM, and Momtaz S collected and/or interpreted the data and wrote the manuscript; Abdolghaffari AH and Momtaz S provided the study material, conceived, designed, and finally approved the manuscript; all authors have read and approved the manuscript.

Conflict-of-interest statement: The authors have no conflicts of interest to disclose.

Country/Territory of origin: Iran

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

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Abstract

Inflammatory bowel diseases (IBD) refer to a subgroup of chronic, progressive, long-term, and relapsing inflammatory disorders. IBD may spontaneously grow in the colon, and in severe cases may result in tumor lesions such as invasive carcinoma in inflamed regions of the intestine. Recent epidemiological reports indicate that old age and underlying diseases such as IBD contribute to severity and mortality in patients with coronavirus disease 2019 (COVID-19). Currently, the ongoing COVID-19 pandemic caused serious morbidity and mortality worldwide. It has also been shown that the transmembrane serine protease 2 is an essential factor for viral activation and viral engulfment. Generally, viral entry causes a 'cytokine storm' that induces excessive generation of proinflammatory cytokines/chemokines including interleukin (IL)-6, IL-2, IL-7, tumor necrosis factor-α, and interferon-γ. Future research could concentrate on developing inflammatory immunological responses that are efficient to encounter COVID-19.

Grade D (Fair): 0
Grade E (Poor): 0

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Received: March 16, 2021
Peer-review started: March 16, 2021
First decision: May 1, 2021
Revised: May 12, 2021
Accepted: November 28, 2021
Article in press: November 28, 2021
Published online: December 14, 2021

P-Reviewer: Moreira TMM, Soares RLS
S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Fan JR

Current analysis elucidates the role of inflammation and immune responses during IBD infection with COVID-19 and provides a list of possible targets for IBD-regulated therapies in particular. Data from clinical, *in vitro*, and *in vivo* studies were collected in English from PubMed, Google Scholar, Scopus, and the Cochrane library until May 2021.

Key Words: Inflammatory bowel diseases; COVID-19; Transmembrane serine protease 2; Inflammation; Pro-inflammatory; Immunological responses

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Core Tip: This article provides clinical evidence on synthetic or natural-based transmembrane serine protease 2 (TMPRSS2) and angiotensin-converting enzyme 2 (ACE2) inhibitors, which are able to reduce coronavirus disease 2019-induced inflammation and cytokine storms in inflammatory bowel disease patients. Hence, targeting TMPRSS2 and ACE2 could be noticed as a novel approach for inflammatory bowel diseases treatment.

Citation: Lashgari NA, Momeni Roudsari N, Momtaz S, Abdolghaffari AH. Transmembrane serine protease 2 and angiotensin-converting enzyme 2 anti-inflammatory receptors for COVID-19/inflammatory bowel diseases treatment. *World J Gastroenterol* 2021; 27(46): 7943-7955
URL: https://www.wjgnet.com/1007-9327/full/v27/i46/7943.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i46.7943

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a primarily respiratory ailment that is caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), additionally named as 2019 novel COVID. It is profoundly overwhelming with case casualty rates of 2%-3%. Since its appearance in December 2019 in China, COVID-19 has quickly spread and influenced populaces in virtually all areas of the world[1,2]. Old-age patients and those with chronic conditions are more prone to dreariness and mortality in COVID-19. This high mortality is implicated in misrepresented and misled invulnerable reactions that cause cytokine storms. In brief, SARS-CoV-2 infects the angiotensin-converting enzyme 2 (ACE2) expressing epithelial cells in the lung and/or the intestine, leading to a massive production of mediators that induce the immune cell activation. Overactivation of immune cells leads to severe complications including acute respiratory distress syndrome, shock, and multiorgan failure[3,4].

Inflammatory bowel diseases (IBD) include two major types: Ulcerative colitis and Crohn's disease. IBD is characterized with persistent resistant interceded sicknesses that regularly require immunomodulatory and immunosuppressive treatments[5,6]. Therefore, patients with IBD are at high risk to different shrewd viral and bacterial contaminations. There is no solid evidence that patients with IBD are at higher risk for COVID-19 infection, although it has been indicated that patients with IBD who are pregnant are more vulnerable[7]. The current study discusses the impact of COVID-19 on IBD[8,9]. We provide evidence on mediatory effects of the transmembrane serine protease 2 (TMPRSS2) and ACE2 signaling pathways against inflammation and introduces the synthetic or natural TMPRSS2 and ACE2 inhibitors as probable approaches for IBD treatment in the COVID-19 situation[9,10].

LITERATURE SEARCH

PubMed, Google Scholar, Scopus, and Cochrane Library were searched and relevant clinical, *in vivo*, and *in vitro* articles (in English) were collected until May 2021. Search terms included "corona virus" OR "COVID-19" AND "inflammatory bowel disease" OR "IBD" OR "inflammation" AND "TMPRSS2" OR "ACE2" AND "TMPRSS2 inhibitors"

OR "ACE2 inhibitors".

COVID-19 PATHOGENESIS

Variations in potency of the SARS-CoV-2 cell entry may account for discovering new solutions to deal with the virus. It has been reported that the entrance of SARS-CoV-2 to the human cells victimizes the SARS-CoV receptor ACE2 and TMPRSS2 for the spike (S) supermolecule priming. It is debatable whether the metallopeptidase domain seventeen [a disintegrin and metalloprotease domain 17 (ADAM17), also referred to as the tumor necrosis factor (TNF)-α-converting accelerator] located in the ACE2 ectodomain shedding may or may not counteract the virus entry by increasing the number of soluble ACE2, or it solely contributes to the ACE1/ACE2 unbalancing, inflammation, and occlusion[11]. The ACE2-receptor/S-protein interaction could be a key factor for success of virus infection and willingness. Similarly, single ester polymorphisms located inside the TMPRSS2 factor (21q22.3) can play a more important role in respiratory disorder[12]. ACE1 and ACE2 collaborate with the renin-angiotensin system to balance the native vasoconstrictor/proliferative ACE1/angiotensin II/angiotensin II type 1/angiotensin (Ang) II/Ang type 1 receptor (ACE1/Ang-II type 1/AT1-axis), and vasodilator/antiproliferative (ACE2/Ang1-7/mitochondrial assembly-axis) actions. This ends up in the protection of organs and blood vessels by the decoagulants, medicinal drugs, anti-proliferation, anti-fibrosis, anti-alveolar vegetative cell caspase-mediated cell death, and anti-oxidative stress activities that are able to antagonize the Ang-II effects[11,13].

TMPRSS2 AND ACE2 STRUCTURE AND RELATED SIGNALING PATHWAYS

In a complex pathophysiological condition like COVID-19, the ACE2 cytoplasmic tail cleavage intervened by TMPRSS2 is a significant event to be considered (Figure 1). Cleavage of the ACE2 tail by TMPRSS2 increases viral load in objective cells, and TMPRSS2 could facilitate the SARS-CoV-2 passage *via* the SARS-S cleavage, which induces the S protein for film combination. The ACE2 cleavage may enhance viral uptake through the cathepsin L-subordinate pathway, resulting in viral integration with the endosomal layer and eventually cell contamination[11,14]. In spite of similar explicitness of TMPRSS2 and ADAM17 for ACE2, they act opposite for cleavage of ACE2. To start with, the divisions produced by the cleavage of these proteases have distinctive subatomic sizes, mainly due to various cleavage locales. Second, cleavage of ACE2 by ADAM17 forms the ACE2 ectodomain, which is shed into the extracellular medium, as the soluble ACE biologically dynamic structure[15,16]. *In vitro* studies have shown that the ACE2 ectodomain does not separate from the TMPRRS2-induced ACE2 cleavage. This was evidenced by a C-terminal intracellular cleavage. In this manner, the distinctions in the cleavage destinations and its organic outcomes might be basic. For sure, just the soluble ACE2 structure would have a defensive impact on prevention of viral particle aggregations[17]. Therefore, overexpression of ADAM17 and TMPRSS2 could be a primary factor in inflammation storm that is characterized by negative features such as renin-angiotensin system lopsidedness, intense irritation, and intravascular coagulation in older populations with COVID-19 comorbidities. Initiation of inflammation cycles is a key element for SARS-CoV-2 contamination[18,19].

TMRPSS2 AND ACE2 INFLAMMATORY PATHWAY

ACE2 is the main receptor for SARS-CoV-2, providing additional insurance against the destructive impacts of viral diseases. Moreover, as referenced above, solid confirmations indicate that the outflow of ACE2 is dependent on the companion of hormonal, hereditary, and age-related systems[20,21]. Overaction of ADAM17 in both COVID-19 and the plasma level of ACE2 has been confirmed by several reports. Overexpression of the *ADAM17* gene and its protein level have been implicated in several inflammatory conditions including IBD[22,23]. High levels of inflammatory cytokines and chemokines in COVID-19 patients are accounted for by more elevated levels of interleukin (IL)-2, IL-7, IL-10, granulocyte colony-stimulating factor, interferon

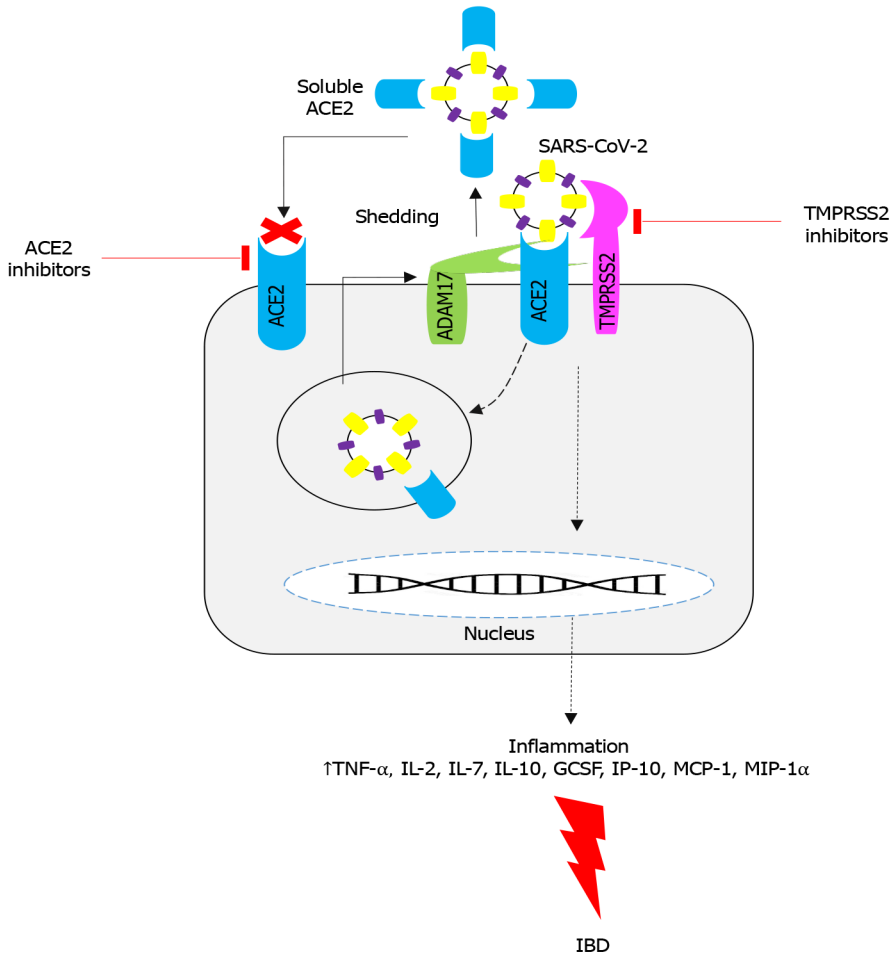


Figure 1 Coronavirus disease 2019 induced inflammatory bowel diseases mechanism. ADAM17: A disintegrin and metalloprotease domain 17; ACE2: Angiotensin-converting enzyme 2; TMPRSS2: Transmembrane serine protease 2; SARS-Cov-2: Severe acute respiratory syndrome coronavirus 2; GCSF: Granulocyte colony-stimulating factor; IP-10: Interferon gamma-induced protein 10; MCP1: Monocyte chemoattractant protein 1; MIP1-α: Macrophage inflammatory proteins-α; IBD: Inflammatory bowel diseases.

gamma-induced protein 10, monocyte chemoattractant protein 1, macrophage inflammatory proteins-1A, and TNF-α. A significant effect of the "fiery wave" in COVID-19 indicates the cytokine storm may be firmly connected with the seriousness of the infection[24,25]. The 'cytokine storm' is a significant target for research about the pathogenic cycles in SARS-CoV–2 contaminations and is a way to recognize new restorative targets. On the other hand, blockade of SARS-CoV–ACE2 in the ACE2 cytoplasmic domain pathway results in upregulation of ADAM17 activity. Upregulated ADAM17 increases the ACE2 ectodomain proteolytic cleavage[26,27]. Similar to the ACE2 tail cleavage, ADAM17 upregulation is essential for SARS-CoV infection. Finally, excessive activity of ADAM17 induces proinflammatory mediators, thus upregulating the inflammatory pathway during SARS-CoV-2 infection. ACE2 can be cleaved by the activity of TMPRSS2 protease[28,29]. TMPRSS2-induced ACE2 cytoplasmic tail cleavage may incite the viral uptake through a cathepsin L-subordinate pathway. Of note, acute respiratory distress syndrome is a delayed consequence of an aberrant generation of proinflammatory cytokines/chemokines or the 'cytokine storm' by effector cells[30-32].

COVID-19 INDUCED IBD: CORRELATIONS AND OVERLAPPING OF INFLAMMATORY PATHOGENESIS

The SARS-CoV-2 receptor ACE2 and TMPRSS2 receptor are central factors in COVID-19-induced IBD pathogenesis (Table 1 and Figure 1). These receptors are often found within the lower respiratory lot of pneumocytes and the gastrointestinal tract[7]. The ACE2 receptors are frequently located within the terminal ileum and colon. It was

Table 1 Clinical evidences of coronavirus disease 2019-induced inflammatory bowel diseases treatment

Ref.	Clinical studies	Model of IBD	Intervention	Duration of treatment	Numbers of animals in intervention group and control group	Outcomes	Adverse effects
Nowak *et al*[25]	Clinical trial	IBD in COVID-19	-	-	138 treatment naïve IBD patients (cases) and 154 controls	↑ACE2/TMPRSS2 expression; ↑ Inflammation	-
Brenner *et al*[67]	18 yr (with IBD), the Pediatric IBD Porto Group	-	TNF antagonist monotherapy (48%), followed by sulfasalazine/mesalamine (23%)	March 2020-October 2020	Hospitalized cases (n = 14); Outpatient cases (n = 195)	Sulfasalazine/Mesalamine and steroid therapy were associated with increased hospitalization risk and TNF antagonist monotherapy was associated with decreased risk parallel those reported in adult IBD patients. PIBD patients have a relatively low risk of severe COVID-19, even when receiving biologic and/or other immune-suppressive therapies for their IBD	-
Norsa *et al*[85]	Clinical trial	Crohn disease and Ulcer colitis	Anti-inflammatory (Salicylates); thiopurines or methotrexate; biologics (Infliximab, Adalimumab, Ustekinumab, Vedolizumab, Golimumab); steroids; Other immunosuppressants (Tacrolimus, Cyclosporin, Mofetil Micofenolate)	February 2020-March 2020	Crohn disease = 186; Ulcer colitis = 336	IBD improvement: ↓TNF-α; ↓ Inflammation; ↓ACE2/TMPRSS2 expression	-
Mazza *et al*[86]	Clinical trial	Ulcerative colitis	Methylprednisolone (40 mg/d); prednisone dosage at the time of patient's death was 25 mg daily	December 2019-February 2020	-	IBD improvement; Improvement in COVID-19 symptoms; ↓ Inflammation	-
Tursi *et al* [87]	Clinical trial	Crohn's disease	Adalimumab	-	-	Maintain of IBD remission during COVID-19; Managing/preventing COVID-driven pneumonia: ↓TNF-α ; ↓Inflammation; ↓ACE2/TMPRSS2 expression	-
Bodini *et al*[88]	Clinical trial	IBD	Immunosuppressants/biological treatment	3 wk	48 patients	IBD improvement; Improvement in; COVID-19 symptoms	Increase the risk of infection
Tursi *et al* [89]	Clinical trial	Crohn's disease	Mesalazine (3 g/d) and Adalimumab 40 mg subcutaneously	-	74 cases	IBD improvement; Improvement in COVID-19 symptoms; ↓TNF-α; ↓ Inflammation; ↓ACE2/TMPRSS2 expression	-
Allocca *et al*[90]	Clinical trial	IBD	Biological treatment	-	162 IBD patients	IBD improvement; Improvement in COVID-19 symptoms	-
Jacobs *et al*[91]	Clinical trial	Ulcerative colitis	Tofacitinib (10 mg twice daily)	5 mo	-	IBD improvement; Improvement in COVID-19 symptoms	Increase the risk of infection
Gutin *et al*[69]	Clinical trial	Ulcerative colitis	Biological treatment	February 2020-March 2020	522 patients	IBD improvement; Improvement in COVID-19 symptoms: ↓TNF-α; ↓ Inflammation; ↓ACE2/TMPRSS2 expression	
Taxonera *et al*[92]	Clinical trial	Crohn's disease	Immunomodulatory/biologics	-	n = 12	IBD improvement; Improvement in COVID-19 symptoms; ↓TNF-α; ↓ Inflammation; ↓ACE2/TMPRSS2 expression	
Allocca *et al*[93]	Clinical trial	-	Immunosuppressant or biologics	-	n = 15	IBD improvement; Improvement in COVID-19 symptoms; ↓TNF-α; ↓ Inflammation	-
Mak *et al* [94]	Clinical trial	IBD in COVID-19	Thirty (75%) were on 5-Aminosalicylates acid, 15 (37.5%) on	-	n = 63	IBD improvement; Improvement in COVID-19 symptoms; ↓	-

			immunosuppressants (14 Thiopurine, one Tacrolimus), 11 (27.5%) on corticosteroids and 7 (17.5%) on biologics (3 Infliximab, 1 Adalimumab, 2 Vedolizumab and 1 Ustekinumab)			Inflammation	
Bardasi and Alvisi [95]	Clinical trial	Crohn's disease in COVID-19	Subcutaneous administration of 40 mg Adalimumab	6 mo	-	IBD improvement; Improvement in COVID-19 symptoms ↓ Inflammation	-
Ashton *et al*[96]	Clinical trial	IBD in COVID-19	Anti-TNF therapy (Infliximab or Adalimumab)	-	*n* = 122	IBD improvement; Improvement in COVID-19 symptoms: ↓TNF-α; ↓ Inflammation	-

ACE2: Angiotensin-converting enzyme 2; TMPRSS2: Transmembrane serine protease 2; IBD: Inflammatory bowel diseases; TNF: Tumor necrosis factor.

shown that the convergence of these receptors was higher in IBD patients, in both the energetic and calm stages of the disease[33]. The ACE2 receptors are a part of the renin angiotensin-aldosterone system that is assumed to play critical roles in controlling the provocative handle. Terminal ileum and the colon are the most affected areas in IBD [34]. IBD is also correlated with upregulation of inflammatory cytokines and the ACE2 receptors. As we discuss in this article, patients with IBD do not seem powerless against COVID-19[35]. In this context, a few theories have been proposed. For instance, the renin angiotensin-aldosterone system has two specific pathways involved in irritation course. Multiple studies confirmed that ACE2 is upregulated in IBD, and in the SARS-CoV-2 condition ACE2 exacerbates the disease symptoms. Accordingly, prevention of the ACE2 protein expression has been suggested for controlling both COVID-19 and IBD[36,37]. While the ACE-angiotensin receptor 1 pathway is favorable for inflammation, the ACE2 pathway helps in tissue security. Given the enteric inflammation in IBD, it has been suggested that the ACE2 receptors and the host cell surface proteases like TMPRSS2 may suppress SARS-CoV-2[38,39]. The ACE2 level was shown to be downregulated in colonic aggravation in animal models; thereby, some IBD drugs such as steroids and biologics were found useful for cutting down the ACE2 in infected cells. Another report declared no change in ACE2 receptors or TMPRSS2 in IBD patients when diverged from controls[40,41].

IBD IN COVID-19: TREATMENT APPROACH

As mentioned, there are limited data on the possible impact of SARS-CoV-2 contamination on patients with IBD. Various methodologies can be utilized alone or concurrently to conquer the infection. Blockage of the ACE2 receptors and the viral S protein are the main focus of current investigations on SARS-CoV-2 regulation. So far, we discussed that blockage of the TMPRSS2 receptor and/or the ACE2/TMPRSS2 complex is likewise a plausible approach to modulate this infection. In this context, a number of synthetic or natural TMPRSS2 and ACE2 inhibitors that are able to mediate the TMPRSS2 and ACE2 signaling have been explored.

Natural agents targeting TMRPSS2 and ACE2 to manage the COVID-19 and IBD overlap

Medicinal plants are the greatest age-old wellspring of remedially valuable phytochemicals that are utilized to keep up human's wellbeing and to forestall and treat numerous infections. Medicinal plants and spices are used in Ayurveda, a conventional and optional restorative treatment in light of comprehensive body recuperating, which began in the Indian subcontinent[42,43]. Enormous investigations are right now centered on understanding the remedial viability and the activity of these phytochemicals. An improved dietary regimen along with natural medicinal formulations may provide preventive strategies for intense respiratory diseases, aspiratory fibrosis, pneumonia, sepsis, and numerous organ failure, which are hallmarks of serious COVID-19 contamination[44]. Also, a significant number of these phytochemicals help the insusceptible framework and instills insurance against infective diseases. It was shown that oxidative stress and many other reasons, notwithstanding existing comorbidities, add to a large number of difficulties related to coronavirus disease. Herein, we introduce plant species that contain various phyt-

ochemicals with antiviral, antifibrotic, cell reinforcement, mitigating, and immunomodulatory properties[45]. These phytochemicals, when used in blend, could have synergistic impacts, either as prophylactic or as steady specialists to limit certain clinical manifestations observed in COVID-19-contaminated patients. Moreover, certain types of microscopic organisms, green growth, and parasites may have remedial impacts against pneumonic fibrosis and intense lung injury[46].

ACE2 is found in the outer layer of the human cell that is accounted as a likely coupling site for the S protein. A couple of experiments have shown that there is a strong link between ACE2 and the S protein. Thus, blockade of ACE2 by phytochemicals is a strategy to fight SARS-CoV-2[47]. Several studies reported that SARS-CoV-2 is able to infect the central nervous system through TMRPSS2 and ACE2 receptors. It was also shown that ACE2 participates in neuroprotective responses, hence playing a critical role in treatment of COVID-19. Phytochemicals such as baicalin, scutellarin, and hesperetin can bind to ACE2 and prevent neurological impairments caused by COVID-19.

It was shown that hesperidin, chrysin, and emodin are also effective for COVID-19 treatment by attenuating the harmful effect of viral infection within cells[48]. Kaempferol, quercetin, and fisetin can bind with human angiotensin-converting enzyme-S-protein. In silico studies demonstrated that quercetin, quercetin 3 glucuronide-7-glucoside, quercetin 3-vicianoside, absinthin, glabridin, and gallic acid have strong affinity toward ACE2 to suppress COVID-19. Nuclear docking examination elucidated that dithymoquinone (aquinone) encounters the COVID-19 neurological side effects through blockade of ACE2. An in silico study reported that two chalcones namely azobechalcone and isolophirachalcone and some alkaloids (*i.e.* fangchinoline and tetrandrine) had high limiting proclivity to the S protein of SARS-CoV-2[49]. Flavonoids reduce the ACE2 expression through inducing the nuclear factor erythroid 2-related factor 2, thus fighting SARS-CoV-2 by means of their antioxidant properties. Kaempferol, quercetin, and fisetin are promising flavonoids against COVID-19-induced adverse neurological effects. Stilbenes, in particular resveratrol, are promising candidates for COVID-19 treatments, mainly by disturbing the formation of the S protein and the ACE2 receptor complex[50]. A variety of phenolic compounds including naringenin, hesperetin, hesperidin, and baicalin (alone or in combination) showed inhibitory effects on ACE2 activity and can be considered as potential treatments for COVID-19[51].

Different studies exhibited that some other phenolic compounds such as cinnamaldehyde as well as terpenoids such as carvacrol, geraniol, anethole, L-4-terpineol, cinnamyl acidic, thymol, and pulegone possess antiviral activities through blockade of the viral S protein[52,53]. It was reported that the binding affinity of ACE2 linkage with scutellarin (a flavonoid glycoside) and glycyrrhizin (a triterpenoid) was stronger than baicalin, hesperetin, and nicotianamine[54].

Limonoids and triterpenoids also displayed similar inhibitory effects on ACE2. Another in silico study similarly demonstrated that limonin, obacunone, ursolic destructive, glycyrrhizin destructive, 7-deacetyl-7-benzoylgedunin, maslinic acid, and corosolic acid effectively target SARS-CoV-2 proteins[55]. In this line, nimbin (a triterpenoid) and curcumin exhibited high limiting proclivity on ACE2 and the S protein [56]. Epigallocatechin-3-gallate and theaflavin gallate were shown to have inhibitory effects on the S-protein central channel of SARS-CoV-2. Moreover, three alkaloids, including cepharanthine, fangchinoline, and tetrandrine, inhibited the S protein of Human coronavirus Subtype OC43 (Human-CoV-OC43) expression, while tetrandrine exhibited moderating effects on viral sicknesses. An indazole alkaloid isolated from the seeds of *Nigella sativa*, called nigellidine, was shown to bind the dynamic areas of SARS-CoV-2, thereby paralyzing the virus. In another study, anthraquinone emodin blocked the ACE2 and S protein conjunction[57,58].

Chemical agents targeting TMRPSS2 and ACE2 to manage the treatment of COVID-19 and IBD overlap

Various classes of medications, with different powers and immunosuppressive potentials, are used for IBD treatment (Table 1 and Figure 1). At present, limited data are available for the utilization of different medications in IBD under the COVID-19 condition, henceforth the level of proof is not yet certain[59]. Current suggestions, proposed by specialists and different social orders, are overwhelmingly based on the recounted proof from the utilization of these medications during other viral pandemics like SARS and Middle East respiratory syndrome coronavirus or a few distributed case reports[60]. By and large, usage of intense immunosuppressants in IBD patients should be limited, except if totally essential. Notwithstanding, patients

who are on stable upkeep portions may keep on doing as such with close contact with their physicians[61,62].

Salicylates: Salicylates are usually utilized in either oral form or as a bowel purge. They have a neighborhood activity and are improbable to influence the course of COVID-19 when are used in IBD patients, thereby they may be securely proceeded in dosages[63].

Corticosteroids: Corticosteroids are the most common drugs that are used in IBD, mainly due to their intense calming effects. Therefore, steroids may be valuable in suppression of COVID-19, particularly in conditions like intense lung injury, intense respiratory trouble disorder, and septic shock. During the SARS and Middle East respiratory syndrome pandemic, corticosteroids treatment helped to postpone viremia [64,65], while there were no general improvement in terms of septic shock or psychosis, *etc.*[66]. Given the absence of adequacy, the World Health Organization suggested that routine corticosteroids ought to be avoided except in explicit circumstances. Steroids are possibly kept away from the first line therapies in recently analyzed IBD patients. Notwithstanding, considering their tremendous advantages in IBD, it was suggested that the main steroids might be beneficial at low doses in patients with COVID-19 and IBD, specifically in patients that are already on treatment [67,68]. Steroids with limited site of action, for example budesonide, seem harmless to be used. Infliximab might be a therapeutic option for COVID-19 positive patients with mild respiratory symptoms[61,69].

Cyclosporin: Cyclosporin is used for serious ulcerative colitis as an option in contrast to steroids. Although, some data pointed out that cyclosporine can inhibit the coronavirus replication proteins *in vitro*, its prescription is controversial in patients with COVID-19 due to its strong immunosuppressive properties[70-72].

Azathioprine and methotrexate: Azathioprine is a thiopurine that is often used for IBD treatment, particularly for upkeep treatment. Curiously, past investigations have demonstrated that thiopurine analogs have both immediate and roundabout activities on smothering antiviral movement. They also hinder viral proteases once the host proteins were engaged with viral replication[73,74]. Depending on the perception of genuine viral contaminations in IBD patients who are using thiopurine, the treatment time can be estimated. Interruption in treatment up to 14 d after recuperation from COVID-19 has been suggested. Methotrexate can perhaps continue without issues[75, 76].

Biologics: Current data show that infliximab and adalimumab (TNF-α inhibitors) have no unfavorable effects on the clinical course of COVID-19[62,77]. One reason speculated is the strong mitigating impact of TNF blockage, which may indeed constrict the cytokine storm in serious types of COVID-19[78,79]. Co-administration of medicines (*i.e.* thiopurine and infliximab) might be an option. Also, monotherapy with natural products may be considered[60,80,81]. Vedolizumab (an adversary of α4β7 integrin) is significantly explicit for movement on the gut, hence it is favorable for fundamental or pneumonic responses in COVID-19[62,82]. Ustekinumab is an approved clinical therapy for patients with IBD. Ustekinumab is a cytokine antibody and an inhibitor of IL-12 and IL-23. Currently, there are no major concerns about usage of ustekinumab in patients with IBD and COVID-19. Vedolizumab or ustekinumab might be the primary therapeutic options for individuals at higher risk of COVID-19 if biological treatments are thought of[79,83,84].

CONCLUSION

Information on the physiologic and pathophysiologic functions of ACE2/TMPRSS2 is still scant. ACE2/TMPRSS2 is very much described in the cardiovascular and renal frameworks. Yet little data exist regarding other organ frameworks, for example the gastrointestinal system. Moreover, specific function of the ACE2/TMPRSS2 axis in pathologic conditions was traditionally restricted to cardiovascular illnesses. Although, considering the ACE2/TMPRSS2 as a multifunctional protein has accomplished significance as of late.

The current COVID-19 pandemic has featured the importance of ACE2/TMPRSS2 as a receptor for SARS-CoV-2, yet research is expected to determine whether the ACE2/TMPRSS2 levels enhance the pathogenesis of COVID-19 or could benefit the course of illness by diminishing the malicious impacts of Ang II. Moreover, the

relationship between ACE2/TMPRSS2, the intestinal amino corrosive vehicle, and IBD merits further consideration in patients with IBD. At last, association of ACE2/TMPRSS2 to integrins raises concerns and expectations, particularly because there were just two articles regarding the matter. Taking everything into account, investigating the multifunctional nature of ACE2/TMPRSS2 in IBD (by describing its appearance/movement in the blood, gut, as well as excrement of patients with IBD and solid control patients) will develop the knowledge on the pathophysiology of this illness. In accordance with this objective, recognizable proof of other biomarkers of infection movement, treatment reaction, and new medication target, as well as setting of the novel helpful alternatives is required to affect tolerant consideration.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 December 14; 27(46): 7956-7968

DOI: 10.3748/wjg.v27.i46.7956

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

MINIREVIEWS

T cells in pancreatic cancer stroma

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Author contributions: Goulart MR and drafted the original manuscript and composed the original figure; Goulart MR, Stasinos K, Fincham REA, Delvecchio FR and Kocher HM contributed to the review and editing of the manuscript and figure.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Supported by PCRF Post-doctoral Fellowship (to Goulart MR); Lewin International Surgical Oncology Fellowship funded by Barts Health NHS Trust and Barts Charity(to Stasinos K); PhD Studentship Awarded by Barts Charity (London, UK) and A*STAR (Singapore) (to Fincham REA); and Cancer Research UK Post-doctoral Fellowship (to Delvecchio FR).

Country/Territory of origin: United Kingdom

Specialty type: Gastroenterology and hepatology

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a highly devastating disease with a dismal 5-year survival rate. PDAC has a complex tumour microenvironment; characterised by a robust desmoplastic stroma, extensive infiltration of immune-suppressive cells such as immature myeloid cells, tumour-associated macrophages, neutrophils and regulatory T cells, and the presence of exhausted and senescent T cells. The cross-talk between cells in this fibrotic tumour establishes an immune-privileged microenvironment that supports tumour cell escape from immune-surveillance, disease progression and spread to distant organs. PDAC tumours, considered to be non-immunogenic or cold, express low mutation burden, low infiltration of $CD8^+$ cytotoxic lymphocytes that are localised along the invasive margin of the tumour border in the surrounding fibrotic tissue, and often display an exhausted phenotype. Here, we review the role of T cells in pancreatic cancer, examine the complex interactions of these crucial effector units within pancreatic cancer stroma and shed light on the increasingly attractive use of T cells as therapy.

Key Words: Immunosuppression; T cell exhaustion; Tumour microenvironment; Pancreatic ductal adenocarcinoma; Pancreatic cancer stroma

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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

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Received: April 9, 2021
Peer-review started: April 9, 2021
First decision: May 17, 2021
Revised: May 18, 2021
Accepted: November 28, 2021
Article in press: November 28, 2021
Published online: December 14, 2021

P-Reviewer: Falasca M
S-Editor: Gao CC
L-Editor: A
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Core Tip: Pancreatic ductal adenocarcinoma (PDAC) is a highly devastating disease with a dismal 5-year survival of less than 5% in patients with metastatic disease, and is predicted to become the second cause of cancer-related death by 2030. Here, we discuss the complexity of the PDAC immunosuppressive tumour microenvironment, the mechanisms involved in T cell dysfunction, and potential immunotherapeutic strategies for treating PDAC.

Citation: Goulart MR, Stasinos K, Fincham REA, Delvecchio FR, Kocher HM. T cells in pancreatic cancer stroma. *World J Gastroenterol* 2021; 27(46): 7956-7968
URL: https://www.wjgnet.com/1007-9327/full/v27/i46/7956.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i46.7956

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a highly devastating disease with a dismal 5-year survival of less than 5% in patients with metastatic disease[1], and is predicted to become the second cause of cancer-related death by 2030[2]. Late detection and incredibly aggressive biology are significant challenges determining therapeutic failure[3,4]. PDAC has a complex tumour microenvironment (TME) characterised by a robust desmoplastic stroma[5], and an expanded pool of immunosuppressive immune cells shielding the malignant cells harbouring aberrant expression of oncogenic pathways. The interplay between various cell types in this fibrotic TME supports tumour cell escape from immunosurveillance, disease progression and spread to distant organs [6,7], highlighting this cancer's ability to evade immune recognition and its extra-ordinary metastatic potential. In this review, we discuss the interactions between T cells and the other components of the PDAC TME and highlight the impact of these interactions on the phenotype and function of T cells. Emerging immune-therapeutic strategies employed in overcoming T cell dysfunction and improve patient survival are also discussed.

PDAC IMMUNE LANDSCAPE

PDAC carcinogenesis is characterised by an abundant fibro-inflammatory reaction and subsequent oncogene activation on epithelial cells, resulting in a pro-tumorigenic microenvironment[8]. At early stages of cancer development, oncogenic *KRAS* expression in pancreatic cells results in the formation of pancreatic intraepithelial neoplasia (PanIN), and drives an inflammatory reaction that modulates the recruitment and infiltration of immunosuppressive myeloid and lymphoid cell subsets. *KRAS*-mutated pancreatic cells regulate the maintenance of immunoregulatory microenvironment by inducing the release of interleukin (IL)-6, IL-10 and transforming growth factor (TGF-β) cytokines. In the setting of sustained chronic inflammation, PanIN progression to malignant lesion is accompanied by mutations in genes such as TP53, CDKN2A and SMAD4 frequently, which further contribute to shape the immune microenvironment. For example, the mutant tumour suppressor gene TP53 are implicated in sustaining the tissue damage and chronic inflammation by enhancing the expression of NF-kB, secretion of vascular endothelial growth factor (VEGF) and activation of fibroblasts. Decreased infiltration of T and B cells and elevated numbers of Tregs were significantly correlated with CDKN2A mutations while SMAD4 mutations are involved with enhanced invasion, metastasis and immunosuppressive effects of TGF-β on immune response[9].

Chemotactic factors associated with the recruitment of dysfunctional bone marrow-derived myeloid cells include granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-colony-stimulating factor (G-CSF), IL-3, VEGF, and the interaction of the C-X-C chemokine ligand 12 (CXCL12)/C-X-C chemokine receptor 4 (CXCR4) or C-C chemokine ligand 2 (CCL2)/C-C chemokine receptor 2 (CCR2), amongst others [10]. Stromal-associated fibroblasts produce C-X-C chemokine ligand 13 (CXCL13), which recruits IL-35-producing regulatory B cells (Breg) into the TME, further contributing to PDAC progression through IL35-mediated stimulation of tumour cell proliferation[10]. Copious infiltration of immature myeloid cells, tumour-associated

macrophages (TAMs), neutrophils and regulatory immune cells ultimately establishes an immune-privileged microenvironment that protects the malignant cells from T cell immunosurveillance and sustains tumour growth[11].

Therefore, pancreatic cancer evolves to establish a complex and heterogeneous immune microenvironment, characterised by high numbers of strongly suppressive immune cells, and a modest infiltration of lymphocytes with anti-tumour properties [12-14]. As such, PDAC tumours are considered to be non-immunogenic or cold, displaying low infiltration of $CD8^+$ cytotoxic lymphocytes (CTLs) that are localised along the invasive margin of the tumour border or trapped in the surrounding fibrotic tissue but are not present within the tumour core. Moreover, infiltrated $CD8^+$ T cells in PDAC tumours often display minimal signs of activation[11,15,16]. T cell exclusion from TME has been demonstrated both in genetically engineered KPC ($KRas^{LSL_G12D/+}$, $Trp53^{LSL_R172H/+}$, Pdx1-Cre) mouse models[16] and PDAC patients[17].

Macrophages compose the most abundant immune cells in PDAC[11]. They play a critical role in the exclusion of T cells from tumours, maintenance of fibrosis through the secretion of pro-fibrotic cytokines[18] and induction of angiogenesis by secreting VEGF[19]. Increases in TAMs correlate with poor prognosis[20,21]. Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immunosuppressive cells, including immature monocytes, granulocytes and dendritic cells (DCs). These cells show potent ability to inhibit proliferation and induce apoptosis of both $CD4^+$ and $CD8^+$ T cells, secrete elevated amounts of immunosuppressive cytokines IL-10 and TGF-β, which collaborate to the recruitment of regulatory $CD4^+$ T cells (Tregs), and decrease the infiltration of natural killer (NK) and NKT cells into the tumour[22]. MDSCs accumulation has been described in the spleen, tumours and metastatic lesions in KPC models of PDAC, and its accumulation negatively correlated with $CD8^+$ T cells infiltration[19].

Likewise, Tregs upregulate the expression of CTL-associated antigen 4 (CTLA-4) [23], interact with DCs suppressing the expression of the co-stimulatory ligands, such as CD80 and CD86, necessary for T cell activation, secrete immunosuppressive cytokines, and directly suppress $CD8^+$ T cells anti-tumour immunity[24]. Infiltration of Tregs occurs at early stages of PDAC formation, and increased numbers of both circulating and intra-tumoural. Tregs have been observed in pancreatic cancer patients [19]. Additionally, the presence of tumour-infiltrating IL-17-producing CD4+ T cells and γδT cells also contribute to tumour immune evasion and progression[11,25].

Similar to PDAC, in inflammatory conditions of the pancreas, such as pancreatitis, the inflammatory reaction leads to the infiltration of myeloid cells, such as monocytes and neutrophils. Although macrophages comprise a significant population within the inflamed pancreas, T cells are also present, and infiltration of $CD4^+$ T cells has been implicated in the progression of acute pancreatitis in mice. As pancreatitis progresses, the ratio of $CD4^+$ and $CD8^+$ T cell increases, with increased numbers of immunosuppressive Tregs observed in patients with chronic pancreatitis.

T CELL INTERACTIONS AND IMMUNE DYSFUNCTION IN PANCREATIC CANCER

T cell infiltration is observed in patients with surgically-resected PDAC and correlates with improved outcomes suggesting the anti-tumour potential of tumour-infiltrating $CD4^+$ and $CD8^+$ T cells[26]. However, as PDAC progresses, tumour-infiltrating T cell composition shifts to a decrease in $CD8^+$ T cells and elevated percentage of Tregs within the $CD4^+$ T cell subset[27]. While $CD4^+$ Tregs are a prominent feature of the immune infiltrate, $CD8^+$ T cells are rare in the PDAC microenvironment[24]. Consequently, PDAC is considered to be a poorly immune responsive cancer, with T cells present within the tumour microenvironment often showing lack of activation, or an exhausted phenotype[28-30]. This observation demonstrates that infiltrated $CD8^+$ T cell may recognise and mount a response against these tumours, but the unfavourable TME halts optimal cytotoxic function.

Spatial localisation of the immune cells in these tumours reflect the challenging biology of PDAC TME. Tumour-infiltrating $CD8^+$ T cells are localised at the periphery, within the surrounding fibrotic stroma in PDAC tissues[6,21,31,32]. $CD8^+$ T cells migrate away from the juxta-tumoural compartment by favouring their movement towards CXCL12-rich stroma laid by activated pancreatic stellate cells (PSCs)[6]. The proximity of intra-tumoral $CD8^+$ T cells to tumour cells correlates with patient survival [32].

PSCs play a central role in shaping the architecture of PDAC by modulating the ECM components and producing a physical barrier that limits T cell infiltration, migration and direct interaction with neoplastic cells[33]. These cells can also act as non-professional antigen-presenting cells (APCs) and secrete cytokines and growth factors that boost the recruitment of immunosuppressive cells and inhibit T cell responses, resulting in increased disease aggressiveness and decreased overall survival[34]. Therefore, in conjunction with the immunosuppressive cells, PSCs are crucial players in the orchestration of an immuno-privileged PDAC microenvironment by combination of secreted cytokines, chemokines and extra-cellular matrix proteins as well as direct cell-cell contact.

Cancer cell-intrinsic factors also impact T cell function. Overexpression of immune checkpoint mediators like programmed death-1 receptor (PD-1)-ligand (PD-L1) is one mechanism by which cancers suppress T cell immunity. PD-L1 is overexpressed in PDAC cells, and this overexpression correlates with worse prognosis[20]. Pancreatic cancer cells can also downregulate Fas, a cell surface receptor associated with the induction of Fas-mediated apoptosis in tumour cells. $CD8^+$ T cells use the Fas-FasL and perforin–granzyme pathways as major effector mechanisms of cytotoxicity, and loss of Fas expression in PDAC tumours result in cancer immune evasion[7,35]. Spatial localisation and T cell interactions within the PDAC tumour microenvironment are shown in Figure 1.

PDAC has a low mutation burden, resulting in low neoantigen burden and the scarcity of tumour-infiltrating effector T cells. Only a few PDAC tumour antigens capable of inducing an anti-tumour immune response have been identified. Low mutation burden with minimal expression of neoantigens, and consequently marginal T cell infiltration is a classical feature in KPC models[36], and in PDAC patients[29,37]. In a recent study aimed to identify T cell neoantigens in long-term survival patients, it appears that the total neoantigen burden does not correlate with increased survival, but the presence of high-quality neoantigens played an essential role in the immunosurveillance of long-term survival patients. This study also highlighted the correlation of prolonged survival with granzyme B^+ $CD8^+$ T cells[26]. In keeping with this hypothesis, the total number of infiltrated $CD8^+$ T cells after vaccine immunotherapy did not show correlation with survival, but the subset of granzyme B^+ $CD8^+$ T cells was associated with long-term survivors[38]. These findings suggested that T cell quality may be more important than the total number of T cells for adequate anti-tumour immunity[39].

Identification of multiple dense lymphocyte aggregates, known as tertiary lymphoid structures (TLS) has also been observed in PDAC[38]. Importantly, detection of TLS in tumour tissue of PDAC patients was an independent prognostic factor for prolonged survival[40-42]. Although TLS can occur intra-tumoral or at the tumour periphery, only the presence of intra-tumoral TLS correlates with survival[41]. TLS aggregates contain T- and B-cell areas co-localised with myeloid and follicular DCs, and high-endothelial venules, displaying similar organisation to secondary lymphoid organs. They comprise ectopic lymphoid sites where T-cell activation and proliferation takes place[41]. Nevertheless, PDAC immune microenvironment is enriched with both exhausted and senescent T cells, and a diverse pool of highly immunosuppressive cells [43].

T CELL PHENOTYPE AND FUNCTIONS

Mature T cells can be classified as $CD8^+$ T cells (CTLs) and $CD4^+$ helper T cells (Th), which further differentiate into Th1, Th2, Th17 and Tregs[17]. $CD4^+$ Th1 cells secrete the pro-inflammatory cytokine interferon-γ (IFN-γ) which activates and supports CTLs cytotoxicity, while $CD4^+$ Th2 cells exhibit tumour-promoting functions by producing a plethora of cytokines, sustaining fibrosis through ECM and collagen deposition, and contributing to the differentiation of macrophages into a M2-immunosuppressive phenotype[44]. Polarisation towards Th2 cell subset is a common trait in pancreatic cancer, and this shift from Th1 to Th2 cells is correlated with decreased patient survival[45]. In PDAC patients, $CD4^+$ Th17 cells functions are mediated by the secretion of IL-17 cytokine. Although not very well understood, infiltration of this population has been associated with immune tolerance and reduced survival in murine models[46]. Tregs are an essential component of the T cell population. PDAC patients have increased numbers of Tregs that are inversely associated with $CD8^+$ T cells, therefore, they are often used as a negative prognostic biomarker in PDAC[45]. These cells can be identified by the expression of $CD4^+CD25^+FOXP3^+$ phenotype[24]

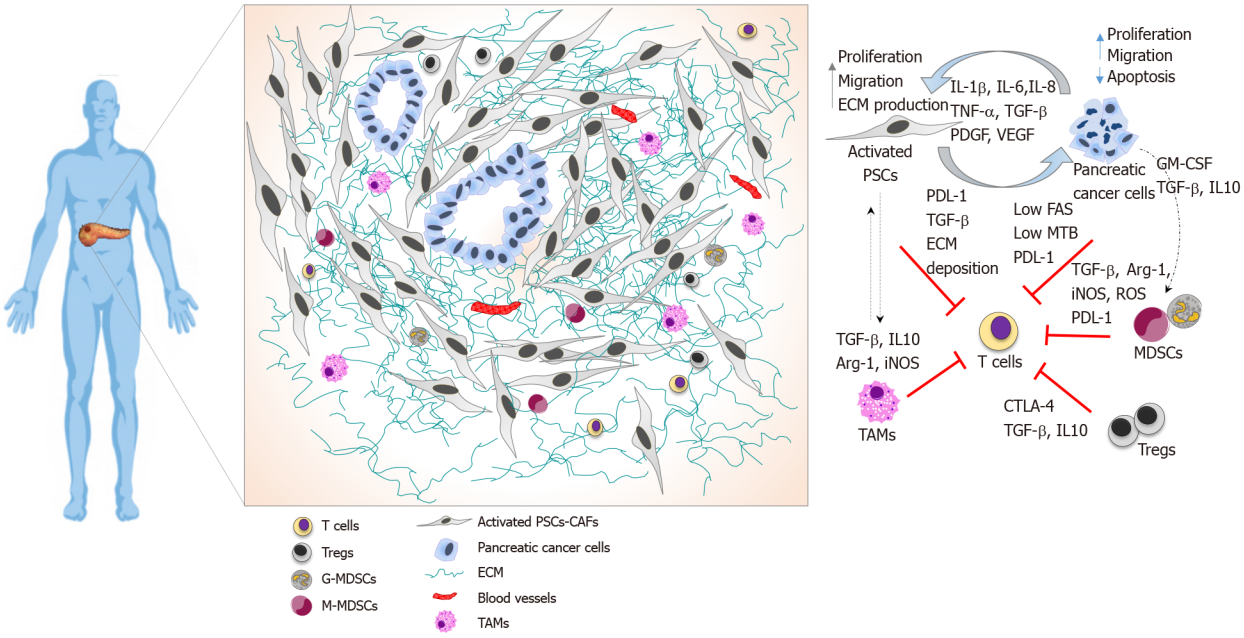


Figure 1 Pancreatic ductal adenocarcinoma immune landscape and T cell immunosuppression. Illustrative image showing spatial localisation of T cells in the pancreatic ductal adenocarcinoma tumour microenvironment and cellular interactions that collectively prevent T cell infiltration and function. T cells are localised at the periphery of tumours preventing direct contact with cancer cells. Pancreatic stellate cells produce elevated amounts of extracellular matrix driving a fibrotic tissue that entraps infiltrated T cells, alongside with immunosuppressive cytokine to and expression of programmed death-ligand 1 (PDL-1). Pancreatic cancer cells avoid T cell killing by downregulating Fas, exhibiting low tumour mutational burden, expressing PDL-1 and secreting growth factors and cytokines that recruits immunosuppressive cells. Myeloid-derived-suppressor cells express PDL-1 and suppress T cells functions by several mechanisms, including depleting of arginase 1, the release of reactive oxygen species, and secretion of cytokines. Tregs directly suppress T cells, express cytotoxic T-lymphocyte-associated protein 4 and secrete cytokines. TAMs play a role in sequestering T cells at the periphery and secrete immunosuppressive cytokines. PSC: Pancreatic stellate cells; TAMs: Tumour-associated macrophages; CTLA-4: Cytotoxic T-lymphocyte-associated protein 4; GM-CSF: Granulocyte-macrophage colony-stimulating factor; Arg-1: Arginase 1; PDL-1: Programmed death-ligand 1; iNOS: Inducible nitric oxide; MDSC: Myeloid-derived-suppressor cells; ROS: Reactive oxygen species; ECM: Extracellular matrix; TBM: Tumour mutational burden.

(Table 1).

CTLs are the preferred immune cells for targeting tumours. For durable and efficient immune responses, naïve T cells are primed in the lymph nodes with tumour antigens through interactions with APCs. Upon activation, they rapidly proliferate, differentiate into antigen-specific CTLs and migrate to tumour sites to perform their cytotoxic functions[47]. Elimination of tumour cells by CTLs occurs *via* the release of cytotoxic granzymes, IFN-γ and tumour necrosis factor α (TNF-α), or by induction of FasL-mediated apoptosis[48]. Following a cytotoxic immune response, the majority of CTLs will undergo apoptosis while a small fraction of them will further differentiate into diverse subsets of multipotent, long-lived memory $CD8^+$ T cells endowed with self-renewal ability[47]. The integration of three coordinated signals regulates T cells activation, expansion, survival, and memory formation: T cell receptor (TCR) stimulation by antigens, engagement of co-stimulatory molecules (CD28, CD27, 4-1BB, and OX40) expressed by $CD8^+$ T cells, and the release of inflammatory cytokines. In the absence of co-stimulatory signals, antigenic stimulation induces tolerance or clonal deletion in peripheral lymphoid organs[49]. The pro-inflammatory cytokines IL-12, IL-2 and IFN-γ, are crucial for satisfactory naïve $CD8^+$ T cell activation, expansion and differentiation whereas IL-7 and IL-15 are predominantly required for formation maintenance of memory $CD8^+$ T cells. In pancreatic cancer patients, both number and functions are altered within the $CD8^+$ T cell population. These patients show a decrease in circulating $CD8^+$ T cells and a decrease in perforin expression within these cells compared to healthy subjects. Moreover, intra-tumoural $CD8^+$ T infiltrates often display abnormal exhausted phenotype[44].

Memory $CD8^+$ T cells immediately proliferate upon antigen stimulation, execute cytotoxic functions, secrete effector cytokines, persist in greater numbers and exist in different metabolic, transcriptional, and epigenetic states[50]. Importantly, while the correlation between the numbers of memory $CD8^+$ T cells and the efficacy of T cell immunity is firmly established, the quality (or functional ability) of memory $CD8^+$ T cells also determines the degree of protection[47,48,50]. While memory T cell population are heterogeneous and consist of multiple subsets, the central memory T cells (T_{CM}) and effector memory T cell (T_{EM}) subsets have been best characterised. T_{CM}

Table 1 T cell phenotype and functions

T cell phenotype	Surface markers	Immune response	Effector functions
Cytotoxic T cell			
CTLs	CD8	Tumour killing	IFN-γ, TNF-α cytokines, granzymes, FasL
Helper T cell			
Th1	CD4 STAT4 T-bet	Tumour killing	IFN-γ, IL-2 cytokines, increase CTL activity
Th2	CD4 STAT6 GATA3	Tumour tolerance	IL-4, IL-5, IL-13 cytokines, decrease CTL activity
Th17	STAT3 RORγt	Tumour tolerance	IL-17 cytokine
γδ T cells	TCRγ/δ	Tumour tolerance	IL-4, IL-10, TGF-β cytokines and CTL activity
Regulatory T cell			
Tregs	CD4 CD25 FOXP3	Tumour tolerance	IL-10, TGF-β cytokines, CTLA-4

CTL: Cytotoxic lymphocyte; IFN-γ: Interferon-γ; TNF-α: Tumour necrosis factor α; IL: Interleukin; TGF-β: Transforming growth factor β.

cells express high levels of CD62L and CCR7 and efficiently home to lymph nodes, whereas T_{EM} cells lack these molecules and reside mainly in non-lymphoid peripheral tissues but are able to migrate rapidy in response to cytokine gradient. T_{CM} and T_{EM} subsets can also be identified along with a terminally differentiated $CD8^+$ T subset that expresses CD45RA (T_{EMRA}). This way, the T_{CM} subset is classified as $CD45RA^-$ $CD27^{high}$ $CCR7^+$ cells and T_{EM} subset as $CD45RA^-$ $CD27^{low}$ $CCR7^-$ cells. In contrast, T_{EMRA} subset can be identified as $CD45RA^+CD27^{lo}$ $CCR7^-$ cells, and naïve T cells as $CD45RA^+CD27^{high}$ $CCR7^+$ cells, but there are other methods of differentiating these sub-types[47,50].

A handful of other markers have been described to differentiate T cell populations during the effector-to-memory transition states. Increased expression of IL-7Rα (CD127) is functionally required for long-term survival and can be used to identify memory precursor $CD8^+$ T cells. Other proteins co-expressing with $CD127^+$ $CD8^+$ T cells include Bcl-2, CD27, CXCR3, and CD28. Cells expressing these set of markers have the most remarkable capacity to develop into central memory $CD8^+$ T cells (T_{CM}), showing elevated ability to proliferate upon antigen stimulation, increased IL-2 secretion, and self-renewal. Conversely, $CD8^+$ T cells with increased expression of KLRG1, CD57 and decreased expression CD127, CD27, CXCR3, and CD28 are associated with effector or memory $CD8^+$ T cells that display cytotoxicity, elevated IFN-γ production and short-life span. Therefore, $KLRG1^+$ $CD127^-$ $CD8^+$ T cells can be considered effector memory $CD8^+$ T cells (T_{EM}), at least in murine models, though human equivalent data is awaited[47,50].

Transcriptional factors promote the development and function of T_{EM} and T_{CM} cells. Expression of T-bet, Blimp1, ID2, and STAT4 is associated with T_{EM} cells, while high expression of TCF1, BCL-6, ID3, and STAT3 is linked to the formation of T_{CM} cells[49,50]. Interesting, in B cells, Blimp-1 and BCL-6 are essential for the development of germinal centre B cells and long-lived plasma cell through reciprocally antagonising each other[51], suggesting that this set of transcription factors acts in a similar fashion, in the regulation of effector- memory T-cell transition. Moreover, Tcf7 and Lef1 transcription factors are found in self-renewing multipotent $CD8^+$ T cells known as memory stem cells[52].

T CELL EXHAUSTION

Exhausted T cells differ from other dysfunctional T cells, including anergic T cells and senescent T cells. Anergic T cells are induced by suboptimal stimulation showing cells with low proliferative capacity and minimal effector function. Senescent T cells initiate from repeated stimulation, resulting in cells with low proliferative capacity, low expression of inhibitory receptors but show high effector functions despite shortened telomeres. Differently, exhausted T cells result from persistent antigenic stimulation causing Tcells with low proliferative capacity, low to moderate effector functions and elevated expression of multiple inhibitory receptors[53].

In cancers such as PDAC, T cells that go through the activation process will later differentiate into memory-like cells and will ultimately become terminally differen-

tiated exhausted T cells. Exhausted T cells result from persistent antigen exposure featuring cells with low proliferative capacity, increased apoptosis, loss of their cytotoxic function, and elevated expression of multiple inhibitory receptors also known as immune checkpoints such as PD-1, CTLA-4, T cell immunoglobulin and mucin-domain containing-3 (TIM-3), lymphocyte activation gene 3 (LAG-3), or T cell immunoreceptor with Ig and ITIM domains (TIGIT)[43,53]. Each inhibitory receptor binds to its ligand, typically expressed by APCs and tumour cells in the TME.

The surface receptor PD-1 (CD279) is the primary receptor involved in T cell inhibitory signalling. PD-1 has two ligands: PD-L1 (CD274) and PD-L2 (CD273) can be found on the surface of antigen-presenting, MDSCs, TAMs and cancer cells. IFN-γ is the main trigger for PD-L1 and PD-L2 upregulation, while induction of PD-1 expression on T cells results from cell receptor (TCR) stimulation or secretion of the cytokines IL-2, IL-7, IL-15, IL-21, and TGF-β. Engagement of PD-L1 or PD-L2 with PD-1 receptor on T cells, inhibits dephosphorylation of TCR signalling components, specifically CD28, resulting in decreased IL-2, IFN-γ, and TNF-α cytokine production, survival, proliferation and effector functions[54].

CTLA-4 (CD152) is a B7/CD28 family member that is constitutively expressed by Tregs. CTLA-4-mediated immunosuppression occurs by limiting signalling through the co-stimulatory receptor CD28 during antigen-presentation, by either binding or deleting CD80 and CD86 from APCs[55]. Thus, indirectly reducing T cell activation and immune responses to tumour antigens. Other T cell subsets such as $CD4^+$ T cells can also upregulate this receptor upon activation[56]. LAG-3 exerts inhibitory effects on T cells through MHCII binding, which results in decreased T cell activation and cytotoxicity, and increased suppressive function in Tregs. In PDAC, upregulation of this receptor is observed in association with upregulation of both PD-1 and CTL-4[57].

The inhibitory receptor TIGIT compete with CD226 to bind the ligands CD112 and CD155 while Tim-3 binds to Galectin-9 and CEACAM1 proteins to inhibit T cell function[58]. Of note, upregulation of Tim-3 in patients with PDAC is correlated with decreased patient survival[57]. Transcription factors involved in the formation of dysfunctional T cells include T-bet, Eomes, Foxo1, Blimp-1, NFAT and IRF-4[53].

A recent study using multiplex immunohistochemistry imaging combined with single-RNA sequencing to evaluate T cell landscape and function in patients with pancreatic cancer, demonstrated that infiltrated $CD8^+$ T cells displayed a senescent phenotype, identified by the expression of $CD57^+CD27^-CD28^-$ or $CD45RA^+$ $CD27^{-/Low}$ $CD28^{-/Low}$ or an exhausted phenotype with elevated expression of $TIGIT^+$ and $CD39^+$ markers alongside PD-$1^{low/intermediate}$ expression[30]. Senescent and exhausted T cells as well as Tregs were also identified within the $CD4^+$ population. Additionally, intra-tumoural Tregs exhibit highly suppressive phenotypes, highlighted by the expression of multiple (TIGIT, ICOS, CD39) inhibitory markers[30].

IMMUNOTHERAPY AND PANCREATIC CANCER

Strategies aiming to leverage the activity of CTLs or the reversal of T cell dysfunction are widespread and have shown clinical success across a variety of cancer[27,29,59]. However, efforts to translate immunotherapy to PDAC, have been met with substantial challenges. The presence of tumour-infiltrating lymphocytes (TILs) with effector and memory functions within the tumour microenvironment and the positive correlation between $CD8^+$ T effector memory cells and patient survival highlight the significance of the T cell immune infiltrate in limiting cancer progression[48]. Hence, the lack of efficacy in existing immunotherapies reflects the challenging non-immunogenic PDAC TME[11,38,48,60].

Chimeric antigen receptor (CAR) T cells or tumour vaccines alone have not demonstrated a survival benefit in PDAC tumours[11,35,48,61]. However, work is ongoing on demonstrating novel targetable antigens or switchable CAR T cells which get activated on reaching the tumour[62,63]. Although most infiltrated $CD8^+$ T cells in the PDAC stroma display features of an exhausted phenotype, demonstrated by cell surface expression of multiple inhibitory receptors, immunotherapy with single-agent immune checkpoint blockade (ICB) has been disappointing. KPC mouse models did not show anti-tumour responses to either CTLA4, PD-1, PDL-1 monotherapy or CTL-4 combined with PD-1/PDL-1 blockade[19,64]. Similarly, human clinical trials using ICB demonstrated insufficient clinical activity and minimal improvement on prognosis, with clinical benefit observed in only highly selected patients[27,39]. Equally, monotherapy with CTLA-4 antibodies and in combination with chemotherapy has not shown ideal clinical activity[59]. Furthermore, exciting avenues for targeting novel

antigens such as CEACAM7 offers hope for CAR-T cell therapy[62,65,66].

The vast majority of trials targeted towards T cells in pancreatic cancer are centred around the use of immune inhibitory receptors against PD-1 and CTLA-4[67]. Most of these trials have enrolled patients with metastatic or borderline resectable pancreatic cancer and assessed the response to either single or double agent immunotherapy or combination therapy with chemotherapy/radiotherapy. The results regarding progression free survival or overall survival have been so far underwhelming[68]. In a meta-analysis on checkpoint inhibitors overall survival and progression-free survival showed no improvement in single agent therapy but a small number of studies on combination therapy have been more promising[69]. It is feasible that the limited tumor mutational burden of pancreatic cancer compared to immunotherapy responsive tumours, such as melanoma or non-small cell lung cancer, may be the key differentiating factor. The phase II KEYNOTE-185 study trying to assess the efficacy of pembrolizumab on patients with non-colorectal microsatellite unstable/mismatch repair deficient cancers enrolled 22 patients with pancreatic cancer, of which four patients showed response to treatment with increase in progression-free survival and median survival[70]. These results, although encouraging, demonstrate that there key barriers around identifying correct groups of patients that would benefit from T cell targeted therapies.

There are various explanations for ICB failure in PDAC tumours including low mutational burden and expression of neoantigens, minimal intra-tumoural infiltration of $CD8^+$ T cells, expression of multiple inhibitory receptors in $CD8^+$ T cells that infiltrate tumours, as well as decreased tumour and myeloid expression cell expression of PDL-1[31,57]. To improve PADC response to ICB, combined approaches have been investigated. Multi-agent immunotherapeutic protocols targeting multiple inhibitory receptors is a promising approach, and has proved more effective than single inhibitory receptor blockade in reversing dysfunctional $CD8^+$ T cells PDAC[27,71]. In the same way, strategies with the goal to prime effector $CD8^+$ T cells to increase their immunogenicity and responsiveness before the use of checkpoint inhibitor treatment represents an exciting opportunity in cancer immunotherapy[12,27,59,72,73]. Combinatory approaches utilising GM-CSF-secreting tumour cells vaccine (GVAX), to induce upregulation of PD-L1 expression into the PDAC TME, prior CTLA-4 and anti-PD-1/PDL-1 blockade has shown promising results in PDAC patients[57,72], and a dual blockade targeting CXCR4 and PD-1 demonstrated improvement in T cell infiltration with a decline in MDSCs[57].

Strategies with the co-stimulatory molecule agonist CD40 used to enhance APC capabilities of macrophages[74] combined with gemcitabine, PD-1 and CTL-4 ICB resulted in increased T-cell priming and infiltration in PDAC tumours[64,72]. Extraction and *in vitro* expansion of TILs from PDAC tumours also have been explored [40] and the results demonstrated autologous T cell killing activity[75,76].

CONCLUSION

The PDAC tumour microenvironment is characterised by complex fibrotic stroma with substantial infiltration of tumour-promoting immunosuppressive cells and pronounced T cell exhaustion, favouring immune evasion that results in immunotherapeutic failures and poor clinical outcome. Therefore, understanding the complexity of PDAC immune landscape and the mechanisms involved in T cell dysfunction may contribute to identifying new immunotherapeutic strategies for treating PDAC and monitoring such response with novel technologies such as ctDNA to assess tumour lysis[77]. As such, unsuccessful immunotherapies could be reversed using combined approaches targeting multiple pathways that obstruct T cell anti-tumour immunity along with other strategies to target stroma[78,79].

A variety of preclinical studies highlighting the influence of PDAC stromal components on T cell anti-tumour responses provided rationale for the development of clinical trials incorporating combined approaches to enhance T cell responses[80]. CXCL12 from cancer-associated fibroblasts synergizes with anti-PD-L1 blockade resulting in activation of T cells and tumour regression in mice[6,81]. Similarly, dual blockade of TGF-β and anti-PD1 resulted in increased T cell responses and tumour regression[82]. Moreover, targeting of myeloid cells with CSF1R in combination with PD-1 or CTLA-4 blockade[83] or focal adhesion kinases inhibitors has been shown to decrease infiltration of suppressive myeloid populations with concomitant activation of T cells, and improved survival in mice models[84].

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World J Gastroenterol 2021 December 14; 27(46): 7969-7981

DOI: 10.3748/wjg.v27.i46.7969

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

MINIREVIEWS

COVID-19 status quo: Emphasis on gastrointestinal and liver manifestations

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Author contributions: Bhurwal A, Minacapelli CD, Orosz E, Gupta K, Tait C, Dalal I, Zhang C, Zhao E and Rustgi VK were involved in data collection, writing the manuscript, and editing the manuscript.

Conflict-of-interest statement: There is no conflict of interest.

Country/Territory of origin: United States

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, B
Grade C (Good): 0

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Abstract

The coronavirus disease 2019 (COVID-19) has caused one of the worst public health crises in modern history. Even though severe acute respiratory syndrome coronavirus 2 primarily affects the respiratory tract, gastrointestinal manifestations are well described in literature. This review will discuss the epidemiology, virology, manifestations, immunosuppressant states, and lessons learned from COVID-19. Observations: At the time of writing, COVID-19 had infected more than 111 million people and caused over 2.5 million deaths worldwide. Multiple medical comorbidities including obesity, pre-existing liver condition and the use of proton pump inhibitor have been described as risk factor for severe COVID-19. COVID-19 most frequently causes diarrhea (12.4%), nausea/vomiting (9%) and elevation in liver enzymes (15%-20%). The current data does not suggest that patients on immunomodulators have a significantly increased risk of mortality from COVID-19. The current guidelines from American Gastroenterological Association and American Association for the Study of Liver Diseases do not recommend pre-emptive changes in patients on immunosuppression if the patients have not been infected with COVID-19. Conclusions and relevance: The COVID-19 pandemic has prompted a change in structure and shape of gastroenterology departmental activities. Endoscopy should be performed only when necessary and with strict protective measures. Online consultations in the form of telehealth services and home drug deliveries have revolutionized the field.

Grade D (Fair): 0
Grade E (Poor): 0

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Received: April 28, 2021
Peer-review started: April 28, 2021
First decision: June 13, 2021
Revised: July 23, 2021
Accepted: November 25, 2021
Article in press: November 25, 2021
Published online: December 14, 2021

P-Reviewer: Amir M, Goenka MK, Xie M
S-Editor: Liu M
L-Editor: A
P-Editor: Liu M



Key Words: COVID-19; Status quo; Epidemiology; Virology; Gastrointestinal manifestations; Liver manifestations; Immunosuppressed states

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Core Tip: The coronavirus disease 2019 (COVID-19) has caused one of the worst public health crises in modern history. Even though severe acute respiratory syndrome coronavirus 2 primarily affects the respiratory tract, gastrointestinal manifestations are well described in literature. This review will discuss the epidemiology, virology, manifestations, immunosuppressant states, and lessons learned from COVID-19.

Citation: Bhurwal A, Minacapelli CD, Orosz E, Gupta K, Tait C, Dalal I, Zhang C, Zhao E, Rustgi VK. COVID-19 status quo: Emphasis on gastrointestinal and liver manifestations. *World J Gastroenterol* 2021; 27(46): 7969-7981
URL: https://www.wjgnet.com/1007-9327/full/v27/i46/7969.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i46.7969

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is considered one of the fastest expanding pandemics since the Spanish flu of 1918, and one of the most impactful public health crises in modern history. As of February 2021, the coronavirus causing COVID-19, also known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), had infected more than 111 million people and caused over 2.5 million deaths worldwide, including 500000 in the United States[1].

It is hypothesized that SARS-CoV-2 originated from animal reservoirs and adapted to human-to-human transmission[2,3]. The first cases of severe pneumonia-like conditions were diagnosed in Wuhan, China at the end of 2019[4]. In February 2020, the international virus classification commission termed the novel coronavirus SARS-CoV-2, and its clinical disease was termed COVID-19 by the World Health Organization (WHO) [5,6]. The case fatality ratio (CFR), defined as the proportion of individuals dying of a disease, was estimated to be up to 3%[7]. Till date, CFR remains the best tool to express the severity of COVID-19 infection among confirmed cases (Table 1). In addition to CFR, many other COVID-19 risk assessment tools have been developed that try to gauge the severity of the novel disease[8] within ethnically diverse populations[9].

SARS-CoV-2 pathogenesis manifests primarily as a respiratory viral syndrome causing symptoms such as cough, fever, general malaise, dyspnea, and respiratory distress, and in a proportion of cases causes severe pulmonary manifestations with respiratory failure and death[10]. SARS-CoV-2 propagates from the respiratory tract to other organs such as the gastrointestinal (GI) tract and liver[10-12].

Due to the availability of the genomic sequence of the viral RNA, scientists and researchers have been able to understand the SARS-CoV-2 virus and develop treatment strategies. This review will discuss the epidemiology, virology, manifestations, immunosuppressant states, and lessons learned from COVID-19.

EPIDEMIOLOGY

The high degree of infectivity of the COVID-19 virus is attributed to its novelty in the human host. It can be can be measured by the basic reproduction number or R0, which is a statistical tool used to describe the contagiousness of a virus. It is estimated that the SARS-CoV-2 R0 is between 2 and 3, signifying that each infected person is likely to spread the infection to 2 to 3 additional people[13,14]. The secondary attack rate characterizes the contagiousness of a virus in the close contact setting, which considers how social behaviors may influence transmissibility[15,16]. Jing *et al*[17] estimated the secondary attack rate of COVID-19 to be 12.4% amongst close relatives and 17.1% amongst those who share the same residential address.

Table 1 Calculated case fatality rate globally and regional according to World Health Organization reports

Region	Cumulative confirmed cases	Cumulative death cases	CFR
Global	111762965	2479678	2.22%
Americas	49700102	1182591	2.38%
Europa	37974729	848644	2.23%
South East Asia	13415064	205814	1.53%
Eastern Mediterranean	6266689	142986	2.28%
Africa	2811106	71159	2.53%
Western Pacific	1594530	28471	1.79%

Estimated calculation with January 24, 2021 Data - World Health Organization Coronavirus (COVID-19) Dashboard, Available from: https://covid19.who.int/. CFR: Case fatality ratio.

There are many medical comorbidities identified as risk factors for increased COVID-19 severity and mortality. In a summary report from China, pre-existing comorbid conditions increase fatality rate by 10.5% for cardiovascular disease, 7.3% for diabetes, 6.3% for chronic respiratory disease, 6.0% for hypertension, and 5.6% for cancer[18]. In a retrospective cohort study of 403 COVID-19 patients from a racially diverse, urban hospital, Rustgi *et al*[9] identified chronic kidney disease, hypertension, congestive heart failure, coronary artery disease, malignancy, dementia, cerebrovascular disease, seizures, and COPD to be associated with increased mortality. The centers for disease control (CDC) lists diabetes and BMI as conditions associated with increased risk of severe illness[19]. In an analysis of nearly 300000 COVID-19 cases in the United States, the mortality rate was 12 times as high among patients with reported comorbidities compared to those with none[20]. Understanding the significance of these risk factors can be vital when triaging and treating patients with COVID-19.

Few GI and liver-specific risk factors have also been identified. In a retrospective study of 2780 COVID-19 patients, Galiero *et al*[21] examined the effect of pre-existing liver disease (including NAFLD, NASH, and cirrhosis) on mortality. Patients with liver disease had a significantly higher risk of mortality. Another gastroenterology-specific risk factor identified is the use of proton pump inhibitors (PPIs). Luxenburger *et al*[22] reported that in hospitalized patients with COVID-19, the use of a PPI significantly increased the risk of developing secondary infection (48.4% *vs* 20.0%, $P \leq 0.001$), ARDS (27.4% *vs* 12.2%, $P = 0.02$), and mortality (19.4% *vs* 5.6%, $P = 0.01$)[22]. One proposed mechanism is that PPI use suppresses gastric acid production leading to increased gastric microbiota which, in turn, can lead to micro-aspiration and subsequent bacterial colonization of the lung[23]. In addition, there is growing evidence that PPIs can also modulate immune responses by inhibiting neutrophil function with anti-inflammatory activity[24].

Age is another notable variable that affects mortality rates with older patients at much higher risk of death. One study in China showed that the mortality rate could be up to 3 times higher in patients who are 80 years or older[18]. Race and ethnicity have also been shown to affect mortality rates, though there is limited literature. Rustgi *et al*[9] showed that White, Blacks, Asians, and Hispanics all have significantly different mortality rates[9]. In a meta-analysis of more than 3 million reported global cases, male patients compared to females had increased odds of ICU admission (OR, 2.84; 95%CI: 2.06-3.92) and mortality (OR,1.39; 95%CI: 1.31-1.47)[25]. COVID-19 mortality rates also vary significantly by country. For example, France had a CFR of 15.2% whereas Korea had a CFR of 2.1%[26]. Unique delivery systems and healthcare infrastructure in these countries play a major role in influencing COVID-19 mortality.

VIROLOGY

SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA virus of the Coronaviridae family[2]. The coronaviruses can infect a wide range of vertebrates, including snakes, bats, pangolins, and humans. Sequence similarities with the bat and pangolin coronavirus virus RaTG13 strains suggest that the SARS-CoV-2 has a

zoonotic origin[2,3,27].

Human infection happens by aerosol droplets or carried on fomites. Upon inhalation, the SARS-CoV-2 enters host respiratory cells *via* the angiotensin-converting enzyme 2 (ACE2) receptor and activating receptors such as the transmembrane protease serine 2 or cathepsin (Figure 1)[27,28]. Viral replication in the infected cells causes immune cells to proliferate and produce large amounts of cytokines and chemokines such as TNF-alpha, interferon-gamma, interleukin 6 (IL-6), IL-8, and IL-10 (Figure 1)[28,29]. This process causes a cascade of inflammatory reactions with toxic damage to the lungs (Figure 2). These mechanisms have also been utilized as targets for therapy. After the initial focus on hydroxychloroquine, emphasis has more recently been on polymerase inhibitors (Remdesivir), binding agents such as convalescent plasma therapy and IL-6 inhibitors such as Tocilizumab[30,31]. Vaccines, such as mRNA-based (Pfizer-BioNTech, Moderna), adenovirus-based (AstraZeneca, Sputnik V, Convidicea, ZF2001), inactivated viral particles (CoronaVac, BBIBP-CorV, Covaxin, CoviVac), non-replicating viral vector (Janssen), and peptide (EpiVacCorona) (Figure 3) are areas of active evolution[30-33]. Adenovirus based intra-nasal COVID vaccines are currently undergoing evaluation *via* clinical trials. These vaccines with different mechanisms of action trigger immune responses and are of great benefit to systematically stop the COVID-19 pandemic[34].

GI symptoms such as diarrhea, nausea, vomiting, or abdominal pain have been reported in approximately 10%-15% of COVID-19 patients before, during or after clinical disease[28,35]. Stool samples from infected patients may test positive for the presence of SARS-CoV-2[36]. In vitro studies have demonstrated that enterocyte organoids may harbor and be capable of supporting SARS-CoV-2 replication[37]. In addition, in-vivo reports indicate that viral RNA is detectable by RT-PCR in biopsies from the esophagus, stomach, duodenum, and rectum[35]. These limited studies suggest that the SARS-CoV-2 virus can actively infect and replicate in the GI tract causing direct organ dysfunction.

The liver may suffer injury in 35%-56% of COVID-19 patients as shown by elevated aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase and/or bilirubin levels[12,38,39]. Interestingly, SARS-CoV-2 RNA sequences have been detected by RT-PCR in liver tissues of infected individuals, suggesting that hepatic injury may be related to direct viral infection[40]. Biopsy findings have demonstrated non-specific inflammatory changes such as hepatocyte swelling and steatosis, mild proliferation of hepatic sinusoid cells, hyperplasia of Kupffer cells and infiltration of lymphocytes[41-43]. SARS-CoV-2 injury in the liver may be mediated by high ACE-2 expression in liver cholangiocytes as well as TROP-2 Liver progenitor cells[41].

Ischemia-perfusion injury has been reported as a complication of COVID-19 in both the GI and liver and has been more frequently observed in those COVID-19 patients admitted to intensive care units[44-46]. This injury may be due to coagulopathy, vasculopathy, hypoxia and shock caused by COVID-19 and thromboembolic events [47-49]. Under these conditions, there is an increase in reactive oxygen species which, in turn, activate transcription factors and initiate the release of various pro-inflammatory factors that lead to tissue damage (Figure 2)[49,50].

GI MANIFESTATIONS

GI symptoms are common in COVID-19[51]. The most commonly reported GI manifestations are diarrhea, nausea, vomiting, and abdominal pain[52]. Loss of appetite and dysgeusia have also been described[53]. Viral particles have been isolated in fecal samples suggesting the possibility of fecal transmission of the virus[54]. A minority of patients with positive stool testing lacked GI symptoms suggesting asymptomatic carriage of disease[55]. These findings highlight the importance of fecal-aerosol-mucosal transmission among individuals exposed to contaminated feces, including public toilets or areas with poor sanitation. This provides a concerning avenue for infectious spread in under-developed regions of the globe, including many regions in Africa and South Asia which lack comprehensive wastewater treatment facilities. Disease control guidelines have emphasized effective management and disinfection of potentially contaminated feces in COVID19 patients, and aggressive vaccination programs in areas at higher risk for fecal-oral spread[43].

Initial case series from China revealed diarrhea as the most prevalent GI symptom, occurring in 2%-36% of cases, followed by nausea (1%-17%), vomiting (1%-6%), and abdominal pain (2%-6%)[56]. As the pandemic has spread, additional reviews and

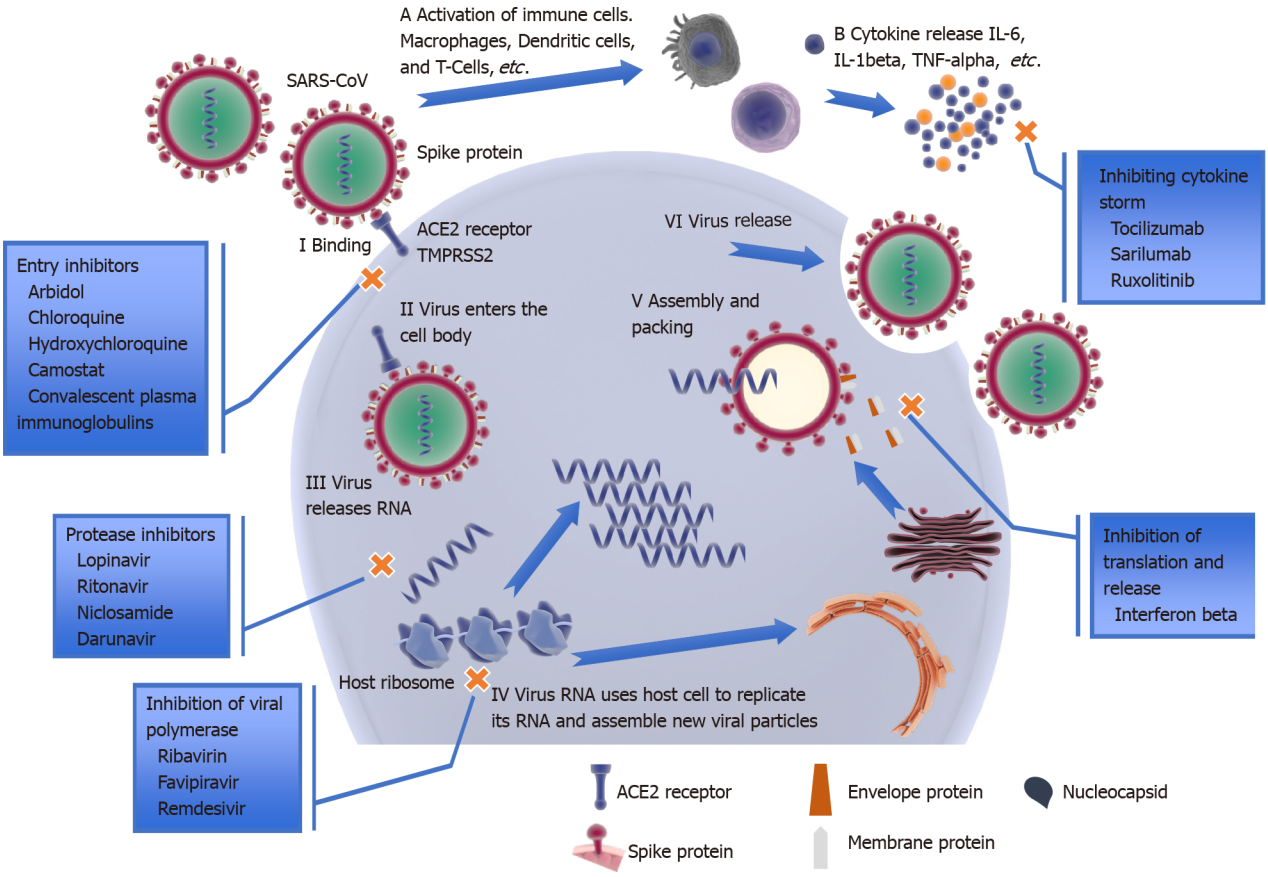


Figure 1 Schematic representation of severe acute respiratory syndrome coronavirus 2 life cycle causing coronavirus disease 2019. Angiotensin-converting enzyme 2 receptors located on the cell surface of ciliated epithelial cells in the respiratory airways, and in type II pneumocytes in the alveoli, bind to virus spike proteins (I). The virus enters the cell body (II) and releases its RNA (III) using host cells to create new virus particles by replication of RNA and translation of polyproteins (IV). New viral particles are assembled (V) and released by exocytosis (VI). Library of Science & Medical Illustrations were utilized in part to create this figure. https://creativecommons.org/licenses/by-nc-sa/4.0/.

meta-analyses have confirmed the prevalence of GI symptoms in other population groups, although much of the reported data remains from cohorts of Chinese patients. A large meta-analysis of 59254 patients predominantly from the Hubei province (75.8), showed that 9% of all patients experience GI symptoms (Table 2)[57]. A more recent meta-analysis by Tariq *et al*[52] focused specifically on GI manifestations of disease, including 12797 patients from 11 countries. Of the patients included, 12.4% reported diarrhea and 9% nausea and/or vomiting. Abdominal pain was also reported in 6.2% of patients. Comparative analysis by patient location revealed a significantly higher proportion of symptoms of diarrhea and nausea/vomiting in the non-China subgroup while loss of appetite was similar between groups.

Anosmia and ageusia are frequently reported with important implications. Ageusia was reported in 20% of patients in one recent review while the rates of anosmia varied greatly across studies from 22%-68%[53]. Anosmia and dysgeusia were more likely to be associated with concurrent nausea or loss of appetite (16.9 *vs* 6.5%, $P = 0.006$).

GI symptoms generally occur with modest frequency compared with respiratory symptoms and fever; however, there are a small number of patients who present with GI symptoms as the only manifestation of disease[58]. Among these patients with isolated symptoms, there is frequently a more delayed hospital presentation compared to respiratory symptoms (9.0 d *vs* 7.3 d)[59]. The American Gastroenterological Association (AGA) has recommended COVID-19 testing in patients with new onset GI symptoms as these may precede pulmonary symptoms[60]. Several studies have attempted to correlate GI symptoms with severity of disease and mortality with mixed results. A recent United States-based case-control series of 150 patients with GI symptoms did not demonstrate increased mortality, intubation, or hospital length of stay compared with controls who lacked GI symptoms[61]. This is in contrast to some initial series from China which linked digestive symptoms to longer length of stay [62]. Further investigation into this area is needed.

Table 2 Gastrointestinal manifestations in coronavirus disease 2019

Ref.	Number of subjects	Diarrhea	Nausea	Vomiting[1]	Abdominal pain
Lin *et al*[35], 2020	95	24%	18%	4%	2%
Wong *et al*[56], 2020	2230	2%-36%	1%-17%	1%-6%	2%-6%
Tariq *et al*[52], 2020	12797	12%	9%[1]	9%[1]	6%

[1]Study presented "nausea and/or vomiting" as one statistic.

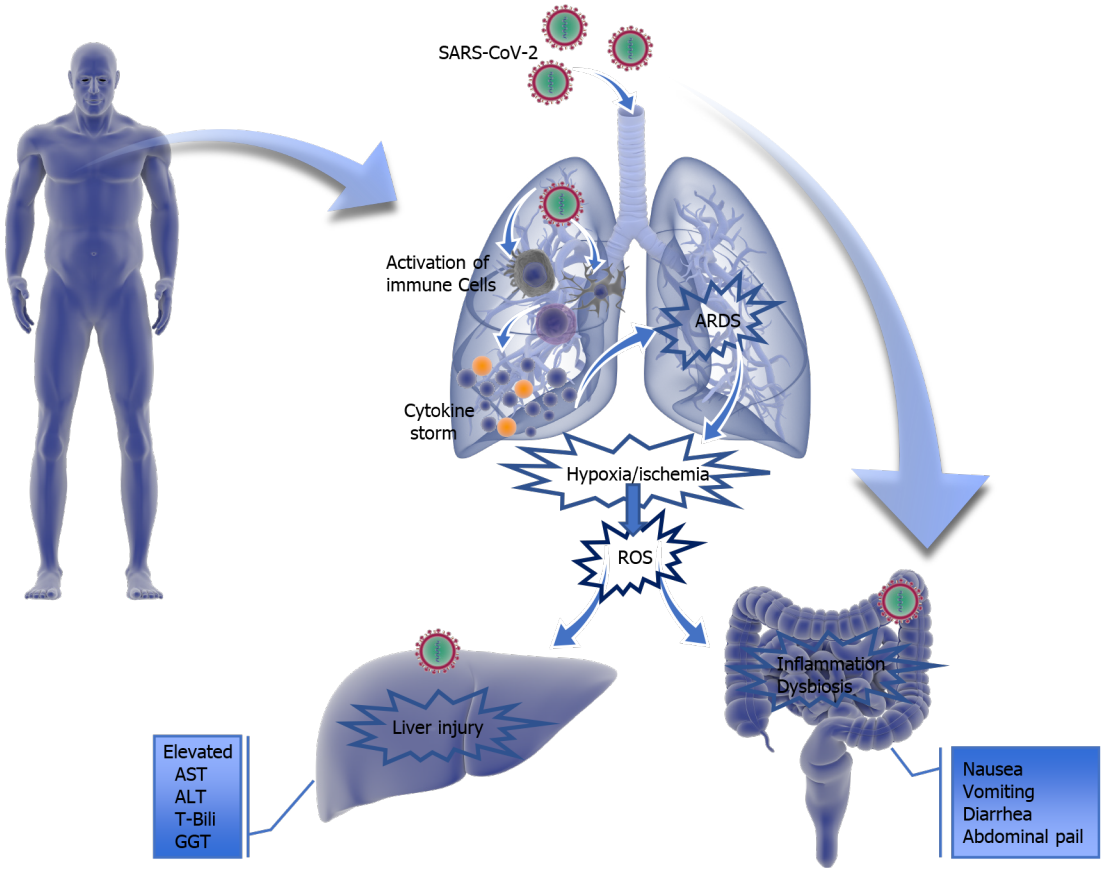


Figure 2 Overview of severe acute respiratory syndrome coronavirus 2 affecting multiple body systems directly or indirectly. Library of Science & Medical Illustrations were utilized in part to create BioNTech this figure. https://creativecommons.org/licenses/by-nc-sa/4.0/.

While critically ill patients still experience many of the same GI symptoms including diarrhea and vomiting, more severe complications noted have included bowel ischemia (3.8%), ileus (55.8%), and Ogilvie-like syndrome (1.9%), as demonstrated in one series of 141 COVID ICU patients(44). A recent review compared GI complications between critically ill patients with COVID and those with non-COVID ARDS and found that the COVID cohort developed more GI complications (74% *vs* 37%, $P < 0.001$)[63]. While many symptoms have been identified, data describing the significance of the GI symptoms in predicting disease course and outcomes has been limited, variable, and sometimes contradictory.

COVID-19 AND LIVER MANIFESTATIONS

COVID-19 infection has been shown to directly affect the liver and cause laboratory abnormalities *via* previously described mechanisms based on abundant ACE2 receptors found on hepatocytes and cholangiocytes to directly enter cells and cause significant liver dysfunction and injury[55,63]. Hospitals in China reported abnormal liver enzymes in approximately 14%-76% of cases hospitalized for COVID-19[64,65].

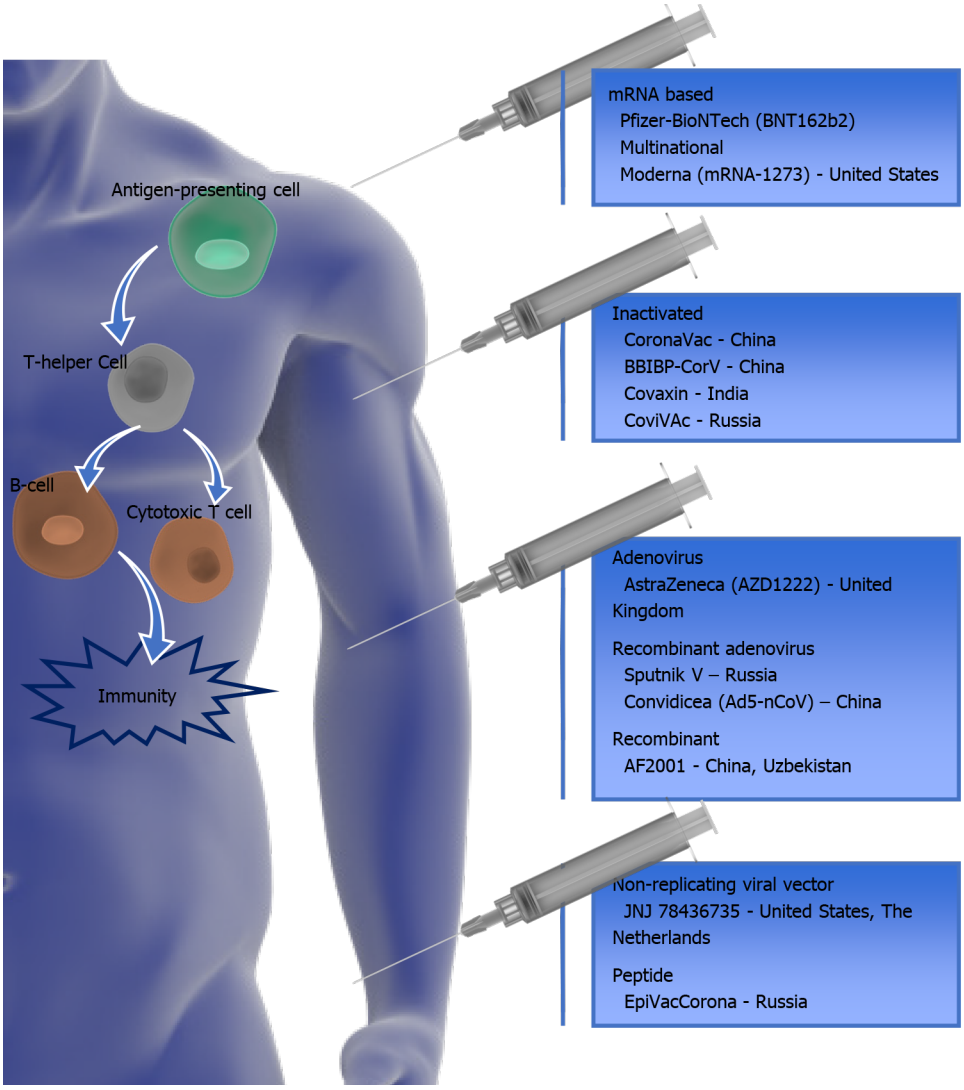


Figure 3 Summary of authorized/approved coronavirus disease 2019 vaccines around the world and their mechanism. The immune response starts by antigen-presenting cells engulfing the virus and activating T-helper cells. These T-helper cells enable an immune response *via* B cells (antibodies) and Cytotoxic T cells to destroy virus-infected cells. Library of Science & Medical Illustrations were utilized in part to create this figure. https://creativecommons.org/licenses/by-nc-sa/4.0/.

According to a systematic review and meta-analysis of 47 studies with 10890 total patients with COVID-19, approximately 15% to 20% were identified to have abnormal liver enzymes with a higher prevalence identified in studies performed outside of China (Table 3)[60].

Hospitalized patients found to have abnormal liver enzymes, may have also shown a higher likelihood of developing severe disease, as well as increased risk of intensive care admission and death[57]. This lack of data has recently prompted the AGA to recommend obtaining baseline liver enzymes and consider monitoring liver enzymes in patients throughout the course of their infection[60].

Treatments for COVID-19 have been associated with elevated liver enzymes and subsequent injury, most notably with remdesivir use. Remdesivir use in early trials and series was associated with 10%-50% of patients developing transient, mild to moderate (< 5 times upper limit or normal) elevations in AST and ALT within 5 da of therapy. Nine percent of patients in reported trials showed at least moderate elevations, but resolved with discontinuation and were not associated with clinically significant injury. Pharmacology guidelines recommend close monitoring of liver enzymes and early discontinuation of infusions if elevations rise > 10 times the upper limit of normal[66,67]. Dexamethasone remains a treatment for severe COVID-19 infection. It should be acknowledged that prolonged use of corticosteroid therapy can cause hepatic steatosis as well as increase the risk of developing reactivation of latent infections, such as viral hepatitis B.

Table 3 Liver manifestations in coronavirus disease 2019

Ref.	Number of subjects	Abnormal LFTs (any)	ALT	AST	Tbili
Lin *et al*[35], 2020	95	-	5%	4%	23%
Wang *et al*[38], 2020	105	56%	16%	9%	2%
Fan *et al*[39], 2020	148	37%	18%	22%	6%
Zhang *et al*[63], 2020	1628	14%-53%	-	-	-
Cai *et al*[65], 2020	417	76%	-	-	-
Sultan *et al*[60], 2020	10890	-	15%	15%	17%

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

COVID-19 AND IMMUNOSUPPRESSED STATES

Corticosteroids, immunomodulators (thiopurines, methotrexate) and biologic therapies (such as anti-TNF agents) are frequently used to manage inflammatory bowel disease (IBD), liver transplant recipients and patients with autoimmune hepatitis[68]. These medications block the intracellular signals necessary for host immunity and are associated with high rates of viral and bacterial infections including pneumonia[69,70]. Therefore, it is plausible that immunosuppressed patients would increase the risk of infection with SARS-CoV-2. However, it is also plausible that these medications reduce mortality in COVID-19 by blocking the cytokine storm of SARS-CoV-2[71]. Hence, how a gastroenterologist should handle immunosuppressive therapies in patients with suspected or confirmed COVID-19 is a challenging clinical question.

The SECURE-IBD (Surveillance Epidemiology of Coronavirus Under Research Exclusion – IBD) database consists of reported cases of COVID-19 in IBD patients[72]. The data suggests that the prevalence of severe COVID-19 is low in patients on immunomodulator therapy and biologic therapy; 25% needed hospitalization on immunomodulator monotherapy whereas 19% needed hospitalization while on anti-TNF agents[73]. Fortunately, mortality rates were low with 2% in the immunomodulator monotherapy group and 1% in the anti-TNF cohort[72]. Thus, the AGA recommends that IBD therapies be continued with a goal of maintaining remission and adjustment being made as necessary[74].

Similar to SECURE-IBD, SECURE-Cirrhosis (Surveillance Epidemiology of Coronavirus Under Research Exclusion - Cirrhosis) is a registry for all COVID-19 cases in patients with chronic liver disease as well as liver transplant recipients[75]. The data from this registry suggests that patients with chronic liver disease but without cirrhosis have a similar risk of mortality from COVID-19 as patients without liver disease. However, patients with cirrhosis have an increased risk with mortality of 32%[75,76]. The data from the registry also suggests that liver transplantation was not associated with an increase in mortality with SARS-CoV-2 infection[77]. The American Association For The Study Of Liver Diseases (AASLD) recommends that anticipatory changes in immunosuppressive regimen should not be made for post-transplant patients and autoimmune patients without COVID-19[78]. In patients on immunosuppression, AASLD recommends lowering the dosages based on the general principles to manage infections in these patients[78].

In summary, the current data does not suggest that patients on immunomodulators have an increased risk of mortality from COVID-19. The current guidelines from AGA and AASLD do not recommend pre-emptive changes in patients on immunosuppression if the patients have not been infected with COVID-19. However, the dosages may be adjusted in patients with COVID-19 on the basis of general principles[74,78].

CONCLUSION

The WHO and CDC have developed ongoing recommendations to be followed during the COVID-19 pandemic. The mechanism of injury and cascade of events due to COVID-19 (Figure 2) have been studied in great detail. These have helped develop targets for therapy and vaccines. The current literature does not reveal that immunosuppressed patients are at higher risk of COVID-19 infection[74,78]. However,

the impact of the COVID-19 vaccine on specific organs such as liver and GI tract are still uncertain. Further research is necessary to evaluate the long-term effects of the vaccine in relation to GI tract. Despite lack of long-term data, patients and physicians are encouraged to get vaccinated as universal vaccination is of great societal and global benefit.

The pandemic has prompted a change in structure and shape of gastroenterology departmental activities. 27% of the centers in the United States and Canada had implemented routine endotracheal intubation for upper endoscopic procedures[79]. The reshaping has been aimed to address urgent and emergent needs of the community and decreasing patient exposures in the hospital. Most of the practices had altered coverage schedule for the physicians. Strict protective measures during endoscopic procedures such as gowns, gloves, face shields, N95 masks, hairnets, double gloves, shoe covers, have also been implemented[79]. The patients are screened at arrival for symptoms and exposures. During the pandemic, only highly urgent endoscopic procedures are being performed on COVID-19 patients[80]. Endoscopy should be performed only when necessary and in a negative pressure flow room for COVID-19 patients if such a room is available. Where a negative pressure flow room for COVID-19 is not possible, strict sanitation measures are recommended[80].

Online consultations in the form of telehealth services and home drug deliveries have been important. Virtual clinics have been started by majority of the institutions as chronic digestive diseases can be managed *via* online consultations. Approximately 96% (70/73) of the practices had adopted telehealth as revealed in the survey of 62 U.S and 11 Canadian Centers in May 2020[80].

The evaluation of inpatients should be judicious to prevent unnecessary exposure of patients, hospital personnel to COVID-19. Clearly, society as well as healthcare delivery will continue to evolve and adapt for this and future crisis.

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World J Gastroenterol 2021 December 14; 27(46): 7982-7994

DOI: 10.3748/wjg.v27.i46.7982

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

ORIGINAL ARTICLE

Basic Study

Recombinant protein *Schistosoma japonicum*-derived molecule attenuates dextran sulfate sodium-induced colitis by inhibiting miRNA-217-5p to alleviate apoptosis

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Author contributions: Sun X contributed to the experiment design; Wu ZD, Yang RB, and Chen F contributed to the experiment implementation; Liu JH and Hu YY analyzed the data; Zhang LC and Wang LF conceived the experiments, performed the experiments, and wrote the manuscript; all authors participated in critical revision of the manuscript and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the Sun Yat-sen University Institutional Review Board.(Approval No. SYSU-IACUC-2019-B517).

Conflict-of-interest statement: The authors declare no competing interests.

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Abstract

BACKGROUND

Inflammatory bowel disease (IBD) affects millions of people worldwide and has emerged as a growing problem in industrialized nations. The lack of therapeutic targets has limited the treatment of IBD. Studies found that parasitic nematode infections can ameliorate clinical and experimental colitis. Our previous study found that rSj16, a 16-kDa secreted protein of *Schistosoma japonicum* produced by *Escherichia coli*, has protective effects on dextran sulfate sodium (DSS)-induced colitis in mice. Apoptosis is an important factor in the pathogenesis of colitis. However, it is not clear whether the effect of rSj16 on colitis is related to apoptosis.

AIM

To investigate whether the protective effects of rSj16 on colitis is related to apoptosis and its mechanism.

METHODS

In-vivo, colitis was induced by DSS. The severity of colitis was assessed. WB was used to detect the changes of apoptosis-related genes in colon tissues. Q-PCR was

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: I have read the ARRIVE Guidelines, and the manuscript was prepared and revised according to the ARRIVE Guidelines.

Supported by the National Natural Science Foundation of China, No. 81902081 and No. 81871682; the Natural Science Foundation of Guangdong Province, No. 2020A1515011573 and No. 2019A1515012068; China Postdoctoral Science Foundation, No. 2018M640858 and No. 2019T120771; Fundamental Research Funds for the Central Universities, No. 19ykpy170, No. 17ykpy09 and No. 19ykpy29; National Science and Technology Major Project, No. 2018ZX10101002-001; the 111 Project, No. B12003; and the Natural Science Foundation of Guangdong Province, No. 2021A1515010976.

Country/Territory of origin: China

Specialty type: Medicine, research and experimental

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, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

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used to detect the changes of miRNA-217-5p and HNF1B. *In-vitro*, WB was used to detect the changes of apoptosis-related genes in intestinal epithelial cells. TUNNEL staining and flow cytometry were used to detect cell apoptosis.

RESULTS

rSj16 attenuates clinical activity in DSS-induced colitis mice. TUNNEL staining and WB results showed that apoptosis was increased in colon tissue after treatment with DSS, and the apoptosis of colon tissue was significantly reduced after treatment with rSj16. Compared with normal mice, the expression of miR-217-5p was increased in colon tissue of DSS-induced colitis mice. In addition, the miR-217-5p target gene *hnf1b* was decreased after administration of DSS. After treatment with rSj16, the expression of miR-217-5p was decreased and the expression of HNF1B was increased compared with the DSS-treated group. When Etoposide was used in combination with miR-217-5p mimic on MODE-K cells, the expression of cleaved-Caspase-3 and Bax was increased, and Bcl-2 was decreased compared with only Etoposide treatment, the expression of HNF1B was significantly reduced, suggesting that miR-217-5p acts as a pro-apoptotic in colon epithelial cells and down-regulates the target gene *hnf1b*. After rSj16 administration in MODE-K cells, miR-217-5p expression was significantly decreased, HNF1B expression was increased, and apoptosis was reduced.

CONCLUSION

The protective effects of rSj16 on colitis is related to apoptosis and miRNA-217-5p may be a further target for therapeutic intervention against IBD.

Key Words: *Schistosoma japonicum*; rSj16; Inflammatory bowel disease; Apoptosis; miRNA-217-5p

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Core Tip: The lack of therapeutic targets has limited the treatment of inflammatory bowel disease (IBD). Parasitic nematode infections can ameliorate clinical and experimental colitis. Our previous study found that rSj16, a 16-kDa secreted protein of *Schistosoma japonicum* produced by Escherichia coli, has protective effects on dextran sulfate sodium (DSS)-induced colitis in mice. We found that rSj16 can inhibit DSS-induced apoptosis in the colons of mice with colitis. In addition, we found that the inhibitory effect of rSj16 on apoptosis was associated with decreased miR-217-5p, and that hepatocyte nuclear factor 1 beta was increased after treatment with rSj16. These results highlight a novel therapeutic target that may be used to treat IBD.

Citation: Zhang LC, Wu XY, Yang RB, Chen F, Liu JH, Hu YY, Wu ZD, Wang LF, Sun X. Recombinant protein *Schistosoma japonicum*-derived molecule attenuates dextran sulfate sodium-induced colitis by inhibiting miRNA-217-5p to alleviate apoptosis. *World J Gastroenterol* 2021; 27(46): 7982-7994
URL: https://www.wjgnet.com/1007-9327/full/v27/i46/7982.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i46.7982

INTRODUCTION

Inflammatory bowel disease (IBD) affects millions of people worldwide and has emerged as a growing problem in industrialized nations[1]. The two distinct forms of IBD, ulcerative colitis and Crohn's disease, are characterized by intermittent, chronic or progressive inflammation[2]. The etiologies of both forms are multifactorial, including immunoregulatory factors, genetic susceptibility, environmental changes, and abnormalities of gut microbiota. Traditional treatments for IBD include 5-aminosalicylic acid agents, steroids, and antimicrobials. However, as these drugs have limitations and many patients cannot achieve remission, a research focus in this field is to devise biological therapies for the treatment of IBD.

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Received: June 16, 2021
Peer-review started: June 16, 2021
First decision: June 30, 2021
Revised: July 9, 2021
Accepted: November 29, 2021
Article in press: November 29, 2021
Published online: December 14, 2021

P-Reviewer: Prasetyo EP, Saber A, Yang X
S-Editor: Wang LL
L-Editor: A
P-Editor: Wang LL

Recent studies have demonstrated that helminth *Schistosoma* can protect against IBD. In a DSS-induced mouse colitis model, an attenuated inflammatory response was found in those infected with *Schistosoma japonicum* (*S. japonicum*)[3]. *Schistosoma mansoni* (*S. mansoni*) egg antigen has a beneficial modulatory effect in a DSS-induced mice colitis model[4]. In adult male mice with colitis, *S. mansoni* infection modulates the colitis mice immune system, suppressing colitis and limiting dysbiosis of intestinal microbiome[5]. Infection with *S. mansoni* also attenuates disease in rats with trinitrobenzene sulfonic acid (TNBS)-induced colitis[6]. Our previous study confirmed that exosomes derived from dendritic cells treated with *S. japonicum* soluble egg antigen attenuate DSS-induced colitis in mice[7]. Furthermore, we have also shown that rSj16 has protective effects on DSS-induced mouse colitis[8].

Apoptosis is an important factor in the pathogenesis of colitis. Abnormal apoptosis of intestinal epithelial cells (IECs) is frequently found in IBD[9,10]. IEC apoptosis results in disruption of intestinal barrier integrity, and may allow the infiltration of bacteria, triggering an inflammatory cascade[11]. Aberrant IEC apoptosis stimulates the production of tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ), both of which further induce IEC apoptosis[12]. MicroRNAs are critical post-transcriptional regulators of gene expression and key mediators of pathophysiology of inflammatory bowel disease (IBD)[13]. However, the molecular basis of IEC apoptosis in the pathogenesis of IBD remains unclear.

MicroRNAs (miRNAs) are small non-coding RNAs that are about 22 nucleotides long. MiRNAs negatively regulate gene expression at the post-transcriptional or translational level by complementary binding to the 3′-untranslated region(UTR). MiRNAs control genes involved in cellular processes such as inflammation, cell-cycle regulation, stress response, differentiation, apoptosis, and migration[14,15]. Studies have shown that miRNAs play an important role in IBD. For example, miR-301a promotes intestinal mucosal inflammation by inducing IL-17A and TNF-α in IBD[16]. MiR-31 is increased in colon tissues of patients with IBD, reduces inflammatory signaling and promotes colon regeneration[17]. Myeloid-derived miR-223 Limits intestinal inflammation by constraining the nlrp3 inflammasome[18]. Upregulation of miR-665 promotes apoptosis and colitis in inflammatory bowel disease by repressing the endoplasmic reticulum stress components XBP1 and ORMDL3[19]. In Shamran's study, miR-217 may induce Sirt-1 and provide protection against intestinal inflammation[20]. The hepatocyte nuclear factor (HNF) superfamily of transcription factors is essential for the development and maintenance of a variety of humans and mice tissues, and is further classified into four families, HNF1, FOXA, HNF4, and ONECUT, based on their functional domains. In gut, HNFs are expressed in IECs, which regulate a variety of physiological functions, including differentiation, barrier function, and metabolism[21]. Hepatic nuclear factor-4α (HNF4α) mRNA level was also downregulated in mouse model of ileitis (SAMP) compared with control mice[22]. Hepatocyte nuclear factor-1beta (HNF1B) is the most important liver-specific transcription factor, with responsibility for sequence-specific DNA binding. HNF1B is reportedly a target of miR-217, with a role in circ-TTBK2- and miR-217-mediated modulation of malignant glioma progression[23].

In this study, we investigate whether the protective effects of rSj16 on colitis is related to apoptosis and its mechanism. miRNA may function through regulating the expression of encoding genes in IBD[16]. We explore the relationship between rSj16, miR-217-5p and IBD, providing theoretical support for the clinical application of rSj16 in the treatment of IBD.

MATERIALS AND METHODS

Animals and ethics

Male BALB/c mice (aged 6 wk, 18-20 g) were purchased from the Experimental Animal Center of Guangdong. All animal experimental procedures were approved by the Medical Research Ethics Committee of Sun Yat-sen University (SYSU-IACUC-2019-B517) and conformed to the Chinese National Institute of Health Guide for the Care and Use of Laboratory Animals.

Induction and treatment of colitis

Recombinant protein (rSj16) was expressed and purified as described previously[8]. A total of 15 mice were randomly assigned to three groups. Acute colitis was induced by administering water with 3% (wt/vol) DSS (36–50 kDa; MP Biomedicals, Illkirch, France) to mice over a period of 7 d. The control mice (n = 5) received drinking water.

Over the same period, rSj16 was administered to the colitis mice (n = 5) *via* intraperitoneal (i.p.) injection (100 μg per mouse) on each day from 1 to 7. Control groups (n = 5) received the same volume of vehicle (phosphate buffered saline; PBS) over the same time frame. The mice were fed standard mouse chow.

Clinical scoring

During treatment, mice were observed daily. Changes in body weight, occurrence of diarrhea and bleeding were recorded. Blood in the feces was determined using a Hemoccult assay kit (Nanjing Jiancheng Bio-engineering Institute, China). A clinical disease score (disease activity index, DAI) was evaluated based on weight loss, diarrhea, and bleeding as described previously[8].

Macroscopic assessment and histologic analysis

Mice were sacrificed on day 7. Colon length was measured, and the macroscopic scores of colons were assessed by an independent observer who was blinded to treatment status[7]. The colons were fixed in 4% formaldehyde and then embedded in paraffin. Colon sections were prepared and stained with hematoxylin and eosin (H & E). Histopathological scores were determined in a blinded fashion, according to the criteria described in our previous study.

Cell culture and treatment

Mouse intestinal epithelial cell line, MODE-K cells were purchased from the BeNa Culture Collection (BNCC, China). The cells were cultured with Dulbecco's modified eagle medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.), 100 IU/mL penicillin and 100 mg/mL streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.), incubated in a 5% CO_2 environment at 37 °C. The cells were seeded one day prior to transfection in 12-well cell culture plates. Cells were incubated in a serum-free medium for starvation overnight, then stimulated with miRNA mimic (Assay ID: MIMAT0000679) or mimic control (50 nM, Ruibo, Guangzhou, China) using RNAi MAX (Invitrogen, United States). MiRNA mimics are miRNAs that mimic endogenous miRNAs and can be synthesized by chemical synthesis to enhance the function of endogenous miRNAs.

Flow cytometry

MODE-K cells were seeded in 6-well plates and treated with mimic control, miRNA mimic, miRNA mimic + Etoposide (MedChemExpress, United States, 25 μM)[24], and miRNA mimic + Etoposide + rSj16 (4 μg/mL) for 48 h. Adherent and floating cells were collected and resuspended in 100 μl binding buffer. Each group of cells was stained with 2 μl Annexin-V FITC and propidium iodide (PI, BD Biosciences) at room temperature for 15 min. Samples were analyzed using a CytoFLEX S flow cytometer (Beckman Coulter, United States).

Western blot

MODE-K cells were homogenized with a protein extraction reagent buffer (RIPA; Beyotime Institute of Biotechnology) containing protease and phosphatase inhibitors. Protein concentration was measured using the bicinchoninic acid assay (Beyotime Institute of Biotechnology, China). Equal amounts of proteins were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride blotting membrane (GE Healthcare Life Sciences, United Kingdom). The membrane was blocked by 10% milk. The membrane was incubated with a primary antibody (proteintech cleaved-Caspase3, Cat No. 19677-1-AP; Bax, Cat No. 50599-2-Ig; Bcl-2, Cat No. 12789-1-AP; HNF1B, Cat No. 12533-1-AP; GAPDH Sigma-Aldrich G9295) diluted 1:1,000 overnight at 4 °C, followed by incubation with the secondary antibody (ProteinTech Group, Inc.; anti-mouse, cat. no. SA00001-1; anti-rabbit, cat. no. SA00001-2) diluted 1:2000 at room temperature for 2 h. Immunodetection was performed using enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.) and visualized using chemiluminescence gel imaging system (Tanon-5200 Multi, Shanghai China). ImageJ (×64) software (National Institutes of Health) was used to quantify the results[25].

TUNEL staining method

MODE-K cells treated with mimic control, miRNA mimic, miRNA mimic + Etoposide, and miRNA mimic + Etoposide + rSj16 were inoculated into 24-well plates for 48 h then fixed with 4% paraformaldehyde for 15 min at room temperature. Diluted TUNEL staining fluid (Beyotime Institute of Biotechnology) was added to cells and colon histological sections according to the manufacturer's instructions. PBS was used

to wash cells, followed by DNA staining with DAPI at room temperature for 10 min, and the staining was observed using a Leica DMI4000B fluorescence microscope (magnification, ×10), positive cells were quantified by Image J software.

RNA extraction and quantitative reverse-transcription PCR

Total RNA was harvested using TRIzol according to manufacturer's instructions, including MODE-K cells and 50 mg mouse colon tissue samples. Complementary DNA (cDNA) was synthesized from 1.0 μg of total RNA with oligo (dT) primers using a cDNA Synthesis Kit (Takara, Japan). The expression of mRNA and miRNA was determined using a SYBR Green Master Mix kit (Takara, Japan), primer sequences are shown in Table 1. GAPDH or U6 were used as an internal control, and the fold change was calculated by the $2^{-\Delta\Delta CT}$ method.

Dual-luciferase reporter assay

HEK 293T cells were transfected using the HNF1B UTR reporter plasmid together with miR-217-5p mimic or control mimic for 48 h using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc). Following this period, cells were lysed using the Dual-Glo® Reagent (Dual-Glo® Luciferase Assay System; Promega Corporation). Renilla luciferase assay substrate and firefly luciferase detection reagent were added and luciferase activities were detected using the Infinite F500 Multimarker Analyser (TECAN, Austria) according to the manufacturer's instructions. Renilla luciferase was used as an internal reference, with luciferase activity normalized to Renilla luciferase activity[26].

Statistical analysis

All data are expressed as means ± SD. Results were compared between the two groups using an unpaired two-sample *t*-test. Multiple comparisons between more than two groups were analysed by one-way ANOVA test or Kruskal–Wallis test (non-parametric). The value of $P < 0.05$ was considered statistically significant.

RESULTS

rSj16 protects against acute DSS-induced colitis

As found in our previous study[8], after DSS administration, the mice lost weight over time. At the same time, the DAI of mice with colitis also increased with time. After treatment with rSj16, body weight loss and DAI were both significantly alleviated in mice with colitis (Figure 1A and B). Colon length was significantly reduced by application of DSS, and was restored after rSj16 treatment (Figure 1C and D). Mean colon macroscopic scores were significantly suppressed in DSS + rSj16 group compared with DSS + PBS group (Figure 1E). Additionally, H&E histopathology results showed that treatment with rSj16 significantly reduced inflammation (Figure 1F). Consistent with this, histopathological scores after treatment with rSj16 + DSS were significantly lower than after treatment with DSS + PBS (Figure 1G and Table 2).

rSj16 inhibit DSS induced apoptosis of colon epithelial cells

IEC apoptosis is increased in affected areas of IBD[27], leading to the disruption of intestinal barrier integrity that may allow bacteria to penetrate into the intestinal wall from the intestinal cavity and trigger an inflammatory cascade, including the production of pro-inflammatory cytokines, to remove the invading bacteria[28,29]. In the present study, to investigate the mechanism by which rSj16 alleviates DSS-induced acute colitis, 3% DSS was administered to mice daily for 7 d, and western blot was performed to detect the apoptosis of colon epithelial cells in mice. As shown in Figure 2A, the pro-apoptotic protein cleaved-Caspase-3 and Bax were increased, while the anti-apoptotic protein Bcl-2 was decreased after treatment with DSS + PBS compared with the Water + PBS, indicating that DSS can induce apoptosis of colon epithelial cells. In addition, pro-apoptotic Bax was decreased and anti-apoptotic protein Bcl-2 was increased after treatment with rSj16 + DSS compared with DSS + PBS. These results demonstrate that rSj16 may significantly inhibit DSS-induced colon epithelial cells apoptosis (Figure 2A). TUNEL staining, indicating apoptosis, was increased in colon tissue and the number of TUNEL positive cells decreased significantly after administration of rSj16 (Figure 2B).

Table 1 Quantitative real time PCR primer sequences

Gene	Forward (5'-3')	Reverse (5'-3')
hnf1b	CCCATCCTCAAAGAGCTCCA	AGAGGTGGGATTGGTTCAGG
GAPDH(Mouse)	ACTCCACTCACGGCAAATTC	TCTCCATGGTGGTGAAGACA
miR-217-5p	UACUGCAUCAGGAACUGACUGGA	mRQ3' Primer (Takara, Kyoto, Japan)
U6	Takara, Kyoto, Japan	Takara, Kyoto, Japan

Table 2 Values of the evaluation indexes

	Body weight loss on day 7 (%) (mean ± SD)	DAI on day 7 (mean ± SD)	Colon length (mean ± SD)	Macroscopic scores (mean ± SD)	Histopathological scores (mean ± SD)	*n*
Water + PBS	125.30 ± 6.30	0.00 ± 0.00	9.70 ± 0.56	0.00 ± 0.00	0.00 ± 0.00	5
DSS + PBS	89.11 ± 8.02	5.20 ± 0.45	6.58 ± 0.48	7.8 ± 1.30	13.00 ± 2.55	5
DSS + rSj16	106.00 ± 5.97	1.40 ± 0.89	8.12 ± 0.35	3.20 ± 0.84	4.4 ± 1.14	5

DSS: Dextran sulfate sodium.

rSj16 inhibits the expression of miR-217-5p in the colon of mice with DSS-induced colitis

Research has shown that down-regulation of miR-217-5p may reduce the apoptosis of cardiomyocyte derived cell lines[30]. We found that, compared with Water-treated mice, the expression of miR-217-5p was increased in colon tissue of mice with DSS-induced colitis. In addition, the miR-217-5p target gene *hnf1b* was decreased after administration of DSS. After treatment with rSj16, the expression of miR-217-5p was decreased and the expression of *hnf1b* was increased compared with the DSS-treated group (Figure 3A and B). Pearson's correlation coefficient analysis showed a negative correlation between miR-217-5p and HNF1B in in colon tissue of mice ($r = -0.3463$, $P < 0.05$) (Figure 3C). Western blot results also indicated that HNF1B was decreased after DSS treatment, and was increased after rSj16 treatment compared with DSS-treated group (Figure 3D and E).

In order to verify whether miR-217-5p regulates the expression of HNF1B, we generated a luciferase reporter plasmid contained 3'- UTR of HNF1B and located on both sides of the binding site of miR-217-5p. Relative luciferase activity of the reporter containing the predicted miR-217-5p binding sites for 3'UTR of HNF1B mRNA transcript was significantly reduced when co-transfected with miR-217-5p mimic compared with a control mimic (Figure 3F). Studies have shown that miRNA-217-5p is closely related to apoptosis[24,31]. Etoposide (an apoptosis inducer) was used to induce the apoptosis of MODE-K, and qPCR results showed that increased *miRNA-217-5p* expression, and decreased miR-217-5p target gene *hnf1b* expression in the process of apoptosis. However, rSj16 may inhibit the expression of miR-217-5p, and increase the expression of *hnf1b* (Figure 3G and H).

rSj16 anti-apoptotic action via regulation of miR-217-5p/HNF1B axis

We further verified the role of miR-217-5p in the process of apoptosis, and the mechanism of rSj16 in regulating apoptosis. Western blot showed that when Etoposide was used in combination with miR-217-5p mimic on MODE-K cells, the expression of cleaved-Caspase-3 and Bax was increased, and Bcl-2 was decreased compared with only Etoposide treatment, and the expression of HNF1B was significantly reduced. These results indicate that miR-217-5p acts as a pro-apoptotic in colon epithelial cells and down-regulates the target gene *hnf1b*. In addition, the expression of cleaved-Caspase-3 and Bax was decreased, while Bcl-2 and HNF1B were increased in mice treated with Etoposide + miR-217-5p + rSj16 compared with Etoposide + miR-217-5p (Figure 4A and B). TUNEL staining of MODE-K after treatment of Etoposide, Etoposide + miR-217-5p, and Etoposide + miR-217-5p + rSj16, showed that the number of TUNEL positive cells increased with Etoposide + miR-217-5p and decreased after

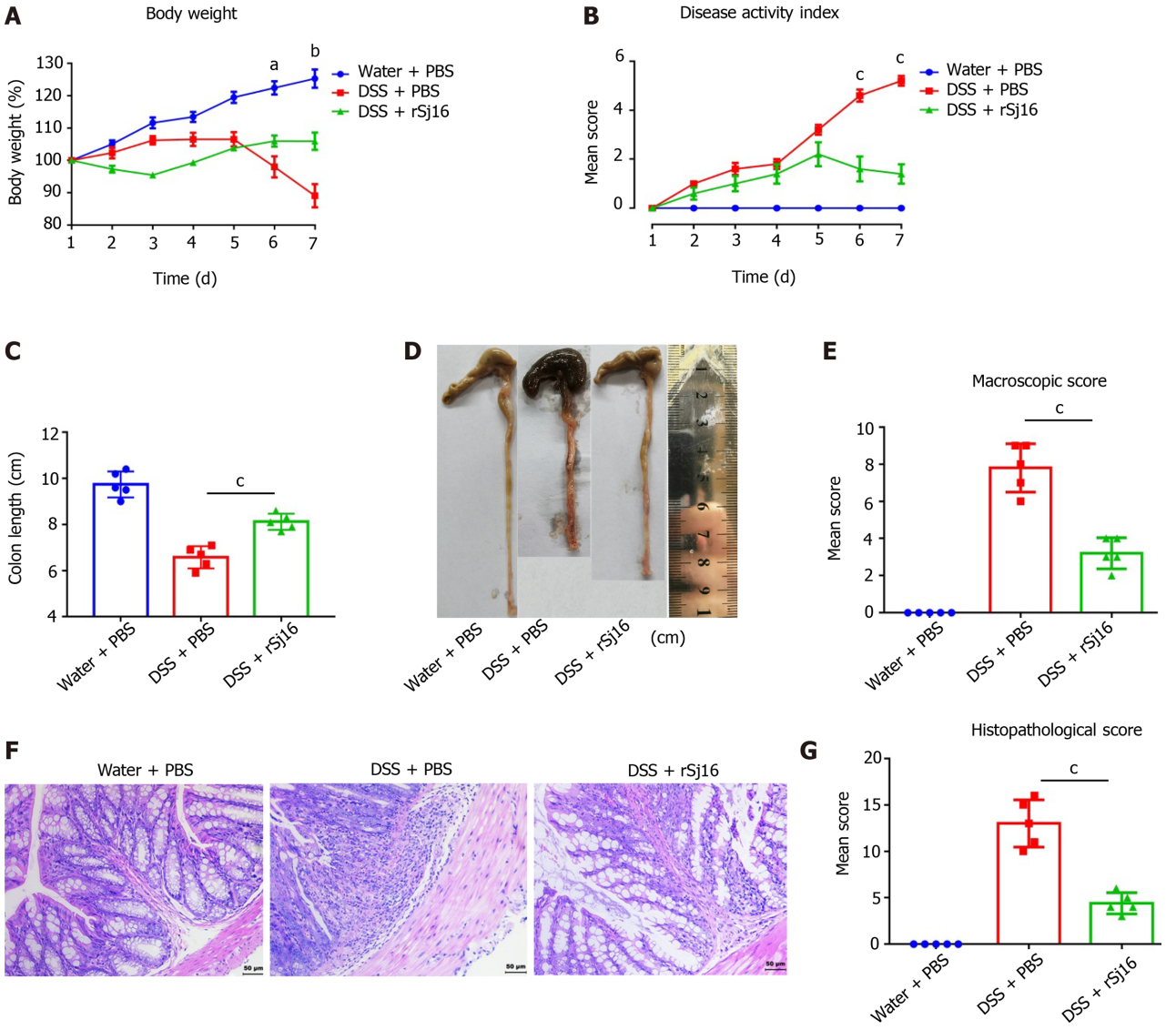


Figure 1 rSj16 protects against acute dextran sulfate sodium-induced colitis. A: Daily changes in body weight [dextran sulfate sodium (DSS) + rSj16 *vs* DSS + PBS]; B: Changes in DAI (DSS + rSj16 *vs* DSS + PBS); C and D: Colon lengths were measured and recorded; E: Macroscopic appearance of the colons; F: The histopathological changes in the colons were examined by H&E staining (20×); G: Histopathological scores of the colons were determined. DSS: Dextran Sulfate Sodium Salt. Statistical analysis was performed using one-way ANOVA. Data are presented as means ± SD; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

treatment with rSj16 (Figure 4C and D). Flow cytometry results also showed that miR-217-5p could obviously promote MODE -K apoptosis. However, rSj16 could significantly inhibit MODE -K apoptosis induced by Etoposide and miR-217-5p. (Figure 4E and F).

DISCUSSION

IBD encompasses Crohn's disease, ulcerative colitis and IBD-unclassified. Although newer treatments have increased the chances of remission, most IBD patients cannot maintain remission, and death is not an infrequent outcome of IBD[32,33]. Therefore, it is very important to explore the pathogenesis of IBD and to find effective therapeutic targets. We have found that rSj16 (a 16-kDa secreted protein of *Schistosoma japonicum*) has protective effects on DSS-induced mouse colitis. Body weight loss was alleviated in mice with colitis after treatment with rSj16. DAI (evaluated based on weight loss, diarrhea, and bleeding) also alleviated in colitis mice after treatment with rSj16. The results of colon length, mean colon macroscopic scores (assessed by hyperemia, wall thickening, ulceration, inflammation extension, and damage), H&E, and histopathological scores (based on extent of inflammation, neutrophil and lympho-histiocyte infiltration, crypt damage, crypt abscess formation, sub-mucosal edema, goblet cell

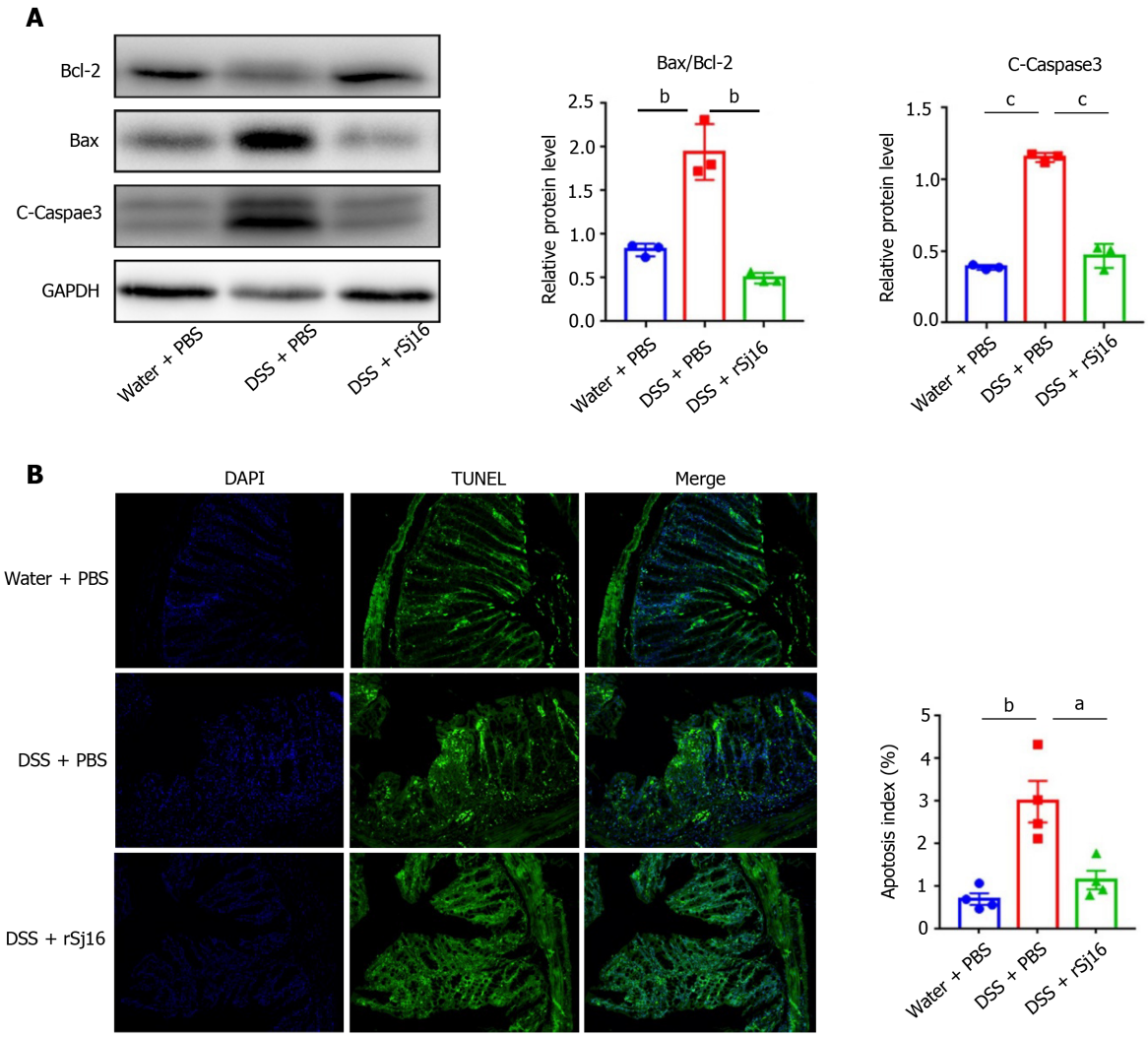


Figure 2 rSj16 inhibits dextran sulfate sodium induced apoptosis of colon epithelial cells. A: Western blot analysis for the expression of apoptosis relative proteins, including Bcl-2, Bax and cleaved-Caspase3; B: The apoptosis of colon tissue of mice treated with dextran sulfate sodium (DSS) + PBS and DSS + rSj16 was detected by TUNEL assay (20×), TUNEL positive cells were apoptotic cells, the number of TUNEL positive cells was quantified. TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling. Statistical analysis was performed using one-way ANOVA (A) and Kruskal–Wallis test (non-parametric) (B). Data are presented as means ± SD; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

loss, and reactive epithelial hyperplasia displayed) indicate that rSj16 protects against acute DSS-induced colitis.

Apoptosis is an important factor in the pathogenesis of colitis. DSS has been shown to initially cause damage in the colon by inhibition of proliferation and induction of apoptosis[34]. In the present study, we found significant apoptosis of colon epithelial cells after DSS administration in mice, and inhibition of the DSS-induced apoptosis after administration of rSj16. Therefore, we hypothesized that rSj16 alleviates DSS-induced colitis, in part by regulating apoptosis.

In recent years, miRNAs have become the key biomarkers and novel therapeutic targets in IBD[16,35]. MiR-217-5p plays dual roles in regulating cell survival and apoptosis. Flum *et al* reported that miR-217-5p could induce apoptosis by regulating multiple target genes involved in the ERK-MAPK signaling pathway including PRKCI, BAG3, ITGAV, and MAPK1[24]. Gao *et al* indicated that upregulation of miR-217-5p significantly inhibited TGF-β1-induced proliferation, migration, extracellular matrix (ECM) deposition, and promoted apoptosis in airway smooth muscle cells[36]. However, Yi *et al* indicated that upregulation of miR-217-5p improved cell viability and attenuated cell apoptosis in SH-SY5Y cells subjected to oxygen–glucose deprivation/reperfusion[37]. The specific regulatory mechanism between miR-217-5p and apoptosis needs to be further studied. In our study, miRNA miR-217-5p was expressed at a high level in IBD mice colon tissues, and was decreased significantly following treatment with rSj16. After inducing MODE-K apoptosis, miR-217-5p expression was significantly increased, after rSj16 treatment, miR-217-5p expression

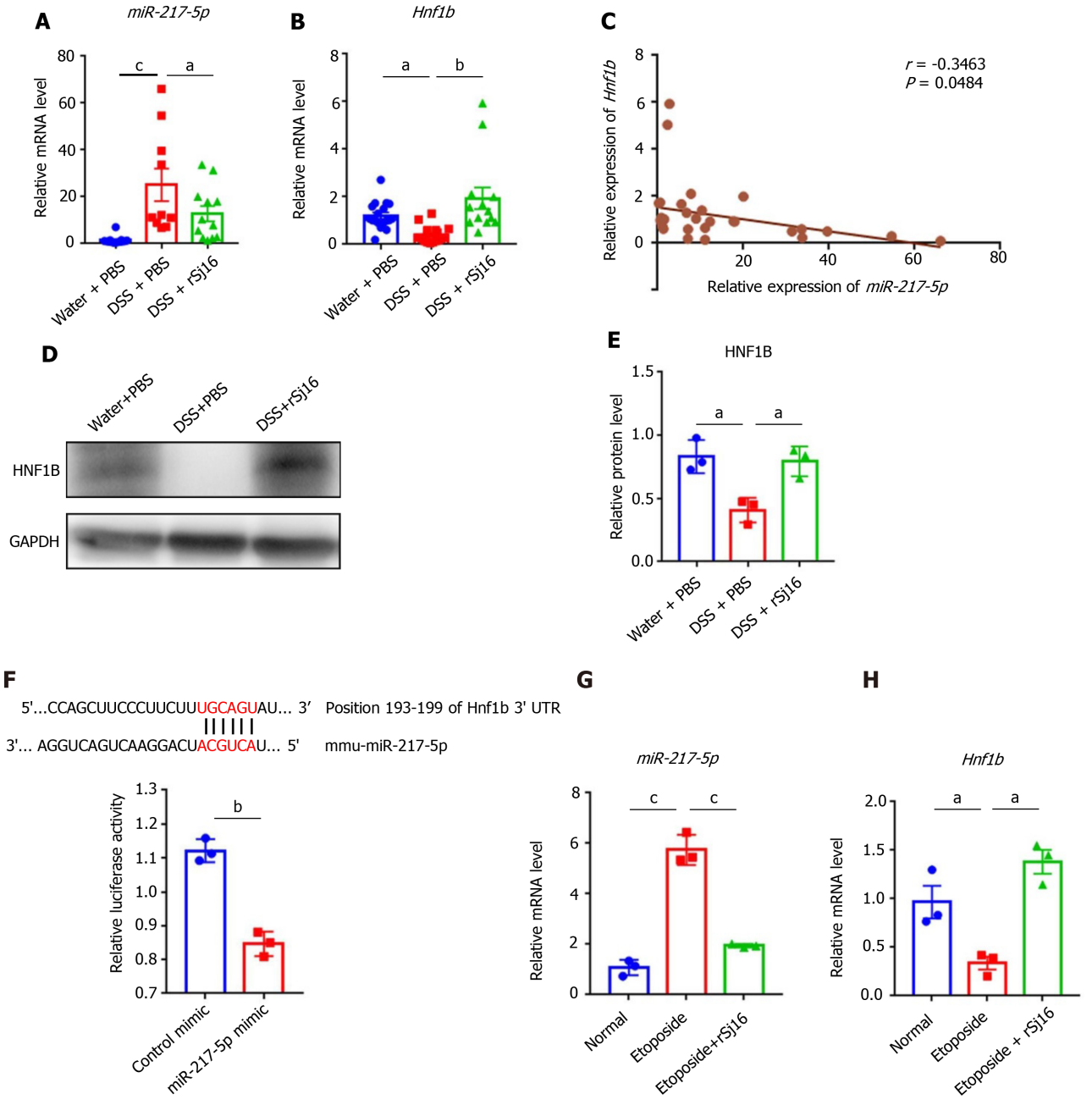


Figure 3 rSj16 can inhibit the expression of miR-217-5p in the colon of mice with dextran sulfate sodium-induced inflammatory bowel disease. A and B: Relative RNA expression of miR-217-5p and *hnf1b* in colon tissue of mice; data were normalized to levels detected in colon tissue of mice after treatment with Water and PBS (control) group; C: Pearson's correlation coefficient analysis showed a negative correlation between miR-217-5p and HNF1B in colon tissue of mice (r = -0.3463, P < 0.05); D and E: Western blot was used to detect the expression of HNF1B in protein levels; F: The wild-type HNF1B -3'- untranslated region (UTR) was cloned into psi-CHECK-2 to predict the binding site of miR-217-5p in the 3'-UTR of *hnf1b* gene. Dual-luciferase reporter assay was performed on HEK 293Tcells transfected with HNF1B UTR reporter plasmid together with miR-217-5p mimic or control mimic; G and H□MODE-K cells were treated with Etoposide or Etoposide + rSj16. The expression of *miR-217-5p* and *hnf1b* were determined using quantitative PCR. HNF1B: Hepatic nuclear factor-1beta. Statistical analysis was performed using one-way ANOVA (B and E) and Kruskal–Wallis test (non-parametric) (A), and unpaired two-sample *t*-test (F, G and H). Data are presented as means ± SD; ^{a}P < 0.05, ^{b}P < 0.01, ^{c}P < 0.001.

was significantly reduced. Therefore, we hypothesized that miR-217-5p is involved in the protective effects of rSj16 on colitis. Bcl-2, caspase-3, and Bax play key roles in cell apoptosis[38]. Caspase-3 is a marker of apoptosis because its activity is required for major apoptosis-related morphological and biochemical events, and its activation and function are regulated by the Bcl-2 family of proteins, among other molecules[39]. In the present study, after overexpression of miR-217-5p in MODE-K cells, cleaved-Caspase-3 and Bax expression were increased, but Bcl-2 was reduced, suggesting that miR-217-5p plays a pro-apoptotic role in MODE-K cells. After rSj16 treatment, the miR-217-5p, cleaved- Caspase-3 and Bax expression were decreased, but Bcl-2 was

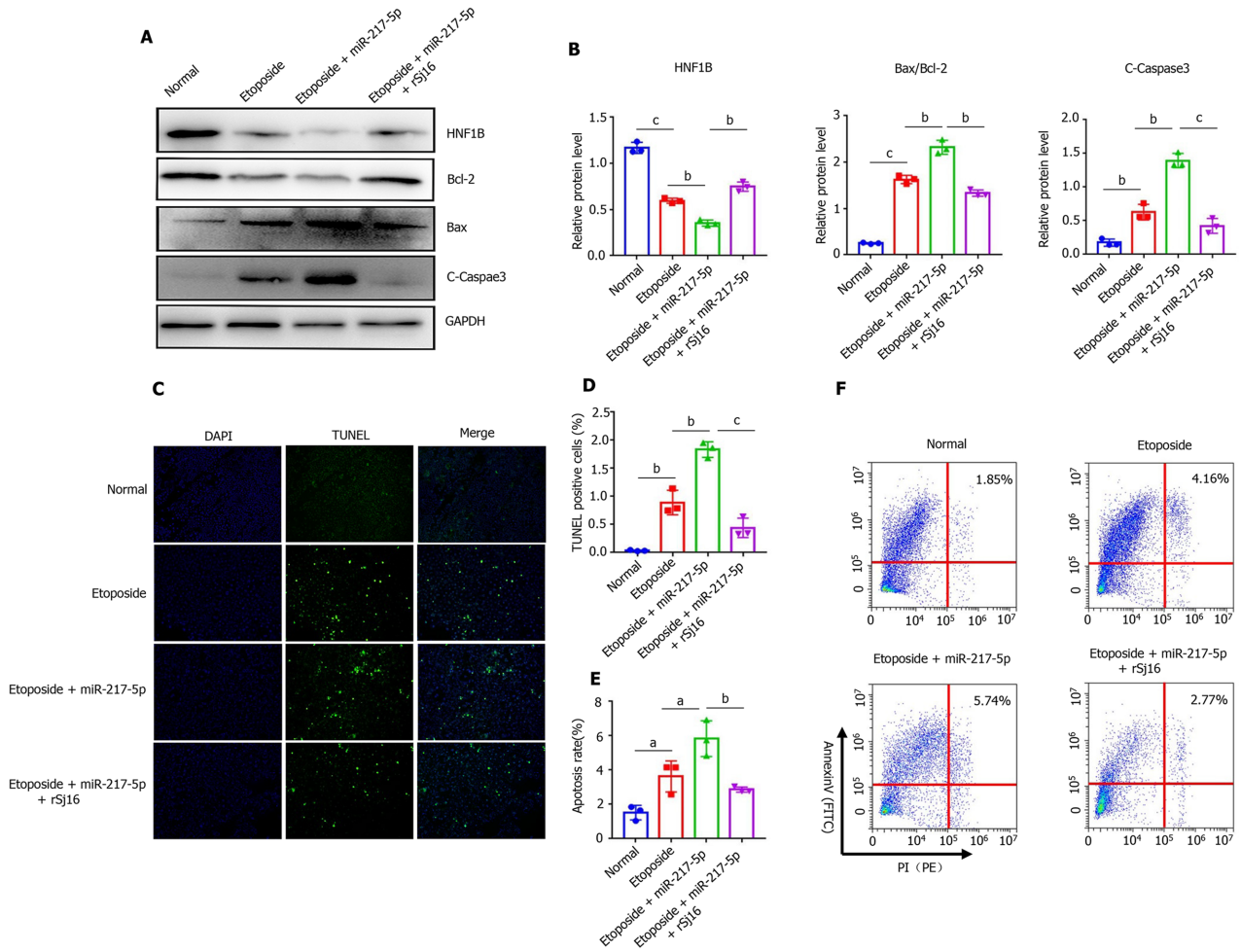


Figure 4 rSj16 have anti-apoptotic action by regulating the miR-217-5p/HNF1B axis. A and B: MODE-K cells were treated with Etoposide, Etoposide + miR-217-5p, and Etoposide + miR-217-5p + rSj16. The expression of apoptosis relative proteins, including Bcl-2, Bax and cleaved-Caspase3 was analyzed by Western blotting; C and D: The apoptosis of MODE-K cells was detected by TUNEL assay after treatment with Etoposide + miR-217-5p, and Etoposide + miR-217-5p + rSj16 (10×), TUNEL positive cells were apoptotic cells, the number of TUNEL positive cells was quantified; E and F: Flow cytometry analysis of MODE-K cells treated with Etoposide + miR-217-5p, and Etoposide + miR-217-5p + rSj16. Statistical analysis was performed using one-way ANOVA. PI: propidium iodide. Data are presented as means ± SD; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

increased, indicating that rSj16 could reduce the apoptosis. Results showed that miR-217-5p aggravated MODE-K cells apoptosis and rSj16 could significantly inhibit the apoptosis by inhibiting miRNA-217-5p expression.

MiRNAs exert pro-apoptotic functions by regulating the expression of target genes [40]. *hnf1b* acts as an oncogene in various tumors, is overexpressed in human prostate cancer and could promote tumor cell proliferation[41]. Early deletion of HNF1B results in a decrease in the number of pancreatic multipotent progenitor cells due to reduced proliferation and increased apoptosis[42]. In our study, we found that *hnf1b* is the direct target gene of miR-217-5p. In the present study, we found that DSS may induce apoptosis of colon epithelial cells, with increased expression of miR-217-5p and decreased expression of its target gene *hnf1b*. We speculated that miR-217-5p/HNF1B was involved in DSS-induced apoptosis of colon epithelial cells. Subsequently, we induced apoptosis and overexpression of miR-217-5p in MODE-K cells. After treatment with rSj16, the expression of miR-217-5p in tissues and MODE-K cells was decreased, the expression of its target gene *hnf1b* was increased, and the apoptosis of MODE-K cells significantly reduced. The results suggested that miR-217-5p exerted pro-apoptotic functions by regulating expression of the target gene *hnf1b*.

As for the limitations of the study, because rSj16 affects the progress of the disease through multiple pathways, we only explore one of them, suggesting that miR-217-5p/HNF1B axis could be used as a potential target for the treatment of enteritis. In addition, rSj16 may attenuate IBD through other pathways which we didn't make a comprehensive exposition, it is still worth exploring. Next, we will conduct a more comprehensive study on the treatment of IBD with rSj16, to provide more possibilities for the development of colitis drugs.

CONCLUSION

In conclusion, rSj16 attenuates IBD in mice by regulating the miR-217-5p/ HNF1B axis to reduce colon epithelial cell apoptosis. These data indicated that miR-217-5p and HNF1B may be potential biomarkers to improve the accuracy of IBD diagnosis and treatment, and that rSj16 may have potential for clinical drug development.

ARTICLE HIGHLIGHTS

Research background

IBD encompasses Crohn's disease, ulcerative colitis and IBD-unclassified. Although newer treatments have increased the chances of remission, most IBD patients cannot maintain remission, and death is not an infrequent outcome of IBD. Therefore, it is very important to explore the pathogenesis of IBD and to find effective therapeutic targets.

Research motivation

Exploring the pathogenesis of IBD and to find effective therapeutic targets.

Research objectives

To apoptosis and its mechanism.

Research methods

In-vivo, after DSS inducing colitis. The severity of colitis was assessed. WB was used to detect the changes of apoptosis-related genes in colon tissues. *In-vitro*, WB, Qpcr and tunel was used to detect the changes of apoptosis.

Research results

rSj16 attenuates clinical activity in DSS-induced colitis mice. rSj16 could reduce the expression of miR-217-5p in MODE-K cells. rSj16 could regulate the apoptosis of MODE-K cells.

Research conclusions

rSj16 attenuates IBD in mice by regulating the miR-217-5p/ HNF1B axis.

Research perspectives

miR-217-5p and HNF1B may be potential biomarkers to improve the accuracy of IBD diagnosis and treatment, and that rSj16 may have potential for clinical drug development.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 December 14; 27(46): 7995-8009

DOI: 10.3748/wjg.v27.i46.7995

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

ORIGINAL ARTICLE

Retrospective Cohort Study

Digestive system involvement and clinical outcomes among COVID-19 patients: A retrospective cohort study from Qatar

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Abstract

BACKGROUND

Coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 virus most commonly presents with respiratory symptoms. While gastrointestinal (GI) manifestations either at presentation or during hospitalization are also common, their impact on clinical outcomes is

Institutional review board statement: The study was approved by the Medical Research Center of Hamad Medical Corporation (MRC-01-20-631).

Informed consent statement: Due to the retrospective design of the study, the requirement of informed consent was waived by the Institutional Review Board.

Conflict-of-interest statement: All authors and study participants declare no potential conflicting interests related to this paper.

Data sharing statement: The authors confirm that the data supporting the findings of this study are available within the article.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

Supported by Hamad Medical Corporation, No. MRC-01-20-631.

Country/Territory of origin: Qatar

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

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controversial. Some studies have described worse outcomes in COVID-19 patients with GI symptoms, while others have shown either no association or a protective effect. There is a need for consistent standards to describe GI symptoms in COVID-19 patients and to assess their effect on clinical outcomes, including mortality and disease severity.

AIM

To investigate the prevalence of GI symptoms in hospitalized COVID-19 patients and their correlation with disease severity and clinical outcomes.

METHODS

We retrospectively reviewed 601 consecutive adult COVID-19 patients requiring hospitalization between May 1-15, 2020. GI symptoms were recorded at admission and during hospitalization. Demographic, clinical, laboratory, and treatment data were retrieved. Clinical outcomes included all-cause mortality, disease severity at presentation, need for intensive care unit (ICU) admission, development of acute respiratory distress syndrome, and need for mechanical ventilation. Multivariate logistic regression model was used to identify independent predictors of the adverse outcomes.

RESULTS

The prevalence of any GI symptom at admission was 27.1% and during hospitalization was 19.8%. The most common symptoms were nausea (98 patients), diarrhea (76 patients), vomiting (73 patients), and epigastric pain or discomfort (69 patients). There was no difference in the mortality between the two groups (6.21% *vs* 5.5%, $P = 0.7$). Patients with GI symptoms were more likely to have severe disease at presentation (33.13% *vs* 22.5%, $P < 0.001$) and prolonged hospital stay (15 d *vs* 14 d, $P = 0.04$). There was no difference in other clinical outcomes, including ICU admission, development of acute respiratory distress syndrome, or need for mechanical ventilation. Drugs associated with the development of GI symptoms during hospitalization were ribavirin (diarrhea 26.37% $P < 0.001$, anorexia 17.58%, $P = 0.02$), hydroxychloroquine (vomiting 28.52%, $P = 0.009$) and lopinavir/ritonavir (nausea 32.65% $P = 0.049$, vomiting 31.47% $P = 0.004$, and epigastric pain 12.65% $P = 0.048$). In the multivariate regression analysis, age > 65 years was associated with increased mortality risk [odds ratio (OR) 7.53, confidence interval (CI): 3.09-18.29, $P < 0.001$], ICU admission (OR: 1.79, CI: 1.13-2.83, $P = 0.012$), and need for mechanical ventilation (OR: 1.89, CI:1.94-2.99, $P = 0.007$). Hypertension was an independent risk factor for ICU admission (OR: 1.82, CI:1.17-2.84, $P = 0.008$) and need for mechanical ventilation (OR: 1.66, CI: 1.05-2.62, $P = 0.028$).

CONCLUSION

Patients with GI symptoms are more likely to have severe disease at presentation; however, mortality and disease progression is not different between the two groups.

Key Words: COVID-19; Gastrointestinal manifestations; Mortality; Intensive care unit admission; Mechanical ventilation; Disease severity

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Core Tip: There is a high prevalence of gastrointestinal symptoms in coronavirus disease 2019 patients both at presentation and during hospitalization. Drugs are associated with the development of gastrointestinal symptoms during hospitalization. The presence of gastrointestinal symptoms in coronavirus disease 2019 patients is associated with disease severity at presentation but is not a predictor of mortality or disease progression.

Citation: Khan MU, Mushtaq K, Alsoub DH, Iqbal P, Ata F, Chaudhry HS, Iqbal F, Balaraju G, Maslamani MAA, Varughese B, Singh R, Ejji KA, Kaabi SA, Kamel YM, Butt AA. Digestive

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Received: April 28, 2021
Peer-review started: April 28, 2021
First decision: June 13, 2021
Revised: June 29, 2021
Accepted: November 28, 2021
Article in press: November 28, 2021
Published online: December 14, 2021

P-Reviewer: Karna ST
S-Editor: Liu M
L-Editor: Filipodia
P-Editor: Liu M



system involvement and clinical outcomes among COVID-19 patients: A retrospective cohort study from Qatar. *World J Gastroenterol* 2021; 27(46): 7995-8009
URL: https://www.wjgnet.com/1007-9327/full/v27/i46/7995.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i46.7995

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) infection presents most commonly as a respiratory illness with symptoms including fever, cough, and shortness of breath. Disease severity ranges from mild disease requiring no intervention to severe illness requiring intensive care unit (ICU) admission and mechanical ventilation[1,2]. While most studies have focused on respiratory manifestations of coronavirus disease 2019 (COVID-19), extra-respiratory manifestations have also been described, including gastrointestinal (GI) symptoms and liver enzyme abnormalities[3,4]. The prevalence of GI symptoms in COVID-19 patients ranges from 7% to 15%[5,6]. Fecal shedding of SARS-CoV-2 has been reported in 40.5%-48.1% of the patients[7,8]. The significant variation in the proportion of patients with GI symptoms among different studies might be related to geographical region[5] and whether symptoms were reported on admission or during hospitalization[3,9].

Diarrhea, nausea, vomiting, and abdominal pain are the most frequently reported GI symptoms[5,8]. The association between GI symptoms and adverse outcomes in patients with SARS-CoV-2 infection is controversial[8,10-13]. Some studies have shown an inverse correlation between GI symptoms and adverse outcomes, including mortality[10,11,14], while others have shown a direct correlation of GI symptoms with disease severity and adverse outcomes[8,12]. Still others, including a recent meta-analysis, have shown that GI symptoms bear no association with adverse outcomes or mortality[13,15]. Studies are needed with consistent standards for describing GI symptoms and distinguishing between GI symptoms on admission *vs* symptoms that develop during the hospital stay to determine whether GI symptoms have correlation to disease severity.

The aim of our study was to evaluate the prevalence of GI symptoms in COVID-19 patients at admission and during hospitalization and their association with adverse outcomes, including mortality.

MATERIALS AND METHODS

Study design and participants

We conducted a retrospective cohort study investigating the epidemiological and clinical characteristics and outcomes among consecutive adult patients with SARS-CoV-2 infection who were admitted to one of the dedicated COVID-19 hospitals in the state of Qatar between May 1-15, 2020. SARS-CoV-2 infection was diagnosed by real-time polymerase chain reaction assays Cobas SARS-CoV-2 Test (Roche Diagnostics, Rotkreuz, Switzerland) or TaqPath COVID-19 Combo Kit (Thermo Fisher Scientific, Waltham, MA, United States) on nasopharyngeal and throat swabs.

The severity of COVID-19 was defined according to the World Health Organization guidelines and categorized into five groups[16] (see Supplementary material, Appendix). Demographic, clinical, laboratory, treatment, and outcome data were retrieved from the electronic medical records. These included a complete blood count, renal function, electrolytes, coagulation profile, liver function tests, and other biochemical markers including creatine kinase, lactate dehydrogenase, C-reactive protein, troponin-T, serum lipase, amylase, procalcitonin, and ferritin. Microbiological investigations, including blood, respiratory, fecal, and urine cultures, were reviewed. Radiologic assessments included chest radiography on admission and subsequent chest computed tomography or abdomen ultrasound according to the patient's clinical care needs. X-ray findings were recorded from the medical records and by examining the films.

Outcomes and definitions

The primary outcome was all-cause mortality during the hospitalization. Secondary outcomes included disease severity at admission, disease progression defined by admission to the ICU, development of acute respiratory distress syndrome, and need

for mechanical ventilation. Other outcomes included the development of septic shock and length of hospital stay. All outcomes were compared between those with and without GI symptoms at admission.

GI symptoms were defined by the presence of at least one of the following symptoms: nausea, vomiting, diarrhea, epigastric pain or discomfort, acid reflux, anorexia, or GI bleeding. GI symptoms were recorded on admission and during hospitalization to determine the influence of medical therapy and other external factors. Diarrhea was defined as the passing of loose stools > three times per day with a negative stool culture for routine bacterial pathogens. Diarrhea that developed during hospitalization was recorded only after recording negative stool culture and absence of *Clostridium difficile* infection. Liver enzyme abnormalities were classified into normal, borderline (< 2 × upper limit of normal), mild impairment (2–5 × elevation), moderate (5–10 × elevation), and severe (> 10 × upper limit of normal).

Travel history in the 3 mo before the presentation and exposure to a confirmed case were recorded. Acute respiratory distress syndrome and shock were defined per the World Health Organization guidelines for COVID-19.

Study oversight

The study was approved by the Medical Research Center of Hamad Medical Corporation (MRC-01-20-631). Due to the retrospective design of the study, the requirement of informed consent was waived, and Institutional Review Board exemption was granted.

Statistical analysis

We summarized continuous variables using mean (standard deviation) and median (interquartile range) for normal and non-normally distributed data, respectively. Categorical variables were expressed as number (%) and compared using the Pearson's χ^2 test or Fisher's exact test, as indicated. Univariate logistic regression analysis was used to identify the risk factors for adverse outcomes. All variables with a P value of < 0.10 from univariate analysis were included in a multivariate logistic regression model with the forward method to identify independent predictors of the adverse outcomes. No adjustment for multiple testing was performed. A two-sided P value of < 0.05 was considered statistically significant. Statistical analyses were performed using Stata Statistical Software Stata/IC 16.1 (StataCorp LLC, College Station, TX, United States).

RESULTS

Demographic and epidemiological characteristics

We identified 601 adult patients hospitalized with confirmed SARS-CoV-2 infection during the study period. The mean age was 46.20 ± 13.66 years, and 85.4% were males. The clinical characteristics at presentation are shown in Table 1. Fever (79.7%), cough (75.7%), and shortness of breath (50.2%) were the most common presenting symptoms. Overall, 163 (27.1%) had at least one GI symptom at presentation. Patients without GI symptoms were more likely to have a cough (77.8% *vs* 68.7%, $P = 0.02$), while patients with GI symptoms had more fatigue (48.0% *vs* 19.0%, $P < 0.001$) and myalgias (38.7% *vs* 27.0%, $P = 0.007$). The patients with GI symptoms had a significantly longer duration of symptoms before the presentation (4.81 ± 2.51 *vs* 4.04 ± 2.51, $P = 0.002$) compared to patients without GI symptoms. There was no difference between the two groups regarding exposure to a sick contact, family clustering, and travel outside the country.

Patients with GI symptoms were more likely to have underlying chronic liver disease (1.2% *vs* 0.0%, $P < 0.001$), malignancy (2.5% *vs* 1.8%, $P < 0.001$), and immunosuppression (4.3% *vs* 3.0%, $P < 0.001$) but were less likely to have chronic lung conditions (4.9% *vs* 6.1%, $P < 0.001$). Severe disease at presentation was more frequent in the patients with GI symptoms compared with those without GI symptoms (33.1% *vs* 22.5%, $P < 0.001$). There was no statistically significant difference in the rest of the general demographics or other epidemiological parameters between the two groups. Of those 163 (27.1%) patients reporting at least one GI symptom at admission, the most common symptoms were nausea (98 patients), diarrhea (76 patients), vomiting (73 patients), and epigastric pain or discomfort (69 patients) (Table 2).

Laboratory and radiological abnormalities

The laboratory parameters of the study participants are presented in Table 3. Alanine

Table 1 Demographic and epidemiological characteristics and presenting symptoms of coronavirus disease 2019 patients with and without gastrointestinal symptoms, *n* (%)

Characteristics	Overall (*n* = 601)	With GI (*n* = 163)	Without GI (*n* = 438)	*P* value
Age (yr)	46.20 13.66	46.45 13.76	46.12 13.64	0.728
Sex, male	513 (85.4)	136 (83.4)	377 (85.9)	0.515
BMI (kg/m^2)	27.55 (24.90-31.00)	27.49 (24.40-30.60)	27.55 (24.90-31.00)	0.811
Duration of symptoms (d)	4.24 2.53	4.81 2.51	4.04 2.51	0.002[a]
Nationality				0.765
Qatar	67 (11.1)	17 (25.4)	50 (74.6)	
India	127 (21.1)	27 (21.3)	100 (78.7)	
Nepal	89 (14.8)	23 (25.8)	66 (74.2)	
Bangladesh	105 (17.5)	33 (31.4)	72 (68.6)	
Pakistan	48 (8.0)	14 (29.2)	43 (70.8)	
Philippines	56 (9.3)	18 (32.1)	38 (67.9)	
Arab countries	60 (9.9)	16 (26.7)	44 (73.3)	
Others	49 (8.15)	15 (30.6)	34 (69.4)	
Presenting symptoms				
Fever	478 (79.7)	130 (79.7)	348 (79.1)	0.147
Cough	455 (75.7)	112 (68.7)	343 (77.8)	0.016[a]
Sputum	75 (12.5)	23 (14.1)	52 (11.8)	0.483
Shortness of breath	303 (50.2)	84 (51.5)	219 (49.7)	0.412
Sore throat	146 (24.3)	37 (22.7)	109 (24.7)	0.747
Nasal obstruction	51 (8.5)	18 (11.0)	33 (7.5)	0.359
Fatigue	163 (27.1)	79 (48.5)	84 (19.1)	< 0.001[a]
Myalgia	182 (30.3)	63 (38.7)	119 (27.0)	0.007[a]
Anosmia	303 (50.2)	86 (52.8)	217 (49.5)	0.424
Exposure history				
Smoking	62 (10.1)	24 (14.9)	38 (8.7)	0.199
Ex-smoker	7 (1.2)	3 (1.9)	4 (0.9)	
Alcohol	44 (7.3)	9 (5.6)	35 (8.3)	0.28
Travel history	43 (7.2)	9 (5.6)	34 (7.8)	0.456
Sick contact	142 (23.6)	34 (20.9)	108 (24.6)	0.533
Family cluster	27 (4.6)	10 (6.3)	17 (3.4)	0.477
Pre-existing conditions				
No. of comorbid conditions				0.39
0	261 (43.4)	72 (43.6)	189 (43.0)	
1-2	203 (33.8)	53 (32.1)	150 (32.2)	
> 2	137 (22.8)	38 (24.9)	99 (22.1)	
Diabetes mellitus	242 (40.3)	72 (44.2)	170 (38.6)	0.327
Hypertension	209 (34.6)	52 (31.9)	157 (35.6)	0.536
Coronary artery disease	46 (7.7)	6 (3.7)	40 (9.1)	0.622
Chronic kidney disease	47 (7.8)	12 (7.4)	35 (8.0)	0.882
Chronic liver disease	6 (1.0)	2 (1.2)	4 (0.9)	< 0.001[a]

Malignancy	12 (2.0)	4 (2.5)	8 (1.8)	< 0.001[a]
Lung disease	35 (5.8)	8 (4.9)	27 (6.1)	< 0.001[a]
Immunosuppression	20 (3.3)	7 (4.3)	13 (3.0)	< 0.001[a]
Disease severity at admission				0.0024[a]
Asymptomatic	48 (8.0)	2 (1.2)	46 (10.5)	
Mild	109 (18.1)	32 (19.6)	77 (17.7)	
Moderate	291 (48.4)	75 (46.0)	216 (49.1)	
Severe	70 (11.6)	26 (15.9)	44 (10.0)	
Critical	83 (13.8)	28 (17.2)	55 (12.5)	
Severe–non severe	153(25.5)	54 (33.1)	99 (22.5)	< 0.001[a]

[a]Statistically significant *P* value.
BMI: Body mass index; GI: Gastrointestinal.

Table 2 Frequency of gastrointestinal symptoms in coronavirus disease 2019 patients at admission and during hospital stay, *n* (%)

GI symptoms at admission (*n* = 601)	Frequency	During hospital stay (*n* = 438)	Frequency
Diarrhea	76 (12.6)	Diarrhea	42 (7.0)
Nausea	98 (16.2)	Nausea	94 (15.6)
Vomiting	73 (12.1)	Vomiting	87 (14.5)
Epigastric pain	69 (11.4)	Epigastric pain	22 (3.6)
GERD	6 (1.0)	GERD	2 (0.3)
Anorexia	66 (10.9)	Anorexia	26 (4.3)
GI bleeding	4 (0.7)	GI bleeding	6 (1.0)
Any GI symptoms	163 (27.1)	Any GI symptoms	119 (19.8)
Any nausea, vomiting, diarrhea	140 (19.0)		

GI: Gastrointestinal; GERD: Gastro-esophageal reflux disease.

aminotransferase levels were significantly higher in patients with GI symptoms (P = 0.04). Overall, the proportion of patients with any liver test abnormality was higher in patients with GI symptoms, but the difference did not approach statistical significance.

All but 2 patients had a chest x-ray at presentation. A normal chest x-ray was observed in 23.5% of the patients, and bilateral lung infiltrates (54.6%) were the most common radiological abnormality at presentation.

Treatment and outcomes

The drug treatment received by the patients is summarized in Table 4. Hydroxychloroquine (HCQ) (90.45%) and azithromycin (90.12%) were the most common drugs administered to the patients followed by broad-spectrum antibiotics other than azithromycin (82.60%). The drug treatment did not differ between the two groups except for broad-spectrum antibiotics, with patients having GI symptoms receiving more antibiotics (92.6% *vs* 78.9%, P = 0.000). We also divided the treatment regimen into six groups based on the different combinations of administered drugs. Group 1 containing hydroxychloroquine plus azithromycin was the most frequently administered treatment combination. COVID-19 patients with GI symptoms received significantly more treatment combination 1 HCQ + azithromycin (92.0% *vs* 85.4%, P = 0.039) and combination 2 HCQ + azithromycin + oseltamivir + antibiotics (78.5% *vs* 69.2%, P = 0.024).

Overall, 34 patients (5.7%) died. Mortality was not different between patients with and without GI symptoms (6.2% *vs* 5.5%, P = 0.741). In addition, 260 (43.6%), 185 (30.8%), and 130 (21.6%) patients needed ICU stay, mechanical ventilation, or developed shock, respectively. There was no statistically significant difference

Table 3 Laboratory and radiological findings of coronavirus disease 2019 patients with and without gastrointestinal symptoms, *n* (%)

Parameters	Total	With GI symptoms (*n* = 163)	Without GI symptoms (*n* = 438)	*P* value
Hematological parameters				
Hemoglobin gm/dL	14.0 (12.7-15.0)	13.9 (12.7-15.0)	14.0 (12.7-15.1)	0.590
Hematocrit	42.1 (39.0-45.2)	42.0 (38.7-45.0)	42.3 (39.1-45.3)	0.010
WBC (10^3/L)	6.3 (4.9-8.6)	6.2 (4.6-8.6)	6.4 (5.0-8.5)	0.595
Neutrophils (10^3/L)	4.3 (3.1-6.6)	4.4 (2.9-6.5)	4.3 (3.1-6.6)	0.617
Lymphocytes (10^3/L)	1.2 (0.8-1.6)	1.1 (0.8-1.6)	1.2 (0.8-1.6)	0.987
Eosinophils (10^3/L)	0 (0-0.02)	0 (0-0)	0 (0-0.05)	0.643
Monocytes (10^3/L)	0.4 (0.3-0.6)	0.4 (0.3-0.6)	0.4 (0.3-0.6)	0.919
Platelets (10^3/L)	211 (169-261)	202 (155-257)	215 (170-261)	0.961
Coagulation function				
INR	1.1 (1.0-1.2)	1.1 (1.0-1.2)	1.1 (1.0-1.2)	0.486
Blood biochemistry				
BUN (mmol/L)	4.0 (3.0-5.3)	3.8 (2.4-5.2)	4.1 (3.1-5.3)	0.758
Creatinine (mol/L)	85 (70-102)	86 (69-101)	84 (70-103)	0.407
Sodium (mmol/L)	136 (133-138)	135 (132-137)	136 (133-138)	0.225
ALT (U/L)	32 (22-51)	34 (22-56)	32 (22-49)	0.043[a]
AST (U/L)	37 (25-59)	42 (28-69)	36 (24-55)	0.116
ALK-P (U/L)	70 (57-88)	71 (58-88)	70 (57-89)	0.199
Bilirubin (mol/L)	9 (6-12)	8 (7-12)	9 (6-12)	0.438
Albumin (gm/L)	36 (31-39)	35 (30-38)	36 (32-39)	0.058
Glucose (mmol/L)	6.6 (5.4-9.0)	6.7 (5.5-9.0)	6.6 (5.4-9.0)	0.708
Lactate (mmol/L)	1.3 (1.0-1.8)	1.4 (1.1-1.9)	1.2 (1.1-1.8)	0.297
CK (U/L)	218 (72-481)	147 (84-518)	237 (68-477)	0.455
Amylase (U/L)	48 (25-124)	47 (23-119)	47 (28-109)	0.604
Lipase (U/L)	55 (35-129)	56 (35-152)	54 (36-103)	0.648
Troponin-T (ng/L)	10 (6-26)	10 (6-17)	11 (6-26)	0.296
LDH (U/L)	436 (305-559)	446 (337-578)	435 (302-547)	0.167
Infection-related biomarkers				
CRP (mg/L)	55.3 (16.0-113.7)	55.5 (24.9-113.7)	55.3 (13.2-113.7)	0.614
Procalcitonin (ng/ml)	0.21 (0.10-0.68)	0.21 (0.10-0.50)	0.21 (0.10-0.70)	0.789
Ferritin (g/L)	659 (327-1229)	805 (450-1475)	618 (289-1154)	0.561
Liver injury at admission				0.059
None	272 (45.87)	65 (40.37)	207 (47.92)	
Abnormality < 2 × ULN	218 (36.76)	61 (37.89)	157 (36.34)	
Mild	85 (14.33)	30 (18.63)	55 (12.73)	
Moderate	15 (2.53)	5 (3.11)	10 (2.31)	
Severe	2 (0.34)	0 (0.00)	2 (0.46)	
Liver injury during hospitalization				0.302
None	107 (19.60)	26 (16.56)	81 (20.88)	
Abnormality < 2 × ULN	163 (29.91)	51 (32.48)	112 (28.87)	

Mild 2-5 ×	171 (31.40)	44 (28.03)	127 (32.73)	
Moderate 5-10 ×	63 (11.56)	21 (13.38)	42 (10.82)	
Severe > 10 ×	41 (7.52)	15 (9.55)	26 (6.70)	
Radiological findings				
X-ray chest				0.286
Not done	2 (0.3)	0 (0.0)	2 (0.5)	
Normal	141 (23.5)	35 (21.6)	107 (24.3)	
Unilateral PNA	100 (16.6)	24 (14.8)	76 (17.2)	
Bilateral PNA	328 (54.6)	96 (59.3)	232 (52.6)	
Ground glass	29 (4.8)	7 (4.3)	22 (5.0)	

WBC: White blood cells; BUN: Blood urea nitrogen; INR: International normalized ratio; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase, ALK-P: Alkaline phosphatase; CK: Creatine Kinase; LDH: Lactate dehydrogenase; CRP: C-reactive protein; ULN: Upper limit of normal; PNA: Pneumonia; GI: Gastrointestinal.

between the two groups. Seven (1.2%), 29 (4.8%), and 48 (8.0%) patients developed acute liver failure, renal failure requiring renal replacement therapy, and multiorgan failure, respectively, with no significant difference between the two groups. Patients with GI symptoms had a longer total hospital length of stay compared with patients without GI symptoms (15 d *vs* 14 d, $P = 0.036$).

GI symptoms during hospitalization

An additional 119 patients (19.8%) developed GI symptoms during hospitalization. Nausea (15.64%) and vomiting (14.48%) were the most commonly reported GI symptoms, followed by diarrhea (6.99%) and anorexia (4.32%) (Table 2). Regarding the treatment administered, use of HCQ was associated with vomiting (28.52%, $P = 0.009$), ribavirin use was associated with diarrhea (26.37%, $P < 0.001$) and anorexia (17.58%, $P = 0.02$), while the use of lopinavir/ritonavir was independently related to the development of nausea (32.65%, $P = 0.05$), vomiting (31.47%, $P = 0.004$), and epigastric pain (12.65%, $P = 0.05$) (Table 5).

The frequency of GI symptoms in different treatment combinations is shown in the Supplementary Table 1, along with significant *P* values. Specifically, treatment group 3 had more chances of developing nausea and vomiting, while treatment groups 5 and 6 had a significant association with anorexia.

Prediction of risk factors for severe/critical COVID-19 and adverse outcomes

In the multivariate regression analysis, age > 65 years was the only significant factor associated with increased mortality risk [adjusted odds ratio (aOR) 7.53, confidence interval (CI): 3.09-18.29, $P < 0.001$]. For disease severity at presentation, presence of GI symptoms (aOR: 1.66, CI: 1.09-2.52, $P = 0.02$), diabetes (aOR: 1.92, CI: 1.28-2.87, $P = 0.002$), hypertension (aOR: 1.68, CI: 1.08-2.60, $P = 0.02$), and smoking (aOR: 1.62, CI: 1.01-2.63, $P = 0.05$) were independent predictors in multivariate regression. Risk factors for ICU admission included age > 65 years (aOR: 1.79, CI: 1.13-2.83, $P = 0.012$), male sex (aOR: 1.82, CI: 1.05-3.15, $P = 0.033$) fever at admission (aOR: 2.14, CI: 1.24-3.69, $P = 0.006$), shortness of breath (aOR: 2.90, CI: 1.99-4.24, $P < 0.001$), and hypertension (aOR: 1.82, CI: 1.17-2.84, $P = 0.008$). Risk factors for mechanical ventilation included age > 65 (aOR: 1.89, CI: 1.94-2.99, $P = 0.007$), male sex (aOR: 1.88, CI: 1.02-3.45, $P = 0.043$), vomiting (aOR: 2.03, CI: 1.10-3.75, $P = 0.023$), fever (aOR: 3.16, CI: 1.63-6.09, $P < 0.001$), shortness of breath (aOR: 2.36, CI: 1.57-3.55, $P < 0.001$), and hypertension (aOR: 1.66, CI: 1.05-2.62, $P = 0.028$) (Table 6). The univariate analysis of risk factors for severe coronavirus disease 2019 at presentation and clinical outcomes is shown in Supplementary Table 2.

DISCUSSION

COVID-19 disease caused by the novel coronavirus SARS-CoV-2 started in China in December 2019 and soon became pandemic, causing unprecedented global public health challenges. COVID-19 mainly presents with respiratory symptoms; however, GI

Table 4 Clinical outcomes and treatment in coronavirus disease 2019 patients with and without gastrointestinal symptoms, *n* (%)

	Total	With GI symptoms *n* = 163	Without GI symptoms *n* = 438	*P* value
Outcomes				
ICU admission	260 (43.55)	78 (48.45)	182 (41.74)	0.143
ARDS	206 (34.30)	61 (38.12)	145 (33.30)	0.268
Shock	130 (21.60)	40 (25.00)	90 (20.64)	0.254
MOF	48 (8.00)	15 (9.32)	33 (7.59)	0.491
ALF	7 (1.20)	1 (0.62)	6 (1.38)	0.447
MV	185 (30.80)	55 (34.80)	130 (29.90)	0.252
ECMO	4 (0.70)	1 (0.62)	3 (0.69)	0.929
CRRT	29 (4.80)	6 (3.73)	23 (5.28)	0.610
Death	34 (5.70)	10 (6.21)	24 (5.50)	0.741
LOS (d)	15 (8-21)	15 (10-22)	14 (7-21)	0.036[a]
Treatment				
Azithromycin	538 (90.12)	151 (93.80)	387 (88.80)	0.068
HCQ	540 (90.45)	150 (93.20)	390 (89.40)	0.170
Chloroquine	38 (6.30)	7 (4.35)	31 (7.11)	0.388
Antibiotics	493 (82.6)	149 (92.6)	344 (78.9)	< 0.001[a]
Steroids	248 (41.61)	67 (41.60)	181 (41.60)	0.831
IFN	62 (10.30)	21 (13.04)	41 (9.43)	0.368
RBV	91 (15.10)	31 (19.25)	60 (13.80)	0.097
Tocilizumab	236 (39.50)	66 (40.99)	170 (38.99)	0.657
Lopinavir/ritonavir	340 (56.6)	96 (59.6)	244 (56.0)	0.422
Oseltamivir	510 (85.43)	139 (86.30)	371 (85.09)	0.702
Darunavir	48 (8.0)	14 (8.7)	34 (7.8)	0.782
Treatment groups				
Group 1	524 (87.2)	150 (92.0)	374 (85.4)	0.039[a]
Group 2	431 (71.7)	128 (78.5)	303 (69.2)	0.025[a]
Group 3	307 (51.1)	88 (54.0)	219 (50.0)	0.409
Group 4	181 (30.1)	50 (30.7)	131 (29.9)	0.920
Group 5	61 (10.1)	22 (13.5)	39 (8.9)	0.128
Group 6	56 (9.3)	20 (12.3)	36 (8.2)	0.155

[a]Statistically significant *P* value.

Group 1: Hydroxychloroquine + Azithromycin; Group 2: Hydroxychloroquine + Azithromycin + Oseltamivir + Antibiotics; Group 3: Hydroxychloroquine + Azithromycin + Oseltamivir + Antibiotics + Lopinavir/ritonavir; Group 4: Hydroxychloroquine + Azithromycin + Oseltamivir + Antibiotics + Lopinavir/ritonavir + Steroids; Group 5: Hydroxychloroquine + Azithromycin + Oseltamivir + Antibiotics + Lopinavir/ritonavir + Steroids + Interferon/ribavirin; Group 6: Hydroxychloroquine + Azithromycin + Oseltamivir + Antibiotics + Lopinavir/ritonavir + Steroids + Interferon/ribavirin + Tocilizumab.

ARDS: Acute respiratory distress syndrome; MOF: Multiorgan failure; ALF: Acute liver failure; MV: Mechanical ventilation; ECMO: Extra-corporal membrane oxygenation; CRRT: Continuous renal replacement therapy; LOS: Length of stay; HCQ: Hydroxychloroquine; IFN: Interferon; RBV: Ribavirin; GI: Gastrointestinal; ICU: Intensive care unit.

manifestations were quickly recognized as frequent presenting symptoms. While numerous studies have reported GI symptoms in COVID-19 patients, the criteria of GI symptoms have been variable and inconsistent[3,4,11,17,18]. Furthermore, some studies reported symptoms at presentation only while others described anytime during illness.

Table 5 Association of gastrointestinal symptoms with individual drugs, *n* (%)

	Azithromycin (*n* = 538)	HCQ (*n* = 540)	Antibiotics (*n* = 493)	Steroids (*n* = 248)	RBV (*n* = 91)	Tocilizumab (*n* = 236)	L/r (*n* = 340)	Oseltamivir (*n* = 510)
Diarrhea	78 (14.50)	79 (14.63)	70 (14.20)	37 (14.92)	24 (26.37)*P* < 0.001[a]	35 (14.83)	44 (12.94)	75 (14.71)
Nausea	160 (29.74)	165 (30.60)	147 (29.82)	74 (29.84)	26 (8.57)	66 (27.97)	111 (32.65)*P* = 0.049 [a]	153 (30.00)
Vomiting	148 (27.51)	154 (28.52)*P* = 0.009 [a]	135 (27.38)	68 (27.42)	22 (24.18)	64 (27.12)	107 (31.47)*P* = 0.004[a]	140 (27.45)
Epigastric pain	57 (10.59)	60 (11.11)	54 (10.95)	29 (11.69)	13 (14.29)	24 (10.17)	43 (12.65)*P* = 0.048[a]	55 (10.78)
Anorexia	55 (10.22)	58 (10.74)	50 (10.14)	30 (12.10)	16 (17.58)*P* = 0.022[a]	28 (11.86)	41 (12.06)	56 (10.98)
Any GI symptoms	208 (38.66)	209 (38.70)	191 (38.74)	95 (38.31)	41 (45.05)	92 (38.98)	138 (40.59)	194 (38.04)

[a]Statistically significant *P* value.

HCQ: Hydroxychloroquine; RBV: Ribavirin; L/r: Lopinavir/ritonavir; GI: Gastrointestinal

In our cohort, 27% of the patients had at least one GI symptom at presentation. Our cohort has a similar prevalence of symptoms as reported in the different meta-analyses [5,6,12,15], and slight variation can be attributed to different geographic locations and varied ethnic backgrounds and patients' perceptions of the importance of symptoms. However, our study differentiated clearly between GI symptoms at presentation and those developing during hospitalization. GI symptoms at the time of presentation are more likely attributable to COVID-19 as most patients were not taking any medications before the hospitalization. In contrast, GI symptoms developing during hospitalization may be multifactorial, including nosocomial infection, drug-related side effects, or progression of COVID-19.

Numerous mechanisms have been proposed for the development of GI symptoms in COVID-19 patients. The entry of the SARS-CoV-2 virus into host cells depends on the interaction of the virus spike protein with the receptor angiotensin-converting enzyme 2 and priming of the spike protein by host cell transmembrane serine protease 2[19,20]. Angiotensin-converting enzyme 2 and transmembrane serine protease 2 have been reported to be coexpressed in the GI tract, including esophageal upper epithelial and gland cells and absorptive enterocytes from the ileum and colon[19,20]. These enterocytes can be damaged, resulting in malabsorption and intestinal secretion abnormalities due to coronavirus or rotavirus infection[21,22]. It is, therefore, possible that GI manifestations in patients with COVID-19 might be associated with direct infection of enterocytes with the SARS-CoV-2 virus[19]. Elevated levels of fecal calprotectin, an inflammatory marker secreted by infiltrated neutrophils, in the fecal samples of COVID-19 patients with diarrhea also support this hypothesis[20].

Gut dysbiosis has been proposed as another mechanism to explain GI symptoms in COVID-19 patients. It is characterized by an increase in the opportunistic pathogens and reduction of beneficial commensals and correlates with COVID-19 severity and fecal levels of SARS-CoV-2[23]. It has been shown that the gut microbial signature of COVID-19 patients is different from healthy controls[24]. Although the clinical significance of these findings is still uncertain, it is possible that gut microbiota composition plays a role in modulating the systemic immune response.

The two primary modes of transmission for SARS-CoV-2 are respiratory droplets and direct contact[25], while the possibility of aerosol transmission has been suggested as well[25]. The GI tract has recently been proposed as an alternative transmission route for SARS-CoV-2 infection in a non-human primate model[26], raising the possibility of potential fecal-oral spread of the disease in humans as well. However, our study did not show any significant correlation of GI symptoms in family clusters, thus arguing against the potential fecal-oral transmission of the virus. Studies have reported the SARS-CoV-2 RNA in fecal samples or rectal swabs and fecal shedding of the virus continues even after clearance of respiratory samples[8,27-29]. The risk of transmission secondary to this prolonged shedding is unknown and warrants further studies.

Table 6 Multivariate analysis of risk factors for severe coronavirus disease 2019 at presentation and clinical outcomes, *n* (%)

Risk factors	Multivariate analysis	
	Adjusted odds ratio	*P* value
	Death	
Age > 65 yr	7.53 (3.09-18.29)	< 0.001
	Disease severity at presentation	
Hypertension	1.68 (1.08-2.60)	0.021
Diabetes mellitus	1.92 (1.28-2.87)	0.002
GI symptoms	1.66 (1.09-2.52)	0.017
Smoking	1.62 (1.01-2.63)	0.049
	ICU admission	
Age > 65 yr	1.79 (1.13-2.83)	0.012
Sex	1.82 (1.05-3.15)	0.033
Fever	2.14 (1.24-3.69)	0.006
Shortness of breath	2.90 (1.99-4.24)	< 0.001
Hypertension	1.82 (1.17-2.84)	0.008
	Mechanical ventilation	
Age > 65 yr	1.89 (1.94-2.99)	0.007
Sex	1.88 (1.02-3.45)	0.043
Fever	3.16 (1.63-6.09)	0.001
Shortness of breath	2.36 (1.57-3.55)	< 0.001
Hypertension	1.66 (1.05-2.62)	0.028
Vomiting	2.03 (1.10-3.75)	0.023

GI: Gastrointestinal; ICU: Intensive care unit.

COVID-19 patients with GI symptoms were more likely to have a longer duration of symptoms before diagnosis, more likely to have fatigue and myalgias, and less likely to have a cough. Lack of typical COVID symptoms (cough) and presence of atypical symptoms (GI symptoms) may result in delayed recognition and diagnosis of COVID-19 patients. Our findings are in keeping with those reported earlier[6,14]. The increased prevalence of myalgias and fatigue has been previously reported as well[9] and may be a reflection of the increased inflammatory burden in these patients. Patients with GI symptoms were more likely to have underlying malignancy and chronic liver disease. This finding has also been reported in the literature[30] and warrants careful evaluation of GI symptoms in cancer patients.

The severity of disease at presentation seen in COVID-19 patients with GI symptoms could be related to increased inflammatory activity in the intestines contributing to the systemic inflammatory response and cytokine syndrome[22]. Cytokine release syndrome is considered a leading cause of severe pneumonia and even death during COVID-19 disease[31]. Higher plasma levels of both proinflammatory and anti-inflammatory cytokines interleukin (IL)-2, IL-6, IL-7, IL-10, IL-18, granulocyte-macrophage colony-stimulating factor, C-C motif chemokine ligand 2 (also known as MCP1), tumor necrosis factor, and macrophage inflammatory protein 1 α have been detected in patients with severe disease compared to those with moderate disease[31,32]. The intestine produces high levels of IL-6 normally involved in crypt homeostasis[33] and can potentially contribute to the increased systemic IL-6 concentrations seen in COVID-19 patients with severe disease[32]. Similarly, the intestinal release of another proinflammatory cytokine, IL-18, can also contribute to disease severity and GI manifestations[34,35]. One recent study has shown downregulation of essential inflammatory genes in the small intestine and relative absence of inflammatory response in COVID-19 patients with GI symptoms[36]. These patients also had reduced levels of inflammatory proteins in circulation and reduced disease severity

and mortality, suggesting that gut inflammatory response has a potential role in modulating systemic immune reaction[36].

The new-onset GI symptoms during the hospitalization were most likely related to the use of several repurposed drugs, including hydroxychloroquine, ribavirin, and lopinavir/ritonavir. The GI side effects of these drugs have been reported in the literature and among COVID-19 patients in different clinical studies[37-39]. It must be noted that most of these therapies were administered during the first wave due to the lack of robust clinical evidence. Ribavirin, lopinavir/ritonavir, and hydroxychloroquine have not shown significant efficacy over standard care/placebo in COVID-19 patients[40,41]. This, coupled with potential adverse effects, warrant against the routine use of these medicines for COVID-19 treatment.

Our study has several limitations. It was a retrospective design, and reporting bias might affect the accurate estimates. We did not evaluate the mechanism and pathogenesis of GI symptoms in our patients. We also did not report the fecal viral load as there was no validated test available during the study period.

The strengths of our study include large sample size, a multi-ethnic population representing a real-world cohort, well-defined inclusion criteria, clear definition of GI symptoms, and distinction of GI symptoms at admission from those developing during the hospitalization. We had clearly defined outcomes and estimated the severity of the disease at presentation and later disease progression. We used multivariate regression to identify the independent risk factors.

CONCLUSION

In this current retrospective cohort study conducted in Qatar on a population with diverse ethnic backgrounds, we found a high prevalence (27.1%) of at least one GI symptom at presentation among COVID-19 patients requiring hospitalization. The mortality and disease progression was not different in patients with or without GI symptoms at presentation. However, patients with GI symptoms were more likely to have the severe disease at presentation and longer length of stay in the hospital. Additionally, one-quarter of the patients developed new GI symptoms during hospital admission. The most common culprit drugs associated with new GI symptoms development were lopinavir/ritonavir, ribavirin, and hydroxychloroquine. The lack of efficacy of several repurposed drugs for COVID-19 coupled with side effect profile warrants against their routine use in clinical practice. Further studies are needed to elucidate the mechanism and importance of GI manifestations in COVID-19 patients. Long-term sequelae of GI manifestations in COVID-19 patients remains unknown and needs to be studied.

ARTICLE HIGHLIGHTS

Research background

Gastrointestinal (GI) manifestations are present in 7%-15% of the patients with coronavirus disease 2019 (COVID-19). The association of GI manifestations with adverse clinical outcomes remains controversial, with some studies suggesting protective effects while others have reported adverse outcomes.

Research motivation

Previous studies reporting the association of GI symptoms with clinical outcomes in COVID-19 patients varied in determining the timing of symptoms development. We planned this study to clearly define GI symptoms in COVID-19 patients and distinguish between GI symptoms on admission and symptoms that develop during the hospital stay. We wanted to determine if there is any correlation of GI symptoms with disease severity and adverse clinical outcomes.

Research objectives

We aimed to determine the prevalence of GI symptoms in COVID-19 patients at admission and during hospitalization. We also aimed to study the correlation of GI symptoms with all-cause mortality, and disease severity at admission and disease progression during hospitalization defined by admission to the intensive care unit, development of acute respiratory distress syndrome, and need for mechanical ventilation.

Research methods

We conducted a retrospective cohort study investigating the epidemiological and clinical characteristics and outcomes among 601 consecutive adult patients with SARS-CoV-2 infection who were admitted to one of the dedicated COVID-19 hospitals in the state of Qatar between May 1-15, 2020. Clinical characteristics, laboratory parameters, treatment data, and disease outcome, including mortality, were compared between patients with and without GI symptoms. A multivariate logistic regression model with the forward method to identify independent predictors of the adverse outcomes.

Research results

The prevalence of any GI symptom at admission was 27.1% and during hospitalization was 19.8%. Nausea, vomiting, and diarrhea were the most common GI symptoms on presentation. There was no difference in mortality between the two groups (6.21% *vs* 5.50%, $P = 0.7$). However, patients with GI symptoms were more likely to have severe disease at presentation (33.13% *vs* 22.50%, $P < 0.001$) and prolonged hospital stay (15 d *vs* 14 d, $P = 0.04$). Age > 65 years was the single risk factor associated with increased mortality on multivariate regression analysis.

Research conclusions

Patients with GI symptoms are more likely to have severe disease at presentation. However, there is no difference in mortality between patients with and without GI symptoms.

Research perspectives

Future studies are needed to elucidate the mechanism of GI symptoms development in COVID-19 patients. Long-term effects and follow-up of COVID-19 patients with GI symptoms are needed.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 14; 27(46): 8010-8030

DOI: 10.3748/wjg.v27.i46.8010

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

ORIGINAL ARTICLE

Retrospective Cohort Study

Life prognosis of sentinel node navigation surgery for early-stage gastric cancer: Outcome of lymphatic basin dissection

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Author contributions: Kinami S was responsible for the scientific conception of the study and writing of the manuscript; Kinami S, Nakamura N, Miyashita T, Kitakata H, Fushida S, Fujimura T, and Ito T contributed to the surgery and data collection; Iida Y was responsible for the statistical analysis; Takamura H and Inaki N contributed to the drafting, editing, and critical revision of the manuscript; and all authors contributed to the approval of the final version of the manuscript.

Institutional review board statement: This study was approved by the ethics committee of Kanazawa University Hospital and Kanazawa Medical University (Trial Number R093, M288). ICG mapping was approved by the ethics committee of Kanazawa Medical University (Trial Number

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Abstract

BACKGROUND

Lymphatic basin dissection is a sentinel node biopsy method that is specific for gastric cancer. In this method, the dyed lymphatic system is dissected *en bloc*, and sentinel nodes are identified at the back table (*ex vivo*). Even with lymphatic basin dissection, blood flow to the residual stomach can be preserved, and function-preserving curative gastrectomy can be performed. The oncological safety of function-preserving curative gastrectomy combined with lymphatic basin dissection has not yet been fully investigated. We hypothesized that the oncological safety of sentinel node navigation surgery (SNNS) is not inferior to that of the guidelines.

AIM

To investigate the life prognosis of SNNS for gastric cancer in comparison with guidelines surgery.

METHODS

This was a retrospective cohort study. Patients were selected from gastric cancer

M404).

Informed consent statement: All patients provided written informed consent for surgery and the use of their data. Regarding data use in the retrospective study, the patients were given the opportunity to opt out of the study at any time.

Conflict-of-interest statement: The authors declare no conflicts of interest related to the publication of this study.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement checklist of items, and the manuscript was prepared and revised according to the STROBE Statement checklist of items.

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

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patients who underwent sentinel node biopsy from April 1999 to March 2016. Patients from April 1999 to August 2008 were from the Department of Surgery II, Kanazawa University Hospital, and patients from August 2009 to March 2016 were from the Department of Surgical Oncology, Kanazawa Medical University Hospital. Patients who were diagnosed with gastric cancer, which was preoperatively diagnosed as superficial type (type 0), 5 cm or less in length, clinical T1-2 and node negative, and underwent various gastrectomies guided by sentinel node navigation were retrospectively collected. The overall survival (OS) and relapse-free survival (RFS) of these patients (SNNS group) were investigated. Patients with gastric cancer of the same stage and who underwent guidelines gastrectomy with standard nodal dissection were also selected as the control group.

RESULTS

A total of 239 patients in the SNNS group and 423 patients in the control group were included. Pathological nodal metastasis was observed in 10.5% and 10.4% of the SNNS and control groups, respectively. The diagnostic abilities of sentinel node biopsy were 84% and 98.6% for sensitivity and accuracy, respectively. In the SNNS group, 81.6% of patients underwent modified gastrectomy or function-preserving curative gastrectomy with lymphatic basin dissection, in which the extent of nodal dissection was further reduced compared to the guidelines. The OS rate in the SNNS group was 96.8% at 5 years and was significantly better than 91.3% in the control group (P = 0.0014). The RFS rates were equal in both groups. After propensity score matching, there were 231 patients in both groups, and the cumulative recurrence rate was 0.43% at 5 years in the SNNS group and 1.30% in the control group, which was not statistically different.

CONCLUSION

The oncological safety of patients who undergo gastrectomy guided by sentinel node navigation is not inferior to that of the guidelines surgery.

Key Words: Early gastric cancer; Sentinel node biopsy; Function preserving surgery; Lymph node dissection; Gastrectomy; Lymphatic basin dissection

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Core Tip: The oncological safety of 239 patients with early-stage gastric cancer who underwent sentinel node navigation surgery was investigated. In total, 81.6% of patients underwent modified gastrectomy or function-preserving curative gastrectomy with lymphatic basin dissection, and the extent of nodal dissection was reduced compared to the guidelines. The overall survival rate at 5 years was significantly better, and the cumulative recurrence rate was equal to that of the control group in original data sets and propensity score-matched comparisons. The oncological safety of patients undergoing gastrectomy guided by sentinel node navigation is not inferior to that of the guidelines surgery.

Citation: Kinami S, Nakamura N, Miyashita T, Kitakata H, Fushida S, Fujimura T, Iida Y, Inaki N, Ito T, Takamura H. Life prognosis of sentinel node navigation surgery for early-stage gastric cancer: Outcome of lymphatic basin dissection. *World J Gastroenterol* 2021; 27(46): 8010-8030
URL: https://www.wjgnet.com/1007-9327/full/v27/i46/8010.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i46.8010

INTRODUCTION

The basic treatment for early gastric cancer not indicated for endoscopic submucosal dissection (ESD) is gastrectomy with lymph node dissection[1,2]. The range of prophylactic lymphadenectomy is determined in the greatest common denominator based on past data of lymph node metastasis, because most metastases to regional lymph nodes

Received: May 20, 2021
Peer-review started: May 20, 2021
First decision: June 22, 2021
Revised: June 28, 2021
Accepted: November 29, 2021
Article in press: November 29, 2021
Published online: December 14, 2021

P-Reviewer: Shi Y
S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

in early gastric cancer cannot be determined without pathological specimens. The Japanese Gastric Cancer Treatment Guidelines[3] recommends D1 + and D1 as the range of nodal dissection for cT1N0 cancer. D1 + requires sacrificial resection of most of the feeding arteries, resulting in the need for extensive gastrectomy. However, patients with nodal metastasis account for only approximately 20% of surgical patients with early gastric cancer. Excessive gastrectomy is performed in 80% of patients with early gastric cancer[4].

The preoperative diagnosis of lymph node metastasis is limited[5-11]. If lymph node metastasis can be diagnosed intraoperatively and node-negative patients can be distinguished, excessive dissection and extensive gastrectomy can be avoided. Currently, the most effective method for diagnosing lymph node metastasis is sentinel lymph node biopsy[12-27].

The sentinel lymph nodes of gastric cancer can be identified by administering a tracer with lymph-palatability to the submucosa using a gastroscopic injection needle and regarding the tracer-taking lymph nodes as sentinel nodes[14,15,24-26]. However, intraoperative pathological diagnosis of lymph node metastasis remains difficult[28]. Genetic diagnosis[29-33] is still in the research phase and, at present, we have to rely on intraoperative rapid frozen section diagnosis, but this method is accompanied by false negatives. Unlike breast cancer, reoperation for additional nodal dissection or additional radiation therapy is not acceptable in the case of gastric cancer. Therefore, a certain range of nodal dissection is necessary even in patients who are node negative by sentinel node biopsy. In view of these trends, Miwa[34] proposed lymphatic basin dissection, which is a sentinel node biopsy method specific for gastric cancer. In dye-based sentinel node biopsy, the lymphatic system specific to gastric cancer is stained by a dye tracer that is administered to the stomach and drains into the lymphatic system. The lymphatic system is then dissected *en bloc* and sentinel nodes are identified at the back table (*ex vivo*) in this method. This method not only reduces the difficulty of sentinel node biopsy, but also serves to a certain extent as backup dissection to cover false negatives of rapid intraoperative diagnosis. Even with lymphatic basin dissection, blood flow to the residual stomach can be preserved and function-preserving curative gastrectomy can be performed instead of extensive gastrectomy (Figure 1)[4,35].

Lymphatic basin dissection has been evaluated as a certain sentinel lymph node biopsy for gastric cancer[4,15,36]. However, the oncological safety of function-preserving curative gastrectomy combined with lymphatic basin dissection has not yet been fully investigated. In this study, we investigated the life prognosis of patients who underwent sentinel node navigation surgery (SNNS) for gastric cancer in comparison with standard surgery.

A prospective nation-wide study is currently undergoing in Japan to verify the oncological safety of the tailor-made surgical strategy guided by sentinel node navigation[37]. However, it is not a comparative study, and a control group has not been set due to difficulty in clinical circumference. In contrast, standard surgery performed at our facility complies with the Japanese guidelines has been performed as the routine medical treatment simultaneously and in parallel with the clinical trial of SNNS by the first author, which made it possible for us to compare the prognoses retrospectively. Therefore, we conducted this retrospective comparative study on patients who underwent SNNS and those who underwent the standard surgery performed as per the guidelines. The sentinel node biopsy is a diagnostic method for lymph node metastasis, and its applicability is determined based on the preoperative findings. To reproduce the findings of the prospective study, we selected patients with preoperative findings that were the same as those with indications for SNNS, and verified them using propensity score matching.

MATERIALS AND METHODS

This was a retrospective cohort study. Patients were selected from gastric cancer patients who underwent sentinel node biopsy by the first author (SK) from April 1999 to March 2016. The inclusion criteria were as follows: Age between 20 and 85 years; American Society of Anesthesiologists physical status (ASA-PS) 1-2 and tolerance to general anesthesia and gastrectomy; superficial type (type 0); preoperative diagnosis of 5 cm or less in length; preoperative diagnosis of T1 or T2 (clinical T1-2); node-negative preoperative diagnosis by X-computed tomography (CT); preoperative confirmation of adenocarcinoma by endoscopic biopsy; and reliable medical records. Conversely, patients with synchronous multiple advanced cancers in other organs,

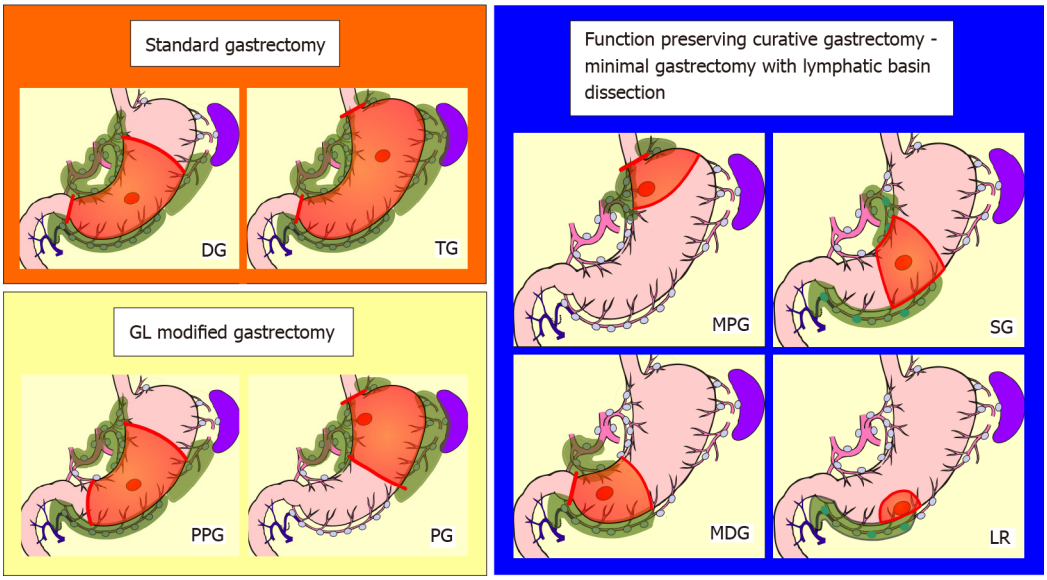


Figure 1 Schemas of standard gastrectomy, modified gastrectomy due to guidelines, and the function-preserving curative gastrectomy with lymphatic basin dissection. The red circle indicates the tumor, the green colored area indicates the extent of lymph node dissection, and the orange area indicates the extent of gastrectomy. The extent of nodal dissection in standard gastrectomy and modified gastrectomy according to the guidelines was D1 +. In contrast, the extent of nodal dissection in lymphatic basin dissection was defined as D0. GL: Japanese gastric cancer treatment guidelines; DG: Distal gastrectomy; TG: Total gastrectomy; PPG: Pylorus-preserving gastrectomy; PG: Proximal gastrectomy; MPG: Mini-proximal gastrectomy; SG: Segmental gastrectomy; MDG: Mini-distal gastrectomy; LR: Local resection.

with severe comorbidities, and those with an Eastern Cooperative Oncology Group Performance Status (ECOG PS) of 3 or higher were excluded. Patients from April 1999 to August 2008 were from the Department of Surgery II, Kanazawa University Hospital, and patients from August 2009 to March 2016 were from the Department of Surgical Oncology, Kanazawa Medical University Hospital.

For patients in the control group, early gastric cancer patients who underwent gastrectomy without sentinel node biopsy were extracted at the same time in the Department of Surgery II, Kanazawa University Hospital, and Department of Surgical Oncology, Kanazawa Medical University Hospital. The inclusion and exclusion criteria were the same as those of patients with sentinel node biopsy. In these patients, the standard surgeries in accordance with the Japanese guidelines[3] were mainly applied without mapping. The choice between mapping and non-mapping patients was mainly determined by the surgeon in charge. However, at both Kanazawa University Hospital and Kanazawa Medical University Hospital, a limited number of surgeons with the same treatment strategies and the same surgical skills were in charge of the gastrectomies.

The sentinel node biopsy methods used at Kanazawa University Hospital were the blue dye method, RI colloid method, and the combination method of blue dye and RI colloid. The dye tracers were patent blue or Lymphazurin, and the RI colloid tracers were ^{99m}Tc-tin colloid or ^{99m}Tc-phytate, which were endoscopically administered into the submucosal layer at four points around the tumor. The RI colloid was administered at 0.5 mL per site the day before surgery, and the blue dye was administered intraoperatively at 0.2 mL per site. The lymphatic basins were defined as the lymphatic system that was stained within 20 min after dye injection. The blue nodes were defined as nodes stained blue, and hot nodes were defined as nodes with radioactivity of more than 10 counts per second by using the gamma probe (Navigator GPS, Tyco Health Care, Mansfield, United States), and these were regarded as the sentinel nodes[14,36].

The indocyanine green (ICG) fluorescence method was used in Kanazawa Medical University Hospital[26]. ICG was adjusted to 50 μg/mL and endoscopically administered at 0.5 mL per site to the submucosal layer at four points around the tumor the day before surgery. Intraoperatively, ICG fluorescence was observed using a photodynamic eye (PDE, Hamamatsu Photonics, Shizuoka, Japan). The lymphatic basins were defined as the lymphatic system that was detected with fluorescent lymphatics, and the obvious fluorescent nodes were regarded as sentinel nodes. According to a previous report[36], lymphatic basins were integrated into the five lymphatic areas, except for the lymphatic flow to the left paracardial lymph node (No.

2 Lymph node, #2). Each of these is called the lymphatic compartment and is classified into five basins: The left gastric artery basin (*l*-GA); right gastric artery basin (*r*-GA); left gastroepiploic artery basin; right gastroepiploic artery basin; and the posterior gastric artery basin (*p*-GA) (Figure 2A). Classifying the lymphatic flow to #2 is challenging because of the multidirectional flow to *l*-GA and No. 19 ahead, and the lymphatic flow to *p*-GA nearby. Therefore, it was excluded from the lymphatic compartment classification and handled separately.

Patients who underwent sentinel node biopsy were divided into two groups: The feasibility phase group and the clinical application phase group. For patients in the former group, sentinel node biopsy was performed to evaluate the diagnostic ability of nodal metastasis; therefore, standard gastrectomy with nodal dissection was performed, and sentinel node identification was also performed postoperatively on the resected specimen. In contrast, in the clinical application phase, function-preserving curative gastrectomy was performed using sentinel node biopsy as a guide[4,26]. First, sentinel node mapping was performed, followed by lymphatic basin dissection, *ex vivo* identification and biopsy of the sentinel nodes, and intraoperative rapid pathology. If the sentinel nodes were diagnosed as metastasis at rapid diagnosis, standard gastrectomy with nodal dissection up to D2 was performed; if the sentinel nodes were diagnosed as node negative, the extent of gastrectomy was reduced and function-preserving curative gastrectomy, such as local resection (LR), segmental gastrectomy (SG), or proximal gastrectomy (PG) was performed according to the preserved blood flow (Figure 2B)[4]. This surgical strategy is generally called SNNS.

The patients were divided into two groups. Patients in the clinical application phase of sentinel node biopsy were designated as the study group (SNNS group). Patients who did not undergo sentinel node biopsy and those in the feasibility phase of sentinel node biopsy were defined as the control group. The control group consisted of patients who underwent guidelines gastrectomy, while the SNNS group consisted of patients who underwent tailor-made gastrectomy guided by sentinel node biopsy (Figure 3).

In this study, we examined and compared the prognosis of patients between the two groups. The prognosis of the patients at Kanazawa University Hospital was investigated in 2013, and that of Kanazawa Medical University Hospital was investigated in 2021. The prognosis was examined up to 10 years after initial gastrectomy, and the investigations included alive or dead, cause of death, presence or absence of recurrence, and the presence of newly detected metachronous multiple gastric cancer (MMGC) in the remnant stomach. Therefore, in this study, the prognosis up to 5 years was generally accurate, but some patients were censored because they did not reach 10 years after surgery at the time of investigation. The causes of death other than gastric cancer recurrence were divided into other cancer deaths (including MMGC) and non-cancer deaths from other diseases. The date of the confirmation of gastric cancer recurrence was also investigated. For cancers found in the remnant stomach, we distinguished between local recurrence and MMGC, and the latter was not judged as gastric cancer recurrence because of its favorable prognosis. In this study, overall survival (OS) treated all-cause mortality as an event, and relapse-free survival (RFS) treated gastric cancer recurrence as an event. All descriptions were described in accordance with the 15th edition of the Japanese classification of gastric carcinoma[38]. In this article, distal gastrectomy (DG) and total gastrectomy (TG) were defined as standard gastrectomy, pylorus-preserving gastrectomy (PPG), and PG were defined as guidelines-modified gastrectomy, and mini-DG (MDG), mini-PG (MPG), SG, and LR were defined as function-preserving curative gastrectomy (Figure 1)[4]. The diagnosis of lymph node metastases was determined by hematoxylin and eosin staining of the permanent slide at the maximum plane. The tumor cells were considered to be metastatic regardless of the size of metastatic foci, so both isolated tumor cells and micrometastases were also considered metastases. The results of immunohistochemical staining and genetic diagnosis were not considered in this study.

The chi-square test was used to compare the background factors of each group. Survival rates were compared by drawing survival curves using the Kaplan-Meier method and certified by using the log-rank test. Multivariate analysis of factors affecting survival was performed using Cox proportional hazards regression with a stepwise variable selection method. The Gray test was used to compare the cumulative incidence of recurrence, incidence of MMGC, other cancer-related deaths, and non-cancer deaths from other diseases, and Fine-Gray proportional hazards regression was used for multivariate analysis. In addition to these comparisons, propensity score matching was performed to adjust for differences in background factors between the two groups. Propensity scores were calculated for the two groups by logistic regression analysis using age, sex, location, circumference, long axis of tumor, macroscopic type, preoperative diagnosis of depth of invasion, and preoperative

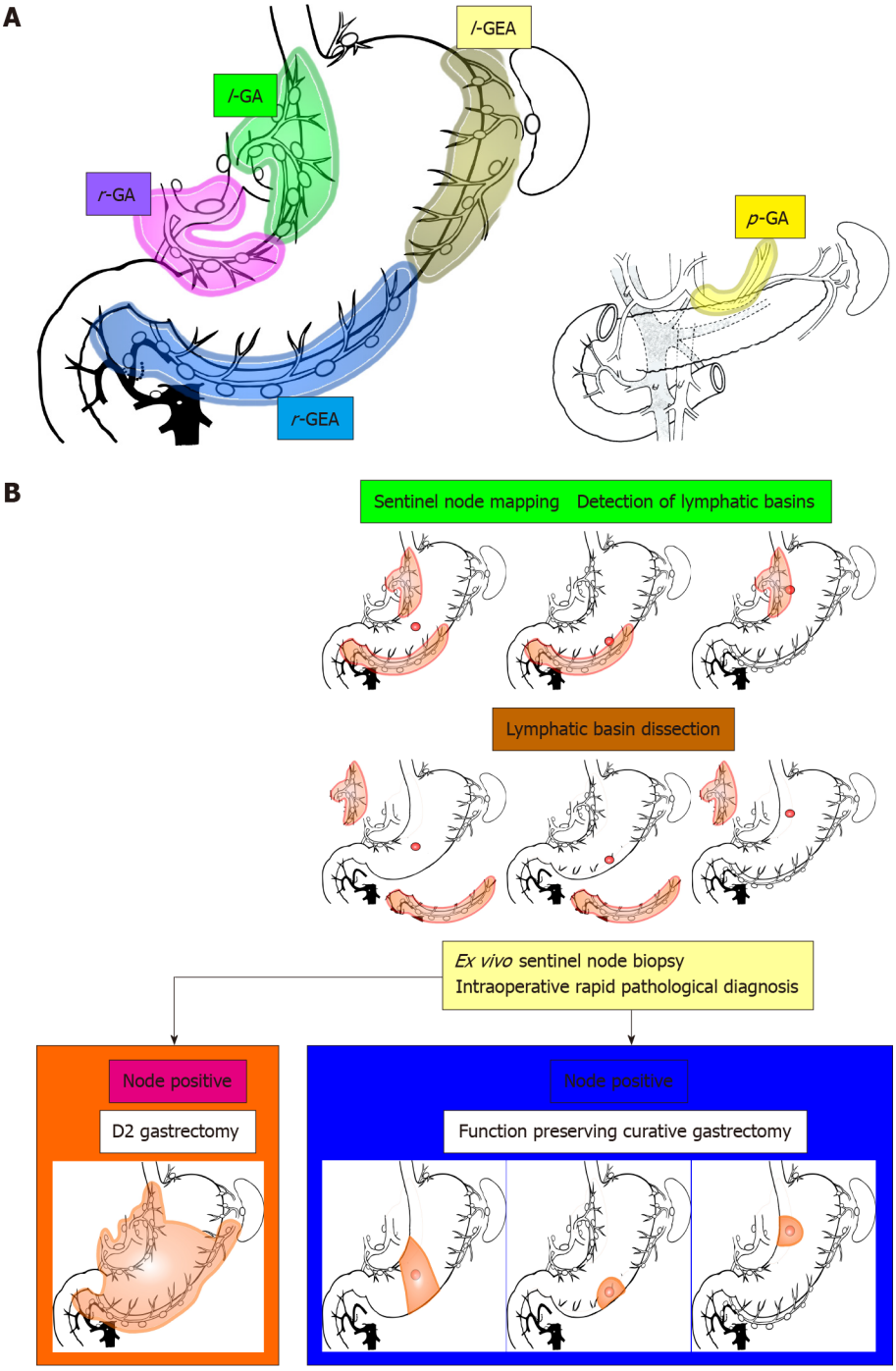


Figure 2 Lymphatic basins, lymphatic compartments, and the strategy of sentinel node navigation surgery. A: The lymphatic basins were defined as the lymphatic system that was detected with dyed or fluorescent lymphatics. The lymphatic basins were integrated into the five lymphatic areas. Each of these was called the lymphatic compartment and was classified into five basins; B: Algorithm for sentinel node navigation surgery for early gastric cancer. First, sentinel node mapping was performed, followed by lymphatic basin dissection, *ex vivo* identification and biopsy of the sentinel nodes, and intraoperative rapid pathology. If the sentinel nodes were diagnosed as metastasis at rapid diagnosis, standard gastrectomy with nodal dissection up to D2 was performed; if the sentinel nodes were diagnosed as node negative, the extent of gastrectomy was reduced and function-preserving curative gastrectomy, such as segmental gastrectomy or local resection, was applied. *l*-GA: Left gastric artery basin; *r*-GA: Right gastric artery basin; *l*-GEA: Left gastroepiploic artery basin; *r*-GEA: Right gastroepiploic artery basin; *p*-GA: Posterior gastric artery basin.

pathological diagnosis as variables. These variables were selected from among the factors that could affect the life prognosis and could be known preoperatively. To adjust for the covariates and estimate the causal effects, we used the nearest neighbor matching method with greedy matching and one-to-one matching with non-restorative extraction. The caliper of the propensity score was calculated by multiplying the standard deviation of the recommended propensity score estimated value by 0.2, after logit conversion. The balance between the groups was evaluated using the standardized difference score.

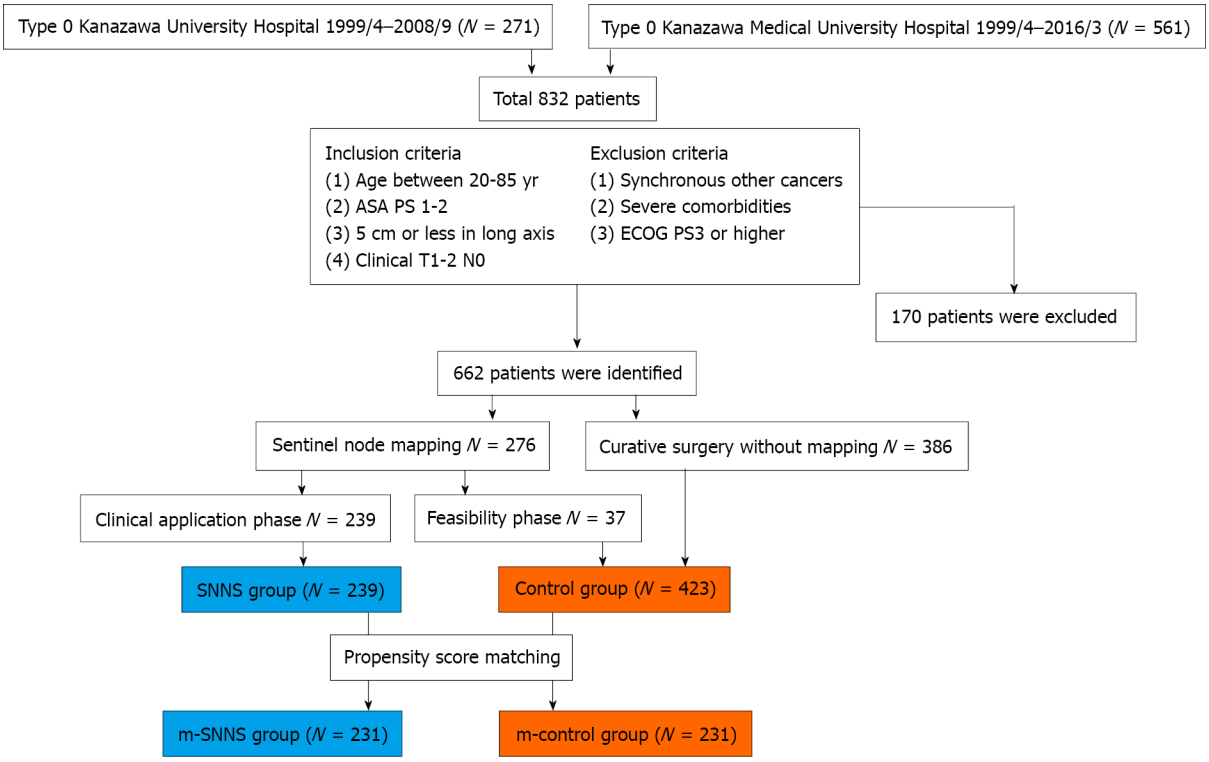


Figure 3 Summary of enrolled patients. The control group consisted of patients who underwent guidelines gastrectomy with standard lymph node dissection, while the sentinel node navigation surgery (SNNS) group consisted of patients who underwent tailor-made gastrectomy guided by sentinel node biopsy. SNNS: Sentinel node navigation surgery; m-SNNS: Propensity score-matched sentinel node navigation surgery; m-control: Propensity score-matched control; ECOG: Eastern Cooperative Oncology Group; ASA PS: American Society of Anesthesiologists physical status.

All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). EZR is a modified version of R Commander designed to add statistical functions that are frequently used in biostatistics[39]. All statistical methods used in this study were reviewed by Yasuo Iida, Department of Mathematics, Division of General Education, Kanazawa Medical University.

This study was approved by the ethics committee of Kanazawa University Hospital and Kanazawa Medical University (Trial Number R093, M288) and registered with the University Hospital Medical Information Network Clinical Trials Registry (trial number UMIN000010154 and UMIN000023828). ICG mapping was approved by the ethics committee of Kanazawa Medical University (Trial Number M404 and jRCTs041180006 https://jrct.niph.go.jp/Latest-detail/iRCTs041180006).

This study was conducted in accordance with the Good Clinical Practice guidelines and the Declaration of Helsinki. All patients provided written informed consent for surgery and use of their data. Regarding data use in the retrospective study, the patients were allowed to opt out of the study at any time.

RESULTS

Characteristics of the patients

A total of 276 patients with sentinel node mapping and 386 patients who underwent sur-gery without mapping were collected. Of the sentinel lymph node mapping patients, 37 were in the feasibility phase and 239 were in the clinical application phase. Therefore, there were 239 patients in the SNNS group and 423 patients in the control group (Figure 3). The patient profiles are presented in Table 1. There were differences in age and histological type between the two groups. In the control group, 67.6% of the patients underwent standard surgery (TG, 5.4%; DG, 62.2%), and 26.7% of patients underwent guidelines-modified gastrectomy (PG, 12.1%; PPG, 14.6%). In contrast, only 18.4% of the patients in the SNNS group underwent standard surgery, 14.2% underwent modified gastrectomy, and 67.4% underwent function-preserving curative

Table 1 Patient characteristics

	n	SNNS *n* = 239	Control *n* = 423	*P* value
Age	Median (range)	64 (28-85)	67 (27-85)	0.004
Sex	Male:Female	157:82	289:134	0.491
Location	U:M:L	35:130:74	78:195:150	0.116
Circumference	Less:Ant:Gre:Post	107:37:48:47	201:78:72:72	0.492
Macroscopic type	Elevated:Depressed	62:177	108:315	0.926
Clinical T status (cT)	1a:1b:2	100:111:28	171:192:60	0.678
Clinical N status (cN)	0:1:2-3	239:0:0	423:0:0	1.000
Pathological diagnosis	DF:UDF	130:109	289:134	< 0.001
Sentinel node mapping	BD:RI:CM:ICG:None	39:6:135:59:0	2:1:13:21:386	< 0.001
Surgical procedure	TG:DG:PG:PPG; SG:MDG:MPG:LR	3:41:24:10; 84:33:6:38	23:263:51:62; 8:4:1:11	< 0.001
Nodal dissection	D0:D1(1 +):D2	174:42:23	45:191:187	< 0.001
Long axis (mm)	Median (range)	22 (2-65)	25 (4-87)	0.265
Pathological T (pT)	1a:1b:2:3-4	129:92:10:8	218:145:39:21	0.065
Pathological N (pN)	0:1:2-3	214:13:12	379:34:10	0.072
Recurrent cases		1	8	

SNNS: Sentinel node navigation surgery group; DF: Differentiated type; UDF: Undifferentiated type; BD: Blue dye mapping; RI: Radioisotope colloid mapping; CM: Dye and RI combination mapping; ICG: Indocyanine green fluorescence mapping; TG: Total gastrectomy; DG: Distal gastrectomy; PG: Proximal gastrectomy; PPG: Pylorus-preserving gastrectomy; SG: Segmental gastrectomy; MDG: Mini-distal gastrectomy; MPG: Mini-proximal gastrectomy; LR: Local resection.

gastrectomy (SG, 35.1%; MDG, 13.8%; MPG, 2.5%; LR, 15.9%), in which the extent of resection was reduced further than that recommended by the guidelines.

All patients in this study were preoperatively diagnosed as node negative by X-CT, but pathological nodal metastasis was observed in 10.5% (25 patients) in the SNNS group and 10.4% in the control group. Table 2 lists the 25 patients in the SNNS group.

Recurrence of gastric cancer and results of sentinel node biopsy

Two patients in the control group died after surgery (hospital death); one was due to aspiration pneumonia and the other was due to peritonitis from idiopathic colon perforation. In contrast, no in-hospital deaths were observed in the SNNS group. Gastric cancer recurrence was observed in one patient in the SNNS group and eight patients in the control group. The recurrent patient in the SNNS group is displayed as No. 16 in Table 2. He was diagnosed as node-positive intraoperatively by sentinel node biopsy, and DG D2 was performed. Although postoperative adjuvant chemotherapy with S1 was administered, the patient died of lymph node metastasis 53.2 mo later. The type of recurrence in eight patients in the control group were four of lymph node recurrence, two of liver metastasis, one of lung metastasis, and one patient of local recurrence.

Of the 276 patients with sentinel node mapping, 37 patients in the feasibility phase had no lymph node metastasis. In contrast, of the 239 patients in the clinical application phase, 25 patients had lymph node metastasis (Table 2). Of these 25 patients, 21 (No. 1-21) were diagnosed as positive for metastasis intraoperatively by sentinel node biopsy, and 4 (No. 22-25) were false negative. The diagnostic ability of sentinel node biopsy in this study was calculated to be 84% (21/25) for sensitivity, 100% for specificity, 100% for positive predictive value, 98.4% (251/255) for negative predictive value, and 98.6% (272/276) for accuracy. The reasons for false negatives were misdiagnosis of frozen section diagnosis in three patients (No. 22-24) and macroscopic lymph node metastasis, which was not able to take up tracer in one patient (No. 25). The diagnosis of metastasis in the later patient was easy due to intraoperative findings. Twenty-one patients who were diagnosed as node-positive by sentinel node biopsy during surgery underwent standard gastrectomy with D1 + or D2. On the other hand, two of the false-negative patients with rapid diagnosis underwent SG but were followed up without additional dissection. One patient died of pancreatic cancer (No.

Table 2 List of 25 patients of lymph node metastasis in the sentinel node navigation surgery group

No.	LOC	MAC	LA	cT	cN	sN	MP	LB	INDSN	OP	D	PD	pT	MS	NS	MLB	MOB	NNS	MRSC	PROG[1]
1	M	0 IIa + IIc	20	1b	0	0	ICG	*l*-GA	TP	DG	2	tub2	sm2	#3 #7	4	#7	-	1	82.3	Alive
2	M	0 IIc	25	1b	0	0	CM	*l*-GA	TP	DG	2	por1	sm2	#3	1	-	-	0	71.4	Alive
3	U	0 IIc	25	1b	0	0	ICG	*l*-GA	TP	PG	1 +	tub2	mp	#1 #3	2	#1	-	1	56.1	Trauma
4	M	0 IIa + IIc	40	2	0	0	ICG	*l*-GA	TP	DG	2	tub2	ss	#3	1	-	-	0	63.7	Alive
5	M	0 IIc + IIb	40	1b	0	0	ICG	*l*-GA	TP	DG	2	tub2	mp	#3	1	-	-	0	80.4	Alive
6	U	0 IIc	45	1b	0	0	CM	*l*-GA	TP	PG	1 +	sig	m	#1 #7	2	-	-	0	69.2	Alive
7	L	0 IIc + IIb	45	2	0	1	CM	*l*-GA	TP	DG	2	por2	ss	#3	2	#3	-	1	67.7	Alive
8	L	0 IIc + III	40	2	0	2	ICG	*r*-GEA	TP	DG	2	tub2	mp	#4d	2	#4d #6	#7	5	72.1	Alive
9	L	0 IIa	55	2	0	1	ICG	*l*-GA, *r*-GA	TP	DG	1 +	tub1	sm1	#5	1	-	-	0	67.7	Alive
10	L	0 IIa + IIc	20	1b	0	0	ICG	*l*-GA, *r*-GEA	TP	DG	2	tub2	sm2	#3 #4d #6	4	#6	-	1	62.0	Alive
11	M	0 IIc + III	25	2	0	0	BD	*l*-GA, *r*-GEA	TP	DG	2	por2	ss	#4d	2	-	-	0	120.0	Alive
12	M	0 IIa + IIc	25	1b	0	0	ICG	*l*-GA, *r*-GEA	TP	DG	1 +	tub2	sm2	#3 #7 #4d	4	-	-	0	59.5	CVD
13	M	0 I	32	1b	0	0	CM	*l*-GA, *r*-GEA	TP	TG	2	tub2	sm2	#3 #4d	5	-	-	0	64.7	Alive
14	M	0 IIc + IIb	37	1b	0	0	CM	*l*-GA, *r*-GEA	TP	DG	2	por2	m	#3	3	-	-	0	61.9	Alive
15	M	0 I	55	1a	0	0	ICG	*l*-GA, *r*-GEA	TP	DG	2	por2	mp	#4d	1	#3 #4d	-	4	69.8	Alive
16	M	0 IIc + III	30	2	0	1	BD	*l*-GA, *r*-GEA	TP	DG	2	por2	se	#1 #3 #4d	3	#4d	-	2	53.2	LNR
17	U	0 IIa + IIc	11	1a	0	0	CM	*l*-GA, *l*-GEA, *p*-GA	TP	PG	1 +	por1	sm2	#11d	1	#7	-	1	84.3	Alive
18	U	0 IIc	33	1b	0	0	CM	*l*-GA, *l*-GEA, *p*-GA	TP	PG	1 +	tub2	mp	#1	1	-	-	0	62.9	Alive
19	U	0 IIc	55	2	0	0	CM	*l*-GA, *r*-GEA, *r*-GEA	TP	TG	2	por2	sm2	#1 #3 #4d #10	4	#1	-	1	65.2	Alive
20	M	0 IIc	40	2	0	0	ICG	*l*-GA, *r*-GA, *r*-GEA	TP	DG	1+	tub2	sm1	#3	1	-	-	0	89.6	Alive
21	L	0 IIa + IIc	24	1b	0	2	CM	*l*-GA, *r*-GA, *r*-GEA	TP	DG	2	por1	sm2	#8a	1	#3	-	1	72.6	Alive
22	M	0 IIc	20	1b	0	0	ICG	*l*-GA, *r*-GEA	FN (FD)	SG	0	por2	sm2	(#4d)	1	#4d	-	1	61.2	Alive
23	M	0 IIc + III	23	2	0	0	CM	*l*-GA, *r*-GEA	FN (FD)	SG	0	tub2	mp	(#3)	1	#3	-	2	75.8	PK
24	L	0 IIc	45	1b	0	0	RI	*l*-GA, *r*-GA, *r*-GEA	FN (FD)	DG	2	por2	m	(#5)	1	-	-	0	66.8	Alive

25	L	0 I	25	1b	0	1	CM	*l*-GA, *r*-GA, *r*-GEA	FN (LM)	DG	2	tub2	sm2	-	0	#4d	-	1	63.6	Alive

[1]"PROG" column indicates whether the patients are alive at the time of recent survival confirmation, recurrent status of gastric cancer, or the cause of death.

SNNS: Sentinel node navigation surgery; LOC: Location; MAC: Macroscopic type; LA: Size of long axis (mm); cT: Clinical T status; cN: Clinical N status; sN: Surgical N status; MP; Mapping procedures; ICG: Indocyanine green fluorescence mapping; CM: Combination mapping; BD: Blue dye mapping; RI: Radioactive colloid mapping; LB: Distributions of lymphatic basins; *l*-GA: Left gastric artery basin; *r*-GA: Right gastric artery basin; *r*-GEA: Right gastroepiploic artery basin; *l*-GEA: Left gastroepiploic artery basin; *p*-GA: Posterior gastric artery basin; INDSN: Intraoperative nodal diagnosis by sentinel node biopsy; TP: True positive diagnosis for nodal metastasis; FN (FD): False-negative diagnosis because of frozen section diagnosis; FN (LM): False negative because of obvious macroscopic nodal metastasis; OP: Surgical procedures; DG: Distal gastrectomy; PG: Proximal gastrectomy; TG: Total gastrectomy; SG: Segmental gastrectomy; D: Degree of nodal dissection; PD: Dominant pathological diagnosis; pT: Pathological T status; MS: Metastastic stations of sentinel nodes; NS: Numerical numbers of metastatic sentinel nodes; MLB: Metastatic stations of not sentinel nodes inside the lymphatic basins; MOB: Metastatic stations of not sentinel nodes outside the basins; NNS: Numerical numbers of metastatic nodes of not sentinel nodes; MRSC: Months to recent survival confirmation; PROG: Prognosis, recurrent status or cause of death; CVD: Cerebrovascular disease; LNR: Lymph nodal recurrence; PK: Pancreas cancer.

23), while the other survived for 5 years without recurrence (No. 22). As for the remaining two patients, DG D2 was performed because one had macroscopic lymph node metastasis (No. 25) and the other was suspected from intraoperative findings to be advanced gastric cancer with serosal exposure (No. 24). However, No. 24 was pathologically a mucosal cancer. These patients survived for five years without recurrence.

Therefore, there were no recurrences in the 218 patients diagnosed as node negative by sentinel node biopsy (214 true negative + four false negative). Of these 218 patients, only 11 underwent standard surgery (DG or TG with D1 + or D2). A total of 190 patients underwent modified gastrectomy or function-preserving curative gastrectomy with reduction of the resection area, and 17 underwent gastrectomy with reduction of the nodal dissection.

Of the 25 patients with nodal metastasis in the SNNS group, 11 had metastasis to only the sentinel nodes, 12 had non-sentinel metastatic nodes other than the sentinel nodes, but they remained within the lymphatic basin, and one was a false-negative patient with macroscopic metastasis as described above, with only one metastatic node. Only one patient had a metastatic node outside the lymphatic basin (No. 8). In this patient, macroscopic metastasis was found intraoperatively, and the final pathological diagnosis was fT2(MP)N3a (#4d, 6, 7). The patient was alive 6 years after surgery without any sign of recurrence.

MMGC of the remnant stomach

After surgery, the residual stomach was followed up with periodic endoscopic examinations, and MMGCs were found in 21 patients. Table 3 shows a list of interval times until the diagnosis of MMGC and treatment details. Of the 21 patients, 5 were in the SNNS group and 16 were in the control group. Four patients in the SNNS group (80%) and eight in the control group (50%) underwent ESD; therefore, their remnant stomachs were preserved. In contrast, five patients in the control group required TG, and one patient was unresectable. The cumulative incidence of MMGC is shown in Figure 4, and there was no difference in the incidence of MMGC between the two groups.

Life prognosis of patients in the SNNS group

The OS of all the patients in this study is shown in Figure 5A. The 5-year survival rates were 92.7% and the 10-year survival rate was 83.2%, respectively. The results of univariate and multivariate analyses for factors affecting OS are shown in Table 4, which shows that OS was affected by age, sex, macroscopic type, size, and pathological nodal status, as well as by the SNNS group. The OS of the SNNS group was significantly better than that of the control group (Figure 5B).

RFS in the SNNS group was 99.6% at both 5 and 10 years, and the RFS in the control group was 98.1% at both 5 and 10 years. Since there were a small number of recurrent patients and these recurrences competed with other cancer deaths and non-cancer deaths from other diseases, the evaluation of RFS was difficult and should be examined by cumulative incidence. Figure 6 shows a graph of the cumulative incidence, including other cancer deaths and non-cancer deaths from other diseases. The cumulative incidence of non-cancer deaths from other diseases was lower in the SNNS group than in the control group, and a significant difference was observed in

Table 3 Profiles of metachronous multiple remnant gastric cancer patients

No.	Group	ISP	Treatment	MTMC	Curability[1]	MRSC	PROG[2]
1	SNNS	SG	DG	50.5	Curative	75.6	Pneumonia
2	SNNS	SG	ESD	60.2	Curative	114.9	Alive
3	SNNS	SG	ESD	22.9	Curative	79.9	Alive
4	SNNS	MPG	ESD	13.6	Curative	76.3	Alive
5	SNNS	LR	ESD	30.7	Curative	73.1	Alive
6	SNNS	DG	TG	43.6	Curative	62.9	Alive
7	Control	DG	TG	19.5	Curative	63.6	Alive
8	Control	DG	TG	17.2	Curative	97.4	Alive
9	Control	DG	ESD	44.3	Curative	55.5	AID
10	Control	DG	ESD	220.4	Curative	240.0	Alive
11	Control	DG	ESD	40.6	Curative	120.0	Alive
12	Control	PG	UR	74.3	UR	120.0	Alive
13	Control	PG	TG	76.8	Curative	120.0	Alive
14	Control	PG	ESD	18.5	Curative	120.0	Alive
15	Control	PG	ESD	28.2	Curative	120.0	Alive
16	Control	PPG	TG	50.8	Curative	120.0	Alive
17	Control	PPG	DG	85.0	Curative	116.2	Alive
18	Control	PPG	ESD	77.1	Curative	118.5	Alive
19	Control	PPG	ESD	22.0	Curative	98.2	Alive
20	Control	LR	DG	7.6	Cure	42.8	CVD
21	Control	LR	ESD	64.2	Cure	85.7	Alive

[1]"Curability" column indicates whether treatment for metachronous multiple remnant gastric cancers was curative or not. All but one unresectable patient could be resected radically, and there were no recurrences of metachronous gastric cancer. One unresectable patient was alive with metachronous cancer 10 years after the initial surgery.

[2]"PROG" column indicates whether the patients are alive at the time of recent survival confirmation, or the cause of death.

ISP: Initial surgical procedure; SG: Segmental gastrectomy; MPG: Mini-proximal gastrectomy; LR: Local resection; DG: Distal gastrectomy; PG: Proximal gastrectomy; PPG: Pylorus-preserving gastrectomy; ESD: Endoscopic submucosal dissection; TG: Total gastrectomy; UR: Unresectable; MTMC: Months to treat metachronous gastric cancer; MRSC: Months to recent survival confirmation; PROG: Prognosis or cause of death; AID: Autoimmune disease; CVD: Cerebrovascular disease; SNNS: Sentinel node navigation surgery.

the Gray test. Table 5 shows the results of the multivariate analysis using the Fine-Gray proportional hazard regression test. Age and the SNNS group were independent factors significantly affecting non-cancer deaths from other diseases, while age and macroscopic type were factors that significantly affected other cancer deaths, and pN was the only factor affecting gastric cancer recurrence.

Evaluation of life prognosis by propensity score matching

In the SNNS group, the gastric cancer recurrences might be comparable, and the number of non-cancer deaths from other diseases might be less than that in the control group. However, caution should be exercised when interpreting the results because of the significant difference in age distribution between the two groups. We re-examined the comparison of life prognosis using the propensity score matching method. Propensity score matching was performed for the two groups using the preoperatively recognizable items of age, sex, tumor location, macroscopic type, preoperative T factor, and pathological diagnosis. We added the long axis of the tumor to the items because size is an important factor affecting prognosis. The characteristics of the two groups after propensity score matching (m-SNNS and m-control groups) are shown in Table 6. There were 231 patients in both groups, and the backgrounds of the two groups became uniform. The distributions of the other factors were also examined after matching. There was no significant difference in pathological depth of invasion or

Table 4 Univariate and multivariate analysis for the factors affected to the overall survival

Factors	*vs*	Univariate[1] (Log-rank *P*)	Multivariate[2]		
			Hazard ratio	95%CI	*P* value
Age		< 0.0001	1.092	1.064-1.120	< 0.0001
Sex	Male:Female	0.0009	1.815	1.099-2.998	0.0199
Location		0.0978			
Circumference		0.301			
Macroscopic type	Elevated:Depressed	< 0.0001	1.678	1.131-2.490	0.0101
Clinical T status		0.632			
Pathological type	Diff.:Undiff.	0.0001			
Long axis (mm)		< 00001	0.9815	0.965-0.998	0.0287
Pathological N status		0.0033	1.785	1.288-2.473	0.0005
SNNS	SNNS:Control	0.0014	0.4892	0.298-0.802	0.0046

[1]The log-rank test was used for the univariate analysis of overall survival.
[2]The Cox proportional hazards regression model was used for multivariate analysis of overall survival.
SNNS: Sentinel node navigation surgery; diff.: Differentiated; Undiff.: Undifferentiated; CI: Confidential interval.

Table 5 Multivariate analysis for the factors affected to the cumulative incidence of causes of death or recurrences

Factors	Non-cancer deaths			Other cancers			Gastric cancer recurrence		
	HR	95%CI	*P* value	HR	95%CI	*P* value	HR	95%CI	*P* value
Age	1.101	1.064-1.140	< 0.0001	1.064	1.026-1.103	0.0008	0.992	0.894-1.101	0.88
Sex	1.533	0.823-2.855	0.18	2.126	0.863-5.239	0.1	3.213	0.592-17.43	0.18
Location	1.259	0.890-1.782	0.19	0.9636	0.637-1.457	0.86	1.472	0.482-4.502	0.5
Circumference	1.037	0.798-1.349	0.78	1.184	0.875-1.602	0.27	1.943	0.732-5.158	0.18
Macroscopic type	1.303	0.752-2.256	0.35	2.322	1.241-4.346	0.0084	1.083	0.136-8.636	0.94
Clinical T status	0.9053	0.584-1.403	0.66	0.8587	0.481-1.535	0.61	1.542	0.662-3.595	0.32
Pathological type	1.509	0.762-2.990	0.24	0.5908	0.211-1.657	0.32	1.27	0.165-9.786	0.82
Long axis	0.9802	0.958-1.003	0.086	0.9874	0.958-1.017	0.4	0.9468	0.867-1.034	0.22
Pathological N status	1.263	0.683-2.337	0.46	1.353	0.751-2.440	0.31	5.252	2.043-13.50	0.00058
SNNS	0.4438	0.230-0.855	0.015	0.7224	0.338-1.542	0.4	0.1859	0.030-1.166	0.072

SNNS: Sentinel node navigation surgery; HR: Hazard ratio; CI: Confidence interval.

pathological nodal status, although there was a natural difference in the distribution of sentinel node mapping and surgical techniques, and there were three cases of recurrence in the control group compared to one case in the SNNS group.

Figure 7 shows a graph of OS and the cumulative incidence of death or recurrence after matching. OS in the SNNS group was significantly better than that in the control group. The cumulative recurrence rate in the SNNS group was 0.43% at both 5 and 10 years, and in the control group was 1.30% at both 5 and 10 years, which was not statistically different. In contrast, the cumulative incidence of non-cancer deaths from other diseases was 2.6% at 5 years and 8.6% at 10 years in the SNNS group, and 5.7% at 5 years and 15.5% at 10 years in the control group. In the SNNS group, the cumulative incidence of non-cancer deaths from other diseases tended to be lower than that in the control group (P = 0.089).

Accuracy of preoperative diagnosis

Although all patients were preoperatively diagnosed with a long axis of 5 cm or less,

Table 6 Patient characteristics after propensity score matching

	n	m-SNNS *n* = 231	m-control *n* = 231	*P* value
Age	Median (range)	64 (29-85)	64 (27-85)	0.473
Sex	Male:Female	152:79	147:84	0.697
Location	U:M:L	34:125:72	37:119:75	0.843
Circumference	Less:Ant:Gre:Post	106:37:45:43	103:40:46:42	0.980
Macroscopic type	Elevated:Depressed	57:174	57:174	1.000
Clinical T status (cT)	1a:1b:2	98:105:28	88:108:35	0.528
Clinical N status (cN)	0:1:2-3	231:0:0	231:0:0	1.000
Pathological diagnosis	DF:UDF	130:101	126:105	0.779
Long axis (mm)	Median (range)	23 (2-65)	25 (4-87)	0.547
Sentinel node mapping	BD:RI:CM:ICG:None	38:5:132:56:0	1:1:8:15:206	< 0.001
Surgical procedure	TG:DG:PG:PPG; SG:MDG:MPG:LR	3:40:23:10; 80:31:6:37	14:147:25:32; 4:4:0:5	< 0.001
Nodal dissection	D0:D1(1+):D2	169:39:23	23:97:111	< 0.001
Pathological T (pT)	1a:1b:2:3-4	125:89:10:7	126:72:20:13	0.075
Pathological N (pN)	0:1:2-3	206:13:12	213:13:5	0.251
Recurrent cases		1	3	

m-SNNS: Matched sentinel node navigation surgery group; m-control: Matched control group; DF: Differentiated type; UDF: Undifferentiated type; BD: Blue dye mapping; RI: Radioisotope colloid mapping; CM: Dye and RI combination mapping; ICG: Indocyanine green fluorescence mapping; TG: Total gastrectomy; DG: Distal gastrectomy; PG: Proximal gastrectomy; PPG: Pylorus-preserving gastrectomy; SG: Segmental gastrectomy; MDG: Mini-distal gastrectomy; MPG: Mini-proximal gastrectomy; LR: Local resection.

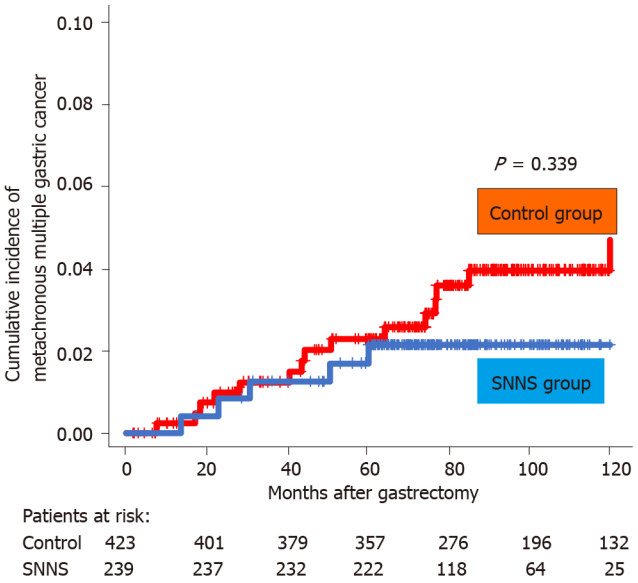


Figure 4 Cumulative incidence of metachronous multiple gastric cancer in the remnant stomach. There was no difference in the incidence of metachronous multiple gastric cancer between the SNNS and control groups. SNNS: sentinel node navigation surgery.

19 patients had a pathological diagnosis larger than 5 cm: Eight patients (3.3%) in the SNNS group and 11 (2.6%) in the control group. All 19 patients had a preoperative diagnosis of sN0, but four had pN1 and two had pN2. There were no recurrences in these 19 patients.

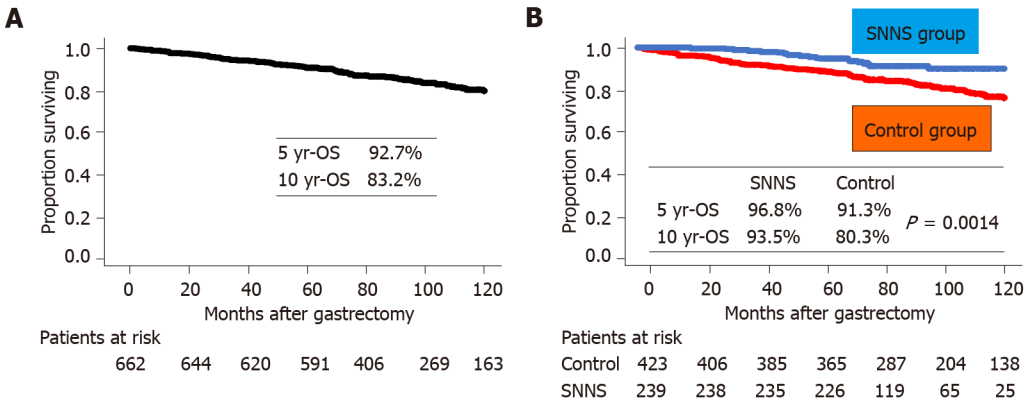


Figure 5 Overall survival curve. A: The overall survival of all patients; B: Comparison of overall survival between the sentinel node navigation surgery (SNNS) and control groups. The overall survival of the SNNS group was significantly better than the control group. SNNS: Sentinel node navigation surgery; OS: Overall survival.

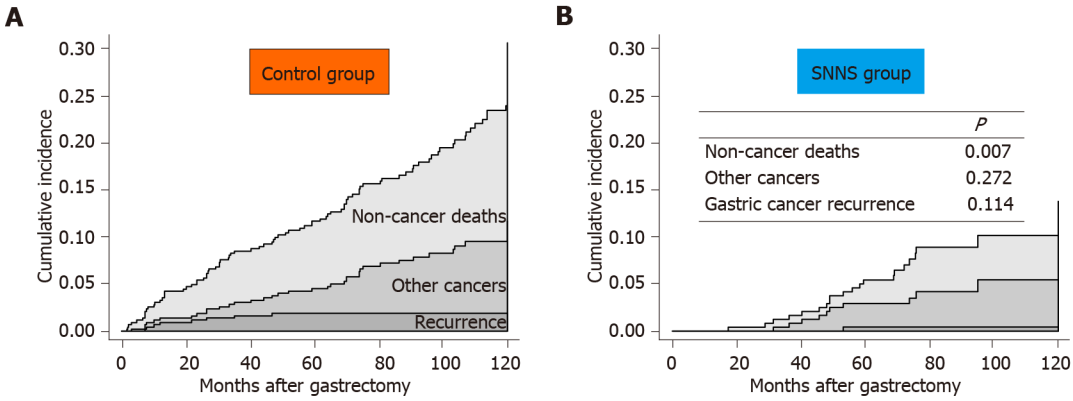


Figure 6 Cumulative incidence of gastric cancer recurrence or reason for death. A: Control group; B: Sentinel node navigation surgery (SNNS) group. The cumulative incidence of gastric cancer recurrence and other cancer-related deaths was almost equal between the two groups; in contrast, that of non-cancer deaths from other diseases was lower in the SNNS group than in the control group. SNNS: Sentinel node navigation surgery.

DISCUSSION

In both original data sets and propensity score-matched comparisons, the OS rate and RFS rate of patients who underwent gastrectomy guided by sentinel node navigation were not inferior to those of standard gastrectomy. In addition, there was no difference in the cumulative incidence of MMGC between the two groups.

Postgastrectomy syndrome (PGS) is a serious drawback after curative gastrectomy for gastric cancer[40-45], and occurs in a certain percentage of patients after standard gastrectomy. Currently, the most common approach for early gastric cancer is laparoscopic gastrectomy (D1 +) worldwide, especially in East Asian countries. Nevertheless, the occurrence rate of PGS and the quality of life (QoL) of patients after laparoscopic gastrectomy after 1 year is similar to that of patients after open gastrectomy[46-51]. To alleviate this, SNNS is a promising treatment strategy for function-preserving curative gastrectomy[4,14-27]. It has been reported that the PGS and QoL of function-preserving curative gastrectomy were less than those of standard gastrectomy[52-61]. However, there are two concerns that must be addressed before SNNS can be applied in clinical practice. One is that reducing the extent of nodal dissection may compromise curability. Another concern is whether preserving a large portion of the stomach will have any disadvantages, especially for an increase in the number of MMGCs of the remnant stomach.

In this study, we investigated the treatment outcome of SNNS from the viewpoint of life prognosis in comparison with the guidelines surgical strategy. In both the original data sets and propensity score-matched comparisons, the OS and RFS of the SNNS group were not inferior to those of the control group. This result supports the hypothesis that the oncological safety of the SNNS group is not inferior to that of the guidelines. Since this is a retrospective study, it is difficult to judge whether the life

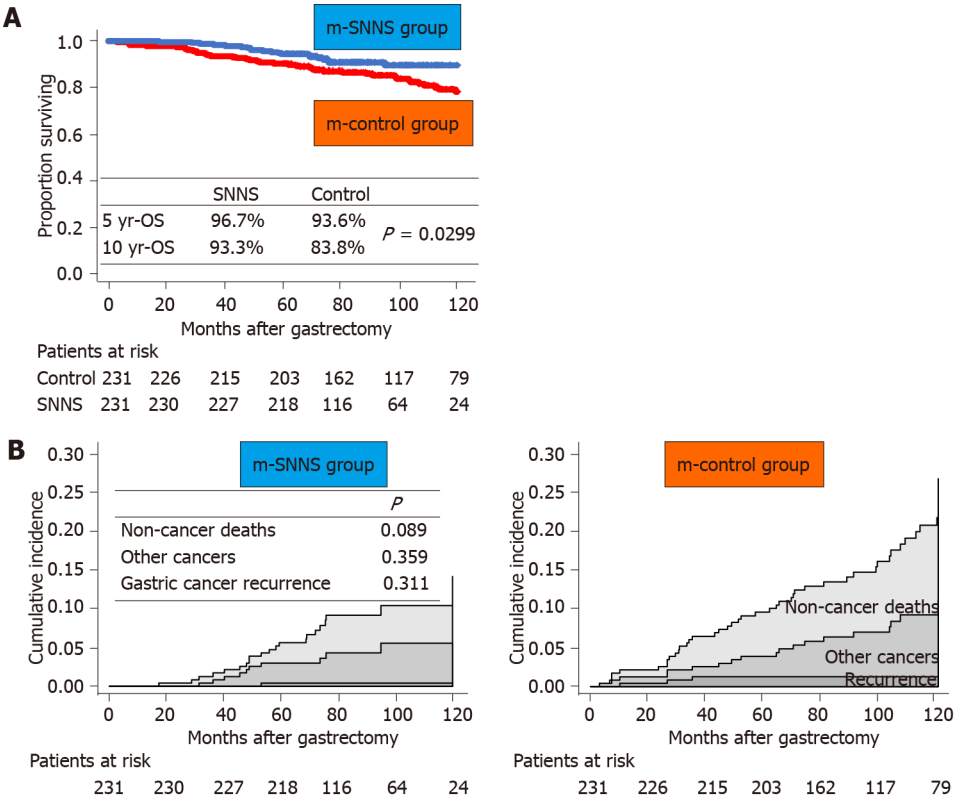


Figure 7 Comparisons of overall survival and cumulative incidence between the two groups after propensity score matching. A: Overall survival curves; B: Cumulative incidence curves of gastric cancer recurrence or the reason for death. The overall survival of the sentinel node navigation surgery (SNNS) group was significantly better than that of the control group. The cumulative recurrence of non-cancer deaths from other diseases in the SNNS group tended to be lower than that in the control group. SNNS: Sentinel node navigation surgery; m-SNNS: Propensity score-matched sentinel node navigation surgery; m-control: Propensity score-matched control; OS: Overall survival.

prognosis of the SNNS group is equivalent to that of the control group based on our results. A prospective non-inferiority trial is needed to make this scientific judgment. A prospective study is currently ongoing by the Japanese Society for SNNS[37]. In the protocol of this study, the expected 5-year recurrence-free survival rate was set at 98%, the non-inferiority margin was set at 10%, and the expected number of patients with sentinel node navigation was set to 225. The number of patients in the SNNS group in our study was 239 and, even after propensity score matching, 231 patients exceeded the number of patients in this prospective study. The result of life prognosis of the SNNS group in our study was one recurrent patient and 99.6% of RFS at both 5 and 10 years, comparable to conventional surgery. Extrapolating from these results, it seems that the curability of the SNNS could be proved to some extent. In addition, in the multivariate analysis, the only significant factor affecting gastric cancer recurrence was pN status, not SNNS grouping. In other words, the concern that reducing the extent of dissection may compromise curative outcomes would be unfounded.

The OS of the SNNS group was better than that of the control group in both comparisons of the original data sets and propensity score-matched groups. There was little difference in RFS between the two groups, and there was no significant difference in other cancer deaths. It was considered that the reason for this difference in OS would be the non-cancer deaths from other diseases. In multivariate analysis, the significant factors affecting non-cancer deaths were age and SNNS grouping. In the prospensity score-matched comparison, age was adjusted between the two groups, and a significantly better trend for non-cancer deaths was observed in the SNNS group. There is a possibility that keeping the gastrectomy area small leads to the maintenance of food volume, dietary habits, and nutritional status and has the effect of suppressing non-cancer death. However, this idea tends to be too advanced, and it may be reasonable to interpret that the survival outcome of patients with SNNS is not inferior to that of standard surgery.

In this study, we distinguished between MMGCs and local recurrence of gastric cancer. One patient with local recurrence of the oral stump was observed in the control group, whereas no local recurrence was observed in the SNNS group. This recurrent

patient was unresectable, underwent chemoradiotherapy, and died due to distant metastasis. Meanwhile, MMGC in the remnant stomach was observed in six patients in the SNNS group and 15 in the control group. One of these patients was unresectable and died after 10 years. However, all other MMGC patients were curatively resectable by gastrectomy or ESD, and there were no recurrent deaths from MMGC during the study period. Although it is sometimes difficult to distinguish between local recurrence and MMGC, we distinguished these two situations because of the favorable outcome of MMGC. A randomized prospective clinical trial of SNNS for gastric cancer was conducted in South Korea[27,62-65], and an interim analysis was recently reported at the American Society of Clinical Oncology (ASCO) annual meeting[63]. They reported that they failed to prove the non-inferiority of RFS in the SNNS group, but they did not strictly distinguish between MMGC and local recurrence. The MMGC and local recurrence should be clearly distinguished.

There was no difference in the cumulative incidence of MMGC[66-69] between the two groups in this study. Therefore, it was speculated that there is not much concern for whether MMGCs increase as the area of the remnant gastric mucosa increases. However, we cannot conclude with this result that there is no need to worry about the increased risk of MMGC in SNNS. Yaguchi *et al*[70] followed the prognosis of 50 SNNS cases and reported that MMGC occurred in 8% of cases. Kinami *et al*[71] conducted a national questionnaire survey and reported that the risk of MMGC increases as the area of the remnant stomach increases. The reason for this discrepancy between the present study and previous reports is unclear. Considering the natural history of early gastric cancer, most MMGC cases may have been caused by misdiagnosis at the time of initial endoscopy. The patients in the SNNS group had more detailed endoscopy than those in the control group to exclude multiple gastric cancers, which may be related to selection bias. However, in the study by Kinami *et al*[71], many MMGCs in surgeries with a large remaining gastric mucosal area were resected by ESD, and it was concluded that there is no need to hesitate to perform function-preserving surgery because of the increased risk of MMGC. The results of the present study also suggest that there is no need to forgo the adoption of SNNS due to concerns about MMGC.

Through this study, the problems of SNNS became apparent, that is, the preoperative diagnostic ability. The precise diagnosis of early gastric cancer is difficult, not only in the depth of invasion but also in the lateral margin. All patients had a preoperative diagnosis of ≤ 5 cm along the long axis; however, 2.8% of the patients were found to have more than 5 cm in the postoperative specimens, including one patient of 87 mm. Six (31.6%) patients > 50 mm had lymph node metastasis. Misdiagnosis of size not only entails a positive margin, but also increases the possibility of lymph node metastasis. It was suggested that the accuracy of preoperative diagnosis, especially accurate extent diagnosis, must be ensured in order to safely perform SNNS.

Standard surgical treatment for early gastric cancer is standard gastrectomy D1 +[1-4]. However, 72.8% of patients in the SNNS group had D0. All SNNS patients underwent lymphatic basin dissection. This result may be interpreted as follows: Early gastric cancer patients do not necessarily require nodal dissection up to D1 +; and in the patients who were node negative, the reduction of the dissection area to the lymphatic basin did not affect the prognosis. On the other hand, 96% (24/25) of nodal metastatic patients in the SNNS group had metastases only within the lymphatic basin; the patient who had nodal metastases that was spread outside the basin was the only one with advanced gastric cancer with macroscopic metastases that could be easily diagnosed intraoperatively. On the other hand, one patient in the SNNS group had nodal recurrence despite being judged to be positive for metastasis during surgery and changed to D2, and recurrence may not have been avoided even if standard treatment was applied initially. Considering these facts, it may be possible to reduce the extent of nodal dissection to only the lymphatic basin for all patients with cT1N0 less than 5 cm in the future.

This study has some limitations. This was a retrospective study. It is possible that there was a selection bias in the SNNS group. Another problem is that the study was conducted over a long period of time. The diagnostic and therapeutic techniques have advanced during this period, and this may have affected the prognosis of patients and the incidence of MMGC. In addition, there were no QoL data of the SNNS group in this study. A nationwide multicenter prospective study is essential to correctly determine the prognosis, rate of non-cancer deaths from other diseases, and QoL assessment data. The results of a Japanese study[37] are awaited.

CONCLUSION

In both original data sets and propensity score-matched comparisons, OS and RFS of patients who underwent gastrectomy guided by sentinel node navigation were not inferior to those of standard gastrectomy. In addition, there was no difference in the cumulative incidence of MMGC between the two groups. The oncological safety of SNNS is not inferior to that of the guidelines. This study also indicates the possibility of reducing the extent of nodal dissection to only the lymphatic basin for all patients with cT1N0 less than 5 cm in the future.

ARTICLE HIGHLIGHTS

Research background

If early gastric cancer patients who are negative for lymph node metastasis can be diagnosed intraoperatively, excessive nodal dissection and extensive gastrectomy can be avoided. Currently, the most effective method for diagnosing lymph node metastasis is sentinel node biopsy. Lymphatic basin dissection is a sentinel node biopsy method that is specific for gastric cancer. The dyed lymphatic system was dissected *en bloc* and sentinel nodes were identified at the back table (*ex vivo*) using this method. This method not only reduces the difficulty of sentinel node biopsy, but also serves to a certain extent as backup dissection. Even with lymphatic basin dissection, blood flow to the residual stomach can be preserved and function-preserving curative gastrectomy can be performed, such as segmental gastrectomy and local resection.

Research motivation

The oncological safety of function-preserving curative gastrectomy combined with lymphatic basin dissection has not yet been fully investigated.

Research objectives

This study aimed to investigate the life prognosis of patients with early gastric cancer who underwent sentinel node navigation surgery (SNNS) in comparison with standard guideline surgery.

Research methods

Gastric cancer patients were retrospectively collected. The inclusion criteria were as follows: Superficial type (type 0); preoperative diagnosis of 5 cm or less in length; clinical T1-2; and node-negative on X-computed tomography. The patients underwent SNNS. First, sentinel node mapping was performed, followed by lymphatic basin dissection and rapid intraoperative pathology. If the sentinel nodes were diagnosed as metastasic at rapid diagnosis, standard gastrectomy with nodal dissection up to D2 was performed; if the sentinel nodes were diagnosed as node-negative, the extent of gastrectomy was reduced, and function-preserving curative gastrectomy was performed. The life prognosis and cumulative incidence of metachronous multiple gastric cancer (MMGC) were investigated. Patients with the same inclusion criteria and who underwent standard gastrectomy and guideline lymph node dissection with or without sentinel node biopsy were selected as the control group.

Research results

There were 239 patients in the SNNS group and 423 patients in the control group. All patients were diagnosed as node-negative preoperatively, but pathological nodal metastasis was observed in 10.5% of patients in the SNNS group and 10.4% in the control group. The diagnostic ability of sentinel node biopsy in this study was 84% and 98.6% for sensitivity and accuracy, respectively. In the SNNS group, 18.4% of patients underwent standard surgery, 14.2% had modified gastrectomy, and 67.4% had function-preserving curative gastrectomy, in which the extent of resection was further reduced than that recommended by the guidelines. The overall survival (OS) rate in the SNNS group was 96.8% at 5 years and was significantly better than 91.3% in the control group ($P = 0.0014$). The relapse-free survival (RFS) rate in the SNNS group was 99.6% at 5 years and 98.1% in the control group. After propensity score matching, there were 231 patients in both groups, and the OS in the SNNS group remained significantly better than that in the control group ($P = 0.030$). The cumulative recurrence rate in the SNNS group was 0.43% in 5 years and 1.30% in the control group, which was not statistically different. There was no difference in the incidence of

MMGC between the SNNS group (1.7% at 5-years) and the control group (2.3% at 5-years).

Research conclusions

In both original data sets and propensity score-matched comparisons, the OS rate and RFS rate of patients who underwent gastrectomy guided by sentinel node navigation were not inferior to those of standard gastrectomy. In addition, there was no difference in the cumulative incidence of MMGC between the two groups.

Research perspectives

The oncological safety of sentinel node navigation surgery for early-stage gastric cancer is not inferior to that of the guideline. This study also indicates the possibility of reducing the extent of nodal dissection to only the lymphatic basin for all patients with cT1N0 less than 5 cm in the future.

ACKNOWLEDGEMENTS

The authors are most grateful to Dr. Koichi Miwa, an ex-professor at Kanazawa University. We would also like to thank Dr. Takeo Kosaka, an ex-professor at Kanazawa Medical University.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 14; 27(46): 8031-8032

DOI: 10.3748/wjg.v27.i46.8031

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

CORRECTION

Erratum: Author's research-fund support notation correction. Mass forming chronic pancreatitis mimicking pancreatic cystic neoplasm: A case report *World J Gastroenterology* 2018; Jan 14; 24 (2): (297-302)

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Author contributions: Jee KN designed the report, collected the patient's clinical data, drafted the article and reviewed the manuscript, and approved the final manuscript as submitted.

Conflict-of-interest statement: The author has no conflicts of interest to disclose.

Supported by Dankook University Research Fund, No. R201600314.

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, B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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Abstract

Correction to "Mass forming chronic pancreatitis mimicking pancreatic cystic neoplasm: A case report" *World J Gastroenterology* 2018; 24 (2): 297-302. This article had accidentally omitted the fact of research-fund support notation. It should be added as supported by Dankook University Research Fund (R201600314).

Key Words: Correction; Supported by; Research fund

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Core Tip: This manuscript is a research-fund support notation correction for Mass forming chronic pancreatitis mimicking pancreatic cystic neoplasm: A case report *World J Gastroenterology* 2018; Jan 14; 24 (2): (297-302).

Citation: Jee KN. Erratum: Author's research-fund support notation correction. Mass forming chronic pancreatitis mimicking pancreatic cystic neoplasm: A case report *World J Gastroenterology* 2018; Jan 14; 24 (2): (297-302). *World J Gastroenterol* 2021; 27(46): 8031-8032

URL: https://www.wjgnet.com/1007-9327/full/v27/i46/8031.htm

DOI: https://dx.doi.org/10.3748/wjg.v27.i46.8031

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Received: September 7, 2021
Peer-review started: September 7, 2021
First decision: September 29, 2021
Revised: September 30, 2021
Accepted: November 29, 2021
Article in press: November 29, 2021
Published online: December 14, 2021

P-Reviewer: Cao ZF, Herraiz JL, Peng JY
S-Editor: Wu YXJ
L-Editor: A
P-Editor: Wu YXJ

TO THE EDITOR

Correction to "Mass forming chronic pancreatitis mimicking pancreatic cystic neoplasm: A case report" *World J Gastroenterology* 2018; 24 (2): 297-302. This article had accidentally omitted the fact of research-fund support notation. It should be added as supported by Dankook University Research Fund (R201600314).

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 14; 27(46): 8033-8039

DOI: 10.3748/wjg.v27.i46.8033

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

LETTER TO THE EDITOR

Surveillance strategies for precancerous gastric conditions after *Helicobacter pylori* eradication: There is still need for a tailored approach

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Author contributions: Shahini E conducted study conceptualization, drafting the manuscript, data collecting, and curation; Maida M supervised; Shahini E and Maida M edited paper, critically reviewed the data entries for important intellectual content, checked for completeness of information; and both authors approved the final draft.

Conflict-of-interest statement: All the authors have no conflict of interest related to the manuscript.

Country/Territory of origin: Italy

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, C
Grade D (Fair): 0
Grade E (Poor): 0

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Abstract

Prevailing evidence declares that *Helicobacter pylori* (*H. pylori*) eradication therapy could shift precancerous gastric conditions (PGC) and positively confines gastric cancer (GC) risk during long-term endoscopic follow-up. Nonetheless, there is a yet unsolved controversy regarding the best-individualized surveillance strategies following *H. pylori* eradication, based on malignant risk stratification. This last dispute is due to the uncertainty of contemporary evidence and the role of *H. pylori* inflammatory changes in underestimating PGC at the index endoscopy. However, the current state of the art suggests that it is reasonable that high-quality endoscopy with histological assessment for the most accurate diagnosis of PGC may be delayed in selected high-risk patients without alarm signs for malignancy, following the eradication of *H. pylori*. Notwithstanding, these aspects need to be further examined in the next future to establish and optimize the most beneficial and cost-effective strategies for recognizing and managing *H. pylori*-positive patients with PGC in the short- and long-term follow-up. Accordingly, additional studies are yet required to sharpen the hazard stratification of patients with the greatest chance of GC evolution, also recognizing the evolving racial, ethnic, immigration factors and the necessity of novel biomarkers to limit GC development or accomplish a diagnosis of malignancy at an early stage.

Key Words: *Helicobacter pylori*; Endoscopic surveillance; Atrophic gastritis; Intestinal metaplasia; Dysplasia; Gastric cancer

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Received: September 4, 2021
Peer-review started: September 4, 2021
First decision: October 2, 2021
Revised: October 3, 2021
Accepted: November 25, 2021
Article in press: November 25, 2021
Published online: December 14, 2021

P-Reviewer: An T, Jin CH, Katayama Y
S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

Core Tip: Prevailing evidence affirms that *Helicobacter pylori* (*H. pylori*) eradication therapy could shift precancerous gastric conditions and positively confines gastric cancer risk during long-term endoscopic follow-up. Nonetheless, there is a yet unsolved dispute concerning the most useful individualized surveillance strategies following *H. pylori* eradication, based on malignant risk stratification. These aspects should be examined in the next future to establish and optimize the most cost-effective strategies for recognizing and managing *H. pylori*-positive patients with precancerous gastric conditions in the short- and long-term follow-up. Accordingly, new studies are required to sharpen the hazard stratification of patients with the greatest chance of progressing into gastric cancer.

Citation: Shahini E, Maida M. Surveillance strategies for precancerous gastric conditions after *Helicobacter pylori* eradication: There is still need for a tailored approach. *World J Gastroenterol* 2021; 27(46): 8033-8039
URL: https://www.wjgnet.com/1007-9327/full/v27/i46/8033.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i46.8033

TO THE EDITOR

We read with great interest the review of Weng *et al*[1], pointing out the most recent literature supporting the impact of *Helicobacter pylori* (*H. pylori*) on the gastric mucosa alterations. Specifically, the authors assumed that, despite some controversy, current evidence suggests that *H. pylori* eradication treatment could reverse atrophic gastritis (AG) and intestinal metaplasia (IM) and favorably limits the appearance of gastric cancer (GC), particularly in long-term surveillance[1].

However, there is still unresolved debate regarding the best-individualized follow-up strategies, based on malignant risk stratification, due to uncertainty of current evidence and the role of *H. pylori* inflammatory changes in underestimating IM extension and dysplastic lesions at the index endoscopy (Table 1)[2-12].

In a recent article focused on the crucial role of high-resolution endoscopy with narrow-band imaging (NBI) for the optimal detection of IM, Dinis-Ribeiro M *et al*[13] criticized the recent U.S. guidelines that discourage short-interval endoscopic surveillance of patients with IM[14]. They supported and elaborated on the rationale behind the suggested 3-year-interval endoscopic surveillance of high-risk subjects with more extensive IM[13,14], for detecting early gastric neoplasia that, due to dismal prognosis of GC and increased aging of the population, can improve patient's survival [14]. Additionally, they stated that "*The majority of patients with gastric IM, those who during high-quality endoscopy were shown to have IM of limited severity and extent, confined to the antrum, and have a negative family history for GC do not require surveillance*"[13]. Notwithstanding, maybe this affirmation seems to neglect genetic/epigenetic/racial factors, personal habits and underlying comorbidity roles (*i.e.*, alcohol consumption, smoking, autoimmune and metabolic diseases) that can hold distinctive malignant potential, theoretically affecting subsequent endoscopic surveillance.

Notably, a recent prospective cohort study[12], including 85 Italian patients with *H. pylori*-related active gastritis, undergoing upper gastrointestinal endoscopy 6 mo following eradication therapy, demonstrated that high-resolution endoscopy with NBI doubled the rate of identifying histological low-grade dysplasia (LGD) missed at pre-treatment endoscopy, in a high-risk subgroup which had extensive atrophy and IM at baseline. In over 40% of patients, visible gastric lesions with LGD were found following *H. pylori* eradication was not identified at their first pre-treatment endoscopy, thus suggesting that inflammatory changes associated with active *H. pylori* infection hinder the correct detection of gastric LGD lesions[12].

Of interest, in cases of indefinite gastric dysplasia, or with "not visible" dysplasia diagnosed randomly throughout the stomach without endoscopic evidence of visible lesions, the prevailing guidelines recommend a necessary endoscopic reassessment using high-resolution endoscopy with NBI to rule out dysplasia on missed visible lesions[12,15].

Moreover, some authors consider high-resolution surveillance endoscopy with NBI as "*sufficient for a diagnosis of extensive IM or premalignant stomach even without biopsy sampling*"[13]. There is an established association between the endoscopic grading of

Table 1 Characteristics of patients included in the eleven selected studies applying endoscopic surveillance shorter than two years for the evaluation of precancerous gastric conditions following *Helicobacter pylori* eradication

	van der Hulst RW *et al*[2], 1997	Tucci A *et al* [3], 1998	Sung JJ *et al* [4], 2000	Annibale B *et al* [5], 2000	Ohkusa T *et al*[6], 2001	Oda Y *et al*[7], 2004	Annibale B *et al* [8], 2002	Yamada T *et al*[9], 2003	Iacopini F *et al* [10], 2003	Wambura C *et al* [11], 2004	Panarese *et al*[12], 2020
Study	Prospective	Retrospective	Prospective, randomized, placebo controlled trial	Observational, prospective study	Single-blind, uncontrolled prospective trial	Retrospective	Retrospective	Retrospective	Observational, prospective study	Observational, prospective study	Observational, prospective study
Country	Netherlands	Italy	China	Italy	Japan	Japan	Italy	Japan	Italy	Japan	Italy
Mean age, yr	49.2	50	51 (Median)	48.7	54	51	46 (Median)	52.6	55	51.2	56.1
Male, %	54	50	49.5	14.3	73	89.8	22.5	64.4	75	74.7	37.6
Overlap AAG	NA	0	NA	48.6	NA	NA	55	NA	NA	NA	26.3
Mean follow-up, mo	12	12	12	6-12	12-15	1-2	6-12	22	12	12	6
Total, *n*	106	10	226	25	115	59	40	87	40	107	85
Resolution of gastric acute/chronic inflammation in the antrum *n* (%)	S	10/10 (100)	S	25/25 (100)	NA	S	S	S	S	S	81/85 (95.3)
Resolution of gastric acute/chronic inflammation in the corpus, *n* (%)	S	NA	S	25/25 (100)	NA	S	S	S	S	S	81/85 (85.3)
Resolution of gastric acute/chronic inflammation in the fundus *n* (%)	NA	10/10 (100)	NA	NA	NA	NA	NA	NA	NA	NA	NA
Improvement of AG in the antrum, *n* (%)	NS	NS	NS	NS	34/38 (89)	NS	NS	NS	NS	S	NS
Improvement of AG in the corpus, *n* (%)	NS	NA	NS	NS	34/38 (89)	NS	8/40 (20) AG reversed	S	NA	S	NS

Improvement of AG in the fundus, *n* (%)	NA	S	NA	NA	NA	NA	NA	S	NA	NA	NA
Improvement of IM in the antrum, *n* (%)	NS	S	S	NS	28/46 (61)	NS	NS	NS	NS	NS	NS
Improvement of IM in the corpus, *n* (%)	NS	NA	NS	NS	28/46 (61)	NS	NS	NS	NA	S	NS
Improvement of IM in the fundus, *n* (%)	NA	NS	NA	NA	NA	NA	NA	NA	NA	S	NA
ECL pattern regression, *n* (%)	NA	NA	NA	8/15 (53.3) patients with AG in the body (12 mo after curing *H. pylori*)	NA	NA	NA	NA	NA	NA	36/39 (92.3)
LGD regression (or progression), *n* (%)	NA	NA	NA	1/1 (100) regression in a patient with AG in the body (12 mo after curing *H. pylori*)	NA	NA	NA	NA	NA	NA	The proportion of patients with histological diagnosis of LGD on random biopsies did not significantly change after *H. pylori* eradication [15 (17.6) *vs* 9 (10.6)]; the detection of LGD on visible lesions significantly increased after *H. pylori* eradication [0 (0) *vs* 19 (22.3)]
Conclusions	The usefulness of *H. pylori* eradication to regress precancerous lesions following 12 mo follow-up is uncertain	The natural history of AG can be modified by the eradication of *H.pylori*	At 12 mo, *H. pylori* eradication can block the histological progression of gastric mucosa alterations	*H. pylori* infection may be cured in patients with AG in the body with a partial reversing of its adverse outcomes on acid secretion and body ECL cell hyperplasia	After successful *H. pylori* eradication, precancerous lesions improved in most patients	After *H. pylori* eradication, neutrophil infiltration in the gastric mucosa improved relatively soon, while AG and IM did not display such tendency	In patients with AG of the body and *H. pylori* infection, the assessment of histological data after eradication is essential. In patients with maintaining body atrophy after *H. pylori* elimination, there is no association with the reversal of body atrophy, even at long-term surveillance	AG in the corpus can be improved after 12 mo following *H. pylori* eradication	*H. pylori* positive patients with AG, the overall oxidative damage of the gastric mucosa is more severe than that in *H. pylori* positive patients with nonatrophic gastritis	Eradication of *H. pylori* may decrease the risk of GC, due to the importance of *H. pylori* infection in the contributory role of gastritis in COX-2 expression and the dissociation between the processes of regression in gastritis and the reduction in COX-2	HR-WLE with NBI can be more reliable in diagnosing LGD on visible lesions after *H. pylori* elimination, presumably due to the removal of the underlying confounding effects of inflammatory and mucosal lymphoproliferative changes induced by *H. pylori* chronically active infection. Aged patients and those with autoimmune diseases (especially AAG) could be at higher risk for *H. pylori* persistent infection

AAG: Autoimmune gastritis; AG: Atrophic gastritis; IM: Intestinal metaplasia; ECL: Enterochromaffin-like cell; LGD: Low-grade dysplasia; *H. pylori*: *Helicobacter pylori*; NA: Not available; NS: Not significant; S: Significant improvement;

HR-WLE: High-resolution wight light endoscopy; NBI: Narrow band imaging.

gastric intestinal-metaplasia (EGGIM) and operative link on gastritis/intestinal-metaplasia assessment (OLGIM) stages in the assessment of the presence/extent of IM [12,13], and EGGIM stages ≥ 5 with OLGIM III/IV predicts early GC risk[12,13], although its reproducibility needs to be further confirmed in larger prospective studies as also expressed in the U.S. guidelines[14].

Nevertheless, even if feasible as a surveillance program in specialized referral centers, this strategy may not be widely applicable in endoscopy units that do not have access to such technologies. A targeted bioptic mapping seems more adequate for identifying mucosal gastric areas at risk of malignant transformation[12], despite the existing risk of overestimating OLGIM in patients with mild/focal IM. Concomitant *H. pylori*-related gastritis may limit the accuracy of EGGIM classification at the time of the initial endoscopy.

Advanced histological atrophy stages, even after *H. pylori* eradication, carry the highest risk for developing gastric neoplasia[12-15]. Nevertheless, recent long-term cohort studies from Eastern countries reported late development of GC during 5-14 years monitoring also in patients with none/mild gastric atrophy or antral IM, irrespectively of *H. pylori* eradication[12,16,17].

Notably, even with a high-resolution endoscope, if morphological changes do not appear, genetic and epigenetic changes in epithelial cells cannot be detected[18]. Specifically, epigenetic alterations (*i.e.*, aberrant DNA methylation), accumulate in cancers and also in normal-appearing tissues surrounding cancers[18]. Indeed, cross-sectional studies prove that aberrant methylation levels in normal tissues may be associated with cancer risk, particularly in chronic inflammation-associated cancers. Additionally, the relationship between miR-124a-3 DNA methylation abnormalities and similar trends for EMX1 and NKX6-1, have been judged extremely relevant predictors of developing authentic metachronous GCs[18].

Therefore, it is reasonable that high-quality endoscopy with histological assessment for the most accurate diagnosis of PGC[12] may be delayed, in selected high-risk patients who are symptomatic but have no alarm hallmarks for malignancy, after eradication of *H. pylori* diagnosed according to prior results of non-invasive tests had been achieved and serological autoimmunity biomarkers had been performed (*e.g.*, autoimmune AG-AAG), rather than applying the prevailing guidelines suggestion of operating targeted biopsies at initial endoscopy for histological estimation and determination of *H. pylori* status[12]. Such an approach is likely to enhance the PGC detection rate, especially for dysplastic lesions, reducing the confounding effect of *H. pylori*-related gastritis or AAG, and complies with the European guidelines[15], which recommend immediate high-quality endoscopy after the diagnosis of dysplasia without endoscopically visible lesions[12,15].

Therefore, we believe that further large prospective multicenter studies are still needed to identify additional risk factors of gastric malignancy development.

Moreover, multiple and evolving racial, ethnic, and immigration factors, may affect the risk of gastric neoplasia[19,20], calling also for the necessity of novel biomarkers for tailoring surveillance strategies to different patients.

These aspects should be considered in the next future to better define and optimize cost-effective strategies for identifying and managing *H. pylori*-positive patients with PGC in the short- and long-term follow-up.

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

World Journal of *Gastroenterology*

World J Gastroenterol 2021 December 21; 27(47): 8040-8200



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World Journal of Gastroenterology

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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: *Yan-Xia Xing*; **Production Department Director:** *Xiang Li*; **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, Subrata Ghosh

EDITORIAL BOARD MEMBERS

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

PUBLICATION DATE

December 21, 2021

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World J Gastroenterol 2021 December 21; 27(47): 8040-8046

DOI: 10.3748/wjg.v27.i47.8040

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

EDITORIAL

Progress in elucidating the relationship between *Helicobacter pylori* infection and intestinal diseases

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Author contributions: Fujimori S contributed to the writing of this paper.

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

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

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Abstract

Helicobacter pylori (*H. pylori*) infection causes changes to the intestinal flora, such as small intestinal bacterial overgrowth, and increases gastric acid secretion-stimulating gastrointestinal hormones, mainly gastrin, due to a decrease in gastric acid caused by atrophic gastritis. In addition, the cellular components of *H. pylori* travel through the intestinal tract, so the bacterial infection affects the immune system. Therefore, the effects of *H. pylori* infection are observed not only in the stomach and the proximal duodenum but also in the small and large intestines. In particular, meta-analyses reported that *H. pylori*-infected individuals had an increased risk of colorectal adenoma and colorectal cancer. Moreover, a recent study reported that the risk of developing colorectal cancer was increased in subjects carrying *H. pylori* vacuolating cytotoxin A antibody. In addition, it has been reported that *H. pylori* infection exacerbates the symptoms of Fabry's disease and familial Mediterranean fever attack and is involved in irritable bowel syndrome and small intestinal ulcers. On the other hand, some studies have reported that the frequency of ulcerative colitis, Crohn's disease, and celiac disease is low in *H. pylori*-infected individuals. Thus, *H. pylori* infection is considered to have various effects on the small and large intestines. However, few studies have reported on these issues, and the details of their effects have not been well elucidated. Therefore, additional studies are needed.

Key Words: *Helicobacter pylori*; Intestine; Colorectal cancer; Intestinal bacterial overgrowth; Inflammatory bowel disease; Intestinal ulcer

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Core Tip: *Helicobacter pylori* (*H. pylori*) infection causes abnormalities in the intestinal flora and increases gastric acid secretion-stimulating gastrointestinal hormones. In

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Received: May 26, 2021
Peer-review started: May 26, 2021
First decision: June 17, 2021
Revised: June 30, 2021
Accepted: December 7, 2021
Article in press: December 7, 2021
Published online: December 21, 2021

P-Reviewer: Krzyzek P, Wu LH
S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

addition, the cellular components of *H. pylori* travel through the intestinal tract, causing an effect of bacterial infection on the immune system. Meta-analyses reported that colorectal adenoma and cancer increase in *H. pylori*-infected individuals, and this bacterium has also been reported to be involved in several other diseases. On the other hand, *H. pylori* infection is considered to suppress inflammatory bowel disease. However, few studies have reported on these issues, and further elucidation is required.

Citation: Fujimori S. Progress in elucidating the relationship between *Helicobacter pylori* infection and intestinal diseases. *World J Gastroenterol* 2021; 27(47): 8040-8046
URL: https://www.wjgnet.com/1007-9327/full/v27/i47/8040.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i47.8040

INTRODUCTION

It is well known that *Helicobacter pylori* (*H. pylori*) infection causes atrophic gastritis, gastric ulcer, duodenal ulcer, gastric cancer, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Many studies have reported that *H. pylori* infection might affect not only the stomach and the proximal duodenum but also the intestinal tract on the anal side. For example, meta-analyses reported that colorectal adenomas and colorectal cancers are more common in *H. pylori*-infected individuals[1-3]. In addition, studies have reported that the symptoms of Fabry's disease are exacerbated[4] and that attacks of familial Mediterranean fever (FMF) are increased[5] in *H. pylori*-infected individuals. Moreover, studies have reported that small intestinal ulcerative lesions in patients are significantly more common in *H. pylori*-infected individuals[6]. Furthermore, it has been suggested that *H. pylori* infection may cause irritable bowel syndrome[7]. On the other hand, the frequencies of ulcerative colitis[8,9], Crohn's disease[8-10], and celiac disease[11] are low in *H. pylori*-infected individuals.

Thus, *H. pylori* infection causes neoplastic and ulcerative lesions not only in the stomach and proximal duodenum but also in a wide range of locations that range from the distal duodenum to the large intestine. Furthermore, *H. pylori* infection is associated with abnormal pathology of immune diseases and abnormal intestinal motility. In the stomach and duodenal bulb where the gastric mucosa is found, various diseases occur due to *H. pylori* infection. Direct infection of the anal side of the duodenum to the large intestine does not occur without ectopic gastric mucosa, such as the Meckel diverticulum[12]. Therefore, the causes of abnormalities of the intestinal tract on the anal side are presumed to be due to *H. pylori* infection; these causes include the effects of *H. pylori* components, abnormalities of the intestinal flora, the effects of immune responses, and the effects of gastrointestinal hormones such as gastrin. In this paper, the effects of *H. pylori* infection on the small and large intestines will be examined and discussed.

EFFECTS OF *H. PYLORI* COMPONENTS

H. pylori DNA is detected in the lowest portion of the small intestine as a bacterial component of *H. pylori*, and this bacterial component is excreted in the stool[13]. Utilizing the fact that bacterial components are excreted in stool, *H. pylori* infection can now be confirmed by a stool test. Studies have reported that the bacterial component of *H. pylori* promotes DNA synthesis in a small intestinal cell line (IEC-6), as evaluated by the labeling index[14]. Similarly, the cancer-related CagA-positive strain of *H. pylori* has been confirmed to stimulate DNA synthesis in IEC-6 epithelial cells *in vitro*, regardless of its ability to produce vacuolating cytotoxin A (VacA) toxin[15].

Butt *et al*[16] recently reported an increased risk of developing colorectal cancer in individuals carrying serum antibodies against VacA of *H. pylori*. Rassow *et al*[17] reported in a review that VacA forms chloride (Cl^-) channels that enter the cell and mitochondrial membranes, and VacA causes loss of mitochondrial membrane potential, mitochondrial fragmentation, formation of reactive oxygen species, autophagy, cell death and gastric cancer. Since Cl^- channel abnormalities are involved in cystic fibrosis, which is known to be associated with colorectal cancer, this VacA-induced Cl^- channel abnormality may be involved in colorectal cancer[18]. Because

Butt *et al*[16] did not directly examine the bacterial cell components of the intestinal tract but examined serum antibodies, the effect of bacterial components could not be determined. However, blood antibodies are unlikely to be carcinogenic. Therefore, bacterial cell components have a high probability of being involved. Whether VacA may be the cause of colorectal carcinogenesis has not been resolved. However, bacterial cell components, such as VacA, can travel through the intestinal tract and could be associated with colon tumors. Thus, *H. pylori* bacterial components that travel through the intestinal tract have a significant likelihood of affecting the intestinal tract.

CHANGES IN THE INTESTINAL FLORA

When atrophic gastritis due to *H. pylori* infection progress, the gastric acid concentration decreases, and the bactericidal ability of the stomach diminishes. The bacterial flora in the stomach changes drastically[19]. This causes abnormalities in the intestinal flora. *H. pylori* often infects the stomach at a young age and significantly reduces the post-infection *Firmicutes* to *Bacteroidetes* ratio at the phylum level[20]. Successful eradication of *H. pylori* increases the amount of *Bifidobacterium* in the intestinal flora [21]. A relationship between *H. pylori* and small intestinal bacterial overgrowth (SIBO) has been reported[22]. SIBO is involved in many gastrointestinal and systemic diseases, and SIBO may be the cause of the increased rate of FMF attack in *H. pylori*-infected individuals[5].

In a systematic review and meta-analysis, Shah *et al*[23] reported a link between irritable bowel syndrome (IBS) and SIBO. Although the authors reported that the overall quality of the evidence was low in the analysis, the relationship between IBS and SIBO had long been strongly suspected. Even recently, there was a report that SIBO plays an important role in IBS[24]. It was also reported that *H. pylori* eradication improves IBS[25]. In the future, *H. pylori* eradication treatment may become an important treatment strategy for IBS patients with *H. pylori* infection.

It has been suggested that dysbiosis may be associated with colorectal carcinogenesis[26], and research on this front is progressing. *H. pylori* causes dysbiosis, including SIBO, which may be the cause of colorectal cancer. Further research could determine whether *H. pylori*-induced dysbiosis is associated with colorectal cancer.

Additionally, intestinal mucosal permeability has been reported to be enhanced in *H. pylori*-infected individuals[27]. We hypothesize that this hyperpermeability of the intestinal mucosa is combined with abnormalities in the intestinal flora, resulting in an increase in small intestinal ulcerative lesions[6]. However, there are very few reports examining the relationship between *H. pylori* infection and the intestinal flora, so future studies are required.

EFFECTS OF GASTROINTESTINAL HORMONES

H. pylori gastritis causes atrophic gastritis and reduces gastric acid secretion. Therefore, the blood gastrin concentration increases. A study in rats reported that *H. pylori* infection altered the levels of gastrin, cholecystokinin, and substance P, resulting in increased colonic motility[28]. This finding suggests the possibility that *H. pylori* infection could cause gastrointestinal motor dysfunction. *H. pylori* infection may also cause IBS due to its effects on gastrointestinal hormones.

Moreover, intestinal tract hormones, especially gastrin, are assumed to cause overgrowth in the large intestinal mucosa and to be closely related to large intestinal tumor development[29]. In addition, progastrin, not gastrin, levels are reported to be high in patients with colorectal cancer[30]. In colorectal cancer, the gastrin receptor is overexpressed, and gastrin-binding capacity is increased 10-fold over that in normal colonic epithelium[31]. It has also been reported that the expression of gastrin and its receptor promotes the progression from colorectal adenoma to cancer[32]. In mice, gastrin treatment enhanced colon cancer cell growth and invasion and decreased oxidative stress and apoptosis[33]. Additionally, G-protein coupled receptor 56, which is expressed in colonic stem and cancer cells, is upregulated in transgenic mice overexpressing human progastrin[34]. Thus, although it is experimentally likely that gastrin is involved in colon tumors, a recent patient study found that gastrin was not associated with colon tumors[35]. At this time, it appears that gastrin and VacA could be potential factors in the development of colorectal tumors due to *H. pylori* infection.

Table 1 Effects and factors of diseases in which *Helicobacter pylori* may affect the small and large intestines

Disease	Impact	Major factors suspected of being involved
Colon adenoma	Increase	Bacterial component, gastrin
Colon cancer	Increase	Bacterial component (especially VacA), gastrin, dysbiosis
Small intestinal ulcer	Increase	Mucosal permeability increased, dysbiosis
Irritable bowel syndrome	Involvement	Gastrointestinal hormones, SIBO
Ulcerative colitis	Decrease	Host immune response, antibacterial drug use
Crohn's disease	Decrease	Host immune response
Fabry's disease	Exacerbation	SIBO
FMF attack	Increase	SIBO
Celiac disease	Decrease	Immunological effects

SIBO: Small intestinal bacterial overgrowth; FMF: Familial Mediterranean fever; VacA: Vacuolating cytotoxin A.

IMMUNITY EFFECTS

H. pylori activates various innate immune system functions[36]. The immune system, especially Peyer's patches in the small intestine, may play an important role in *H. pylori*-induced gastritis because there are reports that gastritis is not induced in *H. pylori*-infected mice lacking Peyer's patches. Peyer's patch dendritic cells phagocytose coccoid forms of *H. pylori*. *H. pylori* transforms into a sphere in the anaerobic small intestine and stimulates the host's immune system *via* Peyer's patches[37]. Most likely, because of the involvement of this immune system response, a meta-analysis has recently evaluated the association between *H. pylori* infection and systemic lupus erythematosus, rheumatoid arthritis, autoimmune atrophic gastritis, and autoimmune pancreatitis. This study suggested that infection with more virulent strains of *H. pylori* (such as CagA positive) may increase the risk of autoimmune diseases[38]. In other words, *H. pylori* infection may be involved in intestinal diseases such as ulcerative colitis and Crohn's disease.

However, the frequency of ulcerative colitis and Crohn's disease is lower in *H. pylori*-infected individuals[8-10]. Meta-analyses have concluded that the risk of inflammatory bowel disease (IBD) is lower in *H. pylori*-infected individuals[39,40]. Furthermore, recent studies have reported that eradication of *H. pylori* under the age of 18 increases the risk of IBD[41]. In other words, *H. pylori* infection may be a potentially protective factor against the development of IBD[42].

In addition, lymphoma is a neoplastic disease of the immune system, and the fact that gastric MALT lymphoma is relieved by *H. pylori* eradication is well known. Small intestinal MALT lymphoma has been shown to be curable by eradication of *H. pylori*[43]. In particular, a study has reported that *H. pylori* eradication is effective in stage 1 MALT lymphoma[44]. However, it is unclear how *H. pylori* is involved in MALT lymphoma in the small intestine.

CONCLUSION

Multiple studies have reported that *H. pylori* has an effect on neoplastic lesions, ulcerative lesions, autoimmune diseases, and the abnormal gastrointestinal motility of the small intestine and large intestine. Table 1 summarizes the diseases in which *H. pylori* may affect the small and large intestines. Unfortunately, the wording in Table 1 is ambiguous because it is not known exactly how *H. pylori* is involved in these diseases. Although there are generally still few reports on this topic, the most advanced of these is the link between colorectal tumors and *H. pylori* infection. These studies show that *H. pylori* infection is involved in the increased rates of colorectal adenoma and cancer. The involvement of gastrin has been suspected as the reason for this increase in colorectal adenoma and cancer; however, recent studies have reported the involvement of bacterial cell components, such as VacA. In addition to the effects of bacterial components and gastrointestinal hormones, *H. pylori* infection may have various effects on the small and large intestines by causing abnormalities in the

intestinal flora and immunological effects. Few studies have reported on this topic, so more studies are needed in the future.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 21; 27(47): 8047-8057

DOI: 10.3748/wjg.v27.i47.8047

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

FRONTIER

Orphan patients with inflammatory bowel disease - when we treat beyond evidence

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Author contributions: Privitera G, Pugliese D, Scaldaferri F, Lopetuso L, Papa A, Rapaccini GL, Gasbarrini A, and Armuzzi A designed the project; Privitera G wrote the manuscript; Pugliese D, Scaldaferri F, Lopetuso L, Rapaccini GL, Gasbarrini A, and Armuzzi A contributed to literature research and revised critically the manuscript; all authors reviewed and approved the final draft of the article before submission; Armuzzi A oversaw the project and guarantees for the integrity of the work.

Conflict-of-interest statement: The authors declare the following conflicts of interest: Giuseppe Privitera received consultancy fees from Alphasigma and speaker fees from Janssen. Daniela Pugliese received speaker fees and/or advisory board from AbbVie, MSD, Takeda and Janssen, Pfizer. Franco Scaldaferri: advisory board

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Abstract

Inflammatory bowel disease (IBD) is a chronic condition that requires continuous medical treatment. To date, the medical management of patients with moderately-to-severely active IBD who develop dependence or resistance to corticosteroids is based on immunomodulator drugs. Such therapies are licenced after passing through three phases of randomized controlled trials (RCTs), and are subsequently adopted in clinical practice. However, the real-life population of IBD patients who require these therapies can significantly differ from those included in RCTs. As a matter of fact, there is a number of exclusion criteria – nearly ubiquitous in all RCTs – that prevent the enrolment of specific patients: Chronic refractory pouchitis or isolated proctitis in ulcerative colitis, short-bowel syndrome and stomas in Crohn's disease, ileorectal anastomosis in both ulcerative colitis and Crohn's disease, and elderly age are some representative examples. In this frontier article, we aim to give an overview of current literature on this topic,

for Abbvie, Janssen, MSD, Sanofi, Takeda. Antonio Gasbarrini reports personal fees for consultancy for Eisai S.r.l., 3PSolutions, Real Time Meeting, Fondazione Istituto Danone, Sinergie S.r.l. Board MRGE, and Sanofi S.p.A, personal fees for acting as a speaker for Takeda S.p.A, AbbVie, and Sandoz S.p.A, and personal fees for acting on advisory boards for VSL3 and Eisai. Alessandro Armuzzi: consulting and/or advisory board fees from AbbVie, Allergan, Amgen, Biogen, Bristol-Myers Squibb, Celgene, Celltrion, Ferring, Gilead, Janssen, Lilly, MSD, Mylan, Pfizer, Samsung Bioepis, Sandoz, Takeda; lecture and/or speaker bureau fees from AbbVie, Amgen, Biogen, Ferring, Giliead, Janssen, MSD, Mitsubishi-Tanabe, Nikkiso, Pfizer, Sandoz, Samsung Bioepis, Takeda; and research grants from MSD, Pfizer, Takeda. The remaining authors declare no competing interests.

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): 0
Grade D (Fair): 0
Grade E (Poor): 0

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in order to address the main knowledge gaps that need to be filled in the upcoming years.

Key Words: Pouchitis; Proctitis; Stoma; Short-bowel; Ileo-rectal anastomosis; Biologics

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Core Tip: Inflammatory bowel disease (IBD) patients with chronic refractory pouchitis, refractory ulcerative proctitis (including those with ileorectal anastomosis), stomas, or short-bowel are routinely excluded from clinical trials, and there is a consequent lack of quality data with regard to their management; however, these patients represent a part of IBD real-life population that needs to be acknowledged. In the present article, our aim is therefore to outline the evidence available so far, and to highlight the main knowledge gaps still present.

Citation: Privitera G, Pugliese D, Lopetuso LR, Scaldaferri F, Papa A, Rapaccini GL, Gasbarrini A, Armuzzi A. Orphan patients with inflammatory bowel disease - when we treat beyond evidence. *World J Gastroenterol* 2021; 27(47): 8047-8057
URL: https://www.wjgnet.com/1007-9327/full/v27/i47/8047.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i47.8047

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic condition, with an immune-mediated pathogenesis, that necessitates continuous medical treatment. It can be divided into three main subtypes: Crohn's disease (CD), ulcerative colitis (UC), and IBD unclassified (IBD-U). Despite this apparently simple classification, it can manifest with a broad range of clinical phenotypes, which are only partially encompassed by Montreal classification. Medical management of patients with moderately-to-severely active IBD who develop dependence or resistance to corticosteroids is based on immunomodulator drugs (*i.e.*, traditional immunosuppressors and targeted therapies, including biologics and small molecules)[1]. Such therapies are licenced after passing through three phases of randomized controlled trials (RCTs), and are subsequently incorporated in clinical practice. However, there is a number of clinical conditions that fall within the spectrum of IBD but are routinely excluded from RCTs. As previously noted by Ha *et al*[2], inclusion/exclusion criteria of RCTs prevent the enrolment of a significant proportion of IBD patients. In their 2012 work, they observed that only 31.1% of 206 IBD outpatients evaluated for RCTs would have been eligible for enrolment. It should be taken in consideration that, at that time, previous anti-tumor necrosis factor α (TNFα) exposure was a nearly ubiquitous exclusion criterion, and this might have substantially contributed to determining such a low percentage of eligible patients; however, many other criteria still stand to date – such as chronic refractory pouchitis, isolated proctitis, ileorectal anastomosis, short-bowel syndrome, and stomas in CD. Age ≥ 65 years old was an exclusion criterion in all RCTs with anti-TNFα drugs. The GEMINI program (which investigated the use of vedolizumab in moderately-to-severely active IBD) was the first RCT to include IBD patients up to 80 years of age[3,4]; many – but not all – subsequent trials also extended their upper limit of age for eligibility. However, elderly patients remain an underrepresented population in IBD clinical trials. Some recent progress needs to be recognized, as some RCTs have been conducted for these underrepresented populations (*i.e.*, trials specifically designed for patients with chronic refractory pouchitis). Nevertheless, there is still a significant gap between the real-life IBD population and the population studied to produce evidence: Evidence-based medicine to treat these "orphan patients" is often lacking, and clinicians rely on treatments that have not been studied in these specific forms of IBD.

In this work, we aim to give a comprehensive review on the topic of "orphan IBD patients", to present evidence available to clinicians and to highlight the main knowledge gaps that need to be fulfilled in the upcoming years.

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Received: March 22, 2021
Peer-review started: March 22, 2021
First decision: June 14, 2021
Revised: July 12, 2021
Accepted: December 8, 2021
Article in press: December 8, 2021
Published online: December 21, 2021

P-Reviewer: Huguet JM
S-Editor: Ma YJ
L-Editor: Wang TQ
P-Editor: Ma YJ

CHRONIC REFRACTORY POUCHITIS

Acute pouchitis occurs in up to a half of UC patients who undergo total proctocolectomy with creation of an ileal pouch-anal anastomosis[5]. Antibiotic therapy (metronidazole and ciprofloxacin) is used as first-line treatment, but patients can develop dependence or refractoriness to those; in a similar fashion to what happens with corticosteroids in other forms of IBD, in these latter cases advanced therapies are frequently used. In a retrospective study including 594 patients with ileal pouch, the cumulative incidence of pouchitis was 48% during a 2-year follow-up (29% of patients had isolated acute pouchitis and the remaining 19% developed recurrent pouchitis): Of note, 40% of patients with pouchitis received non-antibiotic therapy (21% mesalamine, 7% immunomodulatory, and 7% anti-TNFα)[6].

The evidence supporting the use of immunosuppressive drugs for the treatment of antibiotic-dependent or -resistant pouchitis is quite scarce, and mostly derived from real-life experiences. Only one clinical trial designed for chronic refractory pouchitis has been conducted in the last years[7], while a few other phases 2 and 3 RCTs are congoing, either testing novel treatments [faecal microbiota transplantation (FMT), AST-120 (a spherical carbon adsorbent that binds bile acids and bacterial toxins), alicaforsen (an antisense oligonucleotide that targets the mRNA for the production of human intercellular adhesion molecule 1), AMT-101 (a recombinant biologic protein of human interleukin 10)][8-12], or drugs already approved for CD and UC (such as vedolizumab[13], ustekinumab[14], and tofacitinib[15]).

In a randomized, double-blind, placebo-controlled trial including 13 patients, adalimumab did not show any significant benefit: Among nine patients who completed the 12-wk study period, the primary outcome [reduction in clinical pouchitis activity index (PDAI) ≥ 2] was met by 50% and 43% of patients in the drug and placebo arms, respectively ($P > 0.05$); no differences in terms of secondary endpoints were recorded between the two groups[7]. Of note, it should be acknowledged that the sample size might have been insufficient to detect statistically significant differences between the two groups. In a large Canadian cohort of 152 patients (29% with CD-like phenotype of the pouch, and the remaining with chronic refractory pouchitis), the outcomes of those who received infliximab ($n = 42$) were recorded: Post-induction clinical response rate was 74% (48% achieved remission) and 62.6% of patients reported sustained response[16]. In a 2018 meta-analysis, 313 patients who received anti-TNFα treatment (194 infliximab and 119 adalimumab) to treat inflammatory complications of the pouch (*i.e.*, refractory pouchitis and CD-like complications of the pouch) were identified: After induction, rates of clinical remission were significantly higher in CD-like complications of the pouch (0.64, 95%CI: 0.48-0.78) compared to refractory pouchitis (0.10, 95%CI: 0.00-0.35, $P = 0.06$), while such a difference disappeared at 12 mo (0.57, 95%CI: 0.43-0.71 for CD-like complications; 0.37, 95%CI: 0.14–0.62 for refractory pouchitis, $P = 0.57$). Remarkably, no difference between infliximab and adalimumab was observed, besides a numerically higher percentage of patients in clinical remission at 12 mo with infliximab compared to adalimumab (59% *vs* 30%, $P = 0.2$)[17].

Recently, data on the effectiveness of vedolizumab for the treatment of chronic refractory pouchitis have also been presented. A systematic review summarized the results from case-reports and case-series including 44 patients who received vedolizumab therapy due to antibiotic-dependent or -resistant chronic pouchitis. Only 52.3% of those patients had been previously exposed to anti-TNFα therapy. Clinical improvement at week 12 was reported by 75% of patients, and endoscopic improvement within 6 mo was recorded in 28 out of 38 (73.7%) patients[18]. Recently, Gregory *et al*[19] reported the results of a multicentric, retrospective study where 83 patients received vedolizumab for the treatment of chronic refractory pouchitis: 81.9% of patients had a previous diagnosis of UC, the remaining having CD (10.8%) or indeterminate colitis (7.2%), and 68.7% of them had been previously exposed to anti-TNFα therapy. Clinical response and remission were reported by 71.1% and 19.3% of patients, respectively, while endoscopic improvement was recorded in 54.1% and mucosal healing in 17.6% of them[19].

In 2019, Verstockt *et al*[20] reported the results of a cohort of 33 patients who received infliximab ($n = 23$), adalimumab ($n = 13$), or vedolizumab ($n = 15$) for the treatment of refractory pouchitis. Both anti-TNFα and vedolizumab were effective in inducing clinical improvement; interestingly, they observed that patients had a higher risk to discontinue anti-TNFα treatment compared to vedolizumab (HR = 3.0, 95%CI: 1.1-8.8, $P = 0.04$); this might possibly be attributed to the fact that adverse events accounted for 40.7% of anti-TNFα discontinuations, while no patient withdrew vedolizumab due to safety issues[20].

ISOLATED ULCERATIVE PROCTITIS AND ILEORECTAL ANASTOMOSIS

The mainstay of treatment for ulcerative proctitis is represented by topical aminosalicilates and/or topical steroids. Oral steroids are sometimes used in case of unresponsiveness to topical therapy[21]. Moreover, it has been suggested that oral mesalamine can reduce the risk of disease extension[22]. However, for patients who fail (or do not tolerate) conventional therapies, no evidence-based treatment is available, and clinical management is mainly based on the extrapolation of data from RCTs in UC, which include left-sided colitis and pancolitis – indeed, isolated proctitis is a nearly omnipresent exclusion criterion, as the majority of clinical trials require a disease extension of at least 15 cm from the anal verge.

A 2014 systematic review and meta-analysis concluded that there was not enough evidence to support the use of corticosteroids, thiopurines, and anti-TNFα for the treatment of ulcerative proctitis. Since then, only a few more real-life studies have been published[23]. The effectiveness of thiopurines for the treatment of refractory proctitis has been investigated in a 2017 retrospective study: At the last follow-up evaluation (median time of 46 mo), only 5 out of 25 patients were still on azathioprine treatment, while the remaining 20 were considered treatment failures[24]. Dubois *et al*[25] published the results of a retrospective study on a large cohort including 118 patients with ulcerative proctitis: 31% of them were refractory to rectal and oral therapy with aminosalicilates and/or steroids, requiring thiopurine monotherapy (19%) and/or biologics (28%, 25 anti-TNFα and 8 vedolizumab). Long-term outcomes pointed at a superiority of biologics over azathioprine for the treatment of refractory ulcerative proctitis, with a rate of clinical response of 70% *vs* 11% in favour of biologics (P = 0.001)[25]. More recently, a nationwide retrospective study from GETAID investigated the effectiveness of anti-TNFα therapy in 104 patients with refractory proctitis: 50% received infliximab, 39% adalimumab, and 11% golimumab; of note, 45% also received concomitant immunosuppressors. Clinical response was observed in 77% of patients after a median follow-up of 3 mo; the cumulative probability of sustained clinical remission was 86.7%, 74.7%, and 56.4% at 1, 2, and 5 years, respectively. After a median follow-up of 11.7 mo, 60% of the 63 patients with an available endoscopy achieved mucosal healing. Finally, 11 patients with primary nonresponse and 9 with secondary nonresponse to anti-TNFα therapy eventually received vedolizumab (after a second-line therapy with another anti-TNFα) and achieved clinical remission in 82% and 56% of cases, respectively[26].

The evidence becomes even more exiguous when it comes to patients with ileorectal anastomosis who have persistent proctitis. Abdominal colectomy with ileorectal anastomosis represents an alternative to total proctocolectomy with creation of an ileal pouch for the management of refractory UC, to maintain intestinal continuity. To date, ileoanal pouch is considered the gold standard for the surgical management of UC, but ileorectal anastomosis can be proposed to selected patients to postpone the creation of ileal pouch (for instance, in young patients, to postpone pelvic surgery) or when the creation of ileal pouch is not feasible (elderly patients). It has been reported that ileorectal anastomosis seems to be associated with fewer stool movements and night-time evacuations[27,28], and it is usually considered a less complicated procedure, compared to ileo-anal pouch[29]. On the other hand, ileorectal anastomosis comes with two main shortcomings. First, with ileorectal anastomosis, the inflamed rectum is not removed by surgery, so continuation of medical therapy is usually necessary. Indeed, it has been reported, in a retrospective study with 343 patients who received ileorectal anastomosis, that 70% of patients manifested proctitis, 76% of whom developed chronic proctitis[30]. The management of patients with ileorectal anastomosis is similar to those with isolated proctitis, and it is mainly based on topical therapy. In case of refractoriness, proctectomy can be performed to remove the inflamed rectum; otherwise, biologics are sometimes administered, but the experience with their use is extremely limited. The second issue is that ileorectal anastomosis is associated with a non-negligible risk of cancer progression within the rectum[31]. Currently, there are no guidelines for anti-neoplastic surveillance in patients with ileorectal anastomosis, but annual endoscopy is usually recommended.

STOMAS

The presence of an ostomy is an exclusion criterion in RCTs for CD patients. Nevertheless, CD patients with an ostomy are not uncommon: In CD-related surgery, ostomies are usually created in case of urgent procedures (*e.g.*, when the patient has

fistulizing disease complicated by abdominal abscess) or to exclude faecal stream for therapeutic purposes (for instance, in case of medical-refractory perianal disease). Therefore, it would appear reasonable that at least some patients with an intestinal stoma might benefit from the administration of advanced therapies. There are three main scenarios where patients with an ostomy might need advanced therapies. First, when not all of the intestinal segments affected by active CD are resected. These patients usually need medical therapy after surgery, to halt disease progression and prevent additional intestinal resections. Second, to prevent postoperative recurrence. Postoperative recurrence has been usually evaluated in patients with ileocolonic-anastomosis, but can occur after each type of resection in CD[32]. In a 2017 retrospective work, out of 83 CD patients with definitive stoma, 42% of them experienced clinical recurrence after a median follow-up of 28 mo, and 38% needed a subsequent intestinal resection after a median time of 29 mo[33]. Similarly, a recent meta-analysis observed that the median cumulative rate of clinical recurrence, in CD patients with permanent ileostomy after total colectomy, was 23.5% and 40% at 5 and 10 years, respectively[34]. Therefore, even for patients with temporary ileostomy, a prophylactic therapy might be indicated, in order to avoid early recurrences which would make it more difficult for surgeons to restore intestinal continuity. Third, advanced therapies might be required when faecal diversion is used for the management of uncontrollable perianal disease; in such cases, patients might need biological therapy for two reasons: As an additional treatment for perianal disease, or to treat active luminal CD.

The appropriateness of biological therapy in patients with ostomies has not been established. In a 2013 Letter, the outcomes of three patients with acute sever CD colitis and perianal disease managed with ileostomy and anti-TNFα therapy were reported: At the end of follow-up, all patients had undergone total colectomy with creation of permanent ileostomy – of note, one patient achieved complete clinical and endoscopic remission while with stoma, but experienced severe clinical relapse just 1 mo after stoma closure, thus requiring colectomy[35]. In a retrospective study including 21 CD patients undergoing faecal diversion due to severe perianal disease, the authors reported that infliximab use was not associated with an increased likelihood of stoma closure[36]. Similarly, Gu *et al*[37] reported the outcomes of a cohort of 138 CD patients with ostomies created due to perianal CD, among whom 22% managed to achieve stoma closure: Of note, the authors did not observe that biologic therapy was associated with an improved likelihood of restoration of intestinal continuity. Conversely, another retrospective work including 233 patients who had stomas created because of CD colitis reported different outcomes: The incident risk of permanent ostomy was significantly reduced in post-biological era, compared to before the introduction of biologics (19.2% *vs* 60.8%, $P < 0.001$), and biologic use was significantly associated with an higher likelihood of rectal preservation, on multivariate analysis (OR = 3.1, 95%CI: 1.0-9.5, $P < 0.05$)[38]. Finally, a 2017 systematic review with a meta-analysis of 18 studies (including 1438 patients) observed that, in CD patients with permanent ileostomy after total colectomy, there was no difference in the risk of clinical and surgical recurrence between studies published in pre- and post-biological eras[34].

In regard to patients with temporary ostomies, biologics might also have a downside, due to the theoretical risk that pre-operative biologic use might increase the incidence of post-surgical complications, especially infections. However, such a risk has not been unanimously reported. Notably, two recent meta-analyses showed that neither pre-operative anti-TNF[39] nor vedolizumab[40] treatment is associated with an increased risk of postoperative infections. In conclusion, whether and how biologic use before the restoration of intestinal continuity might impact the success rates of permanent stoma closure remains unknown.

SHORT BOWEL

Short bowel and intestinal failure can manifest as rare but severe complications of CD, either in patients with intestinal continuity who had undergone extensive resections, or due to the creation of proximal stomas. Short bowel syndrome represents an exclusion criterion in RCTs; however, it is not uncommon for patients with short bowel to need advanced therapies, as it can be reasonably assumed that these patients have more aggressive diseases. A history of surgical resection is associated with an increased risk of endoscopic recurrence in CD, and biologics – specifically, anti-TNFα – are used to prevent postoperative recurrence.

The effectiveness of biological therapies in patients with short bowel syndrome has not been proven, yet. Limketkai *et al*[41] analysed the National Impatient Sample (a United States national registry of hospitalizations), and evaluated the trends in hospitalizations and small bowel resections in CD patients with short bowel syndrome and intestinal failure (SBS-IF): When comparing the populations before and after the introduction of biologics, the authors did not observe any reduction in the rate of resections among hospitalized patients (0.7 per 1000 CD hospitalizations per year in the pre-biologic era *vs* 0.6-0.7 per 1000 CD hospitalizations per year in the post-biologic era). It should be acknowledged that the main limitation of this study is the method for the identification of patients with SBS-IF, as the authors included all patients with a diagnosis code of "post-surgical malabsorption", which might have led to patient misclassifications in some cases[41]. However, in the same study Limketkai *et al*[41] observed a significant reduction in rates of overall small bowel resections in CD (from 99.0 to 64.6 resections per 1000 CD hospitalizations per year, $P < 0.01$): If this could be applied to patients with CD-related SBS-IF as well, a reduced rate of resections would have been observed in patients with the diagnosis code of post-surgical malabsorption. Another issue that should be taken in proper consideration is that patients with short bowel might have a history of multiple therapeutic failures and multiple resections: As they are not eligible in clinical trials, more advanced therapies, such as the combination of biologics, could also be considered to preserve the residual intestine [42].

Recently, growing attention has been paid to the use of teduglutide – a glucagon-like peptide 2 (GLP2) analogue – in CD patients. Teduglutide is currently licenced for the treatment of SBS-IF patients who are dependent on parenteral nutrition. In a post-hoc analysis of the STEPS study, the efficacy of teduglutide (measured in terms of reduction in weekly parenteral support at week 20) was comparable between patients with SBS-IF secondary to IBD and those who did not have IBD[43]. There has been an initial reluctance to use teduglutide in patients with active IBD, due to the concern that its gut-tropic effect might serve as a pro-inflammatory stimulus and cause IBD exacerbation – indeed, the pivotal STEPS-2 trial only allowed CD patients in stable clinical remission for at least 12 wk and biologics use was an exclusion criterion[44]. A 2017 retrospective study reported the outcomes of 13 CD patients who received teduglutide (8 of whom were on concomitant immunosuppressive therapy): Treatment with teduglutide was effective in reducing the need for parenteral support, but data on gut inflammatory activity were not reported[45]. Contrary to the initial concerns, it has been observed in murine models that teduglutide might actually exert anti-inflammatory effects[46-48]. In 2019, two CD patients who achieved control of intestinal inflammation and reduction of parenteral support while receiving the combination of teduglutide and biologics have been reported[49]. Notably, a CD patient treated only with teduglutide, previously unresponsive to multiple biological therapies, whose CD activity improved in parallel with nutritional status, has also been described[50], suggesting a link between teduglutide administration and clinically relevant anti-inflammatory effects[51].

ELDERLY PATIENTS

The elderly IBD population has been constantly expanding in the last decades. Given that IBD is a chronic disease, as life expectancy is prolonging, the prevalence of IBD in the elderly rises as a direct consequence of the ageing of patients combined with an increase of new diagnosis of late-onset IBD (*i.e.*, IBD diagnosed after 65 years of age). The management of elderly IBD patients can be challenging, since this population is characterized by a higher number of comorbidities and an increased risk of adverse events secondary to immunosuppressive treatment. Data from the Veteran's Health Administration showed that there is an overall reduction of use of both steroids and steroid-sparing agents in elderly IBD patients compared to younger ones[52]. Thiopurine maintenance therapy is usually discouraged in elderly patients, because it correlates with the highest absolute risk of developing lymphomas in patients ≥ 50 years old[53]. Anti-TNFα drugs have not been tested in people older than 65 years old in registration trials; they are generally underused in this population compared to younger patients, and persistence on therapy can be reduced in elderly patients, mostly due to higher rates of serious adverse events.

Vedolizumab was the first IBD drug tested in patients who are 65 to 80 years old; however, only 2-4% of patients enrolled in the GEMINI program were older than 65 years[3,4]. In a post-hoc analysis GEMINI 1 and 2 trials, it was shown that the safety

and effectiveness of vedolizumab were comparable among different groups of patients stratified by age (< 35, 35-54, and ≥ 55 years old)[54]. Due to its gut-selective mechanism of action, anti-integrin therapy seems more appealing to clinicians for the treatment of elderly IBD patients, as non-anti-TNFα treatments seemingly have a more favourable safety profile compared to TNFα inhibitors in IBD. Interestingly, a retrospective study comparing the outcomes of anti-TNFα and non-anti-TNFα treatments in elderly patients did not observe any difference in terms of safety between the two groups, thus questioning whether vedolizumab actually represents a safer choice in elderly patients[55]. A 2020 prospective study conducted on patients starting vedolizumab or ustekinumab showed that Charlson comorbidity index (CCI), but not age, correlated with the occurrence of infections in vedolizumab-treated patients, and with hospitalizations in patients treated with either vedolizumab or ustekinumab[56]. We observed similar results in an Italian multicentric prospective study enrolling over 1000 patients, where elderly CD or UC patients (n = 198) were matched 1:2 to younger ones: In this cohort, a CCI > 2 was associated with a higher risk of developing any adverse events[55]. Finally, in a large cohort study including over 10000 patients, an association between pre-treatment frailty and increased risk of infections was observed in IBD patients treated with TNFα antagonists or immunosuppressors[57]. Evidence on biologic use in patients over 80 years of age is extremely scarce. In a 2020 Letter, Ayoub and colleagues reported the outcomes of 32 patients ≥ 80 years old (median age 82.5 years, range: 80-94) who received anti-TNFα agents (53.1%) or vedolizumab (46.9%): Serious infections occurred in about 15% of patients and three deaths due to cardiorespiratory causes were reported[58].

Another major question regarding the management of elderly IBD patients is whether age might impact treatment efficacy. In a 2020 Italian multicentric study, older patients with both CD and UC showed lower persistence on anti-TNFα treatment compared to younger IBD controls. Lobatón *et al*[59] found reduced short-term effectiveness in patients ≥ 65 years old treated with anti-TNFα therapy, but such a difference disappeared after 6 mo. We recently observed that elderly UC – but not CD – patients had significantly worse outcomes in terms of therapy persistence, steroid-free clinical remission and biochemical remission, when compared to matched younger controls[55]. Conversely, Adar and colleagues reported comparable effectiveness of anti-TNFα and vedolizumab in a retrospective cohort of IBD patients ≥ 60 years old[60].

CONCLUSION

There are several categories of orphan patients in IBD, for whom no (high-)quality evidence is available and whose optimal management has not been established, yet. Accordingly, we can try to identify the main knowledge gaps that need to be filled for these specific populations. Whenever possible, RCTs should be preferred as the optimal source for evidence-based medicine; however, when clinical trials are too difficult to perform due to the relative rarity of the conditions, real-life observational studies become crucial to help clinicians in deciding patients' management. Figure 1 presents the main conditions with "orphan IBD patients" presented in this review and highlights the main issues that need to be addressed in future research.

Chronic refractory pouchitis

Most data on the effectiveness of immunosuppressants come from real-life observational studies, but several RCTs are ongoing; notably, some of these trials include drugs with mechanisms of action that differ from those already licenced for CD and UC. Another major issue is whether a drug that was not effective on IBD before colectomy is ought to be taken in consideration to treat chronic refractory pouchitis in that patient. Finally, the impact of immunosuppressants on pouch failure, as well as their optimal timing for introduction, is yet to be established.

Ulcerative proctitis and ileorectal anastomosis

There is still a significant uncertainty about the appropriateness of biologic use to treat refractory ulcerative proctitis, as most evidence is derived from retrospective studies. It also needs to be addressed whether systemic immunosuppression can help in preventing disease extension: As it has been previously demonstrated that "extenders" tend to have a worse prognosis, preventing disease extension might theoretically have a positive impact on the natural history of some proctitis. However, to avoid unnecessary overtreatment, predictors to stratify patients at higher risk for colitis

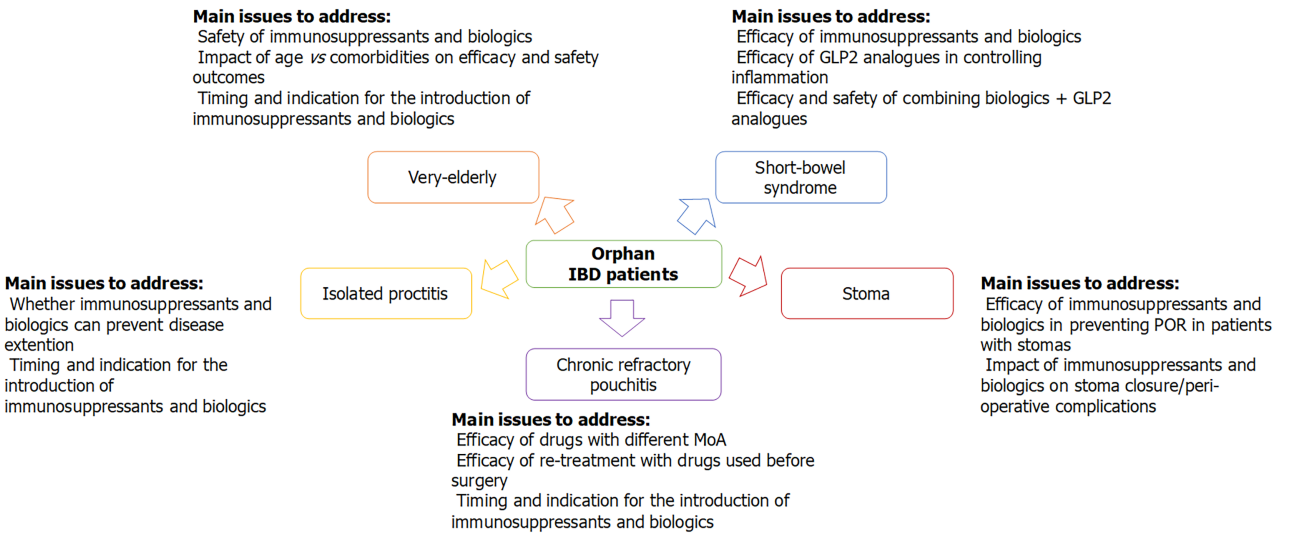


Figure 1 Orphan inflammatory bowel disease patients. IBD: Inflammatory bowel disease; MoA: Mechanism of action; POR: Post-operative recurrence.

extension should be identified.

Stomas

No high-quality data on the effectiveness of biologics in CD patients with ostomies exist. Two main issues need to be addressed: (1) Whether biologic treatment reduces the risk of postoperative recurrence in patients with stomas: Therefore, specific risk factors for precocious recurrence in patients with ostomies should be investigated; and (2) for patients with temporary stomas, the impact of immunosuppressors on the outcomes of surgery for the restoration of intestinal continuity needs to be evaluated to answer the following questions: Do biologics improve the rates of stoma closures? Does pre-operative biologic use worsen the outcomes of intestinal anastomosis?

Short bowel

Data on the effectiveness of biologics in CD patients with short bowel are not available. Crucially, the capability of biologics to prevent further surgery in these patients should be assessed. Encouraging preliminary results on the efficacy of teduglutide in improving the nutritional status of patients who depend on parenteral support have been presented; whether GLP2 analogues might also exert some sort of immunological control over intestinal inflammation is not clear, yet. Finally, more data on safety and effectiveness of combining biologics with teduglutide are required before this strategy can be considered the standard of care for patients with short bowel and active CD.

Elderly patients

Evidence on the use of targeted therapies in the elderly IBD population is scarce. Data on treatment effectiveness suggest that there might be a difference between elderly and younger patients, but more studies are needed before any specific recommendation can be made. The major concern regarding the use of immunomodulators in elderly patients is their safety. Results have not been consistent across different reports on whether age is associated with an increased risk of adverse events, nor on the differences among treatments in regard to safety. The lines of evidence available appear to point out that patient's functional status, rather than chronological age per se, has a clinically meaningful impact on the efficacy and safety profiles of different treatments.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 21; 27(47): 8058-8068

DOI: 10.3748/wjg.v27.i47.8058

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

FRONTIER

Analogies between medusa and single port surgery in gastroenterology and hepatology: A review

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Author contributions: Mittermair C and Weiss H have been involved equally and have read and approved the final manuscript; both authors meet the criteria for authorship established by the International Committee of Medical Journal Editors and verify the validity of the results reported.

Conflict-of-interest statement: The authors declare no conflict of interest

Country/Territory of origin: Austria

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

Open-Access: This article is an open-access article that was selected by an in-house editor and

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Abstract

Single port surgery (SPS) was introduced as an attractive, minimally invasive surgical technique that ensures esthetic results for many types of visceral surgery. Initially, surgeons immediately set about performing SPS without preliminary knowledge or training, which resulted in higher complication rates. Today, current studies conclusively show that SPS is scientifically rehabilitated and indicated for simple and complex laparoscopic procedures. We here describe the astonishing analogies between Greek mythology and modern surgery.

Key Words: Gastroenterology; Single port surgery; Surgical technique; Surgical complication; Gastric or intragastric resections

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Core Tip: Single port surgery (SPS) was introduced as an attractive, minimally invasive surgical technique that ensures esthetic results for many types of visceral surgery. Initially, surgeons immediately set about performing SPS without preliminary knowledge or training, which resulted in higher complication rates. Today, current studies conclusively show that SPS is scientifically rehabilitated and indicated for simple and complex laparoscopic procedures. We here describe the astonishing analogies between Greek mythology and modern surgery.

Citation: Mittermair C, Weiss HG. Analogies between medusa and single port surgery in gastroenterology and hepatology: A review. *World J Gastroenterol* 2021; 27(47): 8058-8068
URL: https://www.wjgnet.com/1007-9327/full/v27/i47/8058.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i47.8058

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Received: June 29, 2021
Peer-review started: June 29, 2021
First decision: August 8, 2021
Revised: August 22, 2021
Accepted: December 7, 2021
Article in press: December 7, 2021
Published online: December 21, 2021

P-Reviewer: Lu C, Palacios Huatuco RM, Zhou S
S-Editor: Wang LL
L-Editor: A
P-Editor: Wang LL

INTRODUCTION

One of the most striking analogies between single port surgery (SPS) and the mythological creature Medusa is of course her hair, reminiscent as it is of the four or more prominent, flexible trocars of SP systems. However, a closer look at the mythological story and the development of SPS brings to light many more surprising similarities.

The following brief resumé is by no means historically all-embracing, but necessary for a better understanding of relationships in Greek mythology: The Greek gods had absolute power over mortals and tended to be jealous, quick-tempered and cunning. Many of them had numerous amours, sexual liaisons and scandals. The children resulting from these dalliances were demigods with impressive skills and an adventurous spirit. The skills or assets were commonly applied by these heroes or the gods themselves to their own advantage. Against this background, the following lines serve to outline Medusa's place in Greek mythology and to bring it into line with developments in SPS.

The modern concept of doing something "under someone's aegis" means doing something under the protection of a knowledgeable and benevolent power. In Greek mythology the aegis, as described by Homer in 735 BCE, was a device carried by Athena and Zeus. It was interpreted as a shield featuring the hideous head of Medusa with live venomous snakes in place of hair[1].

The Roman poet Ovid portrayed Medusa, the only mortal of the three Gorgon sisters, Medusa, Stheno, and Euryale, as an originally beautiful maiden ravished by Poseidon in Athena's temple. As one of the first ideological accounts of rape-victim blaming, Athena punished Medusa by transforming her magnificent hair into horrifying snakes[2].

Anyone who gazed into Medusa's eyes was horror-stricken and turned into stone. Medusa's head was cut off by the Greek hero Perseus, who then used it for its ability to turn someone to stone. Finally, he gifted the head to Athena to be mounted on her royal shield. In Ancient Greece the image of Medusa's head appeared on the Gorgoneion, an amulet worn to avert evil[3]. Although modern interpretations of this myth already range widely from psychoanalysis as an oedipal, libidinous symptom[4] to feminism, which depicts Medusa as a guardian of female power[5], we in all humility would now like to emphasize several parallels between SPS and the mythological story of Medusa.

MEDUSA, THE FAIR MAIDEN

SPS was developed to minimize the surgically traumatic approach to the peritoneal cavity for a variety of indications. The concept of SPS is a modified laparoscopy based on a solitary short skin incision allowing all necessary surgical instruments to be delivered in parallel or crossed at the level of the abdominal wall.

Hans Christian Jacobaeus, a Swedish gastroenterologist, is regarded as the first person to have performed SPS in humans in 1910[6].

After Power and Barnes published their findings with laparoscopic tubal ligation in 1941[7], gynecologists were the first to adopt SPS for routine interval tubal ligations from the 1960s on[8]. Although more demanding SPS procedures such as hysterectomies were introduced by gynecologists in 1991[9], the SPS technique still did not win over the surgical community as transumbilically assisted laparoscopic appendectomies or single-port laparoscopic cholecystectomies using several percutaneous suspension sutures were viewed as not yet fully developed[10,11].

It took another decade of technical development to more appreciably comprehend the beauty of the minimized abdominal wall trauma afforded by SPS. The allure of improved cosmesis roused the surgical community to commence feasibility studies for routine procedures, such as appendectomy[12], cholecystectomy[13], fundoplication [14] or benign colon resection[15]. The publication of scientific reports took off during the next years and peaked in 2012 and 2013 (Figure 1). Compared to conventional multiport laparoscopy, SPS is founded on three fundamental preliminaries: first, a special trocar system; second, a lengthening of the optic and some of the instruments; and third, a modified manipulation of the camera and the non-dominant hand of the surgeon. With regard to the abdominal approach, SPS development began with three to four small trocars used side-by-side after being delivered together through one skin incision[15]. The main disadvantage of this technique was permanent leakage of the pneumoperitoneum, thus impeding safe exposure of the surgical field[16]. In addition,

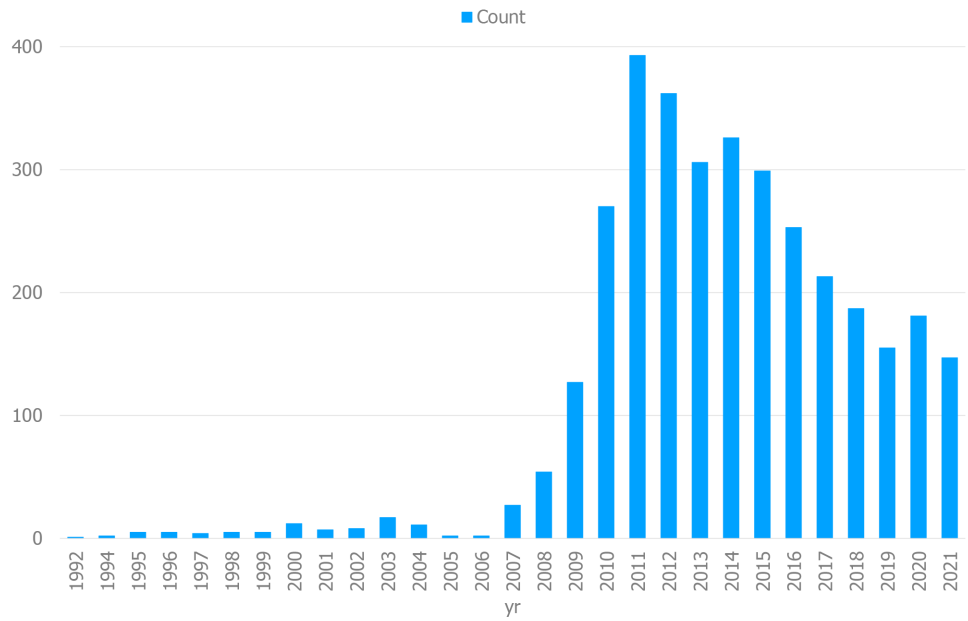


Figure 1 PubMed timeline results per year (search query "single port laparoscopy", article type "journal articles, classical articles, letters, multicenter studies, case reports").

separate fascial defects or specimen retrieval without wound protection significantly increased wound complication rates[16]. The switch from single incision to SPS allowed the pneumoperitoneum to be stabilized. A remarkable variety of single-port devices were introduced, some of them reusable, some of them single-use products. The portfolio ranged from simple home-made ports employing a surgical glove and a wound protector for delivery of all instruments to sophisticated port systems equipped with ambient intraabdominal light, powerful smoke evacuation and routable smart tubing (Figure 2). Delivering all instruments *via* a fulcrum at the incisional site resulted in a clash of trocar valves or a collision of hands during manipulation. This unpleasant side-effect was at least partially averted by arranging the bulky tubing valves at different distances and by elongating the instruments to enable additional degrees of freedom for the surgeon's hands. Last but not least, triangulation between the eyes and hands or the target and instruments is fundamental for spatial orientation and dissection. Delivering instruments and the camera together in one line would make triangulation impossible. For this reason, a 30° to 45° optical view is mandatory in SPS. On the other hand, the use of two instruments close to a target requires at least one of the two instruments to be bent and held preferably in the non-dominant hand in order to enable triangulation (Figure 3). The degrees of freedom are comparable to those for conventional multitrocar surgery. Nevertheless, SPS has been seen to involve an increase in wrist rotating movements[17].

While ensuring all these conditions it was possible for the first time to perform standard surgical procedures solely *via* one nearly invisible incision in the groove of the navel. At first glance, this improved cosmesis would seem to be insignificant in light of the bulk of unsolved problems bothering surgical science. However, esthetic aspects undoubtedly exert an important impact on quality of life[18] and are underestimated by surgeons who focus solely on overall and disease-free survival.

MEDUSA, WITH HER TWO GORGON SISTERS ON THE ISLE OF SARPEDON

SPS was developed at the same time as two novel surgical approaches, namely telemanipulating technologies generally summarized under robotic surgery and intraabdominal or intrathoracic procedures performed *via* a natural orifice, abbreviated as NOTES (natural orifice translumenal endoscopic surgery). In contrast to SPS, which is viewed as an evolutionary development employing standard laparoscopic strategies, the other two concepts revolutionized strategic standards by implementing unconventional instruments and increasing procedural costs. Industrial marketing whipped up



Figure 2 Two examples of single port devices that are currently used. The simple home-made port employing a surgical glove and a wound protector for delivery of all instruments (left) and the JackPort™ equipped with ambient intraabdominal light, smoke evacuation and routable smart tubing (right).

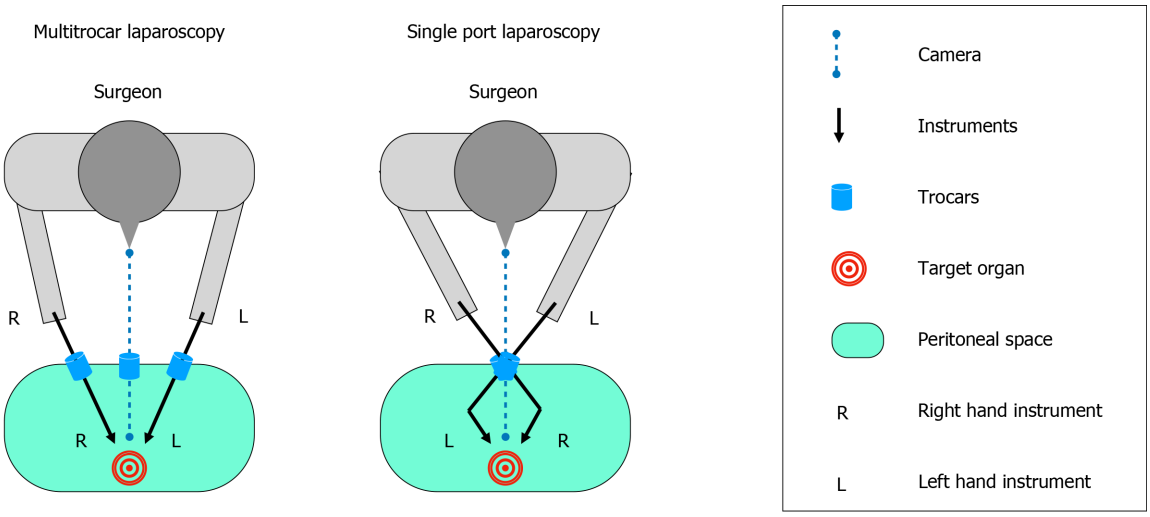


Figure 3 When compared to conventional multitrocar laparoscopy (left) the use of two crossed instruments close to a target requires articulation in order to enable triangulation in single port surgery (right).

a hype particularly for tele-manipulating techniques and NOTES and as a result the tabloid press reported visionary promises long before SPS, robotic and NOTES achieved evidence-based status. Over the last decades all three techniques have gained scientific justification for various surgical indications[19], but are still far from routine daily use because skills remain underdeveloped without additional training and because of the financial burden and prolonged procedural times.

MEDUSA, RAVISHED AND BLAMED

The SPS technique spread to all surgical subdivisions and new applications were created. From 2008, delighted by the first scientific reports[11,20,21], many surgeons immediately felt qualified to perform SPS without in-depth knowledge or training. The resulting disaster saw many procedures fail. Suddenly, SPS was criticized and faulted for providing the negligible benefit of better cosmesis accompanied by an increased hernia rate, prolonged procedural time and possibly increased complication rates in general.

This misunderstanding was prompted by the fallacy that SPS requires a skin incision that is longer than for conventional laparoscopy[20]. A simple comparative

calculation of the incision needed to pass a 10 mm trocar (outer diameter 11mm) *vs* the incision for introduction of three instruments with an external diameter of 5 mm each refutes this assumption: Notwithstanding the elasticity of the fascial sheaths and the skin, a minimum incision length of more or less 17 mm is needed for a "10 mm" trocar and three SPS instruments. The bias against a greater inherent risk for poor wound healing with SPS was fed by numerous publications that reported early learning curve data resulting in meta-analysis similar to that in[23]. A minimized incision is particularly beneficial in those types of surgery where small meshes have to be applied in the abdominal cavity for hernia reconstruction or where no or very small specimens have to be removed. A low rate of less than 1% incisional hernias was achieved in our patient cohort by using a multichannel port or a home-made port and closing the sole fascial incision with running sutures made with non-absorbable monofilament (data submitted for publication).

Insufficient exposure of the surgical field was argued to cause an increased rate of intraoperative complications and prolonged procedural times. This is why an inquiry directed at 600 surgeons from all over the world found that they preferred a standard four-port cholecystectomy rather than SPS if they themselves were undergoing the procedure. However, the prime factors in the decision-making process are the surgeon's inexperience with this technique and the procedure's safety[24]. Similar to other types of visceral surgery, the best approach for each patient is chosen by the surgeon at his own discretion. Our own experience is slightly different as all our surgeons have the freedom to perform SPS, dual- or multiport laparoscopic cholecystectomy, and we have not observed these techniques to have any differences with regard to procedural times or complication rates. However, as these procedures demand good triangulation and adequate exposure, we do not wait long before implementing suspension sutures and additional trocars in patients or pathologies that are challenging. Obviously, part of the hard-core resistance to SPS may be ascribed to many disconcerting examples of surgical bravado and the onerous need to devote time and energy to on-going skills training with a view to learning efficient execution of the SPS technique.

MEDUSA, THE POWER AND PERFORMANCE

SPS has shown equipotent feasibility and safety in many surgical indications as compared to multiport laparoscopy. In particular, some of the following visceral surgery procedures are viewed as standard in our department based on current scientific evaluation.

For appendectomy, recent meta-analyses have demonstrated that patients stay in-hospital less long, return to their jobs sooner and enjoy better cosmesis in the SPS group vis-à-vis briefer operating time and smaller numbers of conversions in the customary three-port groups[25,26]. We incorporated the technique into our clinical routine in 2009 and have meanwhile successfully performed 903 procedures (Table 1) with no significant technical changes.

Cholecystectomy is undoubtedly the most discussed of all SPS procedures. Despite the many prophets of doom and some terrible outcomes produced by so-called early adopters, the results of more recent trials including some randomised controlled trials and a multicenter trial from Korea are more encouraging. Better cosmesis, less postoperative pain, shorter recovery time and a comparably low rate of adverse events [27,28] are proven benefits of the method. This is consistent with our personal experience with more than 2200 performed cases. In this context it should be noted that a low threshold for the implementation of intracorporeal retractors, suspending sutures or additional trocars for optimal exposure or safe dissection is mandatory to prevent adverse events in complex cases.

Colorectal resections are some of the most appropriate procedures for SPS for the following reasons: All parts of the colorectal frame are ideally reachable from the umbilicus, but also even bulky specimens can be removed *via* this incision without destroying the natural shape of the umbilicus. Again, improved cosmesis, less postoperative pain and earlier return to normal life are scientifically proven[29,30] and, more importantly, oncological safety has been demonstrated[31]. Additionally, recent data suggest an even smaller number of postoperative complications[32]. These findings completely match our own experience, but we would like to add that there are key success factors that, amazingly enough, are underestimated. Meticulous handling of the umbilical incision by means of wound protectors and additional retrieval bags (Figure 4), prudent preparation techniques with reduced shear forces

Table 1 Numbers of single port procedures performed at the Surgical Department of the Saint John of God Hospital, Salzburg, Austria (from September 2008 - April 2021)

SPS Procedures	Procedural numbers	Specific surgical methods
Cholecystectomies	2216	Including intraoperative ERCPs/Cholangiographies
Inguinal hernia repairs	1850	TAPP/TEP
Appendectomies	903	
Colorectal resections	798	TME/APR/ ta TME
Liver resections	106	Minor/Major hepatectomies
Small bowel resections	90	
Gastric resections	49	Oncologic surgery: Gastrectomy with D1 Lymphadenectomy, Partial gastric resections, Transgastric resections, Metabolic surgery: Sleeve gastrectomies, RY-gastric bypass, Omega-loop bypass
Pancreas resections	29	Distal pancreas resections, Enucleations
Adrenalectomies	25	Trans-/retroperitoneoscopic approach
Fundoplications	21	Nissen/Toupet reconstructions
Other procedures	256	Abdominal wall reconstructions, Adhesiolysis, Nephrectomies, Lymphadenectomies, Splenectomies, Intraabdominal foreign body removals, Adnexectomies, Hysterectomies, Cyst unroovings, Diagnostic laparoscopies, Ligamentum arcuatum resections, Abscess evacuation, Necrosectomies, Neurectomy
Total	6343	

ERCPs: Endoscopic retrograde cholangio-pancreatographies; SPS: Single port surgery; TAPP: Transabdominal preperitoneal hernia repair; TEP: Totally extraperitoneal hernia repair; TME: Total mesorectal rectal excision; APR: Abdominoperineal rectal resection; taTME: Transanal total mesorectal rectal excision.



Figure 4 Specimen extraction is performed using a tear-proof retrieval bag in order to prevent intraabdominal bacteria or tumor cell dislocation during squeezing.

and thorough closing of the fascial and skin defect are mandatory for successful implementation of SPS in colorectal surgery.

Procedures involving the upper gastro-intestinal tract are meanwhile routinely performed, but exclude esophageal resections because intrathoracic dissection is awkward from the umbilicus in general. Gastric or intragastric resections, bariatric procedures and fundoplications are frequently performed with the SP technique and provide good cosmetic results and less postoperative pain at an acceptable level of postoperative complications[33,34,35]. However, the role of SPS in complex oncologic gastric resections with D2 Lymphadenectomy must be scrutinized, as the current

evidence in no way allows a final conclusion to be drawn on minimally invasive surgery in these cases.

In contrast to the aforementioned indications and procedures with a high level of evidence and a comparably large number of surgeons performing these operations, other procedures have met with less acceptance and distribution among the surgical community.

Despite the probable benefit of reduced trauma to the abdominal wall in patients undergoing inguinal hernia repair, the SP technique did not prevail. While experts demonstrated the safety and feasibility of the technique[36], the complexity of the method, on the one hand, and the very high standard of modern three-port hernia surgery, on the other hand, may have impeded further attempts to implement it in clinical routine. In contrast, we have successfully performed 1850 cases of SP inguinal hernia repair to date. Although the aforementioned high complexity of the procedure is admittedly undisputed and only strong personal convictions allow someone to make a strategic switch to SPS, the pathology of groin or umbilical hernia is closely linked to a disarrangement of micro-tubular tissue assembly. Therefore, minimizing incisional length, which is done better in SPS than in any other type of laparoscopic or open hernia repair, would strongly advocate SP transperitoneal or total extraperitoneal mesh repair.

Larger incisions required for specimen retrieval would speak in favor of SPS. However, the high complexity of minimally invasive liver resection has delayed its implementation. SPS was seen to be superior to multiport surgery in left lateral sectionectomies with regard to operative time[37]. Additionally, our group has demonstrated the feasibility of SP minor liver resection in combination with radiofrequency pre-coagulation[38]. The feasibility of SP major resections has been demonstrated by experienced minimally invasive liver surgeons[39]. These results were confirmed by a series from our department. Nevertheless, we observed an increased risk of substantial blood loss when bleeding occurs in these complex resections[40].

Pancreatic resections generally rank among the most demanding procedures in minimally invasive surgery. This explains the very small number of procedures as well as the small number of published reports, not counting case reports or small case series. Generally, only pathologies at the tail of the pancreas have been treated by means of SPS and even these resections are hampered by reduced degrees of freedom. As a consequence, dual incision laparoscopy was introduced and promoted by some groups to overcome the hurdles of impeded suturing and stapling[41,42].

In addition, some procedures favor SPS for its conceptual features although basically all types of laparoscopy with specimen retrieval are good candidates for exemplary performance *via* a single port.

Intraperitoneal laparoscopic redo surgery is one of the ideal applications for SPS as a single incision permits not only an immediate view, but also delivery of dissection instruments for preexisting adhesions. This efficiency is appreciated to free peritoneal adhesion cords in small bowel obstruction or assist in abdominal wall repair for incisional hernia.

As every incision entails the risk for acute or chronic complications, the SPS concept should support all types of possible repetitive intraperitoneal procedures such as hepatic or lymphonodular metastasectomies in oncologic surgery and preserve the fitness of patients undergoing exhausting polychemotherapies, where staging laparoscopies are required. Indeed, after one century of modern surgery the latter indication has proven a valuable reminder of the early days of SPS. On the other side of the street from oncologic diseases, palliative decompression procedures, such as gastro-enterostomies in gastric outlet obstruction for cancer, can ideally be performed by means of SPS with minimal trauma to patients having limited length and quality of life.

Furthermore, SPS brings momentum to an alternative situation: metabolic surgery for morbid obesity is undoubtedly one of the most important medical interventions available in the fight against the pandemic of obesity and diabetes in the common world. Sadly, society unjustly denounces morbidly obese patients for their psychological weakness. Fear of outing oneself for needing surgical help to cope with obesity as well as fear of invasive surgery itself rob many of these patients of the opportunity to obtain proper treatment. SPS provides a tool for safe metabolic surgery with minimal trauma so as to prevent telltale surgical scars. In this way, patients are protected from the psychological wrath of society for being overweight and for lacking mental or behavioral fortitude.

At the same time, it is mandatory that the limitations of the SPS concept be made known.

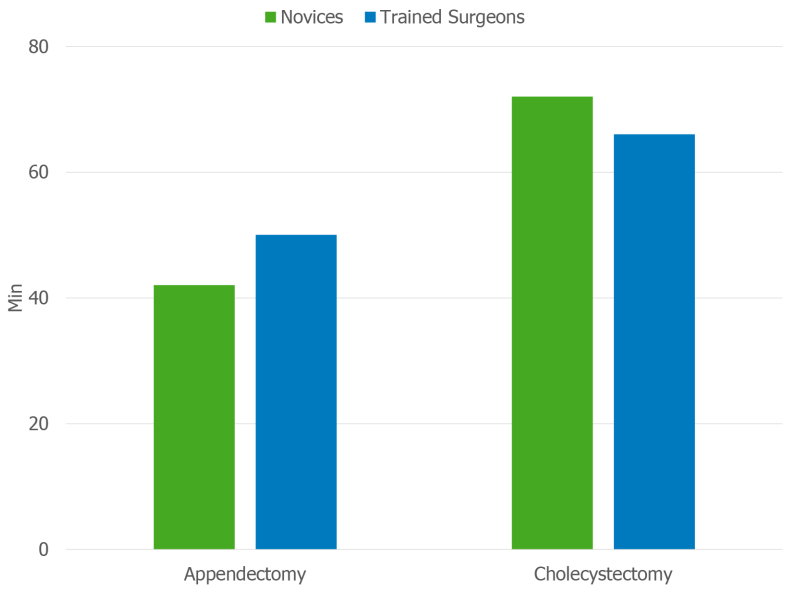


Figure 5 Mean procedural times of appendectomies (left) and cholecystectomies (right) during the learning curve of 10 single port laparoscopies for novices (*n* = 3) and surgeons trained in conventional multitrocar surgery (*n* = 5) did not differ significantly (by means of Two-way ANOVA) between the groups.

First, different targets that are spaced far apart and that can hardly be reached from one incision (*e.g.*, left colonic flexure mobilization in deep rectal resection operated *via* incision of the protective ileostomy or the intrathoracic esophageal dissection required in fundoplication for gastro-esophageal reflux disease) are obvious limitations.

Second, it is recommended that surgical procedures requiring wound drainage not be performed *via* the umbilicus because of the risk of wound complications in the longer term. Therefore, for dual-port laparoscopy in distal pancreas resections an additional port away from the umbilicus is recommended for the drain, as described above[41,42].

MEDUSA, THE AEGIS WITH HER FEARED HEAD

The knowledgeable and benevolent skills that are acquired when performing SPS are described in many reports made by expert surgeons[43]. Better skills for both conventional laparoscopy and SPS are acquired by experienced laparoscopic surgeons, who pass the learning curve for this uncommon minimally invasive technique. On the other hand, SPS training is even less arduous for surgical novices as they are not hampered by the consolidated motion patterns typical of multiport laparoscopy. We have demonstrated that standard SPS procedures can be performed at the same level of expertise by residents and senior surgeons alike (Figure 5).

Furthermore, SPS also generates skills that are useful for other indications. For example, transanal rectal resections, such as transanal total mesorectal rectal excision (taTME) would never have become reality if SPS had not laid the groundwork. Moreover, current techniques of abdominal wall reconstruction (*e.g.*, SPS-enhanced view total extraperitoneal patch hernia repair; eTEP) benefit from small incisions enabled by SPS. Transgastric SPS for oncologic resections of gastrointestinal stroma tumors in rendez-vous technique with flexible endoscopy, on the one hand, and transumbilical resection of hepatic segments 2,3 or 7,8 using inline precoagulation or single-port retroperitoneoscopic adrenalectomy, on the other hand, are the bright and shining pieces of the novel surgical mosaic made possible by SPS skills.

Although marketing experts tout telemanipulation as an unrivalled technique, multiport robotic surgery itself has to date hardly accomplished the aims of improved patient outcome in visceral surgery. Single-port telemanipulation with SP robotics, the newest innovation in this field, is already undergoing clinical evaluation and should soon realize the vision of wide-use reduced port surgery. Advantages over conventional laparoscopy for both patients and surgeons who wish to reduce abdominal wall trauma would be the strongest argument for its large-scale implementation.

CONCLUSION

SPS is an attractive minimally invasive surgical technique that ensures esthetic results for many types of simple and complex visceral surgery. Having traversed the valley of tears when SPS was disdained as inappropriate for use (blame the victim) and criticized by jealous, quick-tempered and big-headed surgical opinion leaders as a rubbish technique, SPS is scientifically rehabilitated when it is performed by surgeons who are willing to tackle the learning curve. Under the aegis of SPS, surgeons have the opportunity to become more accomplished in their professional routine and to finally add the SPS gem to their armamentarium for special indications. Gianni Versace, founder of the Italian luxury fashion label, chose Medusa as his logo because he knew that once people looked his products in the eye, they would be spellbound by their beauty and perfection.

ACKNOWLEDGEMENTS

We gratefully acknowledge the valuable discussions with Univ.-Prof. Dr. Frass M and Wiedergut J for the better understanding of Greek mythology.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 21; 27(47): 8069-8080

DOI: 10.3748/wjg.v27.i47.8069

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

EXPERT CONSENSUS

Chinese expert consensus on neoadjuvant and conversion therapies for hepatocellular carcinoma

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Author contributions: Zhao HT was responsible for investigation, writing-original draft, reviewing, and editing; Cai JQ was responsible for conceptualization, supervision, and writing-review and editing; all authors have read and approve the final manuscript.

Conflict-of-interest statement: The author has nothing to disclose.

Country/Territory of origin: China

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): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Open-Access: This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in

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Abstract

The low resection and high recurrence rates in hepatocellular carcinoma (HCC) are the major challenges to improving prognosis. Neoadjuvant and conversion therapies are underlying strategies to overcome these challenges. To date, no guideline or consensus has been published on the neoadjuvant and conversion therapies in HCC. Recent studies showed that neoadjuvant therapy for resectable HCC and conversion therapy for unresectable HCC are safe, feasible, and effective. Neoadjuvant and conversion therapies have the following advantages in treating HCC: R0 resection with sufficient volume of future liver remnant, relatively simple operation, and wide applicability. Therefore, it was necessary to conduct a widely accepted consensus among the experts in China who have extensive expertise and experience in treating HCC using neoadjuvant and conversion therapies, which is important to standardize the application of neoadjuvant and conversion therapies for the management of HCC. The strategies of neoadjuvant therapy include the selection of the eligible patients, therapy regimen, cycles, effect evaluations, and multidisciplinary treatment. The management of patients with insufficient volume of future liver remnant and patients who cannot achieve R0 resection is the key to the strategies of conversion therapy. Here, we present the resultant evidence- and experience-based consensus to guide the application of neoadjuvant and conversion therapies in clinical practice.

Key Words: Consensus; Hepatocellular carcinoma; Neoadjuvant therapy; Conversion therapy

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Received: September 16, 2021
Peer-review started: September 16, 2021
First decision: October 16, 2021
Revised: October 27, 2021
Accepted: December 8, 2021
Article in press: December 8, 2021
Published online: December 21, 2021

P-Reviewer: Carey I, Thiam A
S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Fan JR

Core Tip: Recent studies showed that neoadjuvant therapy for resectable hepatocellular carcinoma and conversion therapy for unresectable hepatocellular carcinoma are safe, feasible, and effective. It was necessary to conduct a widely accepted consensus among the experts in China who have extensive expertise and experience in treating hepatocellular carcinoma using neoadjuvant and conversion therapies.

Citation: Zhao HT, Cai JQ. Chinese expert consensus on neoadjuvant and conversion therapies for hepatocellular carcinoma. *World J Gastroenterol* 2021; 27(47): 8069-8080
URL: https://www.wjgnet.com/1007-9327/full/v27/i47/8069.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i47.8069

INTRODUCTION

Significance of developing the consensus

Background: Liver cancer is associated with a high recurrence rate and a low resection rate, while neoadjuvant and conversion therapies are important to overcome such challenges.

Hepatectomy is the surgical resection (whole or partial removal) of the liver. Radical therapies, including liver transplantation, resection, and radiofrequency ablation, are highly appropriate for early-stage liver cancer patients [*e.g.*, patients with stage Ia, Ib, and IIa according to the China Liver Cancer (CNLC) staging system], with a median survival of longer than 5 years[1]. Still, the short-term recurrence rate of liver cancer after surgery is relatively noticeable. The 5-year recurrence rate is as high as 70%, and the postoperative survival of the majority of patients is suboptimal[1,2]. About 64% of Chinese liver cancer patients are identified with stage CNLC-II or III [or equivalent to Barcelona Clinic Liver Cancer (BCLC) stage B or C] at the time of diagnosis, and surgical resection is not their first therapeutic option[1,3,4]. For patients with intermediate or advanced liver cancer, surgery following neoadjuvant or conversion therapy could result in higher treatment efficacy[1,5-7]. A previous study that analyzed the surgical data of 10966 patients with primary hepatocellular carcinoma showed that several patients with stages IIb-IIIa could still benefit from surgical resection, whereas the 5-year recurrence rate was as high as 80%[8] (Table 1). Therefore, how to reduce the postoperative recurrence rate and increase the radical resection rate of liver cancer is critical for improving patients' prognoses. Therefore, neoadjuvant and conversion therapies for liver cancer have been developed to tackle such challenges, which could increase the disease-free survival (DFS) rate.

Neoadjuvant and conversion therapies need to be standardized for liver cancer treatment

Neoadjuvant and conversion therapies are already widely applied to treat colorectal cancer, but owing to several clinical and non-clinical barriers, they have not been globally popularized for the treatment of liver cancer[9-11]. To date, the principles and functions of neoadjuvant and conversion therapies have not been fully clarified, and there are still controversies regarding the selection of patients for receiving neoadjuvant or conversion therapy in clinical practice. In addition, variations in treatment targets have also resulted in differences in selecting an appropriate treatment method, treatment duration, and postoperative criteria for follow-up[12]. Several scholars have conducted preliminary studies on neoadjuvant and conversion therapies for liver cancer, while the lack of high-grade evidence and absence of universally acknowledged standard treatments have restricted the popularization of those therapies[2].

Primary objective and significance of developing the present consensus

The *Chinese Expert Consensus on Conversion Therapy for Hepatocellular Carcinoma* (*2021 edition*) published in June 2021 emphasized the broad application of conversion therapy. It provided a reliable basis for the clinical application of conversion therapy. In order to further elucidate the mechanisms of neoadjuvant and conversion therapies and to clarify the treatment targets for different groups of patients, our research group attempted to develop the *Chinese Expert Consensus on Neoadjuvant and Conversion*

Table 1 Analysis of surgical data of 10966 patients with primary hepatocellular carcinoma

Stages and criteria	*n* (%)	Overall survival					Tumor recurrence				
		Median time (mo)	1-yr survival rate (%)	3-yr survival rate (%)	5-yr survival rate (%)	10-yr survival rate (%)	Median time (mo)	1-yr recurrence rate (%)	2-yr recurrence rate (%)	3-yr recurrence rate (%)	5-yr recurrence rate (%)
First round of hepatectomy for HCC	2592	79.4	87.2	71.0	59.1	40.4	36.1	31.8	43.5	9.9	60.7
Stages Ia-IIIa	2549	82.3	88.1	71.9	60.0	41.0	37.0	31.0	42.9	49.3	60.3
Ia	1175	-	96.5	87.5	77.2	55.9	67.2	15.0	27.7	34.1	45.9
Ib	635	79.4	88.2	73.5	62.5	37.0	34.1	32.4	43.7	51.8	63.4
IIa	205	43.5	89.8	56.0	40.8	27.2	15.7	45.7	59.8	65.5	84.3
IIb	119	38.0	83.6	55.3	37.4	23.2	10.0	55.9	67.7	74.0	80.1
IIIa	415	21.9	65.1	38.2	23.8	16.0	7.9	59.9	69.8	73.8	80.2
IIIb	43	8.7	34.1	15.8	0	0	3.9	77.1	77.1	82.8	-

Overall survival and tumor recurrence of patients with different stages of hepatocellular carcinoma after undergoing hepatectomy[8]. Stages and criteria; overall survival; tumor recurrence; *n*; median time (months); 1-yr survival rate (%); 3-yr survival rate (%); 5-yr survival rate (%); 10-yr survival rate (%); median time (months); 1-yr recurrence rate (%); 2-yr recurrence rate (%); 3-yr recurrence rate (%); 5-yr recurrence rate (%); first round of hepatectomy for hepatocellular carcinoma; stages Ia-IIIa. HCC: Hepatocellular carcinoma.

Therapies for Hepatocellular Carcinoma (*2021 edition*) to provide additional reliable suggestions for preoperative decision making according to the features of diagnosing and treating liver cancer in China and to standardize those therapeutic methods for their universal popularization.

CONCEPTS AND TARGETS OF NEOADJUVANT AND CONVERSION THERAPIES

According to the treatment targets, preoperative treatments for liver cancer include neoadjuvant therapy for resectable liver cancer and conversion therapy for unresectable liver cancer. These two treatments are distinguished by the achievement of R0 resection and are different from the aspects of study subjects, treatment targets, and treatment regimens.

Neoadjuvant therapy

Neoadjuvant therapy refers to the interventions including systemic or local treatments for liver cancer patients with technically resectable tumors [R0 resection, with sufficient volume of the future liver remnant (FLR)] and a high risk of recurrence, which could reduce the tumor size, eliminate the undetectable minimal lesions, and increase the negative surgical margins as early as possible, thereby attenuating the incidence of postoperative complications[12].

At present, only limited high-grade evidence for treating liver cancer *via* neoadjuvant therapy is available. Generally, in clinical practice, neoadjuvant therapy is not directly recommended for patients with CNLC stage Ia or Ib and some patients with CNLC stage IIa. However, because such patients are accompanied by a high risk of recurrence and require neoadjuvant therapy, the therapy can be performed in clinical trials after approval of the study protocol by ethic committees. There are substantial controversies regarding whether surgeries can be directly performed for patients with CNLC stage IIb or IIIa (*i.e.* the resection is technically applicable). The current recommendation is to benefit surgery after neoadjuvant therapy to reduce the postoperative recurrence rate.

Conversion therapy

Unresectable liver cancer refers to liver cancer that could not be safely resected, including intolerable liver functions, insufficient volume of FLR, and disability to ensure the negative surgical margins or zero residual lesion (*i.e.* achieving R0

resection).

Conversion therapy refers to the use of interventions to convert unresectable tumors into resectable ones, which includes the conversion of surgically unresectable lesions, such as insufficient volume of FLR, to resectable lesions, and it supports the conversion of R1 and R2 resection to R0 resection.

According to the *Chinese Expert Consensus on Neoadjuvant and Conversion Therapies for Hepatocellular Carcinoma* (*2021 edition*), existing evidence supports conversion therapy, which can be applied in clinical practice after a comprehensive evaluation of patients' clinical conditions.

STRATEGIES FOR CONDUCTING NEOADJUVANT THERAPY

Eligible patients for neoadjuvant therapy

Neoadjuvant therapy aims to reduce cancer recurrence. According to the Chinese guidelines, neoadjuvant therapy can be performed for patients with initially resectable liver cancer (including CNLC stage Ia-IIIa/BCLC stage A, or beyond the BCLC criteria, while being still resectable) and for those patients who are at a high risk of postoperative recurrence. As the high-grade evidence for neoadjuvant liver cancer therapy is limited, neoadjuvant therapy is not recommended for patients with CNLC stage Ia or Ib and for some patients with stage IIa (*i.e.* R0 resection can be directly achieved). However, if the comprehensive evaluation of the patients' clinical conditions (*i.e.* patients at high risk of postoperative recurrence and uncertainty for R0 resection) suggests that neoadjuvant therapy can be performed, the therapy is recommended to be conducted in clinical trials after ethical approval. For patients with technically resectable CNLC stage IIb or IIIa liver cancer, while those are at a high risk of recurrence, neoadjuvant therapy is recommended preoperatively to reduce the incidence of postoperative recurrence.

The documented high risk of liver cancer recurrence includes macroscopic cancer embolus, microvascular invasion, multiple tumors, satellite nodules, and lymph node metastases[13]. With the elevation of the CNLC stage, the risk of recurrence and metastasis after surgical resection also increases[2]. Therefore, multiple examinations should be carried out preoperatively to evaluate the risk of postoperative recurrence. For patients at a high risk of postoperative recurrence, neoadjuvant therapy could control the development of metastatic cancer lesions and improve patients' prognoses with adequate resection margins.

Cycles of neoadjuvant therapy

During neoadjuvant therapy, the patients might not be eligible for surgery due to contraindications to surgery, such as disease progression, postoperative toxicity, and other severe adverse effects[2]. Therefore, the management of preoperative cycles of neoadjuvant therapy is critical to ensure that the treatment targets can be met within a limited period, thereby minimizing the "failure rate" of neoadjuvant therapy.

It is recommended that the duration of neoadjuvant therapy should be 1.5-3 mo (no longer than 4 mo), and surgery should be performed as early as possible after the treatment targets have been met (regardless of the regression of lesions)[2,14]. An appropriate individualized treatment regimen can be selected according to the lesions' locations, general conditions, and hepatic function reserve. Importantly, safe treatment methods are vital to avoid negative influences on the upcoming surgeries (Table 2).

Methods of neoadjuvant therapy

Interventional therapy: Transarterial chemoembolization (TACE): Various studies have shown that preoperative TACE does not improve the survival of patients with resectable liver cancer[15-17]. In a meta-analysis by Qi *et al*[18], 22 randomized and non-randomized studies were included. Their findings showed that preoperative TACE does not improve patient DFS or overall survival (OS). Subgroup analyses showed that the effects of surgery after neoadjuvant TACE were associated with the responses to TACE[18].

Hepatic arterial infusion chemotherapy (HAIC): An embolic agent is not used in the FOLFOX-based HAIC treatment. Thus, the treatment only induces relatively mild inflammatory responses, and certain advancements in the field of neoadjuvant therapy have been achieved[19]. In a Chinese phase III clinical trial, neoadjuvant HAIC was used to treat BCLC stage A/B liver cancer patients that exceeded the Milan criteria for liver transplantation, and it showed that the pathologically complete remission rate was 10.1% and objective remission rate was 63.6%, according to the Modified

Table 2 Evidence of studies on the neoadjuvant therapy

Treatment regimen	Study design	Number of patients	Treatment cycle	Study outcomes
Apatinib + camrelizumab[45]	Phase II	20	6 wk	MPR: 29.4%. PCR: 5.9%
Cabozantinib + nivolumab[46]	Phase I	15	8 wk	12 patients received R0 resection, and MPR or PCR was found in 5 patients (41.7%)
Toripalimab ± lenvatinib	Phase Ib/II	16	21-28 d	3 patients (20%) with MPR
Ipilimumab + nivolumab[47]	Phase Ib	7	6 wk	ORR of 20%; of the 5 patients with pathologically assessable tumors, 3 (60%) were found with pathological remission
Ipilimumab ± nivolumab[48]	Phase II	30	6 wk	Pathological remission rate: 30% (8/27), MPR: 11% (3/27), PCR: 19% (5/27)

MPR: Major pathological response; PCR: Pathological complete response; ORR: Objective remission rate.

Response Evaluation Criteria in Solid Tumors criteria. Compared with the direct resection group (n = 100), the OS and progression-free survival in the neoadjuvant HAIC group (n = 99) both improved significantly (3-year OS rate: 63.5% *vs* 46.3%, P = 0.016; median progression-free survival: 14.1 mo *vs* 8.9 mo, P = 0.017), while the recurrence-free survival was not significantly different between the two groups[20] (grade of evidence: 2A). Another retrospective study showed that neoadjuvant HAIC reduced the risk of recurrence in patients at a high risk of hepatocellular carcinoma and improved patient survival rates. Compared with the control group, the 1-, 3-, and 5-year DFS rates (100%, 78.6%, and 78.6% *vs* 65.8%, 33.7%, and 26.6%, respectively, P = 0.003) and OS rate (100%, 100%, and 100% *vs* 91.7%, 77.8%, and 55.3%, respectively, P = 0.037) in the neoadjuvant HAIC group were significantly higher[21] (grade of evidence: 2B).

Radiotherapy: In a Chinese randomized controlled trial, the patients were randomized to hepatectomy plus neoadjuvant radiotherapy (n = 82) or hepatectomy alone (n = 82). The trial showed that for patients with resectable tumors accompanied with portal vein tumor thrombus, preoperative three-dimensional conformal radiotherapy could effectively improve the postoperative survival of the patients. Regarding patients in the hepatectomy plus neoadjuvant radiotherapy group, partial remission was achieved in 17 (20.7%) patients, and the 6-, 12-, 18-, and 24-mo OS rates were 89.0%, 75.2%, 43.9%, and 27.4% *vs* 81.7%, 43.1%, 16.7%, and 9.4% in the hepatectomy alone group, respectively (P < 0.001). In patients in the hepatectomy plus neoadjuvant radiotherapy group, the 6-, 12-, 18-, and 24-mo DFS rates were 56.9%, 33.0%, 20.3%, and 13.3% *vs* 42.1%, 14.9%, 5.0%, and 3.3% in the hepatectomy alone group, respectively (P < 0.001) [22] (grade of evidence: 2A).

In a Chinese retrospective study, 11920 patients (of whom 134 received neoadjuvant radiotherapy) were included. The adjusted 5-year OS rates in the neoadjuvant radiotherapy and surgery groups were 65.3% and 46.6%, respectively. The results of the adjusted Cox proportional-hazards regression analysis showed that neoadjuvant radiotherapy was significantly associated with longer OS (hazard ratio: 0.549; 95% confidence interval: 0.327-0.921; P = 0.023). In addition, the subgroup analysis showed that patients with N0 disease, alpha-fetoprotein-positive, and aged < 65-years-old could benefit more from the therapy[23] (grade of evidence: 2B).

Another Chinese retrospective study in patients with resectable liver cancer showed that neoadjuvant radiotherapy was associated with long-term patient survival[24] (grade of evidence: 2B).

Systemic therapy: The increasingly abundant systemic therapies have provided new ideas for neoadjuvant therapy of liver cancer. Several studies reported preliminary data regarding applying systemic therapy in neoadjuvant therapy. At the same time, the postoperative consequences of the treatment regimens need to be further verified by large-scale clinical studies.

Recommendations of the consensus

At present, the lack of high-grade evidence for neoadjuvant therapy is noteworthy. In addition, all patients will not benefit from neoadjuvant therapy[2]. In clinical practice,

directly performing neoadjuvant therapy is not recommended for patients with early-stage liver cancer. Assessment of data by multidisciplinary treatment (MDT) is necessary to predict the risk of postoperative recurrence and metastasis and explore whether patients could benefit from neoadjuvant therapy[2]. When the main objective of neoadjuvant therapy is to reduce the risk of recurrence, the treatment cycles are strictly limited, and relatively safe treatment regimens with fewer adverse effects on the surgery are preferred.

STRATEGIES FOR CONDUCTING CONVERSION THERAPY

The main objective of conversion therapy is to eliminate the unresectable factors of liver cancer, thereby satisfying the surgical criteria for safely performing R0 resection.

Specifically, conversion therapy is characterized by active treatment regimens to convert risky surgeries into safe surgeries and change unresectable tumors into radically resectable tumors.

The cycles of conversion therapy could be longer than neoadjuvant therapy to meet the treatment targets, and the treatment duration is not strictly limited. If patients cannot tolerate the therapy or cannot alternatively undergo radical surgeries, the treatment regimen needs to be adjusted after a comprehensive evaluation.

Conversion therapy for patients with insufficient volume of FLR

Patients receiving conversion therapy for an insufficient volume of FLR: The conversion therapy for an insufficient volume of FLR aims to eliminate the unresectable factors from the surgical aspect. Therefore, the target group is the CNLC grade Ia-IIIb liver cancer patients with insufficient volume of FLR after radical therapy.

At present, the criteria for evaluating the functional hepatic reserve for safe resection are generally identical in various centers worldwide. The criteria are as follows: For patients with normal liver function (Child-Pugh class A), indocyanine green-R15 < 10%, and without liver cirrhosis, the FLR/standard liver volume (SLV) needs to be > 20%-30%; for patients accompanied with chronic liver diseases or hepatic parenchyma injuries (*e.g.*, liver cirrhosis, severe fatty liver, and chemotherapy-induced liver injury), the FLR/SLV needs to be > 40%; for patients with liver dysfunction, a higher FLR is required (*e.g.*, for patients with chronic liver diseases or cirrhosis and IGG-R15 of 10%-20%, the FLR/SLV needs to be > 50%)[1,4]. Patients who do not meet these criteria are considered with insufficient volume of FLR.

Considering the contraindications or complications, the treatments should be strictly limited to the following patients: Aged < 65-years-old, with normal liver function (Child-Pugh class A, indocyanine green-R15 < 10%), with insufficient volume of FLR (*e.g.*, FLR/SLV < 30% for patients with normal liver and FLR/SLV < 40% for patients with chronic liver diseases or liver damages), in good general conditions, with good tolerability to surgery, without severe liver cirrhosis, severe fatty liver, or severe portal hypertension[1].

How to reach the target of conversion therapy for an insufficient volume of FLR: Insufficient volume of FLR after tumor resection can lead to an extremely high risk of postoperative liver failure[25]. Therefore, the conversion therapy for an insufficient volume of FLR should reach the following treatment targets: Using specific methods to promote the rapid increase of liver volume and convert the insufficient volume of FLR to a sufficient volume of FLR. After meeting the requirements of hepatectomy, the conversion therapy should convert the liver cancer from unresectable to safely resectable (*i.e.* converting a risky surgery to a safe surgery).

At present, the treatments for insufficient FLR volume include a two-stage hepatectomy procedure combined with portal vein embolization or portal vein ligation, a two-stage hepatectomy procedure combined with portal vein embolization and TACE/hepatic vein embolization, and associating liver partition and portal vein ligation for staged hepatectomy[4]. In addition, the insufficient FLR volume in some patients is caused by giant tumors, and a one-stage radical resection can substantially influence the FLR volume (*e.g.*, possibly damaging blood vessels or bile ducts)[4]. For such patients, a non-surgical therapy (*e.g.*, local therapy or systemic therapy plus local therapy) is effective in reducing tumor size, thereby decreasing the range of resection and increasing the FLR volume [for more details, please refer to section 4.2 (Conversion therapy for patients who are unable to achieve R0 resection)].

For detailed recommendations on selecting appropriate treatment strategies and perioperative management, please refer to the *Chinese Expert Consensus on Conversion Therapy in Hepatocellular Carcinoma* (*2021 edition*). The appropriate treatment methods should be selected according to the tumor type, status of local tumor progression, pathological finding of liver parenchyma, liver functional reserve, and patient tolerability to systemic surgery.

Conversion therapy for patients who are unable to achieve R0 resection

Patients receiving conversion therapy for being incapable of R0 resection: The target patients of the conversion treatment include patients who cannot initially achieve R0 resection due to the tumor burden. Evidence suggests that the efficacy of liver tumor resection in patients who cannot achieve an R0 resection is not significantly higher than non-surgical treatment[7,26-28]. For patients who cannot achieve R0 resection due to causes such as an extremely large tumor volume or blood vessel invasion, conversion therapy can be performed to provide a surgical opportunity for R0 resection, thereby improving long-term efficacy[27].

Reaching the target of conversion therapy for patients who cannot achieve R0 resection: The overall target is to decrease the tumor volume and tumor stage (*i.e.* reducing the volume and number of primary lesions and eliminating portal vein tumor thrombus and metastatic lesions), leading to radical resection[29,30].

The criteria of diagnosing resectable lesions after conversion therapy are as follows: Reduction of tumor volume or tumor stage, complete microvascular tumor thrombus necrosis, evaluation of complete/partial remission, and stable disease lasting for 3-4 mo (according to Modified Response Evaluation Criteria in Solid Tumors criteria)[1]. The details of different conditions are as follows: (1) Intrahepatic lesions: For patients with a giant tumor or a remarkable number of lesions, surgery is risky or is accompanied by surgical difficulties, and the target of a successful conversion is to reduce the number of lesions, ensuring negative surgical margins and decreasing surgical difficulty, according to the Modified Response Evaluation Criteria in Solid Tumors criteria. For patients with vascular invasion who cannot achieve R0 resection, the target of a successful conversion is spontaneous necrosis or regression of malignant tumors, complete microvascular tumor thrombus necrosis, and reduction of tumor stage (*e.g.*, from CNLC stage IIIa to IIb); and (2) Extrahepatic metastasis: For patients with extrahepatic (mainly lung) metastasis (CNLB stage IIIb), the target of a successful conversion is to eliminate the metastatic lesions, reduce the tumor stage, and perform the surgical resection of the intrahepatic lesions.

To date, numerous explorative studies on conversion therapy have been performed, of which the treatment regimens include local therapy[31-36], systemic therapy[37], and combination therapy[38-42] (Table 3). Concerning the absence of a potent systemic treatment, TACE is the major method for conversion therapy[1]. Compared with traditional TACE, drug-eluting bead-TACE continuously releases chemotherapeutic drugs at a fixed dose with outstanding controllability, prolonging the treatment time between the cancer cells and the chemotherapeutic drugs as well as avoiding liver microcirculation injury. A Chinese cohort study on 32 patients with unresectable hepatocellular carcinoma showed that after drug-eluting bead-TACE treatment, the success rate of stage reduction was 59.4%; however, after subsequent radical therapy (surgery or ablation), the rate of complete remission was as high as 81.3%. The successful stage reduction using drug-eluting bead-TACE is associated with longer survival[43]. Several scholars demonstrated the effects of HAIC, selective internal radiation therapy, and radiotherapy for conversion therapy[1]. The rapid advances in systemic therapy also provide new ideas for conversion therapy. A great number of studies are currently available to support the application of systemic therapy plus local therapy in conversion therapy (Supplementary Tables 1 and 2). We recommend that a gradually progressing treatment strategy be performed according to the currently available treatment standards, taking both efficacy and safety of treatment into account. For detailed recommendations on selecting treatment methods and timing of surgical resection following conversion therapy, please refer to the *Chinese Expert Consensus on Conversion Therapy in Hepatocellular Carcinoma* (*2021 edition*)[1].

NECESSITY OF MDT FOR NEOADJUVANT AND CONVERSION THERAPIES

Due to the heterogeneity of liver cancer and the features of the multi-modal MDT,

Table 3 Evidence for systemic therapy plus local therapy in conversion therapy

Ref.	Treatment regimen	Number of patients, *n* (%)	Study results
Zhang *et al*[37], 2020	TKI: Lenvatinib. PD-1 antibody: Pembrolizumab/Sintilimab/Toripalimab	33	Success rate of conversion (imaging): 42.4%. Actual surgery rate following conversion: 30.3%
Li *et al*[40], 2017	TACE + sorafenib	142	Second-stage resection rate following stage reduction: 14.8%
He *et al*[39], 2019	HAIC + sorafenib	125	Surgical resection rate following conversion: 12.8%
He *et al*[41], 2018	HAIC + sorafenib	35	Surgical resection rate following conversion: 14.3%
Zhang *et al*[42], 2021	HAIC + TKI + PD-1 antibody (1, 7, and 17 patients used sorafenib, apatinib, and lenvatinib, respectively)	25	Surgical resection rate following conversion: 56.0%, 7 patients (28.0%) achieved pathologically complete remission
He *et al*[38], 2021	Lenvatinib + toripalimab + HAIC	71	Surgical resection rate following conversion: 12.7%

Grade of evidence in the guideline of Chinese Society of Clinical Oncology diagnosis and treatment. Feature of evidence; grade; level; sources; Chinese Society of Clinical Oncology degree of expert consensus; 1A: High, rigorous meta-analysis, large-scale randomized clinical study, unified consensus (supportive opinion: ≥ 80%); 1B: High, rigorous meta-analysis, large-scale randomized clinical study, generally unified consensus, with slight controversy (supportive opinion: 60%-80%); 2A: Slightly low, fair-quality meta-analysis, generally unified consensus, with slight controversy (supportive opinion: 60%-80%) or small-scale randomized clinical study, well-designed large-scale retrospective study, case-control study, unified consensus (supportive opinion: ≥ 80%); 2B: Slightly low, fair-quality meta-analysis, small-scale randomized clinical study, well-designed large-scale air-quality meta-analysis, generally unified consensus, with slight controversy (supportive opinion: 60%-80%) or small-scale randomized clinical study, well-designed large-scale retrospective study, case-control retrospective study, case-control study; generally unified consensus, with slight controversy (supportive opinion: 60%-80%); 3: Low, non-controlled single-arm clinical study, case report, expert opinion, no consensus, with low substantial controversy (supportive opinion: < 60%). TKI: Tyrosine kinase inhibitor; PD-1: Programmed cell death protein-1; TACE: Transarterial chemoembolization; HAIC: Hepatic arterial infusion chemotherapy.

MDT is essential for neoadjuvant and conversion therapies. Therefore, it is important to form a relatively constant multidisciplinary team to make an individualized treatment decision for liver cancer patients. Before starting a neoadjuvant therapy, preoperative evaluation by a multidisciplinary team is required to predict the risk of postoperative recurrence and metastasis and to indicate whether patients can benefit from the neoadjuvant therapy. During the neoadjuvant and conversion therapies, the treatment methods and measurements, the timing of surgery after treatment, and management of adverse events during the treatment need to be discussed repeatedly among the multidisciplinary team members due to the advantages and disadvantages of different treatment regimens (Figure 1). After a treatment regimen is selected, discussion among the multidisciplinary team members should be regularly carried out, which could ensure the adjustment of treatment regimens according to the changes of disease conditions, enabling patients to benefit from the advantages of the treatment.

FUTURE PROSPECTS

Characteristics of patients requiring neoadjuvant therapy or conversion therapy

The recommended treatment methods are as follows: (1) Surgical resection: It should be performed directly if the patients meet the criteria for R0 resection; (2) Conversion therapy: If patients are eligible for R0 resection according to the defined criteria, conversion therapy can be conducted to eliminate the unresectable hepatic tumors; and (3) Neoadjuvant therapy: Owing to the lack of evidence for neoadjuvant therapy, it should be performed in clinical practice; alternatively, patients can receive immunotherapy, neoadjuvant HAIC therapy, or radiotherapy based on sufficient evidence from discussion performed among members of the multidisciplinary team. Patients' clinical characteristics are worthy of further investigation by experienced clinicians.

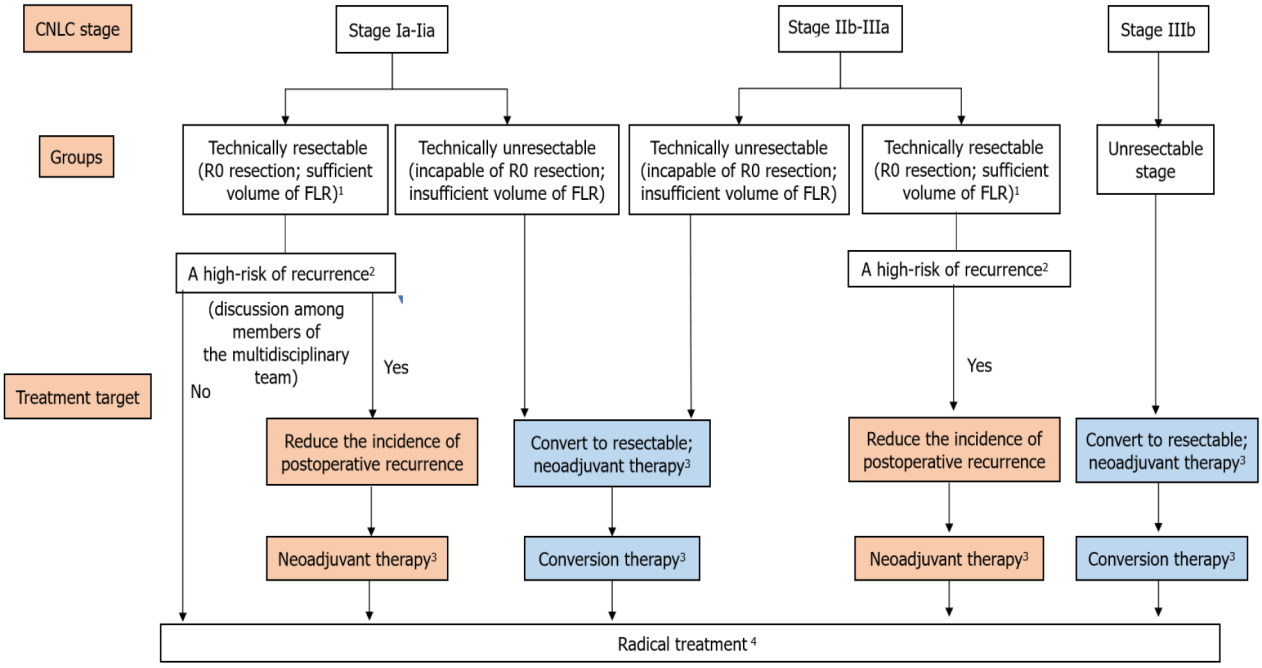


Figure 1 Algorithm of neoadjuvant and conversion therapies for liver cancer. [1]Technically resectable criteria: R0 resection, sufficient volume of the future liver remnant, Child-Pugh class A/B (some patients)[1]. [2]Comprehensive evaluation by preoperative imaging examination findings and serum levels of biomarkers should be performed to assess the risk of postoperative recurrence[44]. Postoperative recurrence risk factors: (1) For patients with stage Ia, Ib, and IIa liver cancer, unclear tumor boundary, closely adjacent of the tumor to blood vessels, and highly suspicious residual tumors are among the high-risk factors of recurrence; (2) For patients with stage IIb-IIIa liver cancer, the high-risk factors of recurrence include the number of tumors ≥ 3, tumor diameter > 5 cm, satellite nodules, macroscopic cancer emboli, microvascular invasion-positive, lymph node metastasis, invasion of adjacent organs, and high alpha-fetoprotein level before surgery[13,44]; and (3) Other recurrence factors include liver diseases (*e.g.*, viral hepatitis and liver cirrhosis)[13]. [3](1) Stage Ia-IIa: Neoadjuvant therapy is not recommended in clinical practice; if the multidisciplinary treatment clarifies the high risk of postoperative recurrence, neoadjuvant therapy can be performed in clinical trials after ethical approval; (2) Stage IIb-IIIa: Radical treatment following neoadjuvant therapy is recommended for patients with technically resectable liver cancer and high risk of recurrence, aiming to reduce the postoperative recurrence; and (3) For patients with unresectable liver cancer who are incapable of R0 resection or insufficient volume of future liver remnant (FLR), conversion therapy can be conducted to eliminate unresectable hepatic tumors. [4]Radical therapies, including liver transplantation, resection, and radiofrequency ablation, are highly appropriate for early-stage liver cancer patients. Liver transplantation: Patients who meet the Milan criteria or the University of California San Francisco (UCSF) criteria after preoperative treatment can be treated with liver transplantation. (1) Milan criteria: Diameter of a single tumor ≤ 5 cm, the number of tumors ≤ 3, in which the diameter of the largest tumor was ≤ 3 cm, and without large blood vessel or lymph node invasion; and (2) The University of California San Francisco criteria: Diameter of a single tumor is ≤ 6.5 cm, the number of tumors ≤ 3, in which the diameter of the largest tumor was ≤ 4.5 cm, and the sum of diameters of all tumors was ≤ 8.0 cm, without a large blood vessel or lymph node invasion[7]. Radiofrequency ablation: Patients reached China Liver Cancer (CNLC) stage Ia or Ib (*e.g.*, a single tumor, the diameter of tumor ≤ 5 cm; or with 2-3 tumors, in which the largest diameter was ≤ 3 cm) after preoperative therapy or without blood vessel, bile duct, and adjacent organ invasion, without distal metastasis, and liver function of Child-Pugh class A/B could be treated by radiofrequency ablation, which can also achieve the effects of radical treatment[7].

CONCLUSION

Neoadjuvant therapy and conversion therapy are important strategies for the preoperative treatment of intermediate or advanced liver cancer. Consensus on conversion therapy has already been achieved, while further evidence for neoadjuvant therapy needs to be presented. With the development of treatment methods, many high-quality clinical trials are in progress, providing further evidence-based support for neoadjuvant therapy. Based on the consensus, Chinese scholars' explorations will scientifically support theories and methods for preoperative liver cancer treatment, thereby improving Chinese liver cancer patient OS.

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World J Gastroenterol 2021 December 21; 27(47): 8081-8102

DOI: 10.3748/wjg.v27.i47.8081

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

REVIEW

Present and future management of viral hepatitis

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Author contributions: Jiménez Pérez M and González Grande R contributed to this paper with the conception and design of the review, critical revision and editing and literature review; López Ortega S and Santaella Leiva I wrote the manuscript; González Grande R designed the figures; All authors approved the final version.

Conflict-of-interest statement: The authors have no conflict of interest to report.

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, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Open-Access: This article is an open-access article that was

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Abstract

Viral hepatitis can result in important morbidity and mortality, with its impact on health conditioned by the specific type of hepatitis, the geographical region of presentation and the development and access to new drugs, among other factors. Most acute presentation forms are self-limiting and may even go unnoticed, with just a small percentage of cases leading to acute liver failure that may necessitate transplantation or even cause the death of the patient. However, when they become chronic, as in the case of hepatitis B virus and C virus, unless they are diagnosed and treated adequately they may have severe consequences, like cirrhosis or hepatocarcinoma. Understanding of the mechanisms of transmission, the pathogenesis, the presence of vaccinations and the development over recent years of new highly-efficient, potent drugs have meant that we are now faced with a new scenario in the management of viral hepatitis, particularly hepatitis B virus and hepatitis C virus. The spectacular advances in hepatitis C virus treatment have led the World Health Organization to propose the objective of its eradication by 2030. The key aspect to achieving this goal is to ensure that these treatments reach all the more vulnerable population groups, in whom the different types of viral hepatitis have a high prevalence and constitute a niche that may perpetuate infection and hinder its eradication. Accordingly, micro-elimination programs assume special relevance at the present time.

Key Words: Hepatitis viral; Diagnosis; Treatment; Trend direct-acting antivirals; Inhibiting recycling

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Core Tip: The various types of viral hepatitis have resulted in important morbidity and mortality for many years. Greater understanding of the pathogenesis as well as the development of new, highly efficient potent drugs mean that we are now faced with a

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Received: January 27, 2021
Peer-review started: January 27, 2021
First decision: March 29, 2021
Revised: April 8, 2021
Accepted: December 7, 2021
Article in press: December 7, 2021
Published online: December 21, 2021

P-Reviewer: Dai K, Hercun J
S-Editor: Liu M
L-Editor: Filipodia
P-Editor: Liu M

new scenario in the approach to this disease. The spectacular advances in the treatment of hepatitis C virus suggest that we can now envisage its eradication, as put forward by the World Health Organization in its objectives for 2030. In this review we comment on the current situation, recent advances and future perspectives in the approach to viral hepatitis.

Citation: González Grande R, Santaella Leiva I, López Ortega S, Jiménez Pérez M. Present and future management of viral hepatitis. *World J Gastroenterol* 2021; 27(47): 8081-8102
URL: https://www.wjgnet.com/1007-9327/full/v27/i47/8081.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i47.8081

INTRODUCTION

The different types of viral hepatitis have resulted in great morbidity and mortality for many years, due to their high prevalence and incidence. Over 250 million persons are estimated to be infected with the chronic form of hepatitis B virus (HBV)[1] and more than 70 million with hepatitis C virus (HCV) in the world[2], with over 1.5 million cases of hepatitis A virus (HAV) annually[3], resulting largely from lack of understanding of the pathogenesis and the absence of efficient treatment. Fortunately, recent years have seen important advances, impacting very positively on the management of viral hepatitis, particularly HBV and HCV, though not so much on HAV and hepatitis E virus (HEV). The most significant advances in these latter types have occurred in aspects related to the epidemiology and pathogenesis of the disease rather than in therapy. Studies have shown new pathways of contagion, especially relevant in certain population groups, such as patients with previous liver disease or immunosuppressed patients where the disease can have an important impact on morbidity and mortality.

Advances in understanding the viral pathogenesis of HBV have mainly led to the development of new drugs. The first significant leap occurred in the early 2000s, with the appearance of the first nucleotide/nucleoside analogs (NAs), like lamivudine and adefovir dipivoxil. This enabled oral treatment, with great efficacy, safety and tolerability, though limited by the development of resistance that was overcome later with entecavir (ETV) and tenofovir (TDF), which have a high genetic barrier to the development of resistance[4,5]. Nevertheless, limitations still exist, such as the need to administer the drugs for prolonged periods of time, even indefinitely, and they are unable to inhibit the initial formation of covalently closed circular (ccc)DNA in newly infected hepatocytes[6]. This has resulted in current research aimed at developing drugs to inhibit viral replication, acting on any of the various phases of the virus replication cycle[7]. Indirectly, all these advances can have a positive impact on the management of hepatitis D virus (HDV), which whilst being a defective virus that needs the presence of the B virus for reproduction, can nevertheless cause important morbidity and mortality. The first drug for the specific treatment of HDV, bulevirtide, has recently been commercialized in Europe[8].

It is, though, in HCV where the most significant advances have been made, with a major impact on improving health over recent years. The efficacy, safety, tolerability and ease of use in clinical practice of direct-acting antivirals (DAA) have led to the rarely seen possibility of eradicating HCV by 2030, an objective set by the World Health Organization[9]. This requires establishing such strategies as micro-elimination based on the active search for cases, simplification of the diagnosis and treatment and prevention measures.

HEPATITIS A

The classic transmission of HAV and HEV is the fecal-oral route, more prevalent in underdeveloped countries. However, new pathways of infection are leading to changes in the epidemiology of these infections. This, perhaps, is the main novelty.

HAV is one of the most common infectious etiologies of acute hepatitis worldwide. Transmission is fecal-oral *via* contaminated food or water, either person-to-person or through consumption of contaminated products. Globally, an estimated 1.5 million

cases occur each year[3]. There are areas of high, intermediate, low and very low HAV endemicity. In low- and middle-income countries, where sanitation and hygiene practices are poor, infection is common, and most children (90%) have it before the age of 10, very often without presenting symptoms[10]. Epidemics are rare because older children and adults are usually immunized. In these areas, morbidity rates are low, and epidemic outbreaks rarely occur. In developed countries with good sanitation and hygiene, infection rates are low. This translates into an increase in the number of adults who have never been infected and who lack immunity. This increased vulnerability in older age groups can increase morbidity rates and lead to large epidemic outbreaks. The disease can appear in adolescents and adults from high-risk groups, such as injection drug users, men who have homosexual relationships and people who travel to high-endemic areas[11]. Because of the epidemiological features, vaccination is recommended for persons at increased risk of exposure or those liable to fulminant disease[12].

Infection with HAV has a mild or asymptomatic course, although it may be fulminant in < 1% of cases[13], especially in patients with chronic hepatitis[14]. Other atypical presentations of acute hepatitis A include renal insufficiency and relapsing hepatitis, which are usually present in children. Some individuals experience a prolonged hepatitis (5.8%)[15] or cholestasis (6.8%), especially in the presence of HBV [16]. HAV accounts for 0.35% of cases of acute liver failure. HAV-related acute liver failure has a spontaneous resolution rate of 70%, with the remaining 30% requiring a liver transplant[17]. Several studies have concluded that acute HAV infection superimposed on chronic liver disease is associated with greater disease severity and a higher case fatality rate, though a review of the literature failed to delineate a relationship between nonalcoholic steatohepatitis and HAV-induced liver failure[18].

Regarding the prevention of hepatitis A, in addition to improving hygiene and sanitation measures, the vaccine is a very effective tool, already being included in the vaccination calendar from childhood in many countries. Nonvaccinated persons traveling to HAV endemic regions should receive a single vaccine dose before departure[19]. The Centers for Disease Control and Prevention 2020 Advisory Committee on Immunization Practices recommends vaccination for all children aged 1 year and older, men who have sex with men and people who use injection and noninjection drugs, have occupational risk factors for infection, travel to high-endemicity areas or those who have an increased risk for complications from hepatitis A (*e.g.*, chronic liver disease, HIV infection and pregnancy, if at risk for infection)[12]. There are currently two single-antigen inactivated vaccines: Havrix™ (GlaxoSmithKline Biologicals, Rixenstar, Belgium) and Vaqta™ (Merck and Co. Inc., West Point, PA, United States)[20,21]. A live-attenuated vaccine is licensed in China and has been used extensively there[22]. A combination HAV-HBV vaccine (Twinrix™; GlaxoSmithKline Biologicals) has been available since 1996[23]. Havrix and Vaqta vaccines are given in a two-dose schedule 6 mo apart and Twinrix requires three doses. The efficacy of both live-attenuated and inactivated vaccines has been well established in a large review[24]. A special population concerns HIV-positive individuals who are susceptible to HAV infection, especially because of low adherence to recommended HAV vaccination. In this group a double dose of HAV vaccination is recommended as an additional dose of the HAV vaccine may improve serological responses and durability of seroprotection. Immunoglobulin is used for both preexposure and postexposure prophylaxis of HAV. Children younger than 12-mo-old, adults with chronic liver disease, adults older than 40 years of age and immunocompromised individuals should receive a single dose of intramuscular HAV immunoglobulin (0.1 mL/kg) in addition to the vaccine, unless either is contraindicated.

HEPATITIS E

Infection with HEV, traditionally considered a disease almost exclusive to developing countries, has now become a worldwide health problem and is endemic in most developed countries, behaving largely as a zoonosis[25]. HEV infection has a greater clinical impact in populations that are especially vulnerable, such as immunosuppressed patients, pregnant women and patients with underlying liver disease. Thus, the World Health Organization places it as one of the leading causes of death from acute viral hepatitis worldwide[26].

HEV is divided into four species (A–D). Genotypes 1 and 2 of species A are strains that infect humans, whereas genotypes 3 and 4 are zoonoses transmitted *via* meat consumption or direct contact with affected animals, mainly pigs or wild boar, or

through contaminated blood products[25]. In recent years, particularly in Eastern China, genotype 1 has become much less common, and genotype 4 is now the most common genotype found in human cases[27]. Person-to-person transmission (direct contact) of HEV is very inefficient; study of close contacts of a case with documented HEV infection is not recommended, unless they share exposure to the source of infection. Blood transfusion is another route of transmission of HEV. The European Association for the Study of the Liver (EASL) recommends that blood donor services screen blood donors for HEV, given the results of local risk-assessment and cost-effectiveness studies[25].

In most cases, contact with HEV produces an asymptomatic infection, mainly in women and young people, followed by spontaneous clearance of the virus[28]. HEV infection during pregnancy (particularly during the third trimester) has been associated with a poorer prognosis compared to other types of viral hepatitis[29-31]. Maternal mortality rates of up to 30% have been observed in different outbreaks of hepatitis due to HEV in pregnant women[32]. The mortality associated with HEV infection during pregnancy is usually associated with infections caused by genotypes 1 and 2, though cases caused by other genotypes have been reported[33,34].

Although the development of acute liver failure in the course of HEV infection is rare (0.5%-4.0%)[35], in some series, like that of Crossan *et al*[36], HEV accounts for 5.0% of all cases of acute liver failure. Those most at risk of developing acute liver failure in the course of HEV are pregnant women and patients with underlying chronic liver disease[37,38]. Because of this, all patients with acute hepatitis, acute liver failure or patients with decompensation of chronic liver disease should be screened for HEV infection. Although isolated cases of chronic HEV infection have been reported in immunocompetent patients[39,40], chronic HEV infection occurs primarily in immunocompromised patients, such as solid organ transplant recipients. In transplant patients, HEV infection may progress to chronicity in up to 2/3 of cases, with rapid progression of fibrosis and development of liver cirrhosis in up to 10% of patients[41, 42]. Cases have even been reported of retransplantation in liver transplant recipients due to acute liver failure from HEV[43]. Extrahepatic clinical manifestations have been described in 2%-5% of patients with HEV infection[44]. In most of these the liver manifestations of the infection are mild or absent. The EASL recommends HEV testing, irrespective of liver function test results, in patients presenting with neuralgic amyotrophy or Guillain-Barré syndrome and suggests HEV testing for patients with encephalitis/myelitis. Patients with proteinuria should also be tested[25]. Currently, screening for HEV is advisable in all cases of acute hepatitis, including those with suspected drug-induced liver injury, particularly if patients have higher levels of transaminases, in which case acute HEV must be excluded systematically[45,46].

The diagnosis of HEV is based on a combination of serology and nucleic acid amplification techniques. Serological techniques measure anti-HEV immunoglobulin (Ig)M antibodies, which appear approximately 4 wk after contact and may be detected up to 6 mo later, and IgG antibodies, which appear at about the same time as the IgM antibodies but can last for years. The presence of anti-HEV IgM indicates recent or acute infection while anti-HEV IgG antibodies indicate recent or past infection. HEV-RNA can be detected in blood or stool 3 wk after infection and a short time before the appearance of symptoms and constitute the gold standard for the diagnosis of active infection[47]. The combination of serological studies and the determination of HEV-RNA increases the diagnostic specificity and sensitivity, though it is necessary to bear in mind the immune-competence status of the patient. The World Health Organization recommends first determining the presence of anti-HEV IgM antibodies and then determining the viral RNA. In immunocompromised patients, the antibodies may be negative despite HEV infection, and it is necessary to determine the viral load in all cases[48].

Current recommendations for the management of HEV infection

Acute HEV infection does not usually require antiviral therapy. In almost all cases HEV infection clears spontaneously. Nevertheless, early therapy of acute hepatitis E may shorten the course of the disease and reduce overall morbidity. Ribavirin treatment may be considered in cases of severe acute hepatitis E or acute-on-chronic liver failure. Corticosteroids have been used in individual cases of acute liver failure, with improvement of liver function parameters. However, there is currently insufficient evidence to support general corticosteroid treatment in this group of patients[25]. The group of transplant patients deserves special mention. The EASL recommends decreasing immunosuppression at diagnosis of chronic HEV infection in solid organ transplant recipients, if possible.

In patients with persisting HEV replication 3 mo after detection of HEV RNA, the EASL recommends ribavirin monotherapy for a duration of 12 wk. Trials have been attempted with other treatments, such as sofosbuvir in single therapy, which showed antiviral activity *in vitro* but was unable to inhibit viral replication in a phase II pilot trial[49].

Future directions

As no efficient therapy exists for HEV and bearing in mind it can cause severe symptoms of liver disease, efforts should be focused on both prevention and research.

Preventive measures should be enhanced in immunosuppressed patients and pregnant women; indeed, screening for HEV should be recommended in pregnant women in endemic areas like Sub-Saharan Africa or South Asia, assessing the risk individually[47].

A few European countries are starting to include HEV-RNA detection in all blood donor samples, although certain aspects remain to be clarified, such as the most cost-effective technique for detection, the viral load considered infectious or the characteristics of the recipient that may influence transmission of the disease, such as the immunological status[50].

Currently only one vaccine against HEV is available (Helicon®). It has only received approval in China, though other vaccines are under development. The availability of an effective vaccine would constitute the main tool for prevention of HEV infection [51].

HEPATITIS B

The discovery of the Australia antigen in 1965 represented a starting point for the identification of the virus of hepatitis B, and for many it was the beginning of the study of viral hepatitis. Infection with HBV is now a public health problem worldwide, with some 257 million persons with chronic infection. HBV is endemic in the western Pacific and Africa, with over 6% of the population infected[1]. In Europe and the United States the prevalence is < 2%, mostly related with immigration[52]. HBV is the leading cause of morbidity and mortality of hepatic origin in the world, despite the availability of an efficient vaccine.

Vaccination programs, control of blood donations, serologic evaluation in pregnant women or persons at risk and activities aimed at limiting invasive procedures in unsafe conditions have all reduced the incidence of acute HBV hepatitis and the prevalence of the disease. Although population screening is not indicated, screening is recommended in risk groups, partners of infected patients and before receiving oncologic or immunosuppressive therapy[1].

The spectrum of the disease varies greatly, from inactive carriers to others with hepatic cirrhosis and hepatocarcinoma. The natural history of hepatitis B is a dynamic process, traditionally differentiated into five phases depending on virus and host factors, like the state of immune competence, age or the duration of the infection[53]. They are classified according to liver inflammation data (alanine aminotransferase), determination of hepatitis B e antigen (HBeAg) and quantification of the HBV-DNA viral load[54] as well as estimation of the degree of hepatic fibrosis, usually by elastography[1] as serologic indices of fibrosis have proven less precise in HBV infection[55]. These phases have traditionally been called: immune-tolerant phase, immune-elimination phase, immune-reactive phase, asymptomatic carrier and hepatitis B surface antigen (HBsAg)-negative phase[56]. However, these phases have recently been grouped into two large spectra of chronic forms of hepatitis B with a new nomenclature: infection, encompassing the phases of immune tolerance and inactive carrier, both HBeAg positive and negative *vs* hepatitis, which refers to the phases in which there exists liver damage, the immune-reactive and the immune-elimination phases[57].

Awareness of the particular phase in which the patient is and its clinical context is important to identify the need to start treatment and the choice of the most suitable antiviral agent.

Evolution of antiviral treatment: Nucleos(t)ide analogs

Initially it was thought that a depressed immune response was involved in the development of HBV infection. Thus, in 1986 the first clinical trial of interferon (IFN) alpha was published, though the findings showed a poor and transitory response[58]. Currently, in its pegylated form it is still considered a treatment option, with PEGα2a

being easier to use and having greater efficacy and tolerance[59]. The duration of treatment with this drug is usually finite and the loss of HBsAg and conversion of HBeAg is relevant, although the drug has to be injected and is contraindicated in patients with decompensated cirrhosis, autoimmune disorders, pregnant women and severe depression or psychosis[59].

Later studies on the lifecycle of HBV found that the virus uses an inverse transcriptase for replication. Accordingly, the use of drugs already available for another virus (HIV), NAs with an inhibitory action on this polymerase, were considered[60]. In 1998 the Food and Drug Administration approved the use of lamivudine for the treatment of HBV, controlling viral replication with oral treatment [61]. Lamivudine was the sole oral treatment available until 2002, when other NAs have become available: adefovir dipivoxil, ETV, telbivudine, TDF in 2008[4] and tenofovir alafenamide in 2016, though the latter is not available in all countries.

All these analogs reduce the pool of cccDNA in infected hepatocytes, inhibiting recycling of the nucleocapsids, although they are unable to inhibit the initial formation of cccDNA in newly infected hepatocytes[5]. The main advantages of NAs are that they can be given orally, they have great efficacy in inhibition of HBV replication (very similar among all of them), their long-term safety and the possibility of being used in any situation, including decompensated cirrhosis and liver transplant or even during pregnancy in the case of TDF. They achieve a virological and biochemical response greater than 95%, for both positive and negative HBeAg[62,63]. The main inconvenience is that they require continued administration over time, even indefinitely, in order to maintain inhibition of viral replication and their efficacy can be affected by the development of drug-resistant HBV mutations, sometimes with cross-resistance between them, partly related with lack of adherence to treatments that have to be taken for such long periods.

Lamivudine is the drug that has shown the greatest risk for development of resistance as compared with the other antiviral agents currently available, particularly ETV and TDF[64]. The main clinical practice guidelines therefore recommend treatment with ETV, TDF and tenofovir alafenami given their great antiviral potency and high resistance barrier[65]. The choice of one over the other treatment strategy depends of the stage of liver fibrosis, virological factors and the comorbidity profile of the patient, in addition to personal preferences[14]. Tenofovir alafenami is a prodrug that reaches higher levels of TDF than TDF in the hepatocytes with lower doses. Lower exposure to the drug is related with less worsening of renal function and less loss of bone mineral density[66].

Recent studies have shown that combined NA treatment with Peg-IFN, simultaneously or sequentially, increases the probability of HBsAg loss, but the benefits are restricted to just a small group of patients with low HBsAg levels. Further studies are therefore needed before it can be recommended[67].

Recent studies have examined whether TDF *vs* ETV could impact the risk of developing hepatocarcinoma. Though this would condition the choice of one over the other, neither has yet been found superior in this respect. Some studies have suggested the benefit of TDF, though the differences were not statistically significant[68]. Retrospective analyses of different series have reported contradictory results, with some showing no difference between the drugs[69] whilst others have found benefits for TDF *vs* ETV in the prevention of hepatocarcinoma[70], a benefit confirmed in a recent meta-analysis[71]. Randomized trials are needed to establish this association.

Nucleos(t)ide analogs and liver transplantation

Patients who required a liver transplant due to HBV initially had a very high risk of recurrence of the infection in the graft. This was particularly so for patients who received their transplant having a high viral load, which in addition was usually a severe, rapidly progressive hepatitis, so much so that HBV infection became considered a contraindication for liver transplantation[1]. With the advent of anti-hepatitis B hyperimmune gammaglobulin during the 1990s, and particularly of the NAs, the perspective changed. The post-transplant results were similar to those of other etiologies. Now that we have potent NAs and a high resistance barrier, the tendency is to use hepatitis B immune globulin for a short time and at lower doses than before and even regimens without hepatitis B immune globulin with NAs in single therapy maintained indefinitely[67]. These advances have thus allowed for an individualized prophylaxis based on the individual risk profile of each patient[72] as well as the use of anti-hepatitis B core positive donors.

Treatment objectives: New definitions of cure

The final aim of antiviral therapy is cure of the infection, eliminating all potential forms of HBV replication[59]. Sustained loss of HBsAg and eradication of the HBV-DNA, including the cccDNA[6], though this is hardly feasible with currently available drugs, is the sterilizing cure. As a result, the American Association of the Study of Liver Diseases and EASL agreed to the definition of functional cure, defined as the sustained loss of HBsAg and HBV-DNA in serum with or without the development of anti-HBs. These two situations are those that allow treatment to be suspended safely and with little risk of relapse. However, they are very rarely achieved. Another concept, partial cure, refers to the persistence of HBsAg but with negativization of HBeAg, with or without seroconversion to anti-HBe, normalization of alanine aminotransferase and a low or undetectable viral load, simulating a phase of inactive carrier and thus susceptible to interruption of treatment[6].

Current guidelines[57,73] recommend that treatment can be suspended with NAs for patients with positive HBsAg but without cirrhosis if there is seroconversion to anti-HBe after 1 year of consolidation. For negative HBeAg, treatment can be suspended for patients whose HBsAg clears and who have had viral suppression for over 2-3 years, requiring strict follow-up[74].

Quantified HBsAg is determined by enzyme immunoassay and reflects the amount and transcriptional activity of the cccDNA. It is very useful in untreated patients with negative HBeAg, with HBV-DNA < 2000 IU/mL, in whom values of quantified HBsAg < 1000 IU/mL are indicative of inactive carrier with a low risk of disease progression and appearance of hepatocarcinoma. Although no cut-off value has yet been established, a level < 100 IU/mL seems to predict this sustained response[1]. Among the new markers is the antigen related with the HBV core that correlates with the transcriptional activity of cccDNA, especially in the negative HBeAg patient, and it could be superior to quantified HBsAg for the identification of inactive carrier patients and to predict viral relapse after suspending treatment[75].

Future directions

Given that treatment with NAs has little impact on the cccDNA, which in addition can be replaced with no need for the entry of new virus[6], numerous studies are in place to develop new drugs that act at the level of the different stages of the life cycle of HBV, about which more and more is being learnt, or that modulate the host immune response. These drugs are at various stages of clinical trials, with up-to-date information available from: http://www.hepb.org/treatment-and-management/drug-watch[7] (Table 1).

The main drugs under development and their therapeutic targets are described below:

Blocking entry of HBV into the hepatocyte: The sodium taurocholate cotransporting polypeptide receptor is a transmembrane protein that cotransports bile acids and participates in the entry of HBV and HDV into hepatocytes[76]. Bulevirtide couples to this receptor and blocks fixation of the preS1 domain on the coat of HBV, inhibiting entry of the virus. Although this does not directly interfere with the formation of the cccDNA, inhibiting virus entry can block the infection of new hepatocytes. It has also been proposed as an option to prevent reinfection in the transplanted liver[77]. It is currently in phase II[7].

Silencers: These are drugs designed to interfere and destroy viral RNA. The process involves small molecules of RNA producing interference in the transcription of viral RNA. They are short non-encoding sequences present in the cells that regulate the post-transcriptional expression of certain genes, resulting in a reduction in the production of multiple viral antigens[77,78]. There are several studies in various phases: preclinical, I and II.

HBsAg release inhibitors: HBsAg is the most abundant circulating viral antigen. It contributes to T-cell tolerance and attenuation of the host immune response. Its inhibition may enable immune regulators to restore the immune response. There are currently two phase II studies with results that need to be validated.

Capsid assembly modulators: As the nucleocapsid contains the viral DNA necessary for replication, inhibiting its formation is an interesting strategy that can prevent the formation of cccDNA during de novo infection[79]. There are currently several studies in various phases, preclinical, I and II and one with vebicorvir in phase II/III.

Antisense molecules: These bind to messenger RNA inhibiting the formation of viral proteins. There is currently one phase II study in the United States.

New NAs: Besifovir has been approved in Korea, with antiviral efficacy comparable to that of TDF after 48 wk of treatment, with effects lasting 96 wk and a better safety

Table 1 Main drugs under development for the treatment of hepatitis B virus (with effect from phase II)

Treatment	Mechanism of action	Country	Phase
Bulevirtide	Inhibitor of HBV entry into the hepatocyte	Germany	Phase II
VIR-2218	Silencers: Interfere and destroy viral RNA	United States	Phase II
JNJ-3989		United States	Phase II
IONIS-HBVRx (GSK3228836)	Antisense molecules: Bind to mRNA to prevent passage by viral protein	United States	Phase II
Vebicorvir	Capsid inhibitors	United States	Phase II/III
Morphothiadin		China	Phase II
JNJ 56136379		Ireland	Phase II
ABI-H2158		United States	Phase II
REP 2139	HBsAg inhibitors	Canada	Phase II
REP 2165		Canada	Phase II
NASVAC	THERAPEUTIC VACCINES: Use stimulation of the immune system as treatment	Cuba	Phase III
GS-4774		United States	Phase II
HepTcell		United States	Phase II
GS9688 (TLR-8 agonist)	TLR	United States	Phase II
GC1102	Monoclonal antibodies	Korea	Phase II
ASC22: Inhibitor PDL1	Checkpoint inhibitors: Stimulate specific T lymphocytes	China	Phase II
IMC-I109V	Other immune modulatorsT-cell receptor	United States	Phase II

Taken from Hepatitis B Foundation[7], Available from: https://www.hepb.org/treatment-and-management/drug-watch/. TLR: Toll-like receptor; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen.

profile than TDF in terms of bone and kidney results[80], though further studies are needed.

Still in the preclinical phase are studies of drugs against HBV-cccDNA. They work *via* degradation of the lymphotoxin beta receptor using specific antibodies or with cccDNA excision enzymes.

Immunotherapy: Immunological dysfunction due to "exhaustion," a phenomenon characterized by lack or absence of specific T cells against HBV associated with poor cytotoxic activity, worsening cytokine production and an increased expression of T-cell inhibitor receptors[81], has resulted in various immunotherapeutic strategies aimed at modulating the innate or adaptive immune response, or both, in an attempt to restore a competent immune response against the virus and infected hepatocytes [59]. New modulatory agents of immunity in clinical development include:

Pattern recognition receptor (PRR) agonists. PRR are proteins that detect pathogen-associated molecular patterns and are present in various cell groups, like hepatocytes and cells of the innate immune system. Among the main PRR are the Toll-like receptors[82]. HBV is recognized by the PRR as it provokes a cytokine-mediated response. The aim of therapy with PRR agonists is to enhance the antiviral response of the cytokines to achieve an adaptive immune response with activation of natural killer cells and T lymphocytes[83]. Agonists of Toll-like receptor-7 and Toll-like receptor-8 are under development and appear to stimulate the immune response satisfactorily.

Molecules to revert the exhausted T lymphocytes by blocking immunoinhibitory signals, mainly programmed cell death protein-1, will be the subject of future studies [59]. There is currently one phase II trial in China (ASC22).

Therapeutic vaccines. Stimulation of specific HBV B and T lymphocytes by vaccines is a way of overcoming immune tolerance in patients with chronic HBV. Several categories exist, based on proteins, DNA and vectors. They are all being studied in clinical trials in combination with current antivirals[84] as, so far, on their own they have been unable to control infection.

In summary, interaction at each stage of the life cycle of HBV may be the therapeutic aim to achieve elimination of the cccDNA, though it is also necessary to standardize

the method to measure this. As therapeutic research in HBV advances new compounds appear, with data on efficacy, although with complex profiles and multiple pharmacological interactions. Given that, the consensus agrees that cure of HBV means clearance of the HBsAg. Clinical studies should demonstrate that this is possible in a high proportion of treated patients. Future studies should include long-term safety results, interactions with common drugs and the pharmacokinetics in special populations[85]. Additionally, the drugs should be safe and easy to administer, as are current NAs, and moreover the clearance of the HBsAg must be sustained after interrupting treatment.

Figure 1 shows the main therapeutic targets and the drugs under development.

HEPATITIS D

HDV was discovered by Rizzetto *et al*[86] in 1977 in patients infected by HBV who had severe hepatitis. Despite being an incomplete virus that requires the presence of HBV it can nevertheless cause severe progressive hepatitis[87]. Progression to cirrhosis may occur in 80% of patients at 10 years[88], and HBV-HDV coinfection increases the risk of hepatocarcinoma compared to infection with just HBV alone[89].

HDV has a worldwide distribution, though with great variations in prevalence (higher in Latin America and eastern Europe but lower in western Europe, Japan and North America, where better socioeconomic conditions and HBV vaccination mean it is virtually restricted to injection-drug users). At least 5% of HBV carries in the world are thought to be infected with HDV, though it is probably even higher as there is no homogenous screening protocol and due to the impossibility of performing diagnostic tests in endemic areas.

The clinical course of HDV infection depends on its mode of transmission[87]. Coinfection with HBV can mean a severe clinical course in up to 15% of persons, though limited courses are more common with cure and immunity. Superinfection (HDV infection in a chronic HBV carrier) is more often associated with chronic hepatitis D that can advance to cirrhosis and liver failure[90]. Accordingly, differentiation between coinfection and superinfection is important in the management and prognosis of liver disease.

HDV infection should be investigated in all cases of acute or chronic hepatitis with a positive HBsAg[57], as any patient with HBV infection can be a carrier of HDV, especially in areas of moderate or high prevalence. HDV infection should also be investigated in patients with chronic HBV infection who present a peak of hypertransaminasemia.

No treatment for acute hepatitis due to HDV has proven useful, the disease being managed with support measures and liver transplant in fulminant cases. The only drug approved to date for the treatment of chronic hepatitis due to HDV is IFNα, which produces a modest response in 23%-57% of those treated[91,92]. NAs are not recommended for the treatment of HDV, although sustained suppression of active HBV infection with NAs can reduce the quantification of HBsAg and thus have a beneficial effect on coinfection with HDV[67].

Several new treatments are being tried aimed at blocking the viral cycle at different points: inhibitors of virus entry into the hepatocyte, assembly and other new strategies based on immune stimulation with cytokines and agonists of the receptors (Table 2):

Bulevirtide is the first drug to be approved by the European Commission specifically for hepatitis D. It acts by blocking entry by binding to the receptor that HBV uses to enter the liver cells (sodium taurocholate co-transporting polypeptide), thereby interfering in the life cycle of HBV and, consequently, preventing replication of HDV. Ezetimibe also acts by blocking entry and is being studied in a trial that is currently in phase II.

Inhibitors of HDV assembly: Lonafarnib is an inhibitor of farnesyl transferase, an enzyme that catalyzes prenylation, which is essential for HDV assembly. It significantly reduces the viral load when compared with placebo[93].

Inhibitor of HBsAg: A study in phase II for REP 2139 shows promising results.

As an immune modulator, IFN lambda binds to a single receptor much expressed on hepatocytes and little on hematopoietic and central nervous system cells. It is therefore well tolerated like IFNα, with a similar effect.

In conclusion, HDV, despite being minor, is in fact an important health problem in many areas of the world, being independently associated with long-term complications[94]. Standardization of diagnostic tests to detect HDV-RNA is needed. Although no specific treatment yet exists, bulevirtide has been approved by the

Table 2 Main drugs under development for the treatment of hepatitis D virus (with effect from phase II)

Bulevirtide	Entry inhibitor	Germany	Approved in Europe
Lonafarnib	Prenylation inhibitor	United States	Phase III
REP 2139	HBsAg inhibitor	Canada	Phase II
Ezetimibe	NTCP inhibitor	Pakistan	Phase II

Taken from Hepatitis B Foundation[7], Available from: https://www.hepb.org/treatment-and-management/drug-watch/. NTCP: Sodium taurocholate co-transporting polypeptide; HBsAg: Hepatitis B surface antigen.

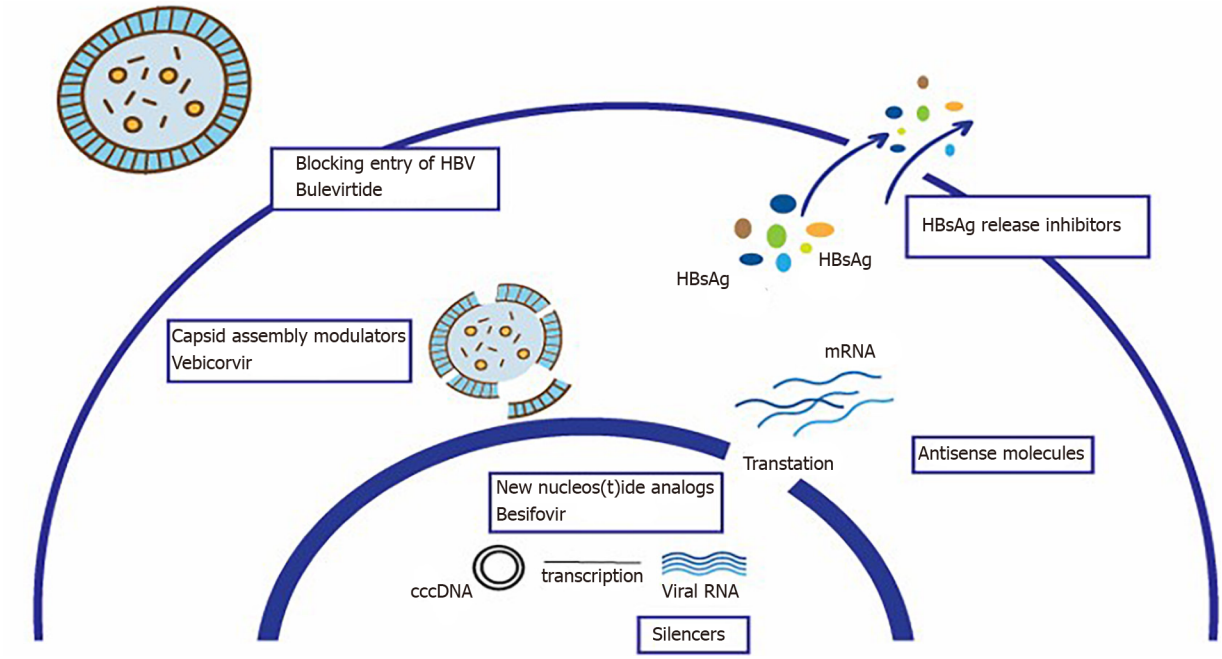


Figure 1 The main therapeutic targets and the drugs under development for hepatitis B. HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; cccDNA: Covalently closed circular DNA.

European Commission, and other drugs are currently being developed whose efficacy and safety will need to be determined.

HEPATITIS C

HCV, an RNA virus with seven genotypes, was discovered in 1989, before which it was referred to as non-A non-B[95]. HCV infection is the type that has undergone most changes over the last 10 years. From a chronic disease with few treatment options, based on IFN ± ribavirin, it has become a curable disease, even before liver damage is produced, as a result of DAA. This has led to a revolution in HCV, impacting the disease and its complications, liver transplant waiting lists and the incidence of HCV-associated hepatocarcinoma as well as the epidemiological and economic situation. In addition, the availability of an efficient therapy, limited in time and with excellent tolerance, has enabled objectives to be established for worldwide elimination of the disease. Nonetheless, HCV is still considered a prevalent disease, with healthcare repercussions and susceptible to management strategies.

The worldwide prevalence of HCV has fallen from 170 million carriers in 1999[96] to 71 million infected persons according to estimates in 2015[2], corresponding to 1% of the population, mainly due to prevention of nosocomial infection and the progressive access to efficient antiviral agents. Previously, most infections were related with transfusions of blood products, persons born between 1945 and 1965 (the so-called "baby boomer generation"), hemodialysis and hemophilia, and a high percentage of patients had advanced or decompensated disease and failure of prior therapy. More

recently, infection has been associated with the use of intravenous drugs, sexual risk-practices like men who have sex with men or chemsex (Party and Play) and certain risk groups such as prisoners or those with severe mental disorders who were not treated during the era of IFN[97], with most being treatment-naïve, having little fibrosis and very often scarce awareness of their condition.

Although hepatitis C is present all over the world, China, Pakistan, India, Egypt, Russia and the United States account for more than 50% of all cases of infection in the world[98], data to be considered with migratory movements.

Despite the reduction in the incidence, approximately 400000 persons die each year from causes related to HCV, mainly cirrhosis and its complications and the development of hepatocarcinoma[2].

Evolution of antiviral therapy and management of chronic hepatitis C from the past to the present: Direct acting antivirals

In 1991 the Food and Drug Administration approved treatment with IFN alpha for HCV infection but with a very low response rate, around 16%, a long treatment period, a parenteral route and important side effects[99]. During the 1990s ribavirin was added, slightly increasing the efficacy though again with more side effects[100]. IFN was later modified to its pegylated form, thus reducing the need for injectable doses, slightly improving tolerability. The combination of pegylated IFN and ribavirin, with cure rates up to 41% in genotype 1 and almost 75% in other genotypes[101], was the only treatment available until 2011 but with important limitations due to its multiple contraindications and frequent important secondary effects.

Better understanding of the life cycle and identification of the structural and non-structural proteins of HCV led to the development of antivirals acting directly on certain targets. The first of these were the protease inhibitors, boceprevir and telaprevir, which are oral antivirals approved by the Food and Drug Administration in 2011 for treatment of HCV genotype 1 both in naïve and pretreated patients in combination with the previous dual therapy[102,103]. This triple therapy increased the success of treatment in patients with genotype 1, but at the same time it also increased the toxicity and pharmacological interactions, the costs and the risk of decompensation of the liver disease in more advanced stages[104] as well as still requiring parenteral administration over long periods.

The real revolution in antiviral treatment arose with effect from 2013 with the successive approval of molecules having different mechanisms of action, mainly inhibition of the proteases NS3/NS4 or the polymerase replication complex NS5A. These drugs provided multiple advantages over the earlier drugs, such as oral administration, high efficacy, good tolerance and the possibility of shorter periods of treatment. The different combinations of "the new antivirals" offered multiple treatment lines depending on the clinical setting[105]. Most were IFN-free, which enabled treatment of populations that had previously been considered difficult to treat, such as the psychiatric population, persons with drug addictions or on replacement therapy, patients with kidney failure or solid organ transplant recipients [106].

The main protagonist of this change was sofosbuvir, a polymerase NS5B inhibitor acting on genotypes 1, 2, 3 and 4, though associated with other antivirals or traditional therapy. Soon after came simeprevir, a protease inhibitor with pan-genotype action [107] and daclatasvir, able to inhibit the non-structural protein NS5A and acting on genotypes 1, 3 and 4[108]. Combinations of antivirals were also produced, with different mechanisms of action, like sofosbuvir/ledipasvir[109], ombitasvir paritaprevir/ritonavir and dasabuvir[110] or grazoprevir/elbasvir[111], each with preferences for certain genotypes and different treatment durations. All of these strategies clearly increased cure rates, increasing sustained viral response (SVR) rates globally above 95% in all genotypes and around 85% in patients with advanced cirrhosis[112,113]. However, in these early IFN-free years the clinical management of HCV became increasingly complex. Before choosing the best treatment option in each case it was necessary to identify the genotype as well as the stage of the liver disease and the particular degree of fibrosis, as the drugs did not have a pan-genotype action and their duration and combination were conditioned by these factors. This situation was reflected in the clinical guidelines of the time from the main scientific societies, showing multiple complex tables of recommendations for usual clinical practice[114-117].

The initial high costs also conditioned the slow access to treatment, such that many countries drew up specific protocols for the progressive approach to patients with hepatitis C, with the first patients to be treated being those with more advanced liver disease or in special situations[118]. This, together with the high risk of drug

interactions and the outlines for these new therapies necessitated an exhaustive follow-up of the patient during antiviral treatment, with regular measurements of laboratory values and even excessive determinations of the HCV viral load, despite the lack of general rules concerning stopping treatment[119].

A qualitative leap occurred in 2016 with the advent of antiviral therapy with pan-genotypic and pan-fibrotic action plus their universal access. Combinations of sofosbuvir/velpatasvir[120,121] and glecaprevir/pibrentasvir[122] in single tablets have pan-genotypic and pan-fibrotic action in short-duration treatments of just 12 wk or 8 wk with the latter combination[123] and with SVR rates above 97% in practically all clinical contexts. With these two lines, plus the already available association of grazoprevir / elbasvir, also pan-genotypic, the approach is much more simplified for patients who now need treatment. This latter combination is indicated in all cases of active infection, the choice depending on certain determinants or conditioning factors, such as the renal function, which may limit the use of sofosbuvir, decompensated cirrhosis, which discourages protease inhibitors, or individual drug interactions, though these are much less common with the latest DAA compared to the earlier antiviral agents[124].

The pan-genotypic action, the high efficacy and the wide safety margin of currently available strategies have all led to simplification of the requirements to start antiviral therapy. These can be limited to determining the existence of viral replication (presence of RNA or HCV antigens), whether there is cirrhosis, which can be estimated with non-invasive methods like the APRI or FIB-4 indexes and ruling out possible interactions[125]. At the same time, monitoring during treatment has also become simpler, with the general recommendation (but not essential) of just determining the viral load 12 wk after completing treatment to confirm the SVR, with earlier safety controls when necessary due to a particular circumstance, like the cirrhotic patient [125].

An additional advantage of the current panorama is the existence of rescue therapy for the few cases that fail to achieve an SVR. The association of sosfosbuvir / velapatasvir / voxilaprevir is indicated for patients who fail with DAA therapy, with a high SVR rate in all genotypes[126].

This radical change over recent years in the setting of antiviral therapy has enabled a high percentage of the HCV population to be treated, often resulting in the patient being discharged if the degree of fibrosis is only mild and there is no comorbidity. Nevertheless, although eradication of the virus is associated with a reduction in mortality due to hepatic and extrahepatic causes related with HCV and improvement in liver function (and even a reduction in the degree of fibrosis), those patients who already had advanced fibrosis still have a risk for complications of the liver disease or the development of hepatocarcinoma and should therefore undergo regular follow-up, even if they have an SVR[127,128].

Direct acting antivirals and the current approach to acute hepatitis C

DAAs have not only had an impact on the management of chronic hepatitis C, but the approach to cases of acute HCV infection has also changed. Historically, acute hepatitis C, defined as the first 6 mo after contact, was not generally susceptible to treatment, and pegylated IFN was used in some patients if viral replication continued for longer than 12 wk[129]. During the early phase of infection, DAAs have proven effective and beneficial, especially to eliminate the disease and particularly in such risk groups as injection drug users, men who have sex with men, persons who undertake sex practices of risk, patients with HIV or nosocomial infection. In these groups, DAAs reduce morbidity and mortality and the risk of transmission during the period waiting for the criteria of chronicity to be fulfilled before starting treatment[130,131]. Based on the available evidence the EASL recommends sofosbuvir/velpatasvir or glecaprevir/pibrentasvir for 8 wk for what is now referred to as recently acquired hepatitis C[125]. During the era of DAA post-exposure prophylaxis is still not recommended without documented transmission of HCV[125,132].

Figure 2 shows the timeline of antiviral treatment for HCV.

Impact of direct acting antivirals on liver transplantation

The efficacy of DAA has impacted two great aspects of liver transplantation. First, it has clearly reduced the indication for liver transplant due to HCV, as shown in different series analyzing the current situation of liver transplant waiting lists[133-135]. Even so, the indication for a liver transplant still exists, mainly due to the development of HCV-associated hepatocarcinoma. Second, DAAs have enabled the majority of patients to reach liver transplantation with no viral replication. This, therefore, eliminates the risk of recurrence of hepatitis C in the graft. Additionally,

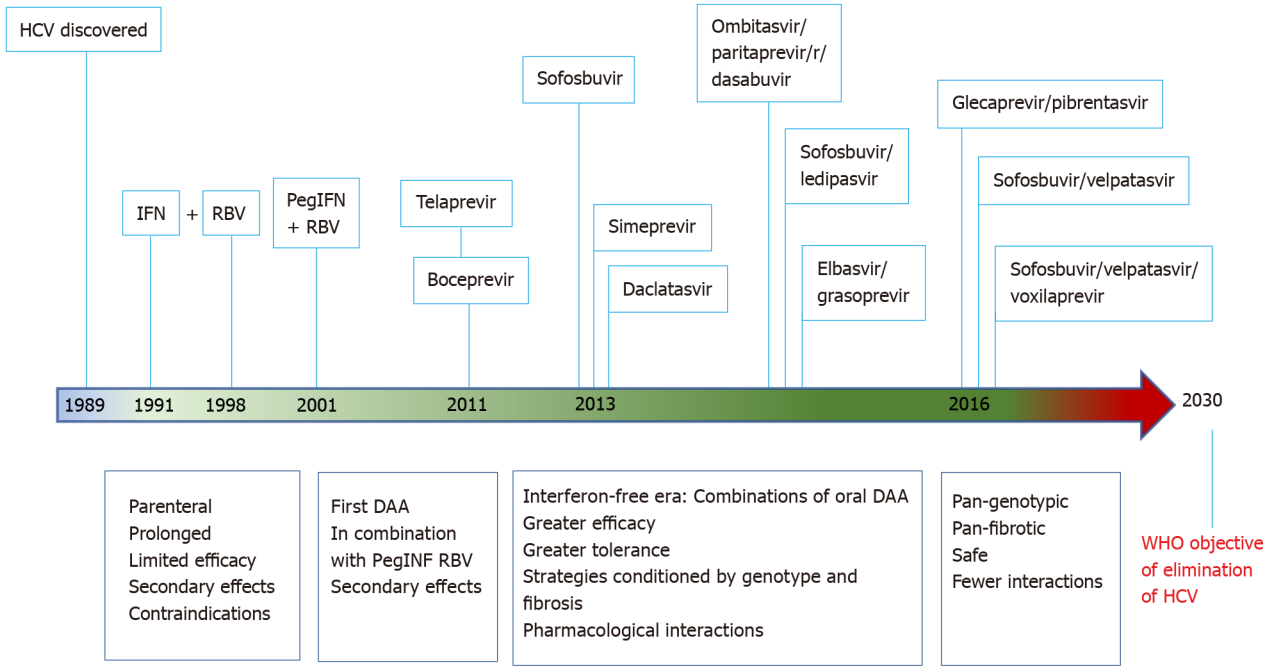


Figure 2 Shows the timeline of antiviral treatment for hepatitis C virus. HCV: Hepatitis C virus; IFN: Interferon; RBV: Ribavirin; Peg: Pegylated; DAA: Direct-acting antiviral; WHO: World Health Organization.

when necessary, DAA can be used safely after transplantation, with little risk of graft rejection and high efficacy, unlike earlier IFN-based treatments[105,136]. This efficacy and safety have been confirmed in recipients of other solid organ transplants, such as kidney transplant patients with chronic hepatitis C[137], thereby endorsing their use in the transplant population.

Impact of direct acting antivirals on solid organ donation

Organ donation from patients with active HCV infection remains controversial, especially for seronegative recipients, but the safety and efficacy of DAAs in solid organ recipients nevertheless allows this option to be contemplated. Different series have shown good results in lung, heart[138] and kidney transplantation. Even in liver transplants[139], provided the benefit of the transplant exceeded the risk of death on the waiting list, and early access to DAA treatment is guaranteed[140,141].

Future directions

The World Health Organization established the objective of elimination of hepatitis C by 2030, defined as a reduction of 80% in cases of de novo infection and a reduction of 65% in death due to HCV[9]. Besides currently available efficient treatment, this objective also requires first making the population aware by means of information campaigns, second, rescuing patients who have already been diagnosed but not treated and third, simplifying the whole diagnostic process and treatment access when necessary in order to guarantee diagnosis and treatment of the greatest number of cases possible.

The active search for already diagnosed patients is one of the main and most feasible strategies for micro-elimination as it permits cases to be rescued for treatment [142].

Reflex testing[143], as well as points of care, will enable diagnosis to be externalized, reaching risk groups with little contact with the healthcare system, like migrants or prisoners[144]. The diagnosis of hepatitis C should be directly linked to starting treatment (test and treat) so that in certain populations it will also be necessary to externalize treatment in the near future. For this, pilot projects have been designed with treatment administered by non-specialized healthcare personnel, like nurses [145], prison doctors, addiction physicians and primary care physicians[146], which has achieved a cost-effective elimination in the groups attended.

Populations still remain in which treatment needs further study, like pregnant women, in whom it is not recommended given the lack of safety data. It may, though, be the optimal time for screening in this group as it is sometimes the only contact they

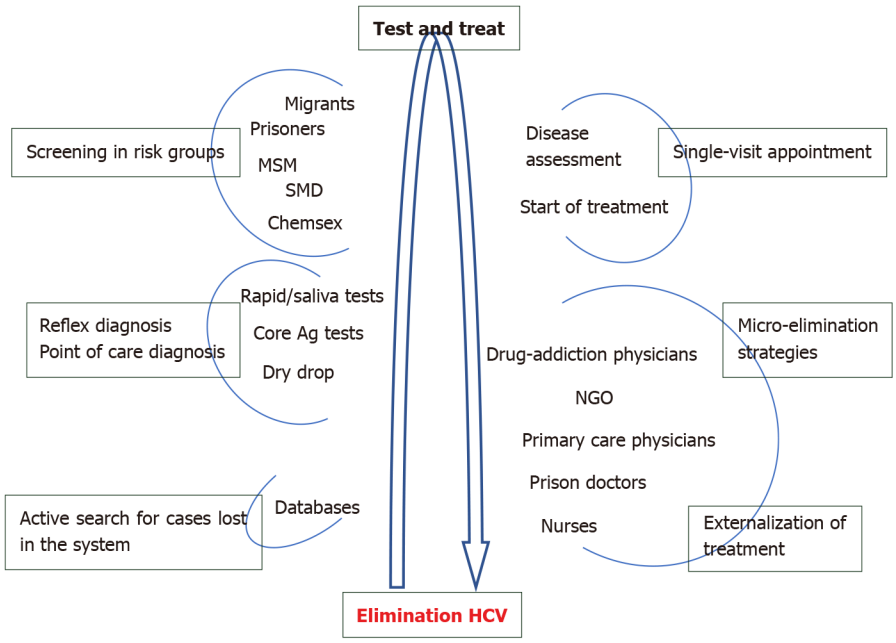


Figure 3 Proposals for the management of hepatitis C. MSM: Men who have sex with men; SMD: Severe mental disorder; Ag: Antigen; HCV: Hepatitis C virus; NGO: Non-governmental organization.

have with healthcare systems[113].

Notwithstanding the above, these emerging models of care of the hepatitis C patient and micro-elimination must not detract from the importance of preventive measures, which necessitate adequate information for the population, particularly the already-mentioned risk groups.

The elimination of hepatitis C is associated with a significant reduction in complications due to liver disease, a reduction in mortality due to liver disease and the risk of developing hepatocarcinoma, in both patients with mild fibrosis and in those with advanced fibrosis[127]. In addition, suppression of viral replication is associated with a reduction in systemic symptoms, mainly diabetes and vascular events, which in turn is associated with a reduction in mortality due to extrahepatic causes[147].

The impact of SVR on portal hypertension and its complications may result in a discrete reduction in pressure gradients in the hepatic veins, particularly in the year of reaching SVR[148]. However, as not all studies have associated an SVR with regression of portal hypertension or regression of esophageal varices[149], the Baveno VI consensus recommends the same cut points for screening of varices in patients with SVR[150].

Several cohort studies have shown that the risk of hepatocarcinoma is reduced after SVR[127], but it does not disappear. Patients with advanced fibrosis and cirrhosis should continue to be screened for hepatocarcinoma as its annual global incidence is 2.5%-4.5%, even with SVR[151]. In patients with mild fibrosis, continued screening for other risk factors, like diabetes, metabolic liver disease, low albumin levels or thrombocytopenia, for the development of hepatocarcinoma should be contemplated [127].

Figure 3 shows the main recommendations for elimination of hepatitis C.

CONCLUSION

Although the various types of viral hepatitis still represent a great public health problem, the great advances over recent years are very positively impacting their management and prognosis. Nevertheless, it is still necessary to persist with, and even boost studies designed to find efficient new therapies, though without forgetting the implementation of preventive measures, as a basis of achieving advances in minimizing the impact of this disease on health.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 21; 27(47): 8103-8122

DOI: 10.3748/wjg.v27.i47.8103

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

MINIREVIEWS

Artificial intelligence-assisted colonoscopy: A review of current state of practice and research

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Author contributions: Taghiakbari M drafted the manuscript under the supervision of von Renteln D; Mori Y and von Renteln D performed the critical revision of the manuscript for important intellectual content.

Conflict-of-interest statement: Mahsa Taghiakbari has no conflicts of interest relevant to this paper to disclose. Yuichi Mori is a consultant for the Olympus corporation and receives a speaking fee from the same corporation. Yuichi Mori has an ownership interest in Cybernet System Corporation. Daniel von Renteln is supported by the "Fonds de Recherche du Québec Santé" career development award and has received research funding from ERBE, Ventage, Pendopharm, and Pentax; he is also a consultant for Boston Scientific and Pendopharm.

Country/Territory of origin: Canada

Specialty type: Gastroenterology and hepatology

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

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Abstract

Colonoscopy is an effective screening procedure in colorectal cancer prevention programs; however, colonoscopy practice can vary in terms of lesion detection, classification, and removal. Artificial intelligence (AI)-assisted decision support systems for endoscopy is an area of rapid research and development. The systems promise improved detection, classification, screening, and surveillance for colorectal polyps and cancer. Several recently developed applications for AI-assisted colonoscopy have shown promising results for the detection and classification of colorectal polyps and adenomas. However, their value for real-time application in clinical practice has yet to be determined owing to limitations in the design, validation, and testing of AI models under real-life clinical conditions. Despite these current limitations, ambitious attempts to expand the technology further by developing more complex systems capable of assisting and supporting the endoscopist throughout the entire colonoscopy examination, including polypectomy procedures, are at the concept stage. However, further work is required to address the barriers and challenges of AI integration into broader colonoscopy practice, to navigate the approval process from regulatory organizations and societies, and to support physicians and patients on their journey to accepting the technology by providing strong evidence of its accuracy and safety. This article takes a closer look at the current state of AI integration into the field of colonoscopy and offers suggestions for future research.

Key Words: Colonoscopy; Adenoma; Artificial intelligence; Computational intelligence; Endoscopy; Surveillance

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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

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Received: March 19, 2021
Peer-review started: March 19, 2021
First decision: August 9, 2021
Revised: August 22, 2021
Accepted: December 3, 2021
Article in press: December 7, 2021
Published online: December 21, 2021

P-Reviewer: Shaikh DH
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR



Core Tip: Artificial intelligence (AI)-assisted decision support systems for endoscopy have shown promising results for the detection and classification of colorectal lesions. However, their integration into clinical practice is currently limited by the lack of design, validation, and testing under real-life clinical conditions. Further work is required to address the challenges of AI integration, to navigate the regulatory approval process, and to support physicians and patients on their journey to accepting the technology by providing strong evidence of accuracy and safety. This article describes the current state of AI integration into colonoscopy practice and offers suggestions for future research.

Citation: Taghiakbari M, Mori Y, von Renteln D. Artificial intelligence-assisted colonoscopy: A review of current state of practice and research. *World J Gastroenterol* 2021; 27(47): 8103-8122
URL: https://www.wjgnet.com/1007-9327/full/v27/i47/8103.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i47.8103

INTRODUCTION

Colorectal cancer (CRC) is the fourth most commonly diagnosed and the third most fatal cancer worldwide in 2018[1]. The prevalence costs of cancer care were estimated to be $14.1 billion for CRC in the United States in 2010[2]. Over the past decade, CRC incidence and mortality have declined as a result of the increase in CRC screening and prevention examinations[3]. Colonoscopy is a screening tool with high sensitivity for the detection of precancerous and cancerous lesions, and may contribute to an approximately 80%, and up to 60% reduction in CRC incidence and mortality, respectively[4-8]. Colonoscopy prevents CRC by breaking the adenoma-carcinoma sequence through detection and removal of premalignant colorectal polyps[3]. Furthermore, it is a cost-effective procedure that often allows surgery to be avoided in patients with adenomas or CRCs that do not invade deeper than the superficial submucosa[9]. However, the quality of colonoscopy procedures depends on the experience of the endoscopists and the techniques and technology used[10]. A suboptimal colonoscopy examination can result in interval cancers, which are CRCs that occur after a colonoscopy and before the next surveillance examination, and are usually due to non-detection and/or incomplete resection of premalignant polyps. Recent research has shown that CRC precursor lesions are incompletely resected in about 14% of colonoscopy procedures [11]. Quality indicators have been established to describe and measure the quality of colonoscopy examinations[12], and the use of pre- and intraprocedural quality metrics has been shown to result in both an increase in colonoscopy quality and standardization of procedures[12,13]. One of the most recognized quality metrics is the adenoma detection rate (ADR), which is the proportion of an endoscopist's patients undergoing screening colonoscopy who have at least one adenoma detected; every 1% increase in the ADR has been shown to result in a 3% decrease in the risk of post-colonoscopy CRC[10].

Over 90% of colorectal polyps are diminutive (≤ 5 mm) or small (≤ 10 mm), and most of these polyps are non-neoplastic[10]. Recent advances in image-enhanced endoscopy [IEE; *e.g.*, blue-light imaging, narrow-band imaging (NBI), and i-Scan] have resulted in enhanced visualization of the polyp surface pattern. IEE can be employed for the optical classification of colorectal polyps during colonoscopy, obviating the need for pathology[14,15]. The American Society for Gastrointestinal Endoscopy (ASGE) Technology Committee, in its Preservation and Incorporation of Valuable endoscopic Innovations (PIVI) statement, has recommended the optical evaluation of diminutive polyps, adopting a "resect and discard" strategy for all diminutive colorectal polyps, and a "diagnosis and leave" strategy for diminutive rectosigmoid polyps, if the endoscopist can reach the recommended threshold of ≥ 90% agreement with histopathology results for surveillance interval assignment and ≥ 90% negative predictive value (NPV) for diagnosis of adenomatous histology, respectively[14,15]. Optical diagnosis can distinguish between neoplastic and non-neoplastic polyps and therefore deliver clinical and cost benefits by reducing the number of unnecessary histopathology examinations and providing immediate surveillance interval recommendations to patients. However, despite the demonstrated high accuracy of

optical diagnosis for diminutive polyps, endoscopists have been reluctant to support its broad implementation because of concerns about incorrect diagnoses, assignment of inappropriate surveillance intervals, and related medicolegal issues[16].

To address the shortcomings in current colonoscopy practice, research has been directed at standardizing colonoscopy procedures among endoscopists through the integration of artificial intelligence (AI) into colonoscopy practice. AI could provide real-time support to physicians by automatically recognizing specific polyp patterns in colonoscopy images and/or videos, as well as suggesting the most probable histology and providing a confidence level for the predicted histology. The use of such technology would help to mitigate the effects of endoscopist experience in optical diagnosis. Computer-assisted, or most recently, AI-assisted colonoscopy diagnostic systems (CAD) for detection (CADe) and classification (CADx) of colorectal polyps are currently the two main areas of research and implementation of AI in clinical practice. AI-assisted colonoscopy improves ADR and allows for reliable, operator-independent pathology prediction of colorectal polyps. However, there is still a substantial communication gap between computer and medical fields, with scientists in these two disciplines divided in terms of background knowledge, available resources, research typology, and awareness of unmet needs in clinical practice. In this review, we summarize the most important aspects of the application of CADe and CADx in routine colonoscopy practice.

DEVELOPMENT OF COMPUTER-ASSISTED DIAGNOSTIC SYSTEMS

Pairing colonoscopy devices with image-enhanced technology (*i.e.*, white-light endoscopy and chromoendoscopy) has improved the quality of care to patients by increasing the precision of colonoscopy procedures[4]. Recently, research efforts have focused on integrating computational power and previously collected data to enhance the simultaneous detection and classification of colonoscopy images or videos and support endoscopists in their decisions about the presence and/or histology of a polyp.

Machine learning is a subset of AI that allows mathematical methods to develop an algorithm based on given data (*e.g.*, polyp images or videos) to predict the same pattern or a specific task in unseen or unknown data[17]. The final output of these systems (*e.g.*, detection or classification of polyps) is based on pre-defined features or extraction of the most relevant image features (*e.g.*, polyps), which may help in the specification, detection, or classification of a new image. In conventional machine learning (*i.e.*, handcrafted models), a researcher manually introduces the clinically relevant polyp features to the machine learning algorithm. In contrast, in the most advanced machine learning method, which is called deep learning, polyp features, clinically relevant or not, are automatically extracted by the algorithm without prior introduction by a researcher. As a result, the output is based on the capture and summary of complex polyp characteristics, either for detection (*i.e.*, discrimination of polyp from background mucosa) or prediction of histopathology (*i.e.*, neoplastic or non-neoplastic)[17]. Deep learning employs deep neural networks (DNNs), which imitates the complex interconnected neural network in the human brain. These artificial neurons are positioned in several detections and pooling layers, taking weighted data (from the precedent layer), processing it, and passing the output (processed data) to the next layer. Each layer performs as a "step of abstraction[17]", which forms a hierarchy of common features that grow in complexity throughout the layers (*i.e.*, edge- > basic shape- > object- > class prediction). In other words, each layer would extract useful and relevant features from a given data that would facilitate the classification of the images. When data are presented, the DNN performs the repetitive iterations of a previously chosen model (*i.e.*, support vector machines, random forests, or neural networks) throughout the deeper layers, so-called hierarchical feature learning[17]. For computer-assisted colonoscopy, the development of the AI model is primarily based on supervised data, where data are retrospectively labeled by one or a group of expert endoscopists. For example, in CADx, colonoscopy images or videos will be labeled as neoplastic or non-neoplastic based on the reference standard of pathology results (Figure 1), which would have been reviewed and finalized following consensus by several pathologists. In CADe, however, polyp images or videos will be reviewed by experienced endoscopists, and polyp borders will be delineated based on consensus by endoscopists. Ultimately, the output of the AI algorithm will identify the presence of a polyp, or be able to discriminate between a neoplastic and non-neoplastic polyp (Figure 2)[17]. However, there are some shortcomings and barriers to

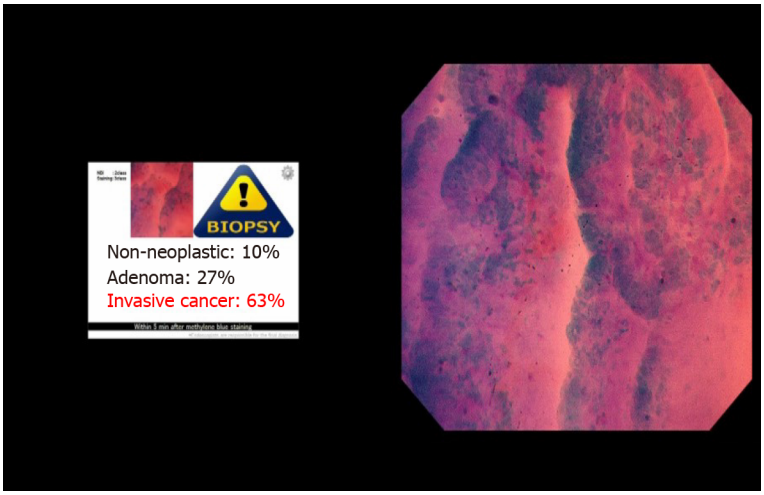


Figure 1 Prediction of colorectal polyp histology by the ENDOBRAIN computer-aided classification system for colonoscopy.

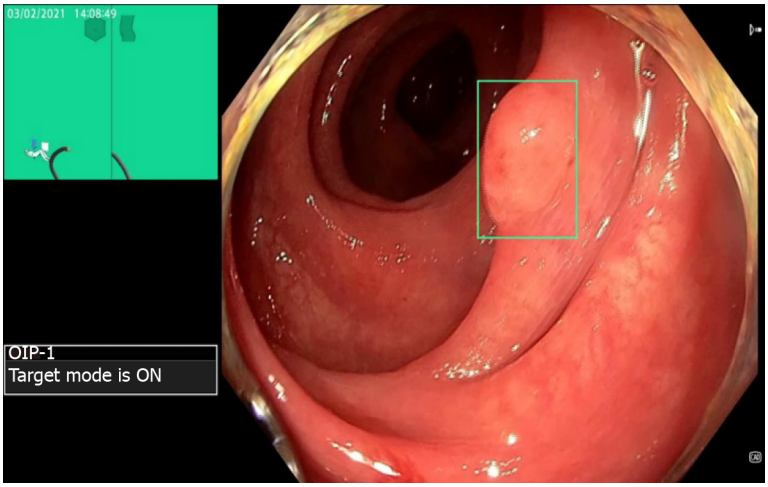


Figure 2 Detection of a colorectal polyp by the ENDOAID computer-aided detection system for colonoscopy. The green box delineates the area containing a polyp.

the development and implementation of CAD systems in real-time endoscopy practice, as discussed below.

Datasets

The data used to develop a CAD system will be divided into three or more datasets: One training dataset to build the AI model, one validation dataset to check the generalizability of the model, and at least one test dataset from another source of data to test the performance of the model[17]. Commonly, training and validation data are derived from the same source (*i.e.*, colonoscopies performed at a single center); however, it is crucial to avoid overlap of data; otherwise, evaluation of the model hyperparameters would be flawed and would lead to "model overfitting." Model overfitting is an error in modeling that occurs when the model is too tightly fitted to the training data and random fluctuations in the training data are learned as concepts by the model. The problem is that the fitted model does not generalize to new data due to its low bias and high variance. Overfitting can be avoided by tight monitoring of the model during the training by constantly evaluating the model performance in the training and validation data[17].

Researchers should use large and heterogeneous data, including normal and abnormal colonoscopies. A sufficient number of colonoscopy images or video frames would ensure a robust evaluation of model performance. Data should ideally be collected from multiple centers and diverse patients in terms of race, age, sex, and medical issues.

A lack of ground truth data or reliable annotated "big data" for generating effective and high-performance AI models could limit the broad application of CAD systems in clinical settings[18]. This is a challenging goal to achieve as it requires millions of colonoscopy images and videos to be annotated by multiple highly experienced experts to ensure a consensus on ambiguous images. Annotation and data labeling by experts should follow a uniform and standardized protocol, otherwise, the generalizability and performance evaluation of the model will be unreliable.

Gold standard comparison

The absence of a "gold standard" for diagnosing polyp histology would affect the accuracy of CAD performance. Although pathology results are currently regarded as the reference standard, the interobserver agreement among pathologists is not 100%; polyp histology determined by one pathologist might be different from that of another pathologist when reassessing the same specimen slides[19-22]. Therefore, the pathology data used for AI models must be re-evaluated by several pathologists prior to inclusion to ensure agreement on polyp pathology.

Technical transparency

The application of CAD in routine practice is a product of an interdisciplinary collaboration between medical and AI researchers. A recent review demonstrated that researchers failed to report the AI model characteristics effectively[23]. Researchers should ensure that they clearly define and report the AI model architecture or hyperparameters, including the number of deep layers and learning rate. The definition and testing of hyperparameters are crucial to the validation process owing to their direct effect on the model's performance; optimal model generalizability in the validation step implies the correct choice of hyperparameters. Researchers should briefly explain the source of data, the process of data selection, and the number of patients, including images/videos frames, normal colonoscopies (*i.e.*, without polyp identification), colonoscopy centers, and participating endoscopists together with their level of expertise[17].

Furthermore, researchers should adopt appropriate techniques to prevent model overfitting. Data leakage may occur when the testing dataset results are used to tune the model parameters instead of using the results derived from the validation dataset. Therefore, the model may over-fit toward the unseen data, risking a biased estimate of model performance. The stringent use of high-quality still images instead of videos that contain large variability in colonoscopy images may increase the risk of overfitting.

Computer-assisted polyp detection system

In the context of CAD, although the shift from separate engineering and medical disciplines to combined medical and engineering research has gained momentum over the last decade, pilot studies established the idea of CADe as early as 2003[24,25]. The primary hand-crafted AI models used the pre-described polyp features (*e.g.*, color and/or texture-based features) and annotated colonoscopy videos for the detection of colorectal polyps[25-29]. Other studies used the same idea and developed several AI models that resulted in up to 90% sensitivity[30-32]. However, these studies used small and homogeneous datasets to develop and validate the AI models, raising doubts over the model's optimal performance. The hand-crafted features used to build the model led to suboptimal performance, probably because of impaired feature recognition and description, and a high level of false-positive detection owing to the presence of colonic folds, blood vessels, and feces in the lateral view.

After the invention of DNNs, important polyp features could be automatically recognized. Subsequently, the accuracy and sensitivity of models improved, signaling the great potential for CADe application. Recently, Yamada *et al*[33] developed a CADe system using a supervised DNN, and validated the system using a dataset of 705 still images of 752 lesions and 4135 still images of noncancerous tissue. This system performed well, with a sensitivity and specificity of 97.3% and 99.0%, respectively, and an area under the curve (AUC) of 0.975 in the validation set. Misawa *et al*[34] developed a model based on 546 short colonoscopy videos, comprising 155 polyp-positive and 391 polyp-negative videos. Two experts retrospectively annotated videos for polyp presentation to provide a gold standard for comparison. The model presented sensitivity, specificity, and accuracy of 90.0%, 63.3%, and 76.5%, respectively. The polyp detection rate and false-positive detection rate were 95% and 60%, respectively. Other significant research used a large dataset for training an AI model, which comprised 8641 annotated images from over 2000 colonoscopies[35]. The

model generated excellent detection capability, with an AUC of 99% and an accuracy of 96.4%. The performance of this model was also superior to that of experts. The authors tested model performance in 20 colonoscopy videos with a total duration of 5 h, during which colonoscopists removed 28 polyps. After reviewing the videos by four independent experts, eight additional polyps were identified (36 polyps) without the use of AI assistance and 17 additional polyps were detected with AI assistance (total 45 polyps). The model had a false-positive rate of 7%.

Research with a prospective design and focusing on the evaluation of the real-time performance of CADe is scarce. Wang *et al*[36] conducted a prospective non-blinded clinical trial, which aimed to measure ADR with and without the application of CADe. Using 522 and 536 colonoscopies in the control and intervention arms, respectively, the authors found a statistically significant increase in ADR (29.1% *vs* 20.3%) and an increased number of adenomas *per* patient (0.53 *vs* 0.31) when CADe was used. The false-positive rate was 7.5% *per* colonoscopy, and there was no significant difference in the procedure time. CADe could detect a higher number of diminutive adenomas and hyperplastic polyps, which represent a higher risk of unnecessary polypectomies, pathology examinations, and longer procedure times. To date, the generalizability of this system has not been tested in Western clinical settings.

In contrast to the results of the latter study, Klare *et al*[37] prospectively evaluated endoscopist performance using CADe assistance during the real-time colonoscopy procedures of 55 patients. However, the endoscopists only observed the regular monitor, and an independent investigator observed the monitor dedicated to representing the real-time outputs of the CADe system in a separate room, which was blinded from the endoscopists' sight. Therefore, the endoscopists were blinded to the real-time CADe outputs. This system did not increase the precision of polyp detection in real-time practice: In per-patient analysis, the application of CADe resulted in endoscopists achieving a lower ADR (29.1% *vs* 30.9%); in per-polyp analysis, CADe could only detect 55 out of 73 polyps previously detected by endoscopists. Tables 1 and 2 shows the summary of the recent studies evaluating a CADe system.

Computer-assisted polyp classification system

Computer-assisted diagnosis of the histopathology of colorectal polyps has become an area of significant research interest because of its potential to prevent the resection of low-risk polyps and reduce the number of unnecessary histopathology examinations. Many studies have successfully developed and validated CADx models, the use of which would allow the "diagnosis and leave strategy" to be implemented. In a prospective pilot study, in which the data from 128 patients undergoing colonoscopy using NBI were used to test a CADx system (209 polyps detected and removed), three polyp features were used to build the AI model: Mean vessel length, vessel circumference, and mean brightness within detected blood vessels[38]. The results showed that the endoscopists' ability to predict polyp histology was superior to that of CADx, which had a sensitivity of 90% and specificity of 70.2% in differentiating neoplastic from non-neoplastic images compared with histopathology as the gold standard. The system's diagnostic performance was compared with that of endoscopists, who were blinded to the histopathology reference standard. Endoscopists accurately predicted polyp histology with a sensitivity of 93.8% and specificity of 85.7% when there was interobserver agreement. In cases of disagreement between endoscopists, the suggested safe prediction of polyp histology (*i.e.*, classification as neoplastic) produced a sensitivity of 96.9% and specificity of 71.4%. Overall, CADx could predict polyp histology with an approximate sensitivity and specificity of 90% and 70%, respectively; however, the overall correct classification rate was moderate (85.3%). Notably, this AI algorithm was not fully automated; thus, its real-time performance in a clinical setting remains to be determined. Another limitation of this study was the use of data from NBI colonoscopies. Although NBI may assist polyp classification, its use may cast doubt on the generalizability of the model, especially in clinical settings where NBI is not available.

The real-time evaluation of CADx is important if the technology is to be integrated into clinical practice. Some studies have used the real-time decision outputs from support vector machines for building CADx algorithms, with promising results[39-43]. Moreover, Chen *et al*[44] demonstrated that an AI model could accurately predict the histopathology of 284 diminutive polyps, comprising 96 hyperplastic and 188 neoplastic polyps diagnosed using NBI, with 96.3% sensitivity, 78.1% specificity, 91.5% NPV, and 89.6% PPV. This study and the study by Byrne *et al*[45] that used the combination of CADe and CADx systems (described below), are remarkable in that they achieved the threshold NPV of ≥ 90% recommended by the ASGE PIVI statement, favoring the implementation of the "diagnose and leave" strategy for diminutive

Table 1 Summary of the randomized controlled trials involving computer-aided detection for colonoscopy

Ref.	Year	Study design	Study aim	CADe system	Image modality	Number of patients in the CADe group	Number of patients in the control group	Number of polyps (CADe *vs* control group)	Adenoma detection rate (%) (CADe *vs* control group)	Polyp detection rate (%) (CADe *vs* control group)	Number of false-positive rate (%) (CADe *vs* control group)	Withdrawal time (CADe *vs* control group), min ± SD; minute
Wang *et al* [36]	2019	Non-blinded prospective randomised controlled study	To investigate whether a high-performance real-time CADe system can increase polyp and adenoma detection rates in the real clinical setting	The real-time automatic polyp detection system (Shanghai Wision AI Co., Ltd.) based on artificial neural network-SegNet architecture	Real-time Video stream	522	536	767 (498 *vs* 269)	29.1 *vs* 20.3; $P < 0.001$; 95%CI = 1.21-2.135	45.0 *vs* 29.1; $P < 0.001$; 95%CI = 1.532-2.544	39 *vs* 0	6.18 ± 1.38 *vs* 6.07 ± 1.11; $P = 0.15$
Wang *et al* [74]	2020	Double-blind Prospective randomised trial	To assess the effectiveness of a CADe system for improving detection of colon adenomas andpolyps; to analyse the characteristics ofpolyps missed by endoscopists	The real-time automatic polyp detection system (Shanghai Wision AI Co., Ltd.) based on artificial neural network-SegNet architecture	Real-time Video stream	484	478	809 (501 *vs* 308)	34.0 *vs* 28.0; $P = 0.030$; OR = 1.36, 95%CI = 1.03–1.79	52.0 *vs* 37.0; $P < 0.0001$; OR = 1.86, 95%CI = 1.44–2.41	48 in CADe group (control group not reported)	6.48 ± 1.32 *vs* 6.37 ± 1.09; $P = 0.14$
Su *et al* [75]	2020	Single-blind Prospective randomised trial	To develop an automatic quality control system; to investigate whether the system could increase the detection of polyps and adenomas in real clinical practice	Five deep learning convolutional neural networks (DCNNs) based on AlexNet, ZFNet, and YOLO V2	Real-time Video stream	308	315	273 (177 *vs* 96)	28.9 *vs* 16.5; $P < 0.001$; OR = 2.055, 95%CI = 1.397-3.024	38.3 *vs* 25.4; $P = 0.00$; OR = 1.824, 95%CI = 1.296-2.569	62 in CADe system (control group not reported)	7.03 ± 1.01 *vs* 5.6 ± 1.26; $P < 0.001$
Gong *et al* [76]	2020	Single-blind Prospective randomised trial	To evaluate whether the CADe system could improve polyp yield during colonoscopy	ENDOANGEL based on the deep neural networks and perceptual hash algorithms	Real-time video stream	355	349	302 (178 *vs* 124)	16 *vs* 8; $P = 0.001$; OR = 2.30, 95%CI = 1.40-3 ·77	47 *vs* 34; $P = 0.0016$; OR = 1.69, 95%CI = 1.22-2.34	For endoscope being inside = 0.8; For identification of the caecum = 2; for prediction of slipping = 0	6.38 ± 2 ·48 *vs* 4.76 ± 254; $P < 0.0001$
Liu *et al*[77]	2020	Double-blind Prospective randomised trial	To study the impact of CADe system on the detection rateof polyps and adenomas in colonoscopy	The convolutional threedimensional (3D) neural network	Real-time video stream	508	518	734 (486 *vs* 248)	39.1 *vs* 23.9; $P < 0.001$; OR = 1.637, 95%CI = 1.201□2.220	43.7 *vs* 27.8; $P < 0.001$; OR = 1.57, 95%CI = 1.586□2.483	36 in CADe system (control group not reported)	6.82 ± 1.78 *vs* 6.74 ± 1.62; $P < 0.001$
Luo *et al*[78]	2021	Non-blinded Prospective randomised trial	To explore whether CADe could improve the polyp detection rate in the actual clinical environment	A CNN algorithm based on a YOLO network architecture	Real-time Video stream	150	150	185 (105 *vs* 80)	38.7 *vs* 34.0; $P < 0.001$	-	52 in CADe system (control group not reported)	6.22 ± 0.55 *vs* 6.17 ± 0.52; $P = 0.102$
Repici *et al* [79]	2020	Singles-blind Prospective randomised trial	To assess the safety and efficacy of a CADe system for the detection of colorectal neoplasia	The CNN (GI-Genius; Medtronic)	Real-time Video stream	341	344	596 (353 *vs* 243)	54.8 *vs* 40.4; $P < 0.001$; RR = 1.30, 95%CI = 1.14-1.45	279/341 (82) 214/344 (62)	-	417 ± 101 seconds for the CADe group *vs* 435 ± 149 for controls; $P = 0.1$

Wang[1] et al [80]	2020	Singles-blind Prospective randomised trial	To investigate the impact of CADe on adenoma miss and detection rate	The artificial neural network (EndoScreener, Shanghai Wision AI Co,Ltd, Shanghai, Chin)	Real-time Video stream	184 (CADe-routine group)[2]	185 (Routine-CADe group)[3]	529 (244 *vs* 285)	42.39 *vs* 35.68; *P* = 0.186; OR = 1.327, 95%CI = 0.872–2.018	63.59 *vs* 55.14; *P* = 0.09; OR = 1.421, 95%CI = 0.936–2.157	67 in CADe system (control group not reported)	6.55 (5.34–7.77) *vs* 6.51 (5.45–7.57); *P* = 0.745[4]

[1]The total adenoma miss rate by computer-assisted detection system (CADe) [colonoscopy = 13.89%, 95% confidence interval (CI) = 8.24%–19.54%]; by routine colonoscopy = 40.00%, 95%CI=31.23%–48.77%, $P < 0.0001$. The total polyp miss rate by CADe colonoscopy = 12.98%, 95%CI = 9.08%–16.88%; by routine colonoscopy = 45.90%, 95%CI = 39.65%–52.15%, $P < 0.0001$). Visible adenoma miss rate: Routine-CADe group = 24.21% *vs* CADe-routine group = 1.59%, $P < 0.001$; Visible polyp miss rate: Routine-CADe group = 30.89% *vs* CADe-routine group = 2.36%; $P < 0.001$.

[2]It means that the colonoscopy was performed by the CADe system and then the conventional method.

[3]It means that the colonoscopy was performed by the conventional method and then the CADe system.

[4]Median (interquartile range).

CADe: Computer-assisted detection system; CNN: Convolutional neural network; DCNN: Deep learning convolutional neural network; SD: Standard deviation; OR: Odds ratio; RR: Relative risk; CI: Confidence interval.

rectosigmoid polyps[46]. However, the results of the former study need to be confirmed in a prospective study, ideally in a controlled trial, where the probability of selection bias is less, and the AI model can be compared with a conventional setting (without using AI).

More prospective studies assessing CADx are required to support the integration into clinical practice. The existing prospective studies resulted in a high and favorable diagnostic performance, which provided strong evidence to support the real-time application of CADx[47,48]. In contrast, the AI models developed and tested in a prospective trial by Kuiper *et al*[49] did not show sufficient power for differentiating adenomatous from non-adenomatous lesions. Another CADx model in a prospective study by Rath *et al*[50] could only produce moderate accuracy, sensitivity, and specificity (84.7%, 81.8%, and 85.2%, respectively), although the NPV was relatively high at 96.1%. This model would therefore allow diminutive rectosigmoid polyps to be diagnosed and left *in situ* without resection. The authors suggested that the low prevalence of neoplastic polyps could explain the model's moderate diagnostic performance compared with hyperplastic polyps in their dataset, which might proportionately result in an overestimation of the NPV, and an underestimation of the accuracy and PPV of the model. Table 3 shows the summary of the recent studies evaluating a CADe system.

Combined CADe and CADx models

The ideal CAD system would support the simultaneous detection and classification of polyps to optimize colonoscopy outcomes and achieve the best level of CRC prevention. A recent study evaluated the real-time application of CADx in combination with CADe[45]. The validated model was tested on a series of 125 diminutive polyps, comprising 51 hyperplastic polyps and 74 adenomas. The combined model could not detect histopathology in 15% of polyps. For the remaining 106 polyps histologically predicted with high confidence, the AI model demonstrated an accuracy of 94%, sensitivity of 98%, specificity of 83%, NPV of 97%, and positive predictive value (PPV) of 90%. In a significant study, Byrne *et al*[51] developed a new

Table 2 Summary of the non-controlled studies involving computer-aided detection for colonoscopy

Ref.	Year	Study design	System	Image modality	Number of patients/colonoscopies used for training/test datasets (total)	Number of colonoscopy/polyp images/videos used for training/test datasets	Diagnostic properties
Park and Sargent[81]	2016	Retrospective	CADe based on DCNN using a conditional random field model	Still images	35 (colonoscopy videos)	562/562 (colonoscopy still images)	Sensitivity = 86%; specificity = 85%; AUC = 0.8585
Fernández-Esparrach *et al*[73]	2016	Retrospective	CADe based on energy map	Still images	NA/24 colonoscopy videos containing 31 different polyps	NA/Experiment A: 612 polyp images from all 24 videos. Experiment B: 47886 frames from the 24 videos	Experiment A: accuracy = small *vs* all polyps = 77.5%, 95%CI = 71.5%–82.6% *vs* 66.2%, 95%CI = 61.4%–70.7%; $P < 0.01$. Experiment B: The AUC = high quality frames *vs* all Frames = 0.79, 95%CI = 0.70–0.87 *vs* 0.75, 95%CI = 0.66–0.83
Yu *et al*[82]	2017	Retrospective	CADe based on three-dimensional (3-D) deep learning integration framework by leveraging the 3-D fully CNN (3D-FCN)	Videos	20/18 (colonoscopy videos)	3799 frames with polyps in total	Sensitivity = 71%; PPV = 88%; precision = 88.1%
Billah *et al* [83]	2017	Retrospective	CADe based on CNN and color wavelet features using a linear support vector machine	Still images	100 (colonoscopy videos for combined training and test datasets)	14000 still images (combined for training and test datasets)	Accuracy = 98.65%; sensitivity = 98.79%; specificity = 98.52%
Zhang *et al* [84]	2017	Retrospective	CADe based on DCNN	Still images	NA	2262/150 random, 30 NBI (colonoscopy still images)	Accuracy = 85.9%; sensitivity = 98%; PPV = 99%; precision = 87.3%; recall rate = 87.6%; AUC = 1.0
Wang *et al* [85]	2018	Retrospective	CADe based on DNN	Still images	1290/1138 (2428) patients	27113/5545 (colonoscopy images)	Sensitivity = 94.38%, 95%CI = 93.80%-94.96% in images with polyp; AUC = 0.984
Misawa *et al* [34]	2018	Retrospective	CADe based on CNN	Videos	59/14 (73)	411/135 (colonoscopy videos containing 150 polyps)	Per-polyp sensitivity = 94%; per-frame sensitivity = 90%; specificity = 63.3%; accuracy = 76.5%; false positive rate = 60%; AUC = 0.87
Yamada *et al* [33]	2019	Retrospective	CADe based on DNN	Videos	NA/77 (number of videos)	13983/4840 (colonoscopy videos)	Sensitivity = 97.3%, 95%CI = 95.9%–98.4%; specificity = 99.0%, 95%CI = 98.6%–99.2%; AUC = 0.975, 95%CI = 0.964–0.986)
Urban *et al* [35]	2018	Retrospective	CADe based on deep learning CNN	Videos	Several training and validation sets: (1) Cross-validation on the 8641 images; (2) Training on the 8641 images and testing on the 9 videos, 11 videos, and independent dataset; and (3) Training on the 8641 images and 9 videos and testing on the 11 videos and independent dataset		Sensitivity = 96.9%; specificity: 95%; AUC = 0.991; accuracy = 96.4%; false positive rate = 7%
Klare *et al*[37]	2019	Prospective	Automated polyp detection software ("KoloPol," Fraunhofer IIS, Erlangen, Germany) based on CNN	Live colonoscopy videos	NA	NA/55 (colonoscopy videos)	Per-polyp sensitivity = 75.3%, 95%CI = 62.3%-84.9%; PDR = 50.9%, 95%CI = 37.1%-64.4%; ADR = 29.1%, 95%CI = 17.6%-42.9%
Ozawa *et al* [86]	2020	Retrospective	CADe based on DCNN	Still images	12895 patients	16418/7077	Sensitivity = 92%; PPV = 86%; accuracy = 83%; identified adenomas = 97%

CADe: Computer-assisted detection system; CNN: Convolutional neural network; DCNN: Deep learning convolutional neural network; AUC: Area Under the Receiver Operating Characteristic curve; PPV: Positive predictive value; NPV: Negative predictive value; PDR: Polyp detection rate; ADR: Adenoma detection rate; CI: Confidence interval.

platform using three distinct AI CADe and CADx algorithms to provide endoscopists with a full workflow from detection to classification: An NBI light detector, a polyp detector, and an optical biopsy. The NBI light detector runs throughout the colonoscopy procedure to ensure the detection of all colorectal polyps with white light imaging, and the optical biopsy provides an accurate polyp classification using NBI light. The NBI light model resulted in an excellent accuracy of 99.94% when tested in 21804 unseen colonoscopy video frames. However, the detection mode using white light resulted in a sensitivity of only 79%. The optical biopsy model could accurately classify 97.6% of polyps, which was significantly higher than a previous CADx model tested by the same research team[45], and had a sensitivity of 95.95%, specificity of 91.66%, and NPV of 93.6% for polyp classification.

QUALITY ASSESSMENT OF COLONOSCOPY BY COMPUTER

Few studies have evaluated an AI-assisted system for the ability to accurately and automatically assess the quality of a colonoscopy procedure, including the identification of critical anatomical landmarks, especially when the endoscopic field is blurry[52,53]. Filip *et al*[53] developed a "Colometer" system that could rate colonoscopy quality based on the percentage of the withdrawal time with adequate visualization. This system could detect the factors associated with optimal real-time visualization of the mucosa, including image clarity, withdrawal velocity, and level of bowel cleanliness. A dataset of expert-annotated images and videos was used to train the AI model. The authors compared the quality rated by this system with that of three independent experts. There was a strong correlation between AI and expert quality ratings (ρ coefficient 0.65, $P = 0.01$). In another study, a system comprising two AI algorithms was designed to automatically detect the appendiceal orifice on a colon image or video[54]. The first algorithm was developed to detect the appendiceal orifice on endoscopic images based on the local shape, lighting, and intensity differences from a normal edge direction. The second algorithm was designed to detect the appendiceal orifice in the colonoscopy videos using a frame intensity histogram. The system could detect the orifice in images with an average sensitivity and specificity of 96.86% and 90.47%, respectively, and correctly classified 21 out of 23 colonoscopy videos (accuracy 91.30%).

Table 3 Summary of the non-controlled studies involving computer-aided diagnosis for colonoscopy including studies with combined detection and diagnosis systems

Ref.	Year	Study design	Study aim	System	Number of patients/colonoscopies used for training/test datasets (total)	Number of colonoscopy/polyp images/videos used in training/test datasets	Diagnostic properties
Tischendorf *et al*[38]	2010	Prospective pilot	Distinguishing adenomas from non-adenomas	CADx based on SVMs	NA/128; Colonoscopy videos	NA/209 polyps containing 160 neoplastic and 49 non-neoplastic polyps in the test dataset	CADx: Sensitivity = 90%, specificity = 70%, correct classification rate = 85.3%. Consensus decision between the human. Observers: Sensitivity = 93.8%, specificity = 85.7%, correct classification rate = 91.9%. "Safe" decision, when there was interobserver discrepancy: Sensitivity = 96.9%, specificity = 71.4%, correct classification rate = 90.9%
Aihara *et al* [47]	2013	Prospective	Distinguishing neoplastic from non-neoplastic lesion	CADx based on numerical color analysis of autofluorescence endoscopy as an Adobe AIRapplication	NA/32 patients in the test dataset	NA/102 lesions containing 75 neoplastic lesions in the test dataset	Sensitivity = 94.2%; specificity = 88.8%; PPV = 95.6%; NPV = 85.2%
Mori *et al* [87]	2015	Retrospective pilot	Distinguishing small (≤ 10 mm) neoplastic from non-neoplastic lesion	CADx (EC-CAD) based on CNN	NA/152 patients in the test dataset	NA/176 small polyps in the test dataset containing 137 neoplastic and 39 non-neoplastic polyps for the test dataset	Accuracy = 89.2%, 95%CI = 83.7%-93.4%; Sensitivity = 92.0%, 95%CI = 86.1%-95.9%; specificity of 79.5%, 95%CI = 63.5%-90.7%
Kuiper *et al* [49]	2015	Retrospective	Distinguishing small (≤ 9 mm) neoplastic from non-neoplastic lesion	CADx (WavSTAT) based on CNN	NA/87 patients in the test dataset	NA/207 small lesions in the test dataset	Accuracy = 74.4%, 95%CI = 68.1%–79.9%; sensitivity = 85.3%, 95%CI = 0.78–0.90; specificity = 58.8%, 95%CI = 0.48–0.69; PPV = 74.8%, 95%CI = 0.67–0.81; NPV = 73.5%; accuracy of on-site recommended surveillance interval = 73.7%
Misawa *et al* [34]	2018	Retrospective	Distinguishing neoplastic from non-neoplastic lesion categorized	CADx based on SVMs	NA	979 images containing 381 non-neoplasms and 598 neoplasms in the training dataset/100 images containing 50 non-neoplasms and 50 neoplasms in the test dataset	Accuracy = 90.0%, 95%CI = 82.4–95.1; sensitivity = 84.5%, 95%CI = 72.6–92.7; specificity = 97.6%, 95%CI = 87.4–99.9; PPV = 98.0%, 95%CI = 89.4–99.9; NPV = 82.0%, 95%CI = 68.6–91.4
Byrne *et al* [51]	2018	Retrospective	Distinguishing neoplastic from non-neoplastic lesions	CADx + CADe based on an improved DCNN model using NBI	NA	NA/21804 unseen frames in the test dataset	Accuracy = 99.94%; sensitivity = 95.95%; specificity = 91.66%; NPV = 93.6%; prediction of polyp videos = 97.6%
Mori *et al* [48]	2018	Prospective	Distinguishing diminutive (≤ 5 mm) neoplastic from non-neoplastic lesions	CADx based on SVMs used with NBI and endocytoscope	NA/791 patients in the test dataset	61925/466 polyps from 325 patients in the test dataset	CADx-NBI: Sensitivity = 92.7%, 95%CI = 89.1–95.4; specificity = 89.8%, 95%CI = 84.4–93.9; PPV = 93.7%, 95%CI = 90.2–96.2; NPV = 88.3%, 95%CI = 82.7–92.6. CADx-endocytoscope: Sensitivity = 91.3%, 95%CI = 87.5–94.3; specificity = 88.7%, 95%CI = 83.1–93.0; PPV = 92.9%, 95%CI = 89.3–95.6; NPV = 86.3%, 95%CI = 80.4–90.9
Byrne *et al* [45]	2019	Retrospective	Distinguishing diminutive (≤ 5 mm) neoplastic from non-neoplastic lesions	CADx based on DCNN		Training dataset: 60089 frames from 223 polyp videos (29% NICE type 1, 53% NICE type 2 and 18% of normal mucosa with no polyp)/validation dataset: 40 videos (NICE type 1, NICE type 2 and two videos of normal mucosa)/test dataset: 125 consecutively	Accuracy = 94%, 95%CI = 86%-97%; sensitivity = 98%, 95%CI = 92%-100%; Specificity = 83%, 95%CI = 67%-93%; NPV = 97%; PPV = 90%

						identified diminutive polyps, comprising 51 hyperplastic polyps and 74 adenomas	
Song *et al* [88]	2020	Retrospective	Distinguishing adenomas from SPs	CADx based on DCNN	NA	12480 image patches of 624 polyps/two test datasets of 545 polyp	Agreement between the true polyp histology CADx = 0.614–0.642; accuracy = 81.3%–82.4%; sensitivity = 82.1%; specificity = 93.7%; PPV = 78%; NPV = 95%; the AUC = 0.93–0.95, 0.86–0.89, and 0.89–0.91 for serrated polyps, benign adenoma/mucosal or superficial submucosal cancer, and deep submucosal cancer, respectively
Kudo *et al* [89]	2020	Retrospective	Distinguishing small (≤ 10 mm) neoplastic from non-neoplastic lesions	The EndoBRAIN system (CADx + CADe based on DCNN)	NA/89 patients test set	69,142 images taken at 520-fold magnification and 2,000 polyps/100 lesions (≤ 10 mm) in the test dataset	CADe: Accuracy = 98%, 95%CI = 97.3%–98.6%; sensitivity = 96.9%, 95%CI = 95.8%–97.8%; specificity = 100%, 95%CI = 99.6%–100%; PPV = 100%, 95%CI = 99.8%–100%; NPV = 94.6%, 95%CI = 92.7%–96.1%; CADx: Accuracy = 96%, 95%CI = 95.1%–96.8%; sensitivity = 96.9%, 95%CI = 95.8%–97.8%; specificity = 94.3%, 95%CI = 92.3%–95.9%; PPV = 96.9%, 95%CI = 95.8%–97.8%; NPV = 94.3%, 95%CI = 92.3%–95.9%

CADe: Computer-assisted detection system; CADx: Computer-assisted diagnosis system; CNN: Convolutional neural network; DCNN: Deep learning convolutional neural network; AUC: Area Under the Receiver Operating Characteristic curve; PPV: Positive predictive value; NPV: Negative predictive value; SVM: Support vector machine; SP: Serrated polyps; CI: Confidence interval.

RECOMMENDATIONS FOR FUTURE RESEARCH

Despite potential benefits of AI in colonoscopy, regulatory approval and standardization of AI models are difficult goals to achieve for a number of reasons described below.

Polyp morphology

Datasets might underrepresent particular polyp morphologies that are not common findings during colonoscopy. For example, non-polypoid lesions with Paris classification of flat and/or depressed morphology are more likely to harbor advanced histology or malignancy but are not a common finding during colonoscopy[55]. The endoscopic detection of non-polypoid lesions is problematic because of their surface pattern resemblance to normal mucosa[56]. Moreover, serrated polyps comprise about 30% of colon polyps, with sessile serrated polyp/adenoma (SSA/P) prevalence being less than 10%[57]. It has been proven that SSA/Ps can be responsible for CRC through a serrated (hyperplastic-SSP/A-serrated-CRC) sequence[58]. However, SSA/Ps can hardly be distinguished from normal mucosa or hyperplastic polyps by features of crypt distortion. Research has shown that previously diagnosed hyperplastic polyps might be reclassified as SSAs after pathological reassessment[19-22], particularly for larger (> 5 mm) or right-sided polyps, and co-existing adenomas containing advanced histology[19,21,59]. A recent meta-analysis showed that pathological reassessment of resected polyps led to a significant change in diagnosis from hyperplastic to SSA for polyps in the right colon and polyps ≥ 5 mm (odds ratio 4.401 and 8.336, respectively)[59]. Moreover, there is poor agreement among pathologists in the determination of

high-risk polyp features owing to the various approaches used for preparing biopsy specimens or level of expertise[19,60]. Therefore, the development of an AI platform capable of detecting and distinguishing subtle adenomatous features from normal mucosa with a high level of accuracy would be a valuable clinical tool.

Metadata

Most studies have failed to assess the performance and accuracy of AI models according to polyp size, polyp location, bowel preparation score, or withdrawal time [18]. Patients' information including demographic and clinical characteristics (*e.g.*, colonoscopy indication, disease status), procedure-related quality characteristics (*i.e.*, bowel preparation level, withdrawal time), procedure time and room, endoscopists fatigue (*i.e.*, the procedure performed in the morning or afternoon) are the important factors that are linked with the long-term non-endoscopic outcome of interest. In other words, the detection and classification of colorectal polyps are the intermediate outcomes of the colonoscopy, but the prevention of interval cancer during the surveillance period, or the evaluation of the effectiveness of medical therapy and the need for surgical treatment in patients with inflammatory bowel diseases are the ultimate goals of the colonoscopy depending on the primary indication of the procedure. As mentioned in Kudo *et al*[61], metadata is a critical component in establishing optimal AI platforms that can perform well in real-world practice with suboptimal conditions. For example, SSA/Ps are mainly located in the right colon, where endoscopic access and complete inspection of the mucosa are challenging[58]. Collecting a high number of colonoscopy videos with a high number of SSA/P polyps and cross-linking with patient's data would increase the accuracy and effectiveness of the colonoscopy. Future AI models must incorporate the information of the polyp size and location as well as the clinical, pre-procedural, and polyp morphological characteristics rather than focusing on the polyp images and videos alone.

Prospective real-time studies

The robustness of AI platforms has not been widely estimated in real-time clinical settings through prospective studies. Most studies have been retrospective in design and subject to selection bias. Therefore, the comparison of accuracy between model and endoscopists may falsely deviate in favor of CAD. For example, in CADe, the researcher might exclude unclear colonoscopy or polyp images/videos; a fuzzy or blurred endoscopic view may occur when water or blood obscures the field, or when feces cover the bowel surface preventing a complete examination. There should also be a mixture of polyp-positive and polyp-negative images from abnormal and normal colonoscopies in all training, validation, and test datasets. The development of AI models must be rigorously based on a training dataset that is preferably gathered during real-time colonoscopies. Data should be collected prospectively by both experienced and novice endoscopists to represent the actual state of practice when assessing the model. The elimination of selection bias is most relevant to CADe systems and less so to CADx systems. Studies should be based in several centers to ensure the reproducibility of the results at the testing level. Testing CAD systems in non-academic settings will demonstrate whether the model represents actual real-world practice, where more polyps are missed and/or there is no access to advanced technologies such as NBI. In addition, real-time and multicenter studies may help to clarify the place of AI in the diagnostic process. Prospective studies would provide robust evidence to support the application of CAD and enhance endoscopists' trust in optical polyp classification[62]. Nevertheless, CAD is still an operator-dependent technology as it is the experienced endoscopists who must provide the annotated datasets for the development of the system, and the accuracy of the AI output relies on the endoscopist presenting a clear endoscopic field to the system. Certain challenges such as prolonged procedure times, high positivity rate, and inability to predict the histology in the presence of feces or blood in the visual field should be mitigated to prevent suboptimal diagnosis. Physicians should continue to follow the recommended procedural measures, including sufficient bowel preparation and photo documentation, to avoid legal and insurance issues.

Researchers should prioritize prospective controlled trials to allow a precise comparison between the settings that use and do not use AI platforms, otherwise, the real benefits of the AI system cannot be determined. Crossover studies, where patients act as their own controls and undergo colonoscopy both with and without AI support would be useful as fewer patients would be needed. In practice, the endoscopist would first detect and classify a polyp before using the AI support system to ensure the accuracy of their classification. This process should be performed in a time-efficient manner as the benefit of AI assistance would be irrelevant if the procedure was

significantly prolonged.

Standardization of endpoints

All research evaluating the diagnostic accuracy of CAD systems should use standardized research endpoints derived from the latest guidelines. Similarly to other diagnostic evaluation studies, sensitivity, specificity, PPV, NPV, and AUC must be reported, as well as confusion matrices and mean average precision for multiclass classifications and intersection over union (IoU), or the DICE coefficient for segmentation (*i.e.*, delineation) in particular situations[63,64]. The use of such a comprehensive set of metrics would provide convincing evidence, reassuring physicians about the reliability of AI tools. For example, ADR must be reported for all research related to the evaluation of CADe systems, as such systems aim to achieve complete detection of all colorectal lesions. Similarly, the NPV of CADx systems must be reported to confirm the ability of CADx to achieve the recommended NPV benchmark of ≥ 90% according to the PIVI statement[46]. In addition, for surveillance interval assignment, the agreement between AI-based assignment and that of the histopathology reference standard must reach the ≥ 90% threshold recommended by the PIVI statement[46].

Transparency of AI analyses

We should avoid the black-box phenomenon when the decision-making process of the model by the convolutional neural network cannot be deconvoluted due to the complexity of the process[65,66]. An important aspect of the wide application of AI platforms is the trust that physicians and responsible regulatory officials place in the AI analyses. Research should move toward facilitating extreme transparency in the generation and validation of AI models to avoid hesitancy about their public implementation.

Safety and cost-effectiveness

Finally, as well as CADe and CADx systems, a computer-based support system that aids endoscopists in selecting the most appropriate polypectomy procedure is necessary. Current practice involves the use of forceps to remove diminutive polyps, especially for the resection of polyps up to 2 mm[67]; however, the rate of incomplete resection is lower for the removal of polyps ≥ 3 mm when a snare is used[68]. In addition to providing a suggestion for an appropriate polypectomy device, AI can also help to estimate polyp size, delineate the extent of the lesion and a safe polypectomy margin, and identify post-resection lesion remnants that indicate an incomplete resection and the need for further tissue removal at colonoscopy follow-up. The goal of this system is to provide a complete polypectomy that will reduce the risk of interval cancer, as about 30% of all interval cancers are thought to be caused by incomplete resection of CRC precursors[11,69,70].

In addition to addressing the challenges associated with the development of reliable AI models that can be confidently employed in routine practice with high efficacy, research is needed to assess the cost-effectiveness of these systems related to the reduction in the number of patients diagnosed with interval cancer, reduction in the number of unnecessary pathology evaluations for low-confidence predictions of polyp histology by optical diagnosis, and facilitation of efficient physician-patient communication concerning future clinical arrangements.

Adapting the newly developed AI-based techniques in routine practice and enhancing endoscopists' trust in the new devices is only possible by a symbiotic relationship between academia and industry. It would facilitate obtaining regulatory approval from health authorities regarding research involving human subjects, constructing large "ground truth" data for developing AI models, and transporting knowledge and technology to ultimately access the market[71]. Several manufacturers have obtained the regulatory approvals to launch and commercialize their AI-based colonoscopy devices around the world (Table 4); however, many of them have not provided a detailed report of their devices' performance. Further research should try to compare the performance of different AI-based systems in real-time settings by conducting prospective controlled trials with multiple intervention arms sing different commercially available AI-based colonoscopy systems. Due to the time- and cost-consuming nature of these studies, an alternative method for accelerating research is to test the "benchmarks" using the publicly available datasets such as the ASU-Mayo colonoscopy video database[29], the CVC-ClinicDB database[28], the Kvasir dataset [72], and the ETIS-Larib Polyp database. Nonetheless, these datasets contain a limited number of colonoscopy videos and images and may not reflect the true performance of

Table 4 Commercially available computer-assisted colonoscopy tools that have cleared regulatory approval

Computer assissted system	Product	Manufacturer	Year of regulatory approval	Place of regulatory approval
CADx	EndoBRAIN	Cybernet System Corp./Olympus Corp.	2018	Japan
CADe	GI Genius	Medtronic Corp.	2019 in Europe; 2021 in United States	Europe/United States
CADe	ENDO-AID	Olympus Corp.	2020	Europe
CADe/CADx	CAD EYE	Fujifilm Corp.	2020	Europe/Japan
CADe	DISCOVERY	Pentax Corp.	2020	Europe
CADe	EndoBRAIN-EYE	Cybernet System Corp./Olympus Corp.	2020	Japan
CADe	EndoAngel	Wuhan EndoAngel Medical Technology Company	2020	China
CADe	EndoScreener	WISION A.I.	2020	China
CADx	EndoBRAIN-PLUS	Cybernet System Corp./Olympus Corp.	2020	Japan
CADx	EndoBRAIN-UC	Cybernet System Corp./Olympus Corp.	2020	Japan
CADe	WISE VISION	NEC Corp.	2021	Europe/Japan
CADe	ME-APDS	Magentiq Eye	2021	Europe
CADe	CADDIE	Odin Vision	2021	Europe

CADe: Computer-assisted detection system; CADx: Computer-assisted diagnosis system.

an AI-based system.

CONCLUSION

AI research is a rapidly evolving discipline that promises to enhance physicians' performance. AI models have demonstrated the ability to compete with and outperform endoscopists, suggesting that all endoscopists would benefit from becoming familiar with CAD technology and comfortable with the integration of AI-assisted devices in colonoscopy practice. The decision support systems are being offered as reliable tools for the detection and classification of colorectal polyps, with the primary aim of outperforming endoscopists by detecting all CRC precursors; however, the new era of AI platforms has seen attempts to establish considerably more complex systems, in which the detection and classification of polyps are supported. Despite the recent achievements in designing and validating such systems, the current lack of AI-assisted systems that support endoscopists in monitoring colonoscopy quality, and that automatically annotate colonoscopy videos, suggest appropriate polypectomy devices, and indicate the completeness of polypectomy, limits the role of AI in colonoscopy practice. Through the integration of the most recent advances in computer science into colonoscopy practice, it appears possible to improve the quality of diagnosis, treatment, and screening in patients. However, AI platforms are still in their infancy in terms of clinical establishment and require much more exploration and innovation. They must be trusted by all physicians, regulatory organizations responsible for approval for clinical use, and patients. The AI-assisted colonoscopy is highly dependent on the endoscopist, who must attempt to present the clearest possible image or video to the AI model for analysis, and then take account of other concurrent patient factors such as the family history of CRC or the results of previous colonoscopies. The human qualities of respect and empathy must be apparent when communicating with patients to overcome any mistrust or reservations patients may have toward the new technology. Therefore, at the current stage of AI development, AI models can only "serve as a second observer, or a concurrent observer, but not an independent decision-maker"[73].

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 21; 27(47): 8123-8137

DOI: 10.3748/wjg.v27.i47.8123

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

MINIREVIEWS

Immunotherapies for well-differentiated grade 3 gastroenteropancreatic neuroendocrine tumors: A new category in the World Health Organization classification

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Author contributions: Xu JX designed the review, drafted the manuscript, sorted and analyzed the data, and prepared the table; Wu DH and Ying LW assisted in editing the table and contributed to clinical data collection; Hu HG coordinated the design of the outline and critically revised the paper; all authors have read and approved the final manuscript.

Conflict-of-interest statement: The authors have no conflict of interests to report.

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): 0
Grade C (Good): 0
Grade D (Fair): D

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Abstract

According to the 2019 World Health Organization (WHO) classification, well-differentiated grade 3 (G3) gastroenteropancreatic (GEP) neuroendocrine tumors (NETs) are a new category of cancer of the digestive system. G3 GEP-NET research and treatment are not as robust as those of lower grade (G1/2) NETs and poorly differentiated neuroendocrine carcinomas (NECs). Previously, the management of high-grade NETs was mainly based on NEC therapies, as high-grade NETs were classified as NECs under the previous WHO classification. Despite this, G3 GEP-NETs are significantly less responsive to platinum-based chemotherapy regimens than NECs, due to their distinct molecular pathogenesis and course of pathological grade transition. Patients with advanced G3 GEP-NETs, who have progressed or are intolerant to chemotherapy regimens such as capecitabine plus temozolomide, have limited treatment choices. Immunotherapy has helped patients with a variety of cancers attain long-term survival through the use of immune checkpoint inhibitors. Immunotherapies, either alone or in combination with other therapies, do not have a clear function in the treatment of G3 GEP-NETs. Currently, the majority of immunotherapy studies, both prospective and retrospective, do not reliably differentiate G3 GEP-NETs from NECs. By contrast, a significant number of studies include non-GEP neuroendocrine neoplasms (NENs). Therefore, there is an urgent need to summarize and evaluate these data to provide more effective therapeutic approaches for patients with this rare tumor. The purpose of this mini-review was to screen and

Grade E (Poor): 0

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Received: September 8, 2021
Peer-review started: September 8, 2021
First decision: October 16, 2021
Revised: October 28, 2021
Accepted: November 30, 2021
Article in press: November 30, 2021
Published online: December 21, 2021

P-Reviewer: Ahmed M, Cho JH
S-Editor: Yan JP
L-Editor: Filipodia
P-Editor: Yan JP

summarize information on G3 GEP-NETs from all studies on NENs immunotherapy.

Key Words: Gastrointestinal tract; Pancreas; Immune checkpoint inhibitors; Immunotherapy; Neuroendocrine tumors; Cytotoxic T-lymphocyte-associated protein 4 antigen

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Core Tip: Several evaluations have been published on immunotherapy for neuroendocrine neoplasms. However, this is the first review to specifically focus on the efficacy of different immunotherapy strategies such as immune checkpoint inhibitor (ICI) monotherapy, dual ICI therapy, anti-angiogenesis plus ICI, and chemotherapy combined with ICI for the treatment of advanced well-differentiated high-grade gastroenteropancreatic neuroendocrine tumors.

Citation: Xu JX, Wu DH, Ying LW, Hu HG. Immunotherapies for well-differentiated grade 3 gastroenteropancreatic neuroendocrine tumors: A new category in the World Health Organization classification. *World J Gastroenterol* 2021; 27(47): 8123-8137
URL: https://www.wjgnet.com/1007-9327/full/v27/i47/8123.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i47.8123

INTRODUCTION

Neuroendocrine neoplasms (NENs) are rare and indolent diseases that can manifest in any part of the body where peptidergic neurons and neuroendocrine cells are found. About 65% of neoplasms are found in the gastrointestinal (GI) tract and pancreas, making gastroenteropancreatic (GEP)-NENs the most common type of NENs[1]. Due to advancements in early-stage disease detection techniques such as endoscopy and imaging, the incidence of GEP-NENs has significantly increased to an overall incidence of 3.56 per 100000[2]. Based on the 2010 grading system, the World Health Organization (WHO) in 2019 comprehensively considered the importance of the primary site, morphological differentiation, and grading in the classification of GEP-NENs, and expanded the 2017 grading system by proposing a classification framework for all NENs[3]. One of the key updates in the 2019 classification system is that all grade 3 (G3) NENs (with Ki-67 proliferation index > 20%) are classified as either well-differentiated G3 neuroendocrine tumors (NETs) or poorly differentiated neuroendocrine carcinomas (NECs). Although G3 NETs have more inert biological behavior compared to NECs, they have a poorer prognosis compared to G1/2 NETs [4]. Compared to patients with poorly differentiated NECs, well-differentiated G3 NET patients have a considerably longer median overall survival (mOS) (41-99 mo *vs* 17 mo)[5].

G3 NENs account for 13.4% of all digestive system NENs, whereas G3 NETs account for 18%-20% of G3 GEP-NENs[6,7]. In general, although significant progress has been made in the management of GEP-NENs as a whole, the treatment of G3 GEP-NETs, a new WHO category, has not been well studied. Therefore, more tailored treatment strategies are needed for these disorders.

According to WHO 2010 classification criteria, G3 GEP-NETs were categorized as NECs. However, clinical variations between individuals with G3 GEP-NETs and NECs were discovered. For example, platinum-based chemotherapy was frequently employed for the treatment of G3 GEP-NEN patients in the past. Patients with G3 NETs or Ki-67 < 55% (mostly well-differentiated) were significantly less responsive to treatment than those with NEC or Ki-67 ≥ 55% (mostly poorly differentiated). G3 NET and NEC patients have an objective response rate (ORR) of less than 17% and 35%-70%, a median progression-free survival rate (mPFS) of 2.4-4 mo and 5.0 mo, and mOS of 17 mo and 99 mo, respectively[8-10].

Recently, the first prospective Phase II study of capecitabine with temozolomide in patients with high-grade GEP-NEN and Ki-67 index < 55% yielded results contrary to those received platinum plus etoposide. Patients with G3 NET (n = 23) responded better to treatment than those with NEC (n = 7) in both short-term [ORR 34.8% *vs*

14.3%, P = 0.393; disease control rate (DCR) 87.0% *vs* 42.9%, P = 0.033] and long-term (mPFS 9.3 mo *vs* 3.5 mo, P = 0.005; mOS did not reach *vs* 6.2 mo, P = 0.004) evaluations [11]. In addition, a retrospective study showed that the first-line fluoropyrimidine-based regimens with oxaliplatin (FOLFOX/XELOX) also achieved superior results (mPFS of 7.9 mo and mOS of 30 mo) in 34 patients with G3 GEP-NETs[12].

However, there is no established treatment for G3 GEP-NET patients who are insensitive or resistant to chemotherapy. Peptide receptor radionuclide therapy (PRRT) is a promising alternative approach to chemotherapy. In a retrospective cohort study, PRRT was delivered to G3 GEP NEN patients and their response was examined. The study found that ORR was not significantly different across well-differentiated (n = 60) and poorly differentiated (n = 62) disease subgroups (42% *vs* 43%). However, DCR, mPFS, and mOS were much longer in patients with well-differentiated tumors than those with poorly differentiated cancers (DCR 93% *vs* 68%, mPFS 19 mo *vs* 8 mo, and mOS 44 mo *vs* 19 mo)[13]. Regrettably, this new therapy is only available in a few countries. Therefore, there is an urgent need to compensate for the inadequacies of the aforementioned medications in patients with G3 GEP-NETs.

In recent years, immunotherapy has emerged as a new and intriguing approach for cancer therapy. Cancer cells have the inherent ability to express negative regulatory molecules of immune cells. The cornerstone of immunotherapy in modern oncology aims to improve the ability of the immune system to recognize and kill tumor cells [14]. Currently, this is being achieved through the use of monoclonal antibodies against immune checkpoints such as programmed death-ligand 1 (PD-L1)/programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). Immune checkpoint inhibitors (ICIs), which sit at the forefront of cancer immunotherapy, have revolutionized the management of a variety of solid malignancies. In relation to NENs, immunotherapy has been mainly used to treat lung and skin tumors such as Merkel cell carcinoma, malignant melanoma, and small cell lung cancer (SCLC)[15]. Although an increasing number of clinical trials and retrospective studies are being conducted to investigate the efficacy of ICIs on NENs of the digestive tract, the role of immunotherapy approaches in well-differentiated G3 NETs has not been sufficiently studied.

In this minireview, we briefly describe the overall pathological changes of G3 GEP-NETs and analyze in detail the immunotherapy experience with well-differentiated G3 GEP-NETs from complex investigations.

ROLE OF IMMUNOTHERAPY IN G3 GEP-NETS

A recent systematic review and meta-analysis of 636 NEN patients treated with ICIs reported an ORR of 10% [95% confidence interval (CI): 6%-15%, I^2 = 67%, P < 0.1], a total DCR of 42%, a mPFS of 4.1 mo (95%CI: 2.6-5.4; I^2 = 96%, P < 0.1), and a mOS of 11 mo (95%CI: 4.8-21.1; I^2 = 98%, P < 0.1)[16]. This demonstrated the overall effectiveness of ICIs in treating NEN patients. Among the NEN study subjects, about 37% were patients with NENs originating from the lung or other unknown sites and only 13.4% were patients who had G3 NETs. However, the study did not include a separate subgroup of G3 GEP-NET patients in its analysis. Previous studies have shown that G3 NETs can share a common pathogenesis with G1-2 NETs[17]. Moreover, more than half of G1-2 pancreatic NETs (pNETs) developed progressively into G3 pNENs over time[18]. Some researchers have even speculated that high-grade pNET may develop from the initial low- and medium-grade NET, while pNEC may develop from pancreatic ductal adenocarcinoma[19,20]. Therefore, the response of lower grade NETs to immunotherapy may have some implications for the treatment of G3 NETs. Other aspects that may influence the immunotherapy choices for G3 GEP-NETs include the presence of predictive biomarkers for ICIs in tumors with high proliferative activity as well as changes in pathological grade over time.

Table 1 summarizes the clinical trials of immunotherapy in GEP-NENs. Below, we presented data from clinical trials and retrospective studies that may have included cases with G3 GEP-NETs. Additionally, we analyzed the data to determine the efficacy of different immunotherapy strategies such as PD-1/PD-L1 inhibitors as a monotherapy or in combination with CTLA-4 inhibitors, anti-angiogenesis, and chemotherapy in the management of these rare diseases.

ICIs monotherapy

Pembrolizumab is the most extensively investigated immunotherapy for NENs. For example, phase Ib (KEYNOTE-28) and phase II (KEYNOTE-158) clinical trials were

Table 1 Clinical trials related to gastroenteropancreatic neuroendocrine tumors

ClinicalTrials.gov identifier	Intervention	Study phase	Trial name	Primary outcome	Estimated/actual enrollment, *n*	Estimated/actual date	Trial status	Medical condition related to advanced NENs	Reported assessable n of NENs
NCT02054806[21]	Pembrolizumab	Ib	Phase Ib study of pembrolizumab (MK-3475) in subjects with select advanced solid tumors (MK-3475-028/KEYNOTE-028)	ORR	477	April 30, 2021	Completed	pNETs: PD-L1 (+), well or moderately differentiated	16 pNETs
NCT02628067[22]	Pembrolizumab	II	A clinical trial of pembrolizumab (MK-3475) evaluating predictive biomarkers in subjects with advanced solid tumors (KEYNOTE-158)	ORR	1595	June 18, 2026	Recruiting	NETs: Well or moderately differentiated	107 NETs: Lung, appendix, small intestine, colon, rectum, or pan origin
NCT02939651[23]	Pembrolizumab	II	A phase 2, open-label study of pembrolizumab monotherapy in patients with metastatic high grade neuroendocrine tumors	ORR	21	March 2020	Completed	G3 NENs: Ki-67 > 20%, poorly or well-differentiated, failed for platinum based chemotherapy, excluding MCC, large/small cell NENs of lung/thymus origin	29 G3 NENs: 19 NECs, 9 G3 NET, 14 Ki-67 ≤ 50%, 12 Ki-67 > 50%, 10 pan, 14 GI, 5 unknown origin
NCT03136055[24, 48]	Part A: pembrolizumab alone; Part B: Pembrolizumab + chemotherapy	II	A pilot study of pembrolizumab-based therapy in previously treated high grade neuroendocrine carcinomas	ORR	36	May 31, 2023	Active, not recruiting	EP-PDNECs: Failed for first-line systemic therapy, excluding MCC or well differentiated G3 NET	Part A: 13 EP-PDNECs; Part B: 22 EP-PDNEC
NCT03190213	Pembrolizumab	II	Pembrolizumab for the treatment of recurrent high grade neuroendocrine carcinoma (Pembro NEC)	ORR (irRECIST)	6	March 11, 2019	Terminated	G3 NENs: Failed for platinum-based regimen or temozolomide-based regimen. exluding lung origin	6 G3 NENs
NCT02955069[25]	Spartalizumab	II	An open label phase II study to evaluate the efficacy and safety of PDR001 in patients with advanced or metastatic, well-differentiated, non-functional neuroendocrine tumors of pancreatic, gastrointestinal (GI), or thoracic origin or poorly-differentiated gastroenteropancreatic neuroendocrine carcinoma (GEP-NEC), that have progressed on prior treatment	ORR	116	May 13, 2020	Completed	NENs: Exclude G3 NETs and include G1/2 NET (non-functional, GEP or thoracic origin, failed to prior treatment) and GEP-NEC (progressed on or after one prior chemotherapy regimen)	99 NETs: 30 thoracic, 32 GI-NET, 33 pNET; 21 GEP-NEC
NCT03167853[30]	Toripalimab	Ib	Phase Ib study of safety and efficacy of recombinant humanized anti-PD-1 monoclonal antibody for patients with advanced neuroendocrine tumors following failure of first-line	ORR	40	May 11, 2019	Completed	NENs: Ki-67 ≥ 10%, nonfunctional NENs, well- or poorly-differentiated, failed for first line therapy	40 NENs: 8 well-differentiated, 32 poorly-differentiated
NCT03352934[26]	Avelumab	II	A phase II, open-label, multicenter trial to	DCR	60	January 2024	Active, not	G3 NENs: after first-line	29 G3 NENs: 16 NEC

			investigate the clinical activity and safety of avelumab in patients with advanced, metastatic high grade neuroendocrine carcinomas NEC G3 (WHO 2010) progressive after chemotherapy (AveNEC)				recruiting	chemotherapy, excluding MCC, SCLC	and 11 G3 NETs, 12 pan, 5 GI, 9 other origin)
NCT03278405[58]	Avelumab	IIa	A pilot study of avelumab in unresectable/metastatic, progressive, poorly differentiated grade 3 neuroendocrine carcinomas (NET001)	ORR	10	March 12, 2020	Completed	NECs: Poorly-differentiated, GI or lung origin, have received 0-2 prior lines of systemic therapy, excluding MANEC	10 NECs: 9 GI and 1 lung
NCT03278379[28]	Avelumab	II	A phase II study of avelumab in unresectable/metastatic, progressive grade 2-3 neuroendocrine tumors (NET-002)	ORR	17	September 20, 2021	Active, not recruiting	G2-3 NETs: GEP or lung, received 0-2 prior lines of therapy (excluding SSAs), excluding G1 NET, NEC and MANEC	17 G2-3 NETs (12 GEP, 5 lung)
NCT03147404	Avelumab	II	Phase II study of avelumab in metastatic gastronetro-pancreatic (GEP) neuroendocrine carcinoma (NEC, WHO Grade 3) as second-line treatment after failing to etoposide + cisplatin: integration of genomic analysis to identify predictive molecular subtypes (MS100070-0177)	Best response	14	July 22, 2019	Completed	G3 GEP-NECs: Second-line treatment after failing to etoposide + cisplatin	-
NCT03879057[39]	Toripalimab + surufatinib	I	Phase I trial evaluating the safety, tolerability, pharmacokinetics, and efficacy of surufatinib combined with JS001 in patients with advanced solid tumors	AEs, MTD	24	December 20, 2021	Recruiting	NENs: G1-3 NET, NEC	18 NENs:11 NECs,4 G2 NETs; 4 G3 NETs, 12 GI,4 pan, 1 lung
NCT04169672[40]	Toripalimab + surufatinib	II	A phase II, open-label, single-arm, multi-center study of the efficacy and safety of surufatinib combined with toripalimab in patients with advanced solid tumors	AEs, ORR	200	February 28, 2022	Recruiting	NECs: Refractory to first-line chemotherapy	20 NECs
NCT03475953	Avelumab + regorafenib	I/II	A phase I/II study of regorafenib plus avelumab in solid tumors (REGOMUNE)	Phase I: Recommended dose of regorafenib; Phase II: ORR, PFS	482	May 2022	Recruiting	G2/3 GEP-NETs	-
NCT03290079	Pembrolizumab + lenvatinib	II	Phase II study of pembrolizumab and lenvatinib in advanced well-differentiated neuroendocrine tumors	ORR	35	December 2023	Recruiting	NETs: Well-differentiated, lung, thymus, small bowel or colon origin, including unknown primary, excluding pNENs and poorly differentiated NECs	-
NCT04579757	Surufatinib + tislelizumab	Ib/II	An open-label phase Ib/II study of	DLT, ORR	120	April 30, 2023	Recruiting	G1/2 NETs: Thoracic or	-

			surufatinib in combination with tislelizumab in subjects with advanced solid tumors					GEP origins, have progressed on at least one line of standard therapy	
NCT04207463	AK105 + anlotinib	II	A phase II, open, single-arm, multi-cohort, multicenter study of anlotinib and AK105 (anti-PD-1) injection in subjects with gastrointestinal tumors, urinary system tumors, neuroendocrine tumors	ORR	150	May 30, 2021	Recruiting	G1/2 GEP-NETs	-
NCT03074513[41]	Atezolizumab + bevacizumab	II	A phase II, single-arm open-label study of the combination of atezolizumab and bevacizumab in rare solid tumors	ORR	164	March 31, 2021	Active, not recruiting	G1/2 NETs: pNET cohort and epNET cohort containing typical or atypical carcinoid if originating in lung	20 G1/2 pNETs, 20 G1/2 epNETs
NCT02923934[45]	Nivolumab + ipilimumab	II	A phase II clinical trial evaluating ipilimumab and nivolumab in combination for the treatment of rare gastrointestinal, neuro-endocrine and gynaecological cancers (CA209-538)	CBR	120	December 2023	Active, not recruiting	NENs: G1-3 NETs, NECs, GEP or lung origin	10 GEP-NENs: 7 pNENs (3 G3 pNETs, 2 pNECs, 2 G2 pNETs); 3 GI-NENs (1 gastro-oesophageal junction NEC, 1 colonic NECs, 1 G1 gastric NET)
NCT02834013[15, 46]	Nivolumab + ipilimumab	II	A prospective, open-label, multicenter phase II basket clinical trial of ipilimumab plus nivolumab across multiple rare tumor cohorts (DART)	ORR	818	August 1, 2021	Recruiting	SWOG 1609 cohort: Refractory epNENs. G3 NETs were included in G3 NECs. SWOG S1609 cohort: Dedicated cohort include G3 NENs	SWOG 1609 cohort: 32 epNENs (18 G3, 10 G2, 4 G1, 15 GI, 6 Lung). S1609 cohort: 19 G3 NENs (2 G3 NETs, 11 NEC, 6 unknown differentiation status)
NCT04969887	Nivolumab + ipilimumab	II	Ipilimumab and nivolumab combination therapy in patients with selected immunotherapy sensitive advanced rare cancers (MOST-CIRCUIT)	CBR	240	December 2024	Not yet recruiting	NECs and G3 NETs independent of primary site, excluding SCLC	-
NCT03591731	Nivolumab alone or nivolumab + ipilimumab	II	A GCO trial exploring the efficacy and safety of nivolumab monotherapy or nivolumab plus ipilimumab in pre-treated patients with advanced, refractory pulmonary or gastroenteropancreatic poorly differentiated neuroendocrine tumors (NECs) (NIPINEC)	ORR	180	September 2023	Recruiting	NECs: Poorly differentiated, refractory, pulmonary or GEP, excluding SCLC	-
NCT03095274[47]	Tremelimumab + durvalumab	II	A phase II study of durvalumab (MEDI4736) plus tremelimumab for the treatment of patients with advanced neuroendocrine neoplasms of gastroenteropancreatic or lung origin (DUNE) (GETNE 1601)	Cohort 1-3: CBR at 9 m; Cohort 4: OS at 9 mo	126	July 2021	Recruiting	G1/G2 NETs of GEP and lung, and G3 of GEP or unknown primary site (excluding lung primaries) after progression to standard therapies	123 NENs (Cohort 1: 27 typical/atypical lung carcinoid; Cohort 2: 31 G1/2 GI-NENs; Cohort 3: 32 G1/2 pNENs; Cohort 4: 33 G3 NEN of GEP or unknown primary site)

NCT04079712	Nivolumab + ipilimumab + cabozantinib	II	A phase 2 study of XL184 (Cabozantinib) in combination with nivolumab and ipilimumab for the treatment of poorly differentiated neuroendocrine carcinomas	ORR	30	October 1, 2021	Recruiting	NECs: All variations of poorly differentiated NECs (small cell, large cell and mixed cells) are eligible, excluding SCLC and MCC. Failure of only one line of prior systemic cancer treatment	-
NCT03728361[49]	Nivolumab + temozolomide	II	A phase II, multi-cohort trial of combination nivolumab and temozolomide in recurrent/refractory small-cell lung cancer and advanced neuroendocrine tumors	ORR	55	December 31, 2021	Active, not recruiting	NENs: Any grade or primary site	12 NENs: 1 G1, 8 G2, 3 G3
NCT03980925	Nivolumab + platinum-doublet chemotherapy	II	A phase II study of platinum-doublet chemotherapy in combination with nivolumab as first-line treatment in subjects with unresectable, locally advanced or metastatic G3 neuroendocrine neoplasms (NENs) of the gastroenteropancreatic (GEP) tract or of unknown (UK) origin (GETNE-T1913)	OS at 12 mo	38	December 2022	Recruiting	G3 NENs: GEP or unknown primary site	-
NCT03365791[59]	Spartalizumab + LAG525	II	Modular phase 2 study to link combination immune-therapy to patients with advanced solid and hematologic malignancies. Module 9: PDR001 plus LAG525 for patients with advanced solid and hematologic malignancies	CBR at 24 wk	76	September 17, 2020	Completed	NETs: Well-differentiated, relapsed and/or refractory to available standard of care therapies	7 NETs
NCT03043664[60]	Pembrolizumab + lanreotide depot	Ib/II	Phase Ib/II study of pembrolizumab with lanreotide depot for gastroenteropancreatic neuroendocrine tumors (PLANET)	ORR	22	September 1, 2021	Active, not recruiting	G1-2 GEP-NETs: Had progressed on a prior SSA	22 G1/2 GEP-NETs (14 GI, 8 pan)
NCT04525638	Nivolumab + ^{177}Lu-DOTATATE	II	A phase II single arm trial evaluating the preliminary efficacy of the combination of ^{177}Lu-DOTATATE and nivolumab in grade 3 well-differentiated neuroendocrine tumours (NET) or poorly differentiated neuroendocrine carcinomas (NEC)	ORR	30	September 30, 2024	Recruiting	G3 NENs: GEP or unknown primary site, well-differentiated or poorly-differentiated.	-
NCT04701307	Dostarlimab + niraparib	II	Niraparib (PARP Inhibitor) plus dostarlimab (Anti-PD1) for small cell lung cancer (SCLC) and other high-grade neuroendocrine carcinomas (NEC)	6 mo PFS, 3 mo ORR	48	May 30, 2025	Recruiting	G3 NECs: SCLC (Cohort 1) and other G3 NECs (Cohort 2), had at least one prior line of systemic therapy, excluding prostate origin	-
NCT03457948	Group I: Pembrolizumab + ^{177}Lu DOTATATE; Group II: Pembrolizumab + TAE; Group III: Pembrolizumab + ^{90}Yttrium- Microsphere Radioembolization	II	A pilot study of pembrolizumab and liver-directed therapy or peptide receptor radionuclide therapy for patients with well-differentiated neuroendocrine tumors and symptomatic and/or progressive metastases	ORR	32	March 31, 2024	Recruiting	G1-3 NETs: Well-differentiated, any primary site and unknown primary site, have liver metastases	-

NCT03879694	SVN53-67/M57-KLH peptide vaccine (SurVaxM) +Octreotide	I	A phase I study of safety and immunogenicity of Survivin Long Peptide Vaccine (SurVaxM) in patients with metastatic neuroendocrine tumors (NETs)	AEs	10	June 13, 2024	Recruiting	NETs: GEP or lung origin, positive for survivin	-
NCT04166006	Dendritic cells loaded with autologous tumour (DC vaccine) + IL-2	II	A phase II study on adjuvant vaccination with dendritic cells loaded with autologous tumor homogenate in resected stage iv rare cancers: Head & neck (H & N), neuroendocrine tumors (NET) and soft tissue sarcoma (STS)	Treatment-Emergent AEs	51	December 2026	Recruiting	NET: Stage IV	-

AE: Adverse event; CBR: Clinical benefit rate; DCR: Disease control rate; DLT: Dose-limiting toxicity; EP-PDNECs: Extrapulmonary poorly differentiated neuroendocrine carcinomas; ep: Extra-pancreatic; G1, 2, 3: Grade 1, 2, 3; GEP: Gastroenteropancreatic; GI: Gastrointestinal; irRECIST: Immune-related Response Evaluation Criteria in Solid Tumors; MANEC: Mixed adenoneuroendocrine carcinomas; MCC: Merkel cell carcinoma; MTD: Maximum tolerated dose; NECs: Neuroendocrine carcinomas; NENs: Neuroendocrine neoplasms; NETs: Neuroendocrine tumors; ORR: Objective response rate; OS: Overall survival; p: Pancreatic; pan: Pancreas origin; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-ligand 1; PFS: Progression-free survival; SCLC: Small cell lung cancer; WHO: World Health Organization.

conducted to evaluate pembrolizumab monotherapy in patients with moderately or well-differentiated NETs. In these two studies, no data on tumor grade and Ki-67 index were collected. The results of the KEYNOTE-28 trial showed an ORR of 6.3% in pNETs[21]. The preliminary results of the KEYNOTE-158 study showed that mixed NETs had an ORR of 3.7% and that all reactive tumors were PD-L1-negative[22]. In addition, two prospective randomized phase II trials were performed to evaluate pembrolizumab in 19 patients with NECs and 9 with G3 NETs. There were no responses to pembrolizumab in patients with GI tract or pancreatic diseases[23]. In a trial of 14 patients with extrapulmonary poorly differentiated NECs, only 1 patient achieved complete remission (CR) (ORR 7%) following pembrolizumab monotherapy [24]. From the abovementioned studies, it can be concluded that pembrolizumab alone has a very limited curative effect on the GEP-NENs independent of their proliferative activity or differentiation. The only clinical trial (NCT02955069) of spartalizumab, a PD-1 inhibitor, for the treatment of NENs excluded patients with G3 NETs and achieved low efficacy comparable to pembrolizumab[25]. Avelumab is the only PD-L1 inhibitor used as a single drug in prospective clinical trials for GEP-NENs. Four phase II clinical trials (NCT03352934[26], NCT03278405[27], NCT03278379[28], and NCT03147404) were conducted to evaluate avelumab in patients with G2/3 NETs or NEC. The trials revealed that none of the patients achieved an objective response to avelumab treatment. In addition, none of the 3 G3 NET patients analyzed in a retrospective study from the Mayo Clinic exhibited an objective response to ICI (pembrolizumab, nivolumab, or atezolizumab) monotherapy[29].

Toripalimab (JS001) is a humanized PD-1 IgG4 monoclonal antibody developed in China. In a phase Ib study (NCT03167853) involving 40 NEN patients with Ki-67 ≥ 10%, toripalimab showed moderate efficacy in both well-differentiated NETs and poorly differentiated NECs (ORR: 25.0% *vs* 18.7% per RECIST 1.1, 25.0% *vs* 25.0% per irRECIST)[30]. This suggests that toripalimab may be the most effective ICI

monotherapy currently available for NENs, including G3 NETs. The study also found that patients with PD-L1 expression ≥ 10% or with high tumor mutational burden (TMB) had a better ORR than patients with PD-L1 < 10% (50.0% *vs* 10.7%, $P = 0.019$) or with low TMB (75.0% *vs* 16.1%, $P = 0.03$)[30].

Anti-angiogenesis combined with ICIs

NENs from different tissues are highly vascularized and express a variety of growth factors including vascular endothelial growth factor (VEGF), platelet-derived growth factor, basic fibroblast growth factor, insulin-like growth factor 1, and transforming growth factor-α/β[31]. The high exposure and activation of VEGFs prevent the immune system from recognizing and killing cancer cells killing tumor cells[32,33].

The hallmark of angiogenesis is the uncontrolled development of new vessels from adjacent normal tissues. This results in a network of immature microvessels characterized by structural and functional abnormalities. The normalizing vascular structure can be achieved with antiangiogenic drugs, including large-molecule monoclonal antibodies and small-molecule tyrosine kinase inhibitors. This results in the activation of adhesion molecules and chemokines that recruit and attract cytotoxic T cells and reduce the entry of regulatory T cells. Moreover, it contributes to immune cell mobilization[34,35].

Surufatinib is a small molecule inhibitor that mainly targets VEGF-1, 2, 3 (VEGFR-1, 2, 3), fibroblast growth factor receptor-1, and colony-stimulating factor-1 receptor (CSF-1R). Blocking of CSF-1R can reduce the polarization of tumor-associated macrophages to the M2 type that participates in immunosuppression and promotes tumor growth[36,37]. Two randomized, double-blind phase III clinical trials (SANET-ep and SANET-p) were carried out to evaluate surufatinib *vs* placebo in Chinese patients with G1-2 NETs. The results indicated that surufatinib can significantly prolong PFS in patients with advanced pancreatic and non-pancreatic G1-2 NETs compared with placebo[37,38]. At present, a phase I trial and a phase II trial of surufatinib combined with toripalimab on patients with NENs are underway. In a phase I clinical trial (NCT03879057), as of 2020-1-20, PR occurred in G1/2 NET (2/4) and NECs (2/11) patients; however, none of the 4 patients with G3 NETs achieved disease remission[39], which may be attributed to the small sample size of patients with G3NETs. In the phase II trial (NCT04169672), 20 evaluable patients with NECs and refractory to first-line chemotherapy achieved a moderate ORR of 20% and a DCR of 70%[40]. However, no data for well-differentiated NETs have been reported. In addition, two prospective studies involving G3 GEP-NETs patients are currently recruiting. In one of the trials, the intervention is avelumab plus regorafenib, while in the other study, the intervention is pembrolizumab plus lenvatinib. The studies are expected to be completed in May 2022 and December 2023, respectively. The combination treatment of atezolizumab and bevacizumab in a phase II basket trial (NCT03074513) showed moderate clinical activity and good tolerance in G1-2 pNETs and extra-pNETs (ORR 20% and 15%, respectively) patients with prior therapy[41]. However, the data of G3 NETs have not been reported either.

Given the favorable preliminary results in patients with G1-2 NETs and NECs, researchers might be optimistic about the combination therapy's effectiveness in patients with G3 NETs.

Dual ICI therapy

The United States Food and Drug Administration has approved ipilimumab (anti-CTLA-4) combined with nivolumab (anti-PD-1) (N+I) for melanoma, metastatic renal cell carcinoma, advanced hepatocellular carcinoma, and previously untreated unresectable malignant pleural mesothelioma[42-44]. Response rates with this combination are higher compared to single-agent anti-PD-1 therapy. For NENs, a phase II clinical trial (CA209-538) of N+I demonstrated a moderate overall ORR of 24%, especially in patients with G3 NENs and atypical bronchial carcinoid[45]. In the study, 7 patients with pNENs and 3 with GI-NENs achieved an ORR of 43% and 33.3%, respectively. All responders had a high-grade disease. It is worth noting that 2 of the 3 patients with G3 pNET achieved an objective response. This result is a breakthrough in the application of ICIs in the treatment of G3 GEP-NET. Currently, a phase II study (NCT04969887) on evaluation of N+I in patients with immunotherapy-sensitive cancers including NECs and G3 NETs from CA209-538 has been registered and is expected to be completed in October 2024.

Another phase II basket study of N+I for the treatment of rare tumors called SWOG DART (NCT02834013) was recently reported. In one cohort, 32 patients with epNENs (excluding SCLC, about 50% have GI-NENs) had a significantly higher response rate for high-grade neoplasms than for middle/low-grade neoplasms (ORR 44% *vs* 0%, $P =$

0.004) with no difference in overall ORR between the different primary origins[15]. Based on the results of the cohort, a second and dedicated cohort of 19 individuals with G3 NENs was explored[46]. Although all patients were microsatellite-stable, the results of the cohort revealed a moderate ORR of 26%. Unfortunately, none of the above cohorts performed an analysis of G3 NENs according to differentiation. A similar dilemma existed in a retrospective study at Moffet Cancer Center and the Mayo Clinic, where G3 NEN patients achieved an ORR of 14.75%. Therefore, determining the efficacy of N+I in the treatment of well-differentiated G3 GEP-NETs is challenging.

Durvalumab (anti-PD-L1) in combination with tremelimumab (anti-CTLA-4) (D+T) is another dual ICI therapy that has been studied for advanced NENs of GEP or lung origin. The initial results of DUNE, a prospective phase II multi-cohort study, were presented at the 2020 ESMO Annual meeting[47]. Cohort 4 consisting of 33 G3 GEP-NENs achieved an ORR of 9.1%, and the clinical benefit rate of 36.1% at 9-mo paved the way for a phase III clinical trial of D+T. Regrettably, comparable to N+I, no independent examination of patients with well-differentiated tumors was conducted, although the efficacy of G3 was higher than that of G1-2.

The above results suggest that dual ICIs have moderate overall efficacy in patients with advanced G3 GEP-NENs. Comparatively, the N+I regimen appears to have a greater response rate than D+T. The success of these therapies, however, must be demonstrated in a large number of patients with well-differentiated G3 GEP-NETs.

Chemotherapy combined with ICI

Current research on chemotherapy combined with ICIs, in the field of GEP-NENs, has a small sample size and the majority of them include previously treated NECs. The ORRs vary from 9% for pembrolizumab plus dealers' choice chemotherapy to 36% for nivolumab plus temozolomide[29,48,49]. Since most NEC patients are sensitive to chemotherapy, it is necessary to compare the above results with the data of chemotherapy alone as a second- or later-line treatment option. This comparison will establish whether NEC patients can significantly benefit from the above combination therapy. As previously described, G3 NETs are insensitive to platinum-based chemotherapy. Unfortunately, G3 GEP-NET, as a well-differentiated tumor with high proliferative activity, has not been specifically included in any prospective clinical trials for chemotherapy combined with ICI.

Other immunotherapies

Immunotherapies currently being investigated in prospective clinical trials related to G3 GEP-NET include classic PD-1 inhibitors combined with other therapies such as LAG525 (LAG-3 inhibitor), ^{177}Lu-DOTATATE (PRRT), and liver-directed therapies (Transarterial Embolization or ^{90}Yttrium Microsphere Radioembolization). In addition, the immune tumor vaccines Survivin Long Peptide Vaccine (SurVaxM) and Dendritic Cells Loaded with Autologous Tumor Homogenate (DC vaccine) have entered phase I and phase II clinical trials, respectively. Both vaccines are currently at the subject recruitment stage.

Predictive biomarkers for immunotherapies

The potential of a given patient with G3 GEP-NET to respond to immunotherapies is still largely unknown. NETs can be considered as immunologically "cold" due to their lack of immunoactive cellular components, low tumor antigens, *etc*.[50,51].

Immunohistochemical assessment of PD-L1 expression and its role in predicting response to ICIs is an incredibly hot topic. However, in the KEYNOTE-28 study, pNETs with positive PD-L1 expression achieved a low ORR of 6.3%[21]. In the KEYNOTE-158 study, all 4 GEP-NET patients who achieved PR had negative PD-L1 expression[22]. Besides, in a joint analysis of two prospective, non-randomized trials, no difference in DCR, PFS, or OS was observed between the PD-L1-negative and PD-L1-positive groups with G3 NENs[23]. In contrast, in the phase Ib trial of toripalimab in the treatment of patients with NENs (Ki-67 ≥ 10%) described above, patients with PD-L1 expression ≥ 10% had better ORR than those with PD-L1 < 10% (50.0% *vs* 10.7%, P = 0.019)[30]. Therefore, it appears that considering merely the negative or positive expression of PD-L1 is insufficient for identifying GEP-NET patients who may benefit from ICIs and that quantifying PD-L1 expression appears to be more significant. Furthermore, only 10% of tumors expressed PD-L1 in a large cohort of 136 patients with G3 GEP-NENs and those tumoral cells with positive PD-L1 were all in poorly differentiated cases[52]. Therefore, it is necessary to combine PD-L1 with other predictive biomarkers to better predict the population that may benefit from immuno-

therapy.

For other biomarkers, both high TMB (TMB-H) and microsatellite instability-high (MSI-H)/deficient mismatch repair protein (dMMR) are independent adverse prognostic factors for NENs[53] and also have an important predictive value. Wang *et al*[54] reported that 50% of the 18 Chinese patients with NETs had TMB-H. In a NET cohort analyzed by Patel *et al*[15], found no difference in the PD-L1 positivity rate between G3 and G1/G2 tumors, while the TMB-H rate was significantly higher in G3 NENs independent of tumor origin. Large samples of clinical and genomic data demonstrated that TMB-H was associated with increased survival in patients treated with ICI across various cancer types[55]. Duan *et al*[56] discovered that half of pNEN patients had decreased expression of MMR, another important biomarker. Venizelos *et al*[57] recently reported that MSI occurred in only 5.3% (8/152) of GEP-NEC patients and 3.4% (1/29) of G3 GEP-NET patients.

Pre-treatment assessment of one or more of these biomarkers provides a new perspective for screening good responders to immunotherapy.

CONCLUSION

In this minireview, data from prospective clinical trials and retrospective studies on the role of immunotherapies on G3 GEP-NET has been screened and reviewed. For ICI monotherapy, the efficacy of pembrolizumab, spartalizumab, and avelumab on G3 GEP-NETs is very limited. Only toripalimab has shown a moderate clinical activity on NENs with Ki-67 ≥ 10%, PD-L1 expression ≥ 10%, or high TMB. In addition, the ORR of well-differentiated tumors treated with toripalimab was slightly better than that of poorly differentiated cancers. Toripalimab and surufatinib therapy did not cause disease remission in 4 patients with G3 NETs. However, the treatment did not prevent remission in NEC and G1-2 NETs. Therefore, these regimens could potentially be effective in the treatment of G3 GEP-NETs if a large number of subjects are included. In other studies, the N+I therapy achieved PD in 2 of 3 patients with G3 NET as well as moderate efficacy in high-grade NENs. These results suggest that N+I may represent an extremely promising treatment option for G3 NET.

At present, all clinical trials investigating G3 GEP-NET are either phase I or phase II studies with small sample sizes. In this study, several challenges were encountered when collecting and evaluating data on the efficacy of immunotherapies for G3 GEP-NETs. According to the 2010 WHO classification, the inclusion of high-grade NETs in studies of NECs, the lack of Ki-67 index data in well-differentiated tumors, and the inclusion of tumors derived from lung, esophageal or unknown tissue all contribute to significant heterogeneity in reported results. Additionally, the review mainly focuses on the ORR to evaluate the potential role of immunotherapies in the treatment of G3 NETs. This is because the majority of prospective studies are ongoing and the survival data are in their infancy. Therefore, it is necessary to conduct prospective clinical trials with a large sample size of pathologically confirmed G3 GEP-NETs to evaluate the efficacy of the above immunotherapies. Besides, referencing data from important biomarkers facilitates the screening of patients who may benefit.

ACKNOWLEDGEMENTS

The authors express their gratitude to the scholars who have conducted prospective and retrospective studies on immunotherapies in the treatment of NENs.

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World J Gastroenterol 2021 December 21; 27(47): 8138-8155

DOI: 10.3748/wjg.v27.i47.8138

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

ORIGINAL ARTICLE

Basic Study

Impact of intrarectal chromofungin treatment on dendritic cells-related markers in different immune compartments in colonic inflammatory conditions

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Author contributions: Kapoor K, Eissa N and Ghia JE conceived and designed the experiments; Bernstein CN collected the human tissue and collated the human data; Kapoor K and Eissa N performed the experiments; Kapoor K, Eissa N, Ghia JE and Bernstein CN analyzed the data; Kapoor K, Eissa N, Tshikudi D and Ghia JE wrote and assembled the paper; all authors have read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the University of Manitoba Research Ethics Board [HS14878 (E)].

Institutional animal care and use committee statement: All the experiments were conducted under protocols #15-010 and #19-014, approved by the University of Manitoba Ethics Committee under Canadian animal research guidelines.

Conflict-of-interest statement:

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Abstract

BACKGROUND

Chromofungin (CHR: chromogranin-A 47-66) is a chromogranin-A derived peptide with anti-inflammatory and anti-microbial properties. Ulcerative colitis (UC) is characterized by a colonic decrease of CHR and a dysregulation of dendritic CD11c$^+$ cells.

AIM

To investigate the association between CHR treatment and dendritic cells (DCs)-related markers in different immune compartments in colitis.

METHODS

A model of acute UC-like colitis using dextran sulphate sodium (DSS) was used in addition to biopsies collected from UC patients.

RESULTS

Intrarectal CHR treatment reduced the severity of DSS-induced colitis and was associated with a significant decrease in the expression of CD11c, CD40, CD80, CD86 and interleukin (IL)-12p40 in the inflamed colonic mucosa and CD11c,

CD80, CD86 IL-6 and IL-12p40 within the mesenteric lymph nodes and the spleen. Furthermore, CHR treatment decreased CD80 and CD86 expression markers of splenic CD11c$^+$cells and decreased NF-κB expression in the colon and of splenic CD11c$^+$ cells. In vitro, CHR decreased CD40, CD80, CD86 IL-6 and IL-12p40 expression in naïve bone marrow-derived CD11c$^+$DCs stimulated with lipopolysaccharide. Pharmacological studies demonstrated an impact of CHR on the NF-κB pathway. In patients with active UC, CHR level was reduced and showed a negative linear relationship with CD11c and CD86.

CONCLUSION

CHR has protective properties against intestinal inflammation *via* the regulation of DC-related markers and CD11c$^+$ cells. CHR could be a potential therapy of UC.

Key Words: Chromofungin; Chromogranin-A; Colitis; Cytokines; Dendritic cells; Gut hormones

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Core Tip: Ulcerative colitis (UC) is characterized by a colonic decrease of chromofungin (CHR: chromogranin-A 47-66) and a dysregulation of CD11c$^+$dendritic cells (DC). Using a UC-like model (dextran sulphate sodium) and biopsies collected from UC patients, we demonstrated a protective effect of CHR *via* the regulation of DC-related markers and CD11c$^+$cells at the colonic, mesenteric lymph node and spleen levels, through a potential effect *via* the NF-κB pathway. In patients with active UC, CHR level showed a negative linear relationship with CD11c$^+$marker. CHR could be a potential therapy of UC, but larger samples and additional experiments are needed.

Citation: Kapoor K, Eissa N, Tshikudi D, Bernstein CN, Ghia JE. Impact of intrarectal chromofungin treatment on dendritic cells-related markers in different immune compartments in colonic inflammatory conditions. *World J Gastroenterol* 2021; 27(47): 8138-8155
URL: https://www.wjgnet.com/1007-9327/full/v27/i47/8138.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i47.8138

INTRODUCTION

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease (CD), disorders in which the gastrointestinal (GI) tract becomes ulcerated and inflamed[1,2]. To date, the exact etiopathology and mechanism behind them are unknown. It is believed that genetic predisposition and environmental factors lead to an abnormal mucosal immune response in the intestinal lining[3-5]. Over the last decade, the diseases have increased worldwide, but the greatest prevalence is evident in western countries[6,7]. Although treatments and therapeutic strategies are evolving quickly, treatments are still inadequate for a substantial percent of those with active IBD, and some therapies may have serious adverse side effects. Therefore, IBD needs new therapeutic approaches associated with higher efficacy and limited side effects[8].

IBD is characterized by an ongoing inflammatory process manifested by an increase of immune cell infiltration and accompanied by an up-regulation of pro-inflammatory cytokines, including interleukin (IL)-6 and IL-12p40[9]. The continuing release of mediators damages the intestinal epithelium, leading to abnormal activities of enterochromaffin cells (EC), goblet cells and Paneth cells, and impaired tissue repair[10,11]. UC is represented by an overactivation of the adaptive immune system represented by T cells which is considered a consequence of abnormal activation of innate immune cells[12] such as dendritic cells (DCs)[10,11,13-17].

DCs are produced by the bone marrow and can be located in the mucosal tissues or circulate in the lymph tissues and the blood[18]. DCs interaction and antigen presentation to T cells and their subsequent polarization can be induced within mesenteric lymph nodes (MLNs) and the spleen[19]. In UC, as in other chronic inflammatory diseases, DC activation is represented by an up-regulation of theCD80, CD86, and CD11c markers and is associated with an increased release of IL-6 and IL-12p40 to

Bernstein CN has been on the advisory boards for Abbvie Canada, Amgen Canada, Bristol Myers Squibb Canada, Janssen Canada, Roche Canada, Sandoz Canada, Takeda Canada, Pfizer Canada and consulted to Takeda and Mylan Pharmaceuticals. He has received educational grants from Abbvie Canada, Pfizer Canada, Takeda Canada, Janssen Canada. He has been on speaker's panel for Abbvie Canada, Medtronic Canada and Janssen Canada. The other authors declare that they have no conflicts of interest.

Data sharing statement: All data generated or analyzed during this study are included in this published article.

ARRIVE guidelines statement: The authors have read the ARRIVE Guidelines, and the manuscript was prepared and revised according to the ARRIVE Guidelines.

Supported by Crohn's and Colitis Canada, No. P46601; Canadian Foundation for Innovation, No. P39902; Children's Hospital Research Institute of Manitoba, No. P45040; Natural Sciences and Engineering Research Council, No. P41224; and Manitoba Health Research Council, No. P41118 (to Ghia JE).

Country/Territory of origin: Canada

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): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Open-Access: This article is an open-access article that was selected by an in-house editor and

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Received: March 12, 2021
Peer-review started: March 12, 2021
First decision: May 1, 2021
Revised: May 12, 2021
Accepted: December 7, 2021
Article in press: December 7, 2021
Published online: December 21, 2021

P-Reviewer: Watanabe T
S-Editor: Gao CC
L-Editor: A
P-Editor: Gao CC

promote a Th1 cell polarization[20,21]. In UC, the IL-12p40 pathway's importance has been confirmed by genome-wide association studies, identifying the IL-12 genes as a major contributor[22,23]. Intracellularly, the production of IL-12p40 is regulated by different pathways, including NF-κB. In IBD, a significant increase in NF-κB p65 has been observed in the gut epithelial and immune cells isolated from colonic biopsies [24]. More specifically related to DCs, the NF-κB pathway plays a crucial role in maintaining their intracellular activation responsible for T-cell polarization[25]. Therefore, the excessive pro-inflammatory cytokine production can in part be related to the overactivation of DCs in the gut[26]. Nevertheless, in certain conditions, the level and expression of DCs activation markers are not quite equivalent when comparing the different immune compartments (*i.e.* colon, MLN, spleen)[19].

Chromogranin-A (CHGA) is the second most prominent protein found in the secretory granules of ECs in the GI tract. For decades, CHGA has been known to be associated with IBD, but few data have demonstrated a plausible mechanism of association due to the lack of a specific CHGA receptor[27]. An increase in the total number of endocrine cells defined as CHGA-immunoreactivity has been described in several cohorts of patients with active IBD[28]. In parallel, the serum CHGA concentration has been reported to be significantly increased compared to controls[29-32]. CHGA is considered a prohormone and can be the precursor of several bioactive peptides having a wide variety of functions in the human body affecting the cardiovascular, endocrine, neuroendocrine and immune systems[33]. One of the peptides generated, CHR ($CHGA_{47-66}$), encoded by Exon-IV located at the N-terminal section of CHGA, has been demonstrated to depict anti-fungal and anti-microbial activities[34]. Recently, in the context of UC and experimental colitis, new data highlighted an essential anti-inflammatory role through epithelial cells and peritoneal macrophage regulation[25], but no data exist regarding the broader effect within the immune system and its different compartments.

Taken together, we hypothesized that CHR regulates DCs activation in the context of UC experimental colitis, and we evaluated CHR's effects on different DCs-related markers at various immune compartments and defined a potential intracellular pathway implicated.

MATERIALS AND METHODS

Human participants

Biopsies were collected from participants undergoing colonoscopy with known UC or with no IBD. A total of four biopsies were taken from inflamed sites identified endoscopically in persons with active UC (n = 10) and from normal tissue from healthy participants (n = 10). Biopsy collection was approved by the University of Manitoba Research Ethics Board. Participants were ≥ 18 years. All concomitant IBD therapies, including 5-amino salicylates, corticosteroids, and methotrexate, were permitted in active UC patients. Healthy control individuals had no history of abdominal afflictions, especially IBD. For more details, see Kermarrec *et al*[20]. The individuals in the control group were not on any regular medication at the time of the study. The University of Manitoba Research Ethics Board [HS14878 (E)] approved the study.

Animal and ethics statement

All the experiments were conducted under protocols # 15-010 and #19-014, approved by the University of Manitoba Ethics Committee under Canadian animal research guidelines. In-house groups of 6 male C57BL/6 mice (six to eight weeks old) with bodyweight between 20 g to 25 g were used. All animals were kept in the specific pathogen-free barrier facility maintained at the University of Manitoba animal care facility. Animals were kept in a 12-h dark/light cycle and fed ad libitum.

Peptide

$CHGA_{47-66}$: RILSILRHQNLLKELQDLAL[35-39] were synthesized by a solid-phase method and purified by reverse-phase high-performance liquid chromatography to reach < 98% purity (Pepmic Co., Suzhou, China). As previous studies from our laboratory demonstrated an absence of effect of the scramble version[25], this group was not added to our experimental plan.

Dextran sulphate sodium-induced experimental colitis and experimental plan

Based on previously published data, CHR's dose was adjusted to 2.5 mg/kg per day

[40]. The 1% of phosphate buffer saline (PBS, 1%) (Life Technology, Grand Island, NY, United States) was given intra-rectally to the controls[40], all the injections of both CHR and PBS started one day before the induction of experimental colitis and lasted for 5 d. Dextran sulphate sodium (DSS) (molecular weight, 40 kDa; MP Biomedicals, Soho, OH, United States) was added to regular drinking water and freshly replaced every two days at a concentration of 5% (wt/vol) for 5 d till the mice are sacrificed [41]. The average consumption of DSS was noted per cage each day. Animals were randomized, 6 mice per group were assigned for each experiment. Time matching of the controls was done with mice receiving normal drinking water only.

Assessment of DSS-induced colitis

Disease activity index (DAI) included a combined score of weight loss, stool consistency, and bleeding was recorded daily[42] from day zero to day five during DSS treatment. Blood in the stool was evaluated using the Hemoccult II test (Beckman Coulter, Oakville, ON, Canada). Colon length and macroscopic scores[42] were assessed on sacrifice day.

Spleen and MLN isolation

After the induction of DSS colitis on sacrifice day, the spleen and MLN were collected and digested in 2 mg/mL collagenase D (Roche Diagnostics, Meylan, France) dissolved in RPMI 1640 (Life Technologies) at 37 °C for 30 min associated with a 10 min intermittent shaking. To stop the reaction and disrupt the DC-T cell complexes, the cell suspension was supplemented with 5 mmol/L EDTA (Sigma, Mississauga, ON, Canada) during the last 5 min. The homogenate was then passed through a 70-mm cell strainer (VWR, Mississauga, ON, Canada) and then washed with RPMI-1640 at 1200 rpm for 5 min. Red blood cells (RBC)'s lysis was done using ACK lysis buffer (150 mmol/L NH4Cl, 10 mmol/L KHCO3, 0.1 mmol/L EDTA; Life Technologies).

MACS CD11c sorting

EasySep™ Mouse CD11c Positive selection kit (Stemcell Technologies) was used according manufacturer's instructions. The cells were then collected, counted and used for stimulatory experiments.

Isolation and stimulation of bone marrow-derived DCs

Six to eight weeks naïve mice were sacrificed by cervical dislocation. In sterile conditions, the femur and tibia were removed and soaked in RPMI-1640 medium [containing 10% heat-inactivated fetal bovine serum (FBS), 25 mg/mL gentamicin, 2 mmol/L L-glutamine) (Life Technologies)]. Bone marrow cell extraction was performed by cutting both ends of the bones and flushing them with 1 mL of sterile RPMI-1640 into a sterile Petri dish. The cell suspension was collected and centrifuged for 5 min at 1000 rpm. The supernatant was discarded, and pellets were resuspended in RBC ACK lysis buffer (150 mmol/L NH4Cl, 10 mmol/L KHCO3, 0.1 mmol/L EDTA; Life Technologies) for the lysis of RBC. After the second centrifugation, the pellet was resuspended in RPMI-1640 and counted[43]. The purity of the cells was then verified by flow cytometry. Granulocyte-macrophage induced colony-stimulating factor (GM-CSF, Cedarlane, Burlington, ON, Canada) induced culture of bone marrow-derived DCs (BMDCs) were used for the cell culture. Cells were suspended at a concentration of 1×10^6 cells/mL in culture plates containing complete RPMI 1640 medium and stimulated with 20 ng/mL of GM-CSF. The cell culture was then put in an incubator at 37 °C with 5% CO_2. GM-CSF cultured media was replaced on the 3rd and 6th day. On the 8th day, the semi-suspended cells were collected with gentle pipetting, and the loosely attached cells were scraped using a cell scraper generating mature cells[43,44]. The $CD11c^+$ cells (1×10^6 per well) isolated *via* MACS were cultured in complete RPMI 1640 medium (Life Technologies) containing 10% heat-inactivated FBS, 25 mg/mL, gentamicin and 2 mmol/L L-glutamine (Life Technologies) in 12-well plates in the presence or absence of 10^{-6} M of CHR for 12 h. After 12 h, they were stimulated with 100 ng/mL of lipopolysaccharide (LPS; Sigma) for 24 h. The cell culture was collected for quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis, and ELISA assessed the supernatant. To determine the intracellular pathway, pharmaceutical blocker/activator, betulinic acid (NF-κB activator, 10 μmol/L; Sigma) or BAY 11-7082 (NF-κB inhibitor, 10 μmol/L; Sigma) were added to the culture medium for 24 h at the same time of LPS.

qRT-PCR

Total RNA from colonic tissue, MLN, splenocytes, CD11c+ splenic and $CD11c^+$ BMDC

isolated cells was extracted using TRIzol™ Plus RNA Purification Kit (Life Technologies) and reverse-transcribed using SuperScript VILO cDNA Synthesis Master Mix (Life Technologies). Gene expression was measured by a qRT-PCR using a Roche Light Cycler 96 Real-Time System and Power SYBR green master mix (Life Technologies). Differences in the threshold cycle (ΔCt) number between the target genes and the optimal reference gene Eukaryotic elongation factor 2 (*Eef2*)[45] were used to calculate differences in expression. Human and mouse primer sequences used are provided in Tables 1 and 2.

RT2-profiler qPCR array

RT2 profiler™ PCR array mouse dendritic and antigen-presenting cell (Qiagen Inc, Toronto, ON Ref #330231) was performed for the quantitative PCR according to the manufacture instructions to profile the expression of 84 essential genes. Changes in gene expression between different experimental groups and the heat maps (colour-coded graphs with groups in columns and genes in rows) were generated using the web-based program of RT2 profiler PCR array data analysis.

Protein quantification

Enzyme-linked immunosorbent assays (ELISAs) were used for protein quantification from full-thickness tissue homogenates and/or supernatants from the cell cultures. Mouse commercial ELISA kits were used to detect IL-6 and IL-12p40 (R & D Systems, Inc., Minneapolis, United States).

Statistical analysis

Data are expressed as the mean ± SE. Unpaired Mann-Whitney *U* test was applied to compare between two groups. To compare between more than two groups, One-Way ANOVA followed by a post-hoc test was used. The analysis DAI repeated measure Two-Way ANOVA followed by a post-hoc test was used. To analyze the association between different markers studied, Spearman's correlation test was used. The statistical two-tailed significance level was determined at $P = 0.05$. GraphPad Prism software (version 6; GraphPad Software, Inc, La Jolla, CA, United States) was used to compute the statistics.

RESULTS

In colitic mice, in vivo CHR treatment decreased colonic inflammatory macro- and micro-scopic scores

As reported previously[40], we confirmed CHR's anti-inflammatory effect on experimental colitis induction. CHR treatment (2.5 mg/kg/day, *i.r.*) resulted in a marked reduction in the external DAI (Figure 1A). No difference was seen within the first two days, but starting day 3, a significant difference was detected, culminating at a 3-fold reduction on day 5 when compared with the colitic group. We also confirmed the beneficial effect of the CHR treatment on the macroscopic score ($P < 0.0002$) when distal colonic sections were examined (Figure 1B).

In colitic mice, in vivo CHR treatment decreases whole colonic section DCs-related surface and functional markers

RT²-PCR array analysis of DCs-related membrane and functional markers was conducted on whole colonic sections. As presented in Figure 1C, whole colitic samples isolated from mice treated with CHR demonstrated a significant downregulation of surface and intracellular markers, notably: CD86 4.43-fold ($P < 0.04$, $n = 6$ per group), CD80 4.21-fold ($P < 0.04$, $n = 6$ per group) and NF-κB1 11.01-fold ($P < 0.02$, $n = 6$ per group). To confirm the data, gene expression level using a single target qRT-PCR technique was used. Development of colitis induced a significant colonic increase of mRNA expression of surface markers 86 ($P = 0.0089$, $n = 6$ per group) and CD80 ($P = 0.0005$) when compared with the non-colitic PBS group (Figure 2A and B). Treatment with CHR significantly decreased the two markers ($P = 0.0001$ and $P < 0.0001$ respectively, $n = 6$ per group). Besides, two others DC-related markers not present in our initial array were quantified. Induction of colitis induced a significant colonic increase of CD40 ($P < 0.0001$, $n = 6$ per group) and CD11c ($P < 0.0001$, $n = 6$ per group) when compared with the non-colitic PBS group. The same pattern of significant decrease was found for CD40 ($P = 0.0002$, $n = 6$ per group) and CD11c ($P < 0.0001$, $n = 6$ per group) (Figure 2C and D) in colon treated with CHR when compared with the

Table 1 Human primers sequences

Gene name	Forward (5'-3')	Reverse (3'-5')
INFg	GCCTATCTGTCACCATCTCATC	CCTCTGGCTGCTGGTATTTAT
CCR7	GTTCAAATCTCAGGTCCCTCTC	TACCCTGGTGATCCTCATCTTA
CD74	CCCAAGGAAGAGCCAATGT	CATGGCCCTGAAAGCTGATA
Il12p40	ACCAGAGCAGTGAGGTCTTA	CTCCTTTGTGACAGGTGTACTG
CD86	CAGACCACATTCCTTGGATCA	CCGCTTCTTCTTCTTCCATTTC
TBP	CCCGAAACGCCGAATATAATCC	AATCAGTGCCGTGGTTCGTG
CD11C	ACTCAGATCGGCTCCTACTT	TCGGGTCTGCTCGTAGTAAT
IL12A	ATTCCAGAGAGACCTCTTTCATAAC	CCACCTGGTACATCTTCAAGTC
IL23A	CAGGTCACTATTCAATGGGATGC	GCAGTTCTTAATTGCTGCTTGG

Table 2 Mouse primers sequences

Gene	Forward (5'-3')	Reverse (3'-5')
IL6	CCTGAACCTTCCAAAGATGGC	TTCACCAGGCAAGTCTCCTCA
CD86	TTACGGAAGCACCCACGATG	ACTACCAGCTCACTCAGGCT
CD11c	CCAAGACATCGTGTTCCTGATT	ACAGCTTTAACAAAGTCCAGCA
Eef2	TGTCAGTCATCGCCCATGTG	CATCCTTGCGAGTGTCAGTGA
CD80	TCGGCGCAGTAATAACAGTC	GTTTCTCTGCTTGCCTCATTTC
CD40	GTCACACAGGAGGATGGTAAAG	AAAGCAGTTCCAGGGTTCAG
IL12p40	TGGTTTGCCATCGTTTTGCTG	ACAGGTGAGGTTCACTGTTTCT
CCR7	TGGCTCTCCTTGTCATTTTCCA	CTTGAAGCACACCGACTCGTA
IFNg	CTCTTCCTCATGGCTGTTTCT	TTCTTCCACATCTATGCCACTT

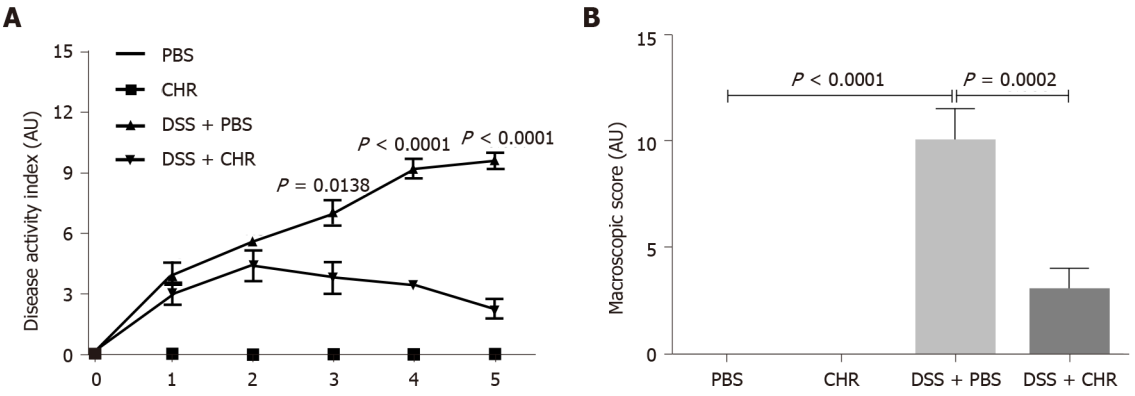
untreated group. Besides, a significant increase in colonic mRNA expression and protein quantification of IL-12p40 ($P < 0.0001$, $n = 6$ per group) were detected in colitic conditions when compared with the untreated group, and CHR treatment decreased those two markers ($P < 0.0001$ and $P < 0.0043$ respectively, $n = 6$ per group) (Figures 2E and F) when compared with the untreated group.

In colitic mice, in vivo CHR treatment decreases MLN DCs-related surface and functional markers

Next, we assessed the regulation of the different markers within the MLN. Induction of colitis was associated with a significant increase of CD86 ($P < 0.0001$, $n = 6$ per group), CD80 ($P < 0.0001$, $n = 6$ per group) and CD11c ($P < 0.0001$, $n = 6$ per group) mRNA expression (Figures 3A-C) when compared with non-colitic PBS group. In colitic mice, when compared with PBS group, CHR treatment decreased significantly CD86 ($P < 0.0001$, $n = 6$ per group) (Figure 3A), CD80 ($P < 0.0001$, $n = 6$ per group) (Figure 3B) and CD11c ($P < 0.0001$, $n = 6$ per group) (Figure 3C) mRNA expression. Also, induction of colitis induced a significant increase of IL-12p40 ($P < 0.0001$, $n = 6$ per group) and IL-6 ($P < 0.0001$, $n = 6$ per group) within the MLN when compared with the PBS group, and CHR treatment significantly decreased those two markers ($P < 0.0001$, $n = 6$ per group) (Figure 3D and E) when compared with untreated group. No significant changes were observed for CD40 (data not shown).

In colitic mice, in vivo CHR treatment decreases splenocytes DCs-related surface and functional markers

We assessed the expression of the different markers within the spleen cells. Induction of colitis was associated with a significant increase of splenocytes CD86 ($P < 0.0001$, $n = 6$ per group), CD80 ($P < 0.0001$, $n = 6$ per group) and CD11c ($P < 0.0001$, $n = 6$ per group) mRNA expression (Figures 4A-C) when compared with PBS group. In colitic



C

Gene symbol	Fold regulation	*P* value
Cd86	-4.43	< 0.04
Cd80	-4.21	< 0.04
Nfkb	-11.1	< 0.02

Figure 1 Chromofungin (chromogranin-A 47-66) treatment decreases dextran sulphate sodium-induced experimental colitis and colonic colonic cluster of differentiation markers and NF-κB gene expression in dextran sulphate sodium-induced experimental colitis. Colitis was induced with 5% dextran sulphate sodium in drinking water to C57BL/6 male mice, and the control group of mice received regular drinking water. Chromofungin (2.5 mg/kg per day, *i.r.*) was given as a preventive treatment or phosphate buffer saline (1%) starting from one day before the induction of colitis till the fifth day when the mice were sacrificed. A-C: External disease activity index recorded over the period of 5 d (A), macroscopic scores at sacrifice day (B), CD86 and 80 and NF-κB gene expression profile by RT^2 profiler PCR array at sacrifice day (C). Unpaired two-tailed Mann-Whitney *U* and Two-way repeated measures or One-way ANOVA followed by multiple comparison tests were used to analyze the data, and adjusted *P* equal to or smaller than 0.05 is believed to be significant. Each value represents the mean ± SE, *n* = 6 mice/group. PBS: Phosphate buffer saline; CHR: Chromofungin; DSS: Dextran sulphate sodium.

mice, when compared with untreated group, CHR treatment significantly decreased splenocytes' CD86 ($P < 0.0001$, $n = 6$ per group), CD80 ($P < 0.0001$, $n = 6$ per group) and CD11c ($P < 0.0001$, $n = 6$ per group) (Figures 4A-C) mRNA expression. Induction of colitis induced a significant increase of splenocytes mRNA expression of IL-12p40 ($P < 0.0001$, $n = 6$ per group) and IL-6 ($P < 0.0001$, $n = 6$ per group) when compared with the PBS group and CHR treatment was associated with a significant decrease of IL-12p40 ($P < 0.0001$, $n = 6$ per group) and IL-6 ($P < 0.0001$, $n = 6$ per group) (Figures 4D and E) when compared with the colitic PBS group. No significant changes were observed for CD40 (data not shown).

In colitic mice, in vitro CHR treatment decreases splenic CD11c⁺ cells DCs-related surface and functional markers

Next splenic CD11c$^+$ cells were isolated from the colitic group treated or not with CHR *via* the MACS technique. As presented in Figure 5, RT^2-PCR array analysis demonstrated a significant downregulation of DC-related surface and intracellular markers after CHR treatment: CD86 (4.0-fold) ($P < 0.04$, $n = 6$ per group), CD80 (3.8-fold) ($P < 0.04$, $n = 6$ per group) and NF-κB (20-fold) ($P < 0.04$, $n = 6$ per group) marker.

CHR decreases DC-related markers in BMDCs treated with LPS or NF-κB activator

Finally, we determined the intracellular pathway implicated. In BMDCs CD11c$^+$ cells, treatment with NF-κB stimulator significantly increased the mRNA expression of CD86 ($P = 0.0088$, $n = 6$ per group), CD80 ($P = 0.0026$, $n = 6$ per group), CD40 ($P = 0.0119$, $n = 6$ per group) and CHR treatment abolished the increase (Figures 6A-C). LPS treatment increased the mRNA expression of the same three markers, and treatment with NF-κB inhibitor or CHR abolished the increase. CHR treatment decreased the harmful effect of LPS stimulation even in the presence of the NF-κB activator.

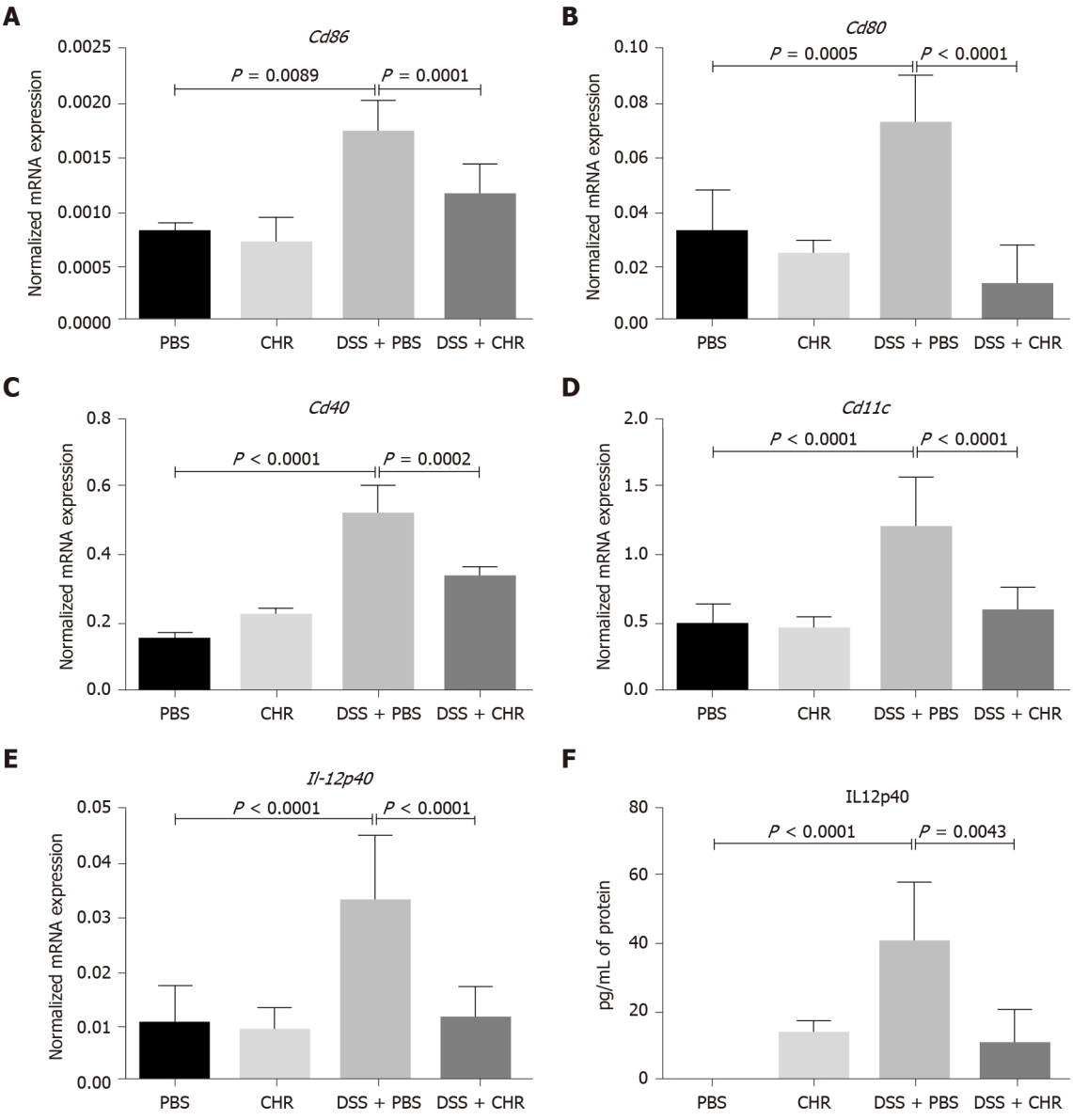


Figure 2 Chromofungin (chromogranin-A 47-66) treatment decreases colonic cluster of differentiation markers and interleukin-12 level in dextran sulphate sodium-induced experimental colitis. Colitis was induced with 5% dextran sulphate sodium in drinking water to C57BL/6 male mice, and the control group of mice received regular drinking water. Chromofungin (2.5 mg/kg per day, *i.r.*) was given as a preventive treatment or phosphate buffer saline (1%) starting from one day before the induction of colitis till the fifth day when the mice were sacrificed. A-F: CD86 (A), CD80 (B), CD40 (C), CD11c (D), interleukin (IL)-12p40 mRNA expression (E), IL-12p40 protein level (F). mRNA expression was quantified by quantitative real-time reverse-transcription polymerase chain reaction and protein level by ELISA. One-way ANOVA followed by multiple comparison tests was used to analyze the data, and adjusted *P* equal to or smaller than 0.05 is believed to be significant. Each value represents the mean ± SE, *n* = 6 mice/group. PBS: Phosphate buffer saline; CHR: Chromofungin; DSS: Dextran sulphate sodium.

In parallel, the NF-κB stimulator increased significantly the protein concentration of IL-6 ($P < 0.0001$, $n = 6$ per group) and IL-12p40 ($P < 0.0001$, $n = 6$ per group) and CHR treatment significantly decreased the level of the two inflammatory proteins (Figure 6D and E). LPS treatment increased significantly the level of IL-6 ($p < 0.0001$, $n = 6$ per group) and IL-12p40 ($P < 0.0001$, $n = 6$ per group), and NF-κB inhibitor or CHR treatments decreased the status of the two markers. CHR treatment decreased the deleterious effect of LPS stimulation even in the presence of an NF-κB activator.

Relationship between CHGA-Exon-IV and DCs-related markers in colonic biopsies of active UC patients

Colon biopsies from UC patients were analyzed using qRT-PCR to assess the relationship between *CHGA-Exon-IV* and $CD11c^+$ cells-related markers. We used our previously published data on *Exxon-IV*[25] to perform a new correlation analysis. Our data demonstrated an upregulation of surface markers related to DCs (Figure 7A and

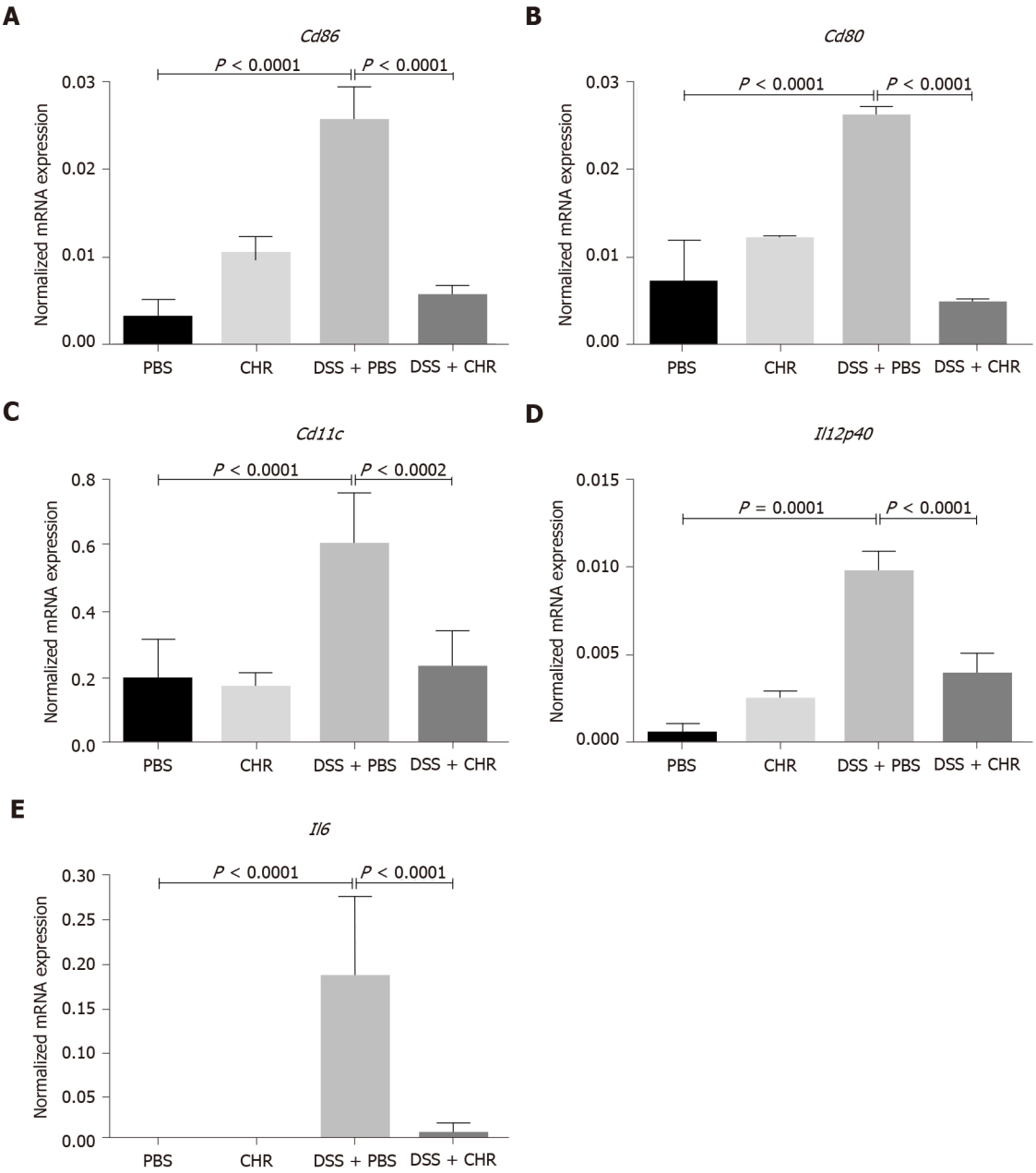


Figure 3 Chromofungin (chromogranin-A 47-66) treatment decreases mesenteric lymph node cluster of differentiation markers and cytokine levels in dextran sulphate sodium-induced experimental colitis. Colitis was induced with 5% dextran sulphate sodium in drinking water to C57BL/6 male mice, and the control group of mice received regular drinking water. Chromofungin (2.5 mg/kg per day, *i.r.*) was given as a preventive treatment or phosphate buffer saline (1%) starting from one day before the induction of colitis till the fifth day when the mice were sacrificed. A-E: CD86 (A), CD80 (B), CD11c (C), interleukin (IL)-12p40 (D), IL-6 mRNA expression (E). mRNA expression was quantified by quantitative real-time reverse-transcription polymerase chain reaction. One-way ANOVA followed by multiple comparison tests was used to analyze the data, and adjusted *P* equal to or smaller than 0.05 is believed to be significant. Each value represents the mean ± SE, *n* = 6 mice/group. PBS: Phosphate buffer saline; CHR: Chromofungin; DSS: Dextran sulphate sodium.

B): CD86 ($P = 0.0002$, $n = 10$ per group), CD11c ($P = 0.0002$, $n = 10$ per group). *Exon-IV* showed a strong to moderate negative correlation with DCs-related surface markers (Figure 7C and D) such as CD86 ($r = -0.6765$, $P = 0.0051$, $n = 10$ per group), CD11c ($r = -0.4647$, $P = 0.0710$, $n = 10$ per group).

DISCUSSION

Overactivation of the immune system through the dysregulation of DCs and its subsequent T-cell polarization is one of the cytokine storm features seen in inflamed mucosa[46]. Over the last five years, a lot of attention has been given to CHGA and its derived peptides in regulating the immune components. However, little is known as to

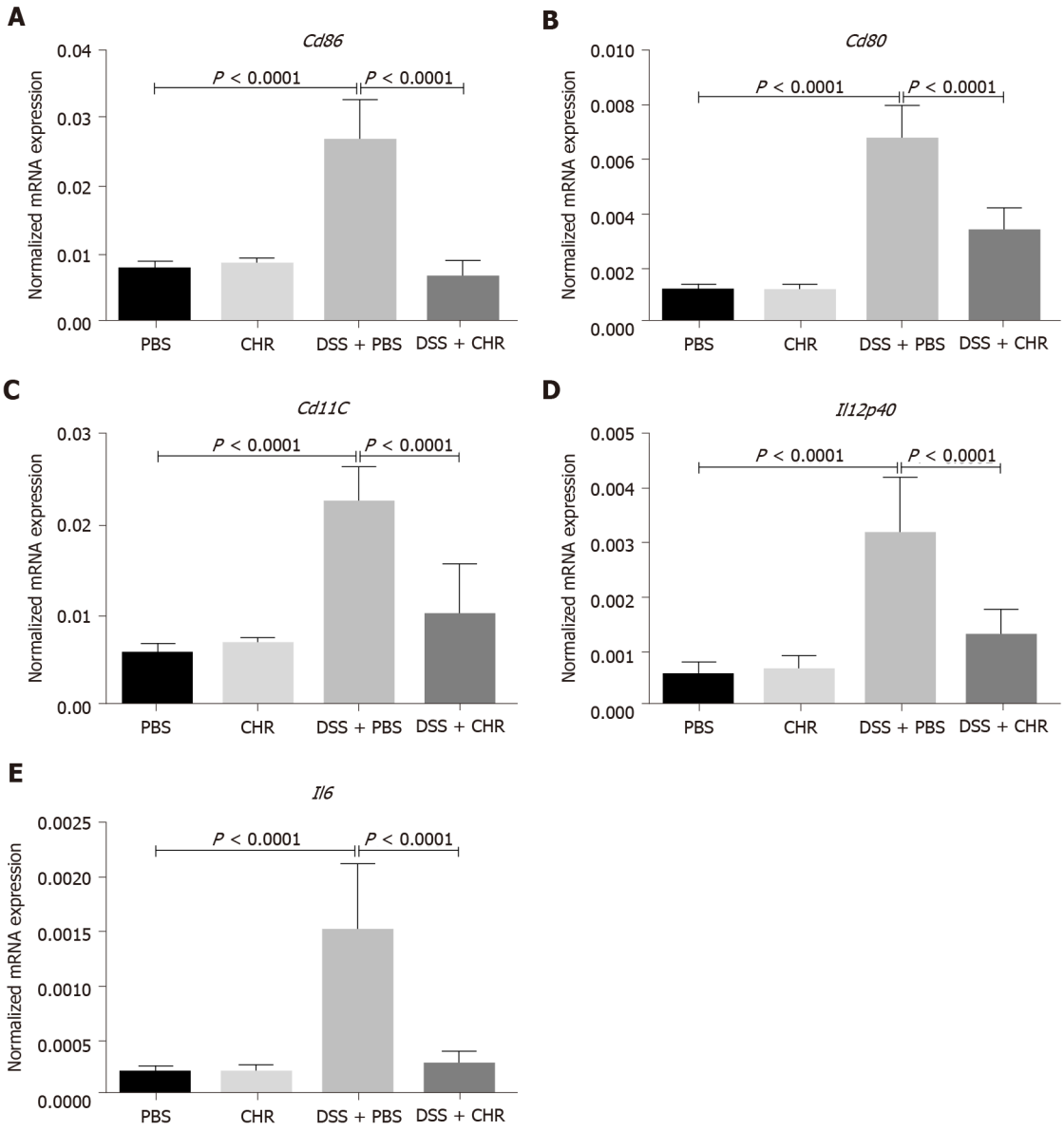


Figure 4 Chromofungin (chromogranin-A 47-66) treatment decreases splenocyte cluster of differentiation markers and interleukin-12 level in dextran sulphate sodium-induced experimental colitis. Colitis was induced with 5% dextran sulphate sodium in drinking water to C57BL/6 male mice, and the control group of mice received regular drinking water. Chromofungin (2.5 mg/kg per day, *i.r.*) was given as a preventive treatment or phosphate buffer saline (1%) starting from one day before the induction of colitis till the fifth day when the mice were sacrificed. A-E: CD86 (A), CD80 (B), CD11c (C), interleukin (IL)-12p40 (D), IL-6 mRNA expression (E) from splenic cells. mRNA expression was quantified by quantitative real-time reverse-transcription polymerase chain reaction. One-way ANOVA followed by multiple comparison tests was used to analyze the data, and adjusted *P* equal to or smaller than 0.05 is believed to be significant. Each value represents the mean ± SE, *n* = 6 mice/group. PBS: Phosphate buffer saline; CHR: Chromofungin; DSS: Dextran sulphate sodium.

how those peptides can modulate innate immune cells in different immune compartments. Using an experimental colitis model mimicking UC and colonic biopsies obtained from participants with active UC compared with biopsies from healthy controls, we assessed CHR's impact in regulating DCs-related markers. Our findings suggest a novel effect by which CHR promotes a down-regulation of DCs-related functional markers *via* the NF-κB pathway within different immune compartments.

In our UC-like experimental colitis model, we first confirmed the beneficial effect of CHR on qualitative and quantitative inflammatory markers described in our previous publications[25,27,40]. Because of the peptide's specific action demonstrated in our previous study on different cytokines like TNF-α[25,40], we did not consider using a scramble version; this can be regarded as a limitation. However, in a few experimental plans (not shown), the scramble version was used, and no significant effect was seen. With CHR treatment, DAI and macroscopic scores were significantly downregulated, and in addition to the decrease previously seen in IL-6[25], there was a significant decrease in the concentration of IL-12p40 at the mucosal level. This decrease was also

Gene symbol	Fold regulation	*P* value
Cd86	-4.40	< 0.04
Cd80	-3.80	< 0.04
Nfkb	-20.01	< 0.04

Figure 5 Chromofungin (chromogranin-A 47-66) treatment decreases dextran sulphate sodium-induced experimental colitis and colonic cluster of differentiation markers and NF-κB gene expression in dextran sulphate sodium-induced experimental colitis. CD86 and 80 and NF-κB gene expression profile by RT^2 profiler polymerase chain reaction array at sacrifice day. One-way ANOVA followed by multiple comparison tests were used to analyze the data, and adjusted *P* equal to or smaller than 0.05 is believed to be significant. Each value represents the mean ± SE, *n* = 6 mice/group.

seen at the mRNA level, suggesting the peptide's ability to down-regulate pro-inflammatory markers at the protein and gene levels. These data confirm GWAS studies, which have shown the importance of several APC-related cytokines, most prominently, the ones related to DCs: IL-6, IL-12 which have a critical role during the development of UC[47].

In our study, experimental colitis induced a significant colonic increase of DCs-associated markers CD86, CD80, CD40 and CD11c mRNA expression, and CHR treatment decreased all of them. DCs play a critical role during inflammation[48] as they activate and differentiate T cells and play an essential role in regulating adaptive immunity by releasing IL12p40[20]. At the colonic level, $CD11c^+$ cells show a crucial role in IBD progression, colitis being associated with an increased level of $CD11c^{hi}$ cells [46]. In parallel, in inflammatory conditions like asthma, arthritis, other markers like CD86, 80 and 40 are highly expressed in $CD11c^+$ cells[49,50]. T-cell activation can be blocked by suppressing CD86 and CD80[51,52] and the release of IL-12p40 or 6[53,54]. Therefore, our data support the hypothesis that disruption of intestinal DCs-related markers can contribute to the perpetuation of the inflammatory process seen in IBD and that attenuation of DC's activation could lead to a better outcome.

DCs are essential in detecting antigens at the mucosal level and presenting them within other immune compartments, where, within the MLN, their roles in the regulation of immune activation are pivotal[55]. Human DCs, specifically myeloid (mDCs) $CD11c^+$ $DC\text{-}SIGN^+$, are characterized by producing many cytokines and further activating the adaptive immune cells[56-58]. In the context of gut inflammation, increased $CD11c^{hi}$ cells' level is found in the draining MLN and associated with a marked enhancement of $CD11c^{hi}HLADR^{int}$ DC[46]. In IBD patients, plasmacytoid DCs isolated from MNL exhibit overexpression of CD86, CD80 and an increase of IL-12p40 and IL-6 is seen[55]. In our study, in the context of experimental colitis, our data confirmed an up-regulation of CD86, 80 and 11c markers and IL-12p40 and IL-6. We demonstrated a significant decrease in CD86, CD80 and CD11c in the group treated with CHR. This highlights CHR's critical role in regulating CD markers and depicts the treatment's effectiveness in controlling IL-12p40 and IL-6 within the MNL. Surprisingly no effects were seen on CD40.

In addition to the MLN, the spleen plays a significant role in the crosstalk between the innate and adaptive immune systems. Within the spleen, $CD11c^+$ DCs act as first responder cells during the immune response development[59]. Studies have shown that surface costimulatory molecules CD86, 80, and 40 present on isolated splenic $CD11c^+$ cells are modified during the inflammatory process[60]. Using splenocytes isolated cells from colitic mice, our study confirmed these elements by depicting a decreased expression of CD86, CD80 and CD11c, and IL-12p40 and IL-6 levels when treated with CHR. We were also able to confirm the importance of the $CD11c^+$ population, as our RT^2-PCR array demonstrated a significant decrease of CD86 and 80 in that population. This was consistent with our data presented in our next experimental plan when we determined the direct impact of CHR on the $CD11c^+$ BDMC cell population. Again here, like within the MLN, no effect was visible on the expression of CD40.

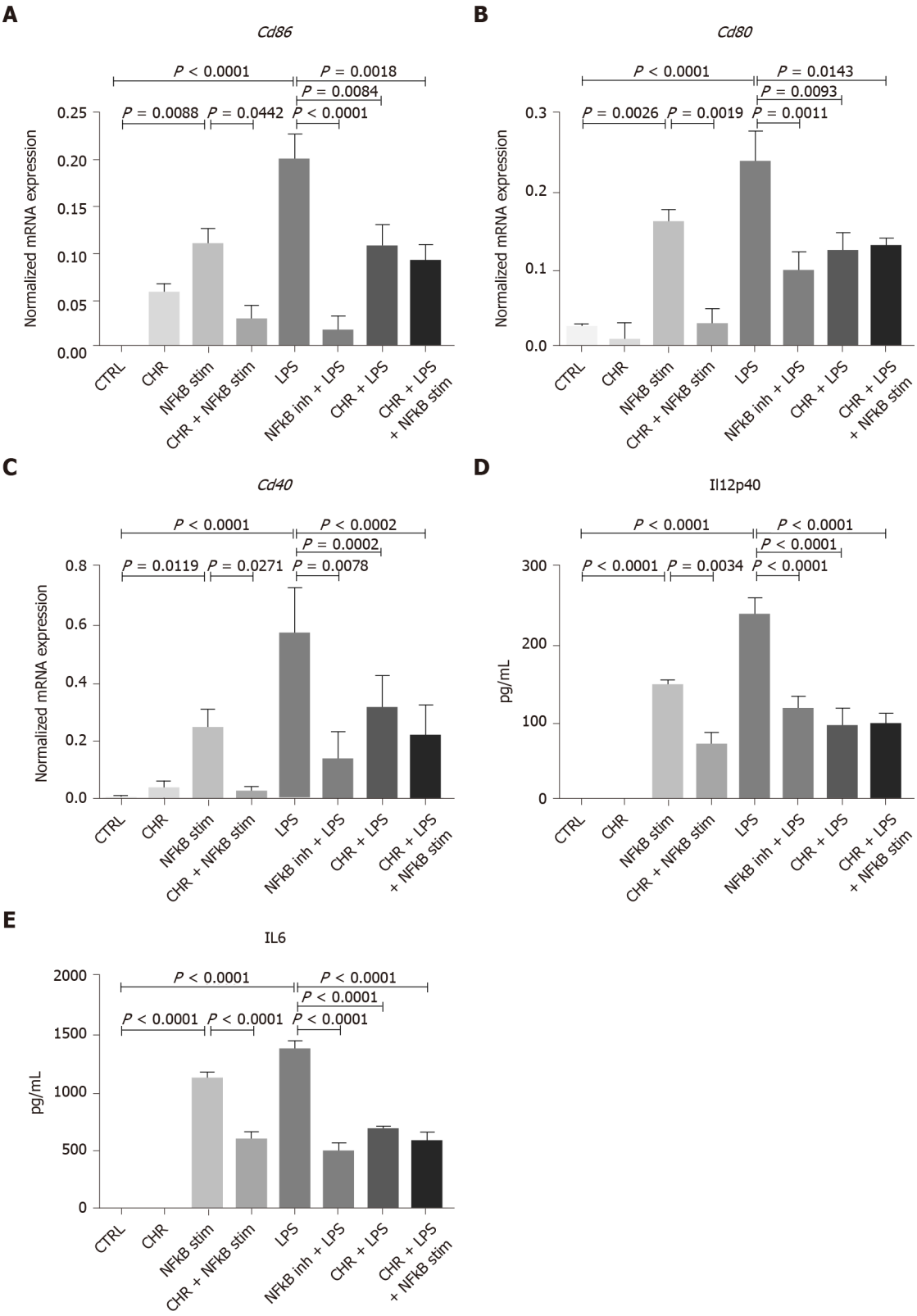


Figure 6 Chromofungin (chromogranin-A 47-66) treatment decreases lipopolysaccharide-stimulated bone marrow-derived CD11c⁺ cells' cluster of differentiation markers and cytokine-associated level *via* the NF-κB pathways. Bone marrow-derived CD11c⁺ cells using MACS technique were isolated and cultured with granulocyte-macrophage induced colony-stimulating factor for 8 d until full maturation. Cells were treated with CHR (10^{-6} M/mL) for 12 h and then stimulated with lipopolysaccharide (100 ng/mL) in the presence or absence of NF-κB activator/stimulator (10 u/mL) for 24 h. A-E: CD86 (A), CD80 (B), CD40 (C), mRNA expression and IL-12p40 (D), IL-6 (E) medium protein level. mRNA expression was quantified by quantitative real-time reverse-transcription polymerase chain reaction and protein levels were quantified by ELISA. One-way ANOVA followed by multiple comparison tests was used to analyze the data, and adjusted *P* equal to or smaller than 0.05 is believed to be significant. Each value represents the mean ± SE, *n* = 6 mice/group. LPS: Lipopolysaccharide; CHR: Chromofungin.

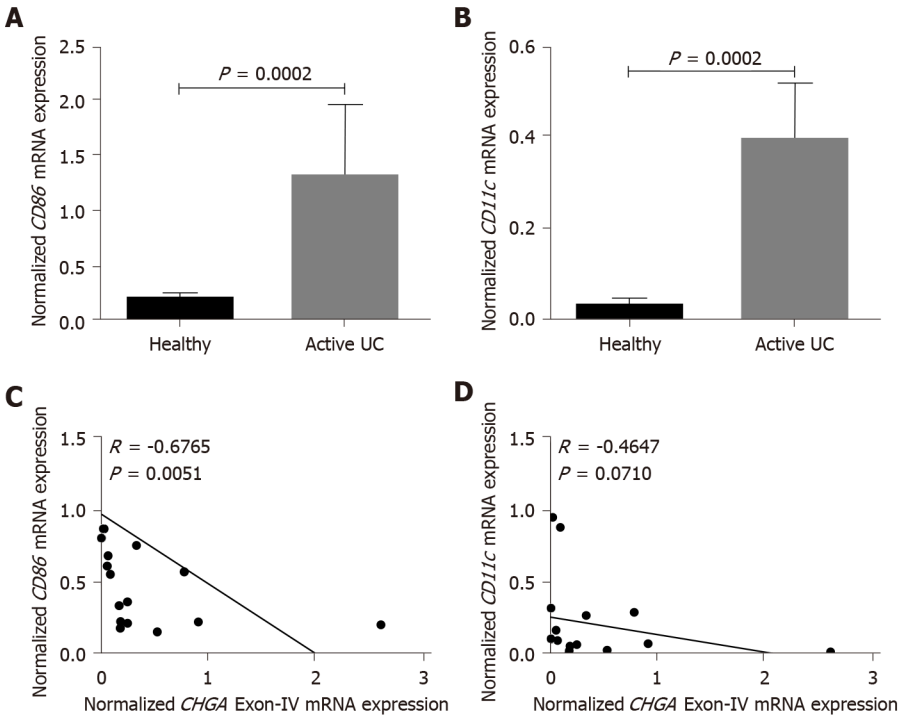


Figure 7 Chromofungin (CHGA Exon-IV) correlates negatively with cluster of differentiation 86 and 80 mRNA expression. A: mRNA expression of CD86 in the colonic tissue of healthy individuals (n = 10) and participants with active ulcerative colitis (UC) (n = 10); B: Correlation analysis between biopsy CHGA Exon IV and CD86 mRNA expression; C: mRNA expression of CD80 in the colonic tissue of healthy individuals (n = 10) and participants with active UC (n = 10); D: Correlation analysis between biopsy CHGA Exon IV and CD80 mRNA expression. mRNA expression was quantified by quantitative real-time reverse-transcription polymerase chain reaction. Student t-test and Mann-Whitney test and Spearman's correlation were used to analyze, and adjusted P equal to or smaller than 0.05 is believed to be significant. UC: Ulcerative colitis.

In the context of acute and chronic inflammatory conditions, several inflammatory cytokines are regulated by NF-κB, and previous studies have reported that blocking NF-κB in mice can be considered as a potential treatment for preventing gut inflammation[61-65]. NF-κB plays an essential role in regulating the expression of CD86, CD80, CD40 and CD11c[66-68]. In our study, the RT²-PCR array performed on colonic colitic samples, and splenic CD11c⁺ cells demonstrated a down-regulation of NFκB1 after CHR treatment. Consistent with our *in vivo* data, mimicking an inflammatory activation state, mouse LPS-stimulated CD11c⁺ BMDCs showed an increase of CD86, 80, 40 mRNA expression. The difference in CD40 expression between the colon, the MLN, spleen, and the CD11c⁺ BMDCs can be explained by a potential weak stimulus of CHR in that marker in an *in vivo* condition in contrast to a strong effect when the peptide had a direct action on the CD11c⁺ BMDCs, as presented by other when deciphering the impact of commensal bacteria on DC cells in different immune compartments[69].

In addition, the presence of CHR abolished colitis in the same way as the NF-κB inhibitor. To confirm NF-κB as a potential target, using an NF-κB stimulator, we demonstrated the impact of CHR's inhibitory effect on the detrimental impact induced by the stimulator. Our *in vitro* evaluation also showed a significant decrease in IL-6 and IL-12p40 in LPS-stimulated CD11c⁺ BMDCs. As CHR can decrease the effect caused by the LPS and NF-κB stimulator, we can postulate that the targeted protein resides at one of the NF-κB targets. These data agree with human data, showing that anti-IL-12p40 monoclonal antibodies have demonstrated excellent clinical efficacy in a group of UC patients[70]. In the clinical and experimental models of colitis, NF-kB has been demonstrated to be a major immune regulator[61,62,65] when inflammation is developed. However, at that stage, we cannot confirm if the effect of CHR is a direct or an indirect effect on NFkB. Many upstream pathways can be modulated, as for example, TLR4[71], ER stress[71]. However, there are some early indications that the phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/Akt) pathway maybe be of interest[72], as CHR can produce a cardioprotective action by regulating the PI3K pathways[37]. Thus, considering the previous literature related to NF-κB and IBD, our results support the concept that CHR can potentially decrease intestinal inflammation by regulating the NF-κB pathway in CD11c⁺ cells.

We previously demonstrated that *CHGA* (*Exon-IV*) was highly down-regulated in UC patients showing that the disease is affecting the levels of *CHGA* (*Exon-IV*)[27,73]. As a novel finding, in colonic biopsies from UC participants, we demonstrated that CD11c and its related costimulatory surface markers CD86 were significantly upregulated, leading to a potential enhancement of colitis. Furthermore, in active UC participants, we observed a negative correlation between the *CHGA* (*Exon-IV*) with CD86 markers. These alterations can be explained by previous data, which demonstrated changes at the level of the ECs during the inflammatory process[32]. Our small sample size likely led to our inability to show a significant difference in CD11c bearing cells.

Some limitations exist in our study. Regarding the overall impact of $CD11c^+$ cell deactivation, we did not demonstrate a direct impact on T-cell activation, a regulation that remains elucidated *via* the use of a co-culture model. Further studies are also required to investigate the functional consequences of CHR on other immune cells or epithelial cells and angiogenesis, all implicated in the pathogenesis of colitis. Moreover, we acknowledge that $CD11c^+$ cells isolated from the lamina propria need to be studied, and new studies should define if DC expressing CD11c markers are the main target of CHR. Additional biopsies need to be added to determine a compelling correlation between various markers, and a proteomics approach should be used for protein quantification in the colon, and all correlation analyses should be repeated accordingly.

Several other factors might lead to CHR's protective effect, which we have observed in our study. For example, previous studies demonstrated a link between gut microbiota and experimental colitis and human IBD, and there is evidence showing that CHR can have anti-microbial function[34,35]. Therefore, in our *in vivo* model, *i.r.* injection could have induced a microbial change in favour of beneficial bacteria; further metagenomic analyses are needed to study this aspect, but that would not account for the beneficial effect seen on $CD11c^+$ BMDCs.

CONCLUSION

In summary, our study shows that treatment with CHR led to lower colonic inflammation, which was associated with decreased levels of $CD11c^+$-associated cytokines through an NF-κB-dependent mechanism. Hence, CHR seems to act as an NF-κB blocker, reducing primary APCs' activation and ultimately decreasing the intestinal inflammatory process. CHR alone had no side effect on the mice or the cells, indicating CHR's relative safety at the studied dose. This previously unknown spatial impact of CHR in colonic inflammation may help broaden research done by other groups on the overall effect of CHGA and its derived peptides on immune regulation in the context of IBD. This might lead to future novel CHR-based therapies in IBD.

ARTICLE HIGHLIGHTS

Research background

Ulcerative colitis (UC) is a disorder in which the gastrointestinal tract becomes ulcerated and inflamed. Although treatments and therapeutic strategies are evolving quickly, treatments are still inadequate for a substantial percentage of those with active UC, and some therapies may have serious adverse side effects. Therefore, UC needs new therapeutic approaches associated with higher efficacy and limited side effects. In UC, there is an overactivation of innate immune cells such as macrophages and dendritic cells (DCs). Recently, in the context of UC and experimental colitis, new data highlighted an essential anti-inflammatory role of chromofungin (CHR), a chromogranin-A derived peptide, on peritoneal macrophages.

Research motivation

To evaluate CHR's effects on different DCs at various immune compartments and to define a potential intracellular pathway implicated.

Research objectives

To investigate the association between CHR treatment and DCs-related markers in colitis.

Research methods

Participants with active UC and a model of acute UC-like colitis using dextran sulphate sodium were used. We used cell culture and quantitative reverse transcription-polymerase chain reaction to analyze the relative expression levels of CD11c, CD80, CD86, interleukin (IL)-6 and IL-12p40 within the colonic samples, mesenteric lymph nodes and the spleen.

Research results

In a preclinical setting, CHR treatment the expression of CD11c, CD40, CD80, CD86 and IL-12p40 in the inflamed colonic mucosa and CD11c, CD80, CD86, IL-6 and IL-12p40 within the mesenteric lymph nodes and the spleen. In addition, CHR treatment decreased CD80 and CD86 expression markers of splenic $CD11c^+$ cells and decreased NF-κB expression in the colon and splenic $CD11c^+$ cells. *In vitro*, CHR decreased CD40, CD80, CD86, IL-6, and IL-12p40 expression in naïve bone marrow-derived $CD11c^+$ DC stimulated lipopolysaccharide. Using a pharmacological approach, we demonstrated the impact of CHR on the NF-κB pathway. In a clinical setting, in patients with active UC, CHR level was reduced and showed a negative linear relationship with CD11c and CD86.

Research conclusions

CHR has protective properties against intestinal inflammation, potentially through the regulation of DC-related markers and $CD11c^+$ cells.

Research perspectives

Although we have demonstrated that CHR may have a potential therapeutic interest, additional markers and detailed mechanisms of action need to be determined in a large sample.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 December 21; 27(47): 8156-8165

DOI: 10.3748/wjg.v27.i47.8156

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

ORIGINAL ARTICLE

Basic Study

Multiparameter magnetic resonance imaging of liver fibrosis in a bile duct ligation mouse model

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Institutional review board statement: The protocol of our study was approved by the Animal Care and Use Committee of the Second Xiangya Hospital of Central South University (Approval No. 2020495).

Conflict-of-interest statement: None of the authors have identified a conflict of interest.

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Abstract

BACKGROUND

Bile duct ligation (BDL) in animals is a classical method for mimicking cholestatic fibrosis. Although different surgical techniques have been described in rats and rabbits, mouse models can be more cost-effective and reproducible for investigating cholestatic fibrosis. Magnetic resonance imaging (MRI) has made great advances for noninvasive assessment of liver fibrosis. More comprehensive liver fibrotic features of BDL on MRI are important. However, the utility of multiparameter MRI to detect liver fibrosis in a BDL mouse model has not been assessed.

AIM

To evaluate the correlation between the pathological changes and multiparameter MRI characteristics of liver fibrosis in a BDL mouse model.

METHODS

Twenty-eight healthy adult male balb/c mice were randomly divided into four groups: sham, week 2 BDL, week 4 BDL, and week 6 BDL. Multiparameter MRI sequences, included magnetic resonance cholangiopancreatography, T1-weighted, T2-weighted, T2 mapping, and pre- and post-enhanced T1 mapping, were performed after sham and BDL surgery. Peripheral blood and liver tissue were collected after MRI. For statistical analysis, Student's *t*-test and Pearson's correlation coefficient were used.

Data sharing statement: No additional data are available.

Supported by the Natural Science Foundation of Hunan Province, No. 2019JJ40444 (to Xiao EH) and 2021JJ30945 (to Luo YH).

Country/Territory of origin: China

Specialty type: Gastroenterology and hepatology

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

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

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Received: April 18, 2021
Peer-review started: April 18, 2021
First decision: June 13, 2021
Revised: June 15, 2021
Accepted: September 19, 2021
Article in press: September 19, 2021
Published online: December 21, 2021

P-Reviewer: Hasan A
S-Editor: Wang LL
L-Editor: Kerr C
P-Editor: Li JH

RESULTS

Four mice died after BDL surgery; seven, six, five and six mice were included separately from the four groups. Signal intensities of liver parenchyma showed no difference on TI- and T2-weighted images. Bile duct volume, ΔT1 value, T2 value, and the rate of liver fibrosis increased steadily in week 2 BDL, week 4 BDL and week 6 BDL groups compared with those in the sham group ($P < 0.01$). Alanine aminotransferase and aspartate transaminase levels initially surged after surgery, followed by a gradual decline over time. Strong correlations were found between bile duct volume ($r = 0.84$), T2 value ($r = 0.78$), ΔT1 value ($r = 0.62$), and hepatic fibrosis rate (all $P < 0.01$) in the BDL groups.

CONCLUSION

The BDL mouse model induces changes that can be observed on MRI. The MRI parameters correlate with the hepatic fibrosis rate and allow for detection of cholestatic fibrosis.

Key Words: Liver; Fibrosis; Magnetic resonance imaging; Pathology; Animal model; Bile duct ligation

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Core tip: Magnetic resonance imaging (MRI) is promising for the noninvasive assessment of liver fibrosis, but the utility of multiparameter MRI in the bile duct ligation (BDL) mouse model has not been assessed. We established an experimental BDL mouse model that mimicked various aspects of cholestatic fibrosis and evaluated the potential of multiparameter MRI for the assessment of cholestatic fibrosis. We found that the BDL mouse model induced a complex cascade of changes that were observed clearly on MRI. The T2 and ΔT1 values were well correlated with the hepatic fibrosis rate and may allow for the detection of cholestatic fibrosis.

Citation: Liu JY, Ding ZY, Zhou ZY, Dai SZ, Zhang J, Li H, Du Q, Cai YY, Shang QL, Luo YH, Xiao EH. Multiparameter magnetic resonance imaging of liver fibrosis in a bile duct ligation mouse model. *World J Gastroenterol* 2021; 27(47): 8156-8165
URL: https://www.wjgnet.com/1007-9327/full/v27/i47/8156.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i47.8156

INTRODUCTION

An appropriate animal model and a reliable detection tool are crucial for investigating liver fibrosis. Cholestatic fibrosis, one of the main types of liver fibrosis, is a widespread disease with broad etiologies including genetics, immunity, infection, calculi, and tumors[1]. Obstruction of the biliary system induces a complex cascade of pathological and functional changes that leads to cholestatic fibrosis. Therefore, bile duct ligation (BDL) in animals has become a classical method for mimicking cholestatic fibrosis. In recent years, different surgical techniques have been described in rats and rabbits[2-4]. However, long-term experiments with rats or rabbits require a considerable work force, materials, and financial resources. The mouse has become the most popular experimental animal because of its higher genetic similarity with humans, lower cost, and more human-like responses to pathophysiological changes [5]. With careful surgical technique and the 3R rule for laboratory animals, the BDL mouse model can be more cost-effective and reproducible when subjected to the same conditions as the rat or rabbit models. The BDL mouse model may be the better choice for investigating cholestatic fibrosis.

Liver biopsy, with its invasiveness and subsequent related complications, is not suitable for screening and long-term monitoring in experimental animals[6]. Magnetic resonance imaging (MRI) is a promising tool for noninvasive assessment of liver fibrosis[7]. During the past decade, MRI has made great advances, and many versatile and powerful MRI sequences and methods have been widely applied, such as T1 mapping[8] and T2 mapping[9]. However, more comprehensive liver fibrotic features

on MR images, including radiological, quantitative and analytical aspects, are important. To the best of our knowledge, this has not been described in the BDL mouse model. Thus, it is appropriate to seek an effective way to evaluate the relationship between BDL mouse models and reliable MRI techniques accurately, particularly for noninvasive monitoring of liver fibrosis.

We hypothesized that the BDL mouse model can be noninvasively characterized radiologically using an MR imager and further validated with radiological, quantitative and analytical aspects. Accordingly, the purpose of our study was to establish and characterize the BDL mouse model and evaluate the correlation between the characteristics of hepatic fibrosis seen in MR images with both the pathological changes of liver fibrosis and the biochemical results matched with the histological specimens.

MATERIALS AND METHODS

The protocol of our study was approved by the Animal Care and Use Committee of the Second Xiangya Hospital of Central South University (2020495). All experiments were conducted in strict accordance with the Chinese law and institutional guidelines.

Animal model

Twenty-eight 7–8-wk-old male balb/c mice that initially weighed 28–30 g were used. The animals were exposed to a regular light–dark cycle and housed in an air-conditioned room at 26°C. Food and water were available at all times. Before surgery, all mice were randomly divided into sham and BDL groups and anesthetized with an intraperitoneal injection of 45 mg/kg pentobarbital sodium. After losing reflexes in response to a toe pinch, they were fixed on a mouse fixator in the supine position, and the skin on the chest and abdomen was prepared. For sham surgery procedures, only an upper abdominal incision with a length of ~2 cm was made before the abdominal suture. For BDL surgery, the liver was lifted using a moisturized (normal saline) cotton swab so that the liver was stuck to the diaphragm, and the gut was caudally moved with a humidified cotton swab to better expose the bile duct. Subsequently, the 4-0 nonabsorbable suture was placed around the bile duct and secured by two surgical knots. After replacing the abdominal organs to their physiological positions and closing the abdominal layers with 6-0 absorbable sutures, the mice were moved to a warm cage until they were fully awake and active.

MRI

All MR images were obtained using a 3.0-T clinical MRI scanner (Magnetom Skyra; Siemens Medical Solutions, Erlangen, Germany) with a custom-built rodent receiver coil with an inner diameter of 7 cm (Zhongzhi Medical Technologies, Suzhou, China). Before imaging, the mice were anesthetized with an intraperitoneal injection of 45 mg/kg pentobarbital sodium solution, and the respiration rate was monitored by periodic visual inspection. Following the acquisition of magnetic resonance cholangiopancreatography (MRCP), T1-weighted, T2-weighted, T2 mapping, and pre- and post-enhanced T1 mapping images were obtained. The sequences and parameters were used as follows: (1) MRCP images were generated using a 3D multi-shot fast spin-echo sequence (repetition time (TR)/echo time (TE) = 4585.0/404.0 ms) to generate heavily T2-weighted 3D volumetric images; (2) Transverse T1-weighted sequence (TR/TE = 3.3/1.7 ms; voxel size, 0.9 mm × 0.9 mm × 0.7 mm; slice thickness, 0.7 mm; and total examination time, 5 min 58 s); (3) Transverse T2-weighted sequence (TR/TE = 5361/111 ms; voxel size, 0.4 mm × 0.4 mm × 1.0 mm; slice thickness, 1.0 mm; and total examination time, 3 min 57 s); (4) T1 mapping sequence (TR//TE = 8.5/3.7 ms; two flip angles = 5°/15°; scan time, 5 min 20 s; and five averages acquired); and (5) T2 mapping sequence (TR =1950 ms, a multiple TE equally spaced from 11.1 ms to 55.5 ms; flip angle, 180°). For contrast-enhanced T1 mapping imaging, the images were collected at the tenth minute after intravenous injection of 0.1 mL gadoxetate disodium.

Image analysis

Two radiologists (Q-L.S, and Y-H.L) with > 5 years of abdominal diagnostic experience independently recorded the image data and resolved any discrepancies by consensus. Both radiologists were blinded to the histopathological results.

The MR data were imported into an image-processing workstation (Syngo; Siemens Medical Solutions). At least three circular regions of interest (ROIs) were randomly placed manually in the liver lobes to measure the signal intensities of routine sequences and mean values of the quantitative parameters. Care was taken to avoid moving artifacts and large vessels. Quantitative parameters for the liver, including T1 and T2 values, were extracted from relaxation pseudocolor maps in T1 and T2 mapping separately. ΔT1 value = (pre-enhanced T1 value) – (post-enhanced T1 value). The semiquantitative parameter, bile duct volume, was segmented by manually delineating each 2D slice and was estimated by adding up the individual voxel volumes inside the ROI.

Biochemic and histological evaluation

All mice were killed under general anesthesia after MRI examination to procure their livers and collect blood at defined time points, as mentioned above. Blood was collected to measure clinical chemistry parameters, including alanine aminotransferase (ALT) and aspartate transaminase (AST) levels. Liver specimens were soaked in a 4% phosphate-buffered formaldehyde solution for 48 h, fixed using paraffin, and transversely sectioned. Liver tissue sections were soaked in a water bath and baked for 1 h at 70°C. The baked sections were then stained with the Masson's Trichrome Staining Kit (G1006; Servicebio, Wuhan, China) and observed under an Olympus microscope (DP72; Olympus Corporation, Tokyo, Japan). The rate of liver fibrosis and fibrotic thickness of the bile duct were analyzed using ImageJ software, which was defined by the area of positive staining with blue color divided by the total area of the background.

Statistical analysis

Statistical analysis was performed using SPSS 22.0, and GraphPad Prism 7.0. Student's *t*-test was used to determine the differences in average values between the sham and surgically treated groups. The relationships between radiological and histological findings were investigated using Pearson's linear correlations. Statistical significance was set at $P < 0.05$. Statistical review of the study was performed by a biomedical statistician.

RESULTS

Animal model

A schematic diagram of BDL is shown in Figure 1. Of the 28 mice, four in the liver fibrosis group died after BDL surgery. Finally, seven, six, five and six mice were included separately from the sham group, week 2 BDL, week 4 BDL and week 6 BDL groups. The bile duct volume ($P < 0.01$) grew dramatically from 0.04 cm^3 in the sham group to 3.92 cm^3 in the week 4 BDL group after BDL surgery (Figure 2A). The mean levels of ALT and AST, biomarkers of hepatocellular injury, in the week 6 BDL group were lower than those in the week 2 BDL group ($P < 0.01$). The BDL mice showed good adaptability after the second week of BDL (Figure 2B).

MRI

Morphological changes in Figure 2B are demarcated on the MR images obtained with all sequences. Swollen bile ducts showed signal intensities similar to that of water on MRCP, and TI-weighted and T2-weighted images. The volume of the bile duct had an obvious rise from 0.04 to 3.92 cm^3 over time after BDL surgery. However, the signal intensities of the liver parenchyma did not show obvious differences upon perusal of the TI-weighted and T2-weighted images.

In MR functional sequences, dynamic documented pseudocolor maps qualitatively and quantitatively showed functional parameter changes (Figure 3A). T2 value increased with aggravation of liver fibrosis between the sham and BDL subgroups ($P < 0.01$, Figure 3B) from 28.9 to 52.1 ms. Aside from this, ΔT1 value for the sham group was lower than that of the week 6 BDL group with 352.1 and 675.8 ms, respectively ($P < 0.01$). In contrast, the pre-enhanced T1 values did not show a significant difference among the four groups.

Histopathological examination

The different areas for measurement of liver fibrosis, including intrahepatic bile duct and hepatic parenchyma, were compared with respect to the model duration

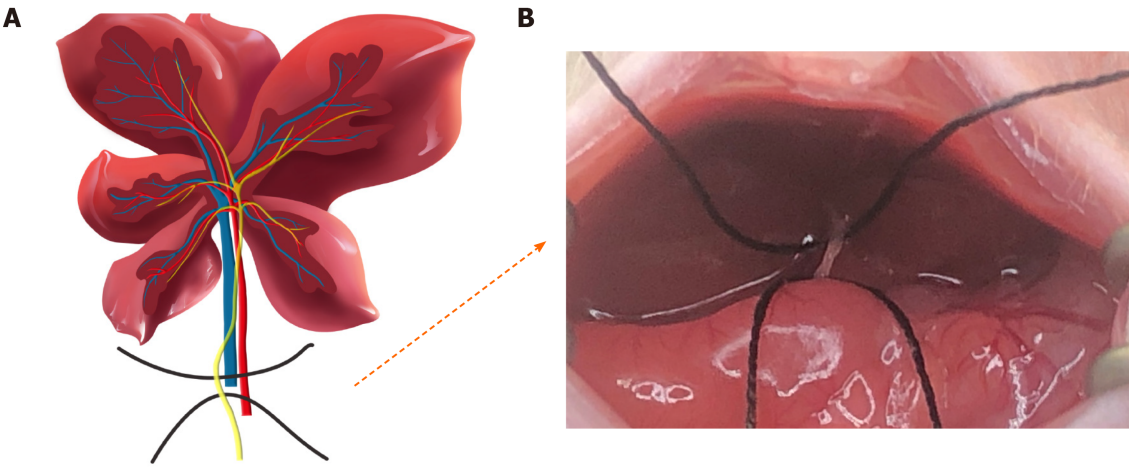


Figure 1 Schematic diagram of bile duct ligation. A: Anatomy of the liver, bile duct, portal vein, and hepatic artery in mice; B: Exposure of the bile duct.

(Figure 4). The area of hepatic fibrosis and fibrotic thickness of the bile duct increased significantly over time. The highest proportion of hepatic fibrosis (12.58%) was found in the week 6 BDL group, which exceeded that in the remaining groups ($P < 0.01$). The fibrotic thickness of the bile duct was highest (54.73 mm) in the week 6 BDL group, compared with that of the other groups ($P < 0.01$).

Correlation analysis

Correlation analysis between the tested parameters and the area of liver fibrosis was calculated (Figure 5). Histopathological examination revealed that the area of hepatic fibrosis showed steady growth. In the tested parameters, the changes in bile duct volume, T2 value, and ΔT1 value were in line with those of hepatic fibrosis. Also, the related coefficients of bile duct volume, T2 value, and ΔT1 value showed the manifest correlation with 0.84 ($P < 0.01$), 0.78 ($P < 0.01$) and 0.62 ($P < 0.01$), respectively. The levels of ALT and AST dramatically surged 2 wk after BDL surgery, followed by a gradual decline to the lowest values at week 6. The changes in ALT and AST levels differed from those in hepatic fibrosis. Thus, the correlation coefficients did not show valuable results ($P > 0.05$).

DISCUSSION

This study established an experimental BDL mouse model that mimicked various aspects of cholestatic fibrosis and evaluated the potential of multiparameter MRI for the noninvasive assessment of cholestatic fibrosis in the BDL mouse model.

The BDL model has been widely used to study cholestatic liver injury and the subsequent fibrosis. Surgical BDL can induce strong proliferation of bile duct cells, while the variable activation of oval cells (*i.e.* hepatic progenitor cells) depends on additional liver damage, leading to extensive bile duct reactions, cholestasis, portal inflammation, and rapid establishment of bile duct fibrosis[2-4]. This model was first established in rats and then successfully applied to rabbits and mice[5]. Unlike the BDL rabbit or rat model, the BDL mouse model may face more challenges, such as accurate operation, postoperative infections, and accidental injuries. In our study, standardized protocols with strict guidelines were followed, thereby enabling us to establish a mouse model of BDL, successfully.

Noninvasive liver fibrosis evaluation, including serum tests, ultrasound elastography, and magnetic resonance elastography (MRE), can overcome many limitations of liver biopsy and, therefore, are now incorporated into specialist clinical practice. These are valuable for ruling out advanced fibrosis or cirrhosis; however, each individual test cannot be fully predictive when used alone[10]. In this study, MRI was utilized in postoperative evaluation to noninvasively image and depict the changes in morphological and functional processes. Our results revealed that the BDL mouse model combined with multiparameter MRI is an innovative way to investigate liver fibrosis, and it induces a complex cascade of changes that can be observed clearly on MRI images.

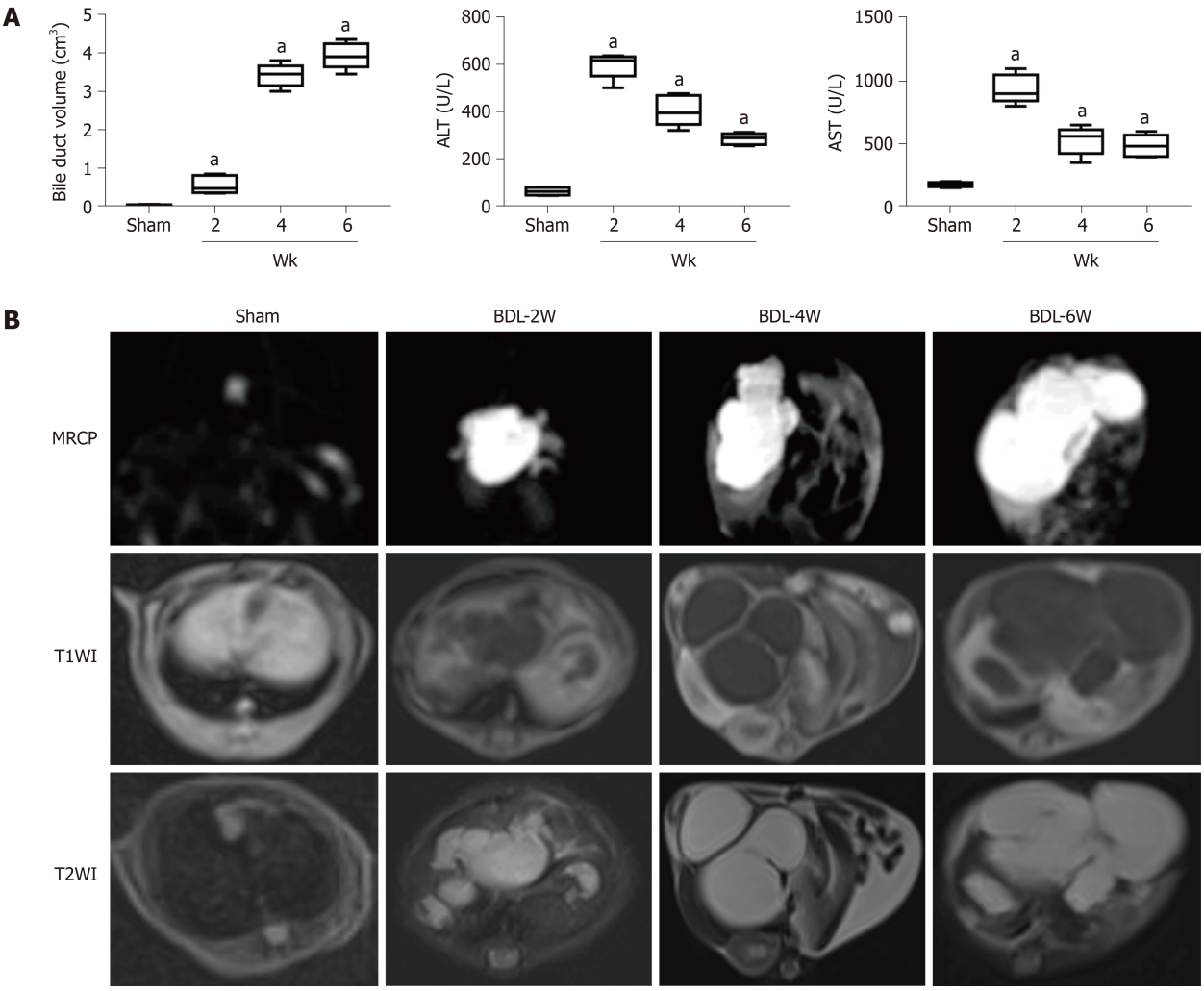


Figure 2 Magnetic resonance imaging findings and quantitative analysis. A: Box diagram of bile duct volume, ALT and AST in sham and BDL subgroups; B: Maximum cross-section of the bile duct or gallbladder on MRCP, T1-weighted, and T2-weighted images in sham and BDL subgroups. BDL: Bile duct ligation; MRCP: Magnetic resonance cholangiopancreatography; ALT: Alanine aminotransferase; AST: Aspartate transaminase. $^{a}P < 0.01$ for comparison between and sham and liver fibrosis subgroups.

Multiparameter MRI sequences including MRCP, T1-weighted, T2-weighted, T2 mapping, and pre- and post-enhanced T1 mapping were performed in the sham and BDL groups. The results from the present study revealed that: (1) Pre-enhanced sequences including MRCP, T1-weighted, and T2-weighted clearly showed the morphological changes in the BDL mouse model; (2) Compared with the sham group, T2 and ΔT1 values showed significant differences in the BDL groups; and (3) Bile duct volume, T2 value, and ΔT1 value also demonstrated a higher correlation with the hepatic fibrosis rate.

The T2 value in our study showed a significant increase with time after BDL surgery and a good correlation between the T2 value and the rate of liver fibrosis in the BDL mouse model. Zhang *et al*[11] have also shown that the T2 value increases with the development of fibrosis, and has a close relationship between inflammation, edema, and liver fibrosis in rat models. Similarly, Luetkens *et al*[9] indicated that T2 values correlated not only with stages of fibrosis but also with total collagen protein and transcription of collagen type I. It is reasonable to conclude that the prolonged T2 value may be attributable to collagen deposition and inflammation.

Owing to the characteristics of MRI data acquisition, the signal intensity obtained from contrast-enhanced MR images is affected by many technical parameters[12]. The signal intensity is not proportional to the gadolinium concentration and cannot be directly compared between sequences collected at different time points. In recent years, increasing attention has been paid to the measurement of T1 relaxation time from T1 mapping images[13]. The T1 value, which is proportional to the concentration of contrast medium *in vivo*, is more objective and reliable than direct measurement of the liver signal[2]. In the present study, an upward trend was observed in the ΔT1

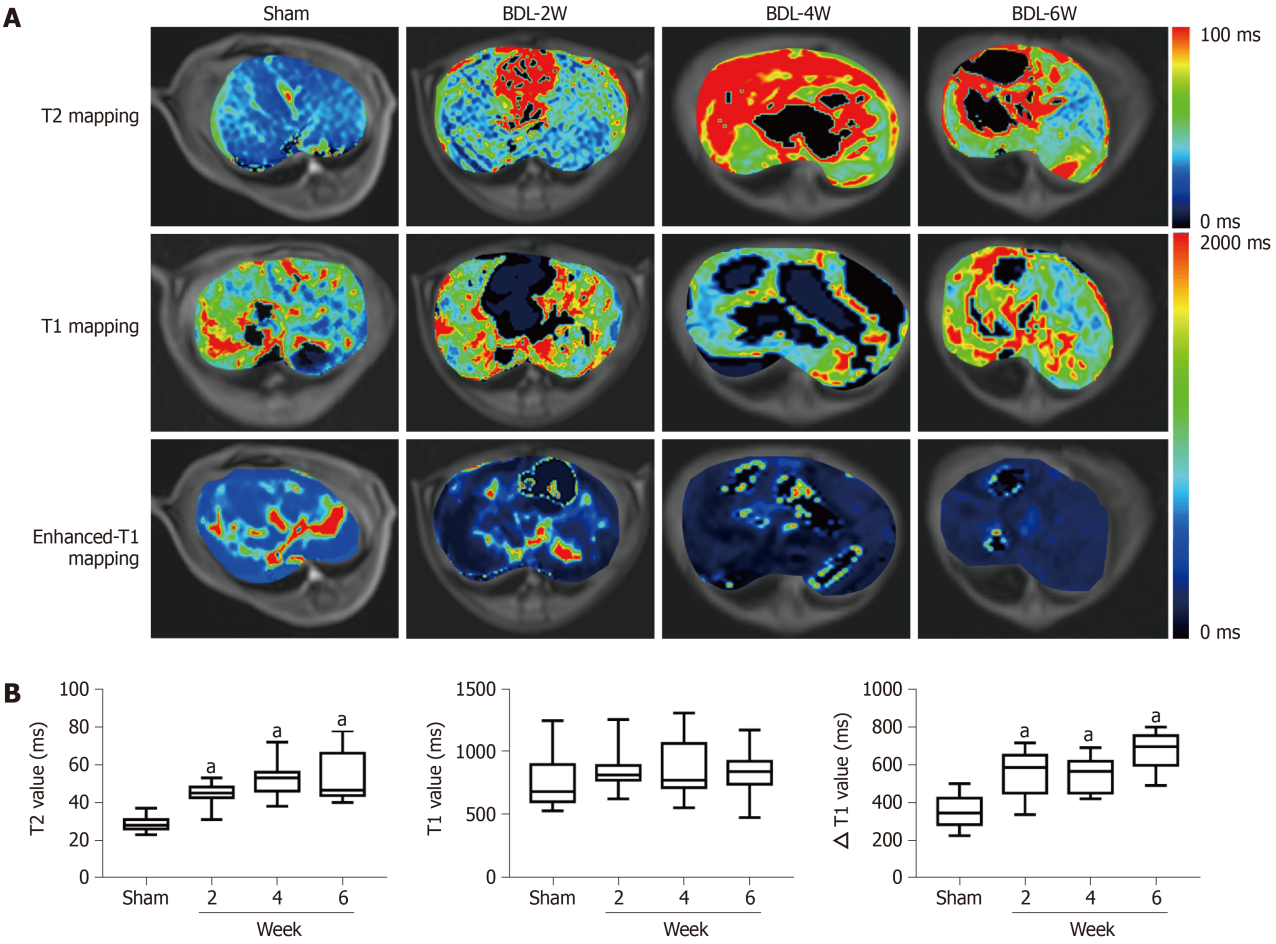


Figure 3 Functional parameter changes of magnetic resonance imaging and quantitative analysis. A: Maximum cross-section of liver parenchyma on T2 mapping, pre-, and post-enhanced T1 mapping in sham and BDL subgroups; B: Box diagram of T2 value, T1 value, and ΔT1 value in sham and BDL subgroups. BDL: Bile duct ligation. $^{a}P < 0.01$ for comparison between and sham and liver fibrosis subgroups.

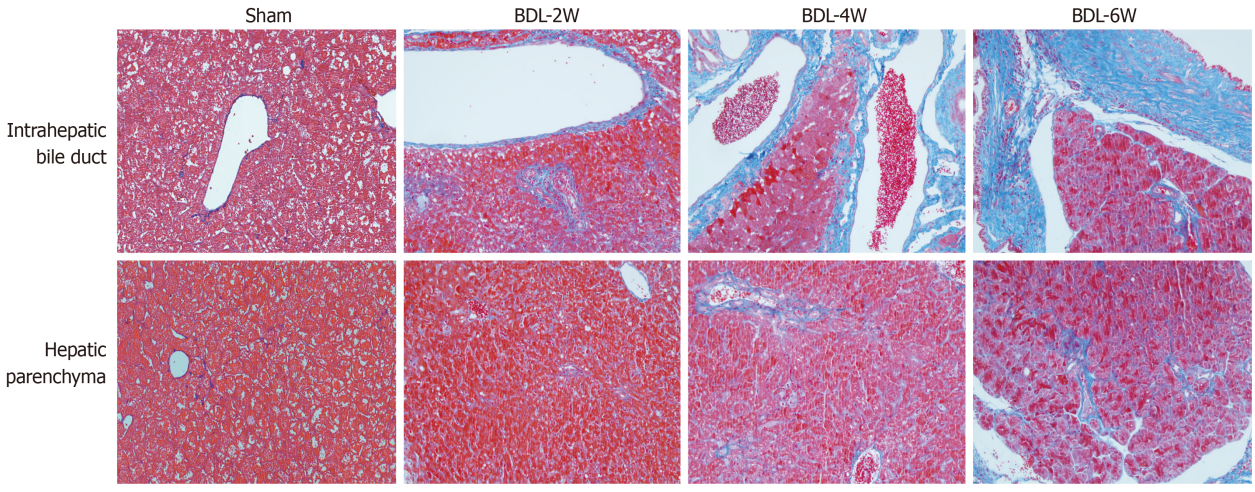


Figure 4 Masson's trichrome stain of the intrahepatic bile duct and hepatic parenchyma in sham and bile duct ligation subgroups (light microscopy, 400×). BDL: Bile duct ligation.

value from the sham group to the BDL group. However, the findings of the current study do not support previous research[14]. This discrepancy could be attributed to the partial obstruction of hepatic arteries as a result of a steady rise in bile duct volume such that the excretion time of the contrast would take longer than that of normal hepatic arteries. These factors may result in a greater amount of intracellular remnant in the form of the contrast agent and a decreased T1 value at 10 min after intravenous

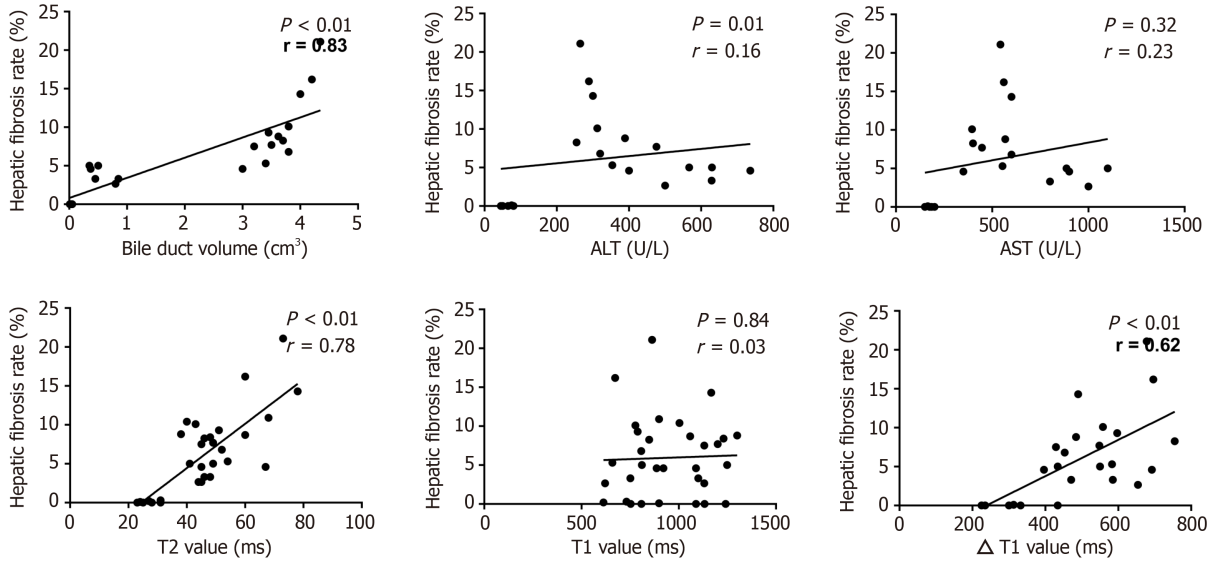


Figure 5 Spearman correlation coefficients between magnetic resonance imaging parameters with hepatic fibrosis rate. ALT: Alanine aminotransferase; AST: Aspartate transaminase.

injection. Furthermore, a moderate correlation between the ΔT1 value and fibrosis rate was found in our study. This combination of findings provides some support for the conceptual premise that T1 mapping is a reliable tool for discriminating liver fibrosis.

This study had several limitations. First, because of the limited number of mice, it was difficult to avoid selection bias and carry out a subgroup analysis. Second, many advanced sequences such as MRE[15] and intravoxel incoherent motion imaging[16] could not be used in our study because of technological limitations in clinical MRI. Third, it is difficult to use the breath-holding technique in mice, and the subsequent respiratory movement in the abdomen might have degraded image quality and caused bias in measurements. Fourth, steatosis is a common hepatic lesion that occurs before the formation of fibrosis in biliary cirrhosis. This could have been a potential confounding factor in the pathological changes.

CONCLUSION

BDL induces a complex cascade of changes that can be clearly observed on MRI. To our knowledge, this is the first study to assess the utility of multiparameter MRI in a BDL mouse model. The results showed moderate to high correlations between ΔT1 value, T2 value, and liver fibrosis, indicating that T1 mapping and T2 mapping could be useful tools for the detection of liver fibrosis in the BDL mouse model. In the future, advanced MR techniques will have great potential for widespread application in both preclinical and clinical fields.

ARTICLE HIGHLIGHTS

Research background

Bile duct ligation (BDL) is a classical method for mimicking cholestatic fibrosis in animals. Magnetic resonance imaging (MRI) has enabled significant advances in noninvasive assessment of liver fibrosis. More comprehensive liver fibrotic features of BDL on MRI are important. However, the utility of multiparameter MRI to detect liver fibrosis in a BDL mouse model has not been assessed.

Research motivation

We hypothesized that the BDL mouse model can be characterized radiologically using MRI, which is a noninvasive method. The model can be further validated regarding radiological, quantitative, and analytical aspects.

Research objectives

Using a BDL mouse model to evaluate the correlation between the pathological changes and several parameters of MRI characteristics of liver fibrosis.

Research methods

Twenty-eight healthy adult male balb/c mice were included. They were randomly divided into four groups: Sham group and week 2 BDL, week 4 BDL and week 6 BDL groups. The MRI sequences included the following parameters: Magnetic resonance cholangiopancreatography (MRCP), T1-weighted, T2-weighted, T2 mapping, and pre- and post-enhanced T1 mapping. All these were performed after sham and BDL surgery. Peripheral blood and liver tissue were collected after the MRI.

Research results

The bile duct volume, ΔT1 value, T2 value, and the rate of liver fibrosis increased steadily in the week 2 BDL, week 4 BDL and week 6 BDL groups compared to those in the sham group ($P < 0.01$). Strong correlations were found between bile duct volume, T2 value, ΔT1 value, and hepatic fibrosis rate (all $P < 0.01$) in all BDL groups.

Research conclusions

The BDL mouse model induces changes that are easily observed using MRI. The MRI parameters correlate with the hepatic fibrosis rate and enable the detection of cholestatic fibrosis.

Research perspectives

We believe that advanced MR techniques have considerable potential for widespread application in preclinical and clinical fields.

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World Journal of Gastroenterology

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World J Gastroenterol 2021 December 21; 27(47): 8166-8181

DOI: 10.3748/wjg.v27.i47.8166

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

ORIGINAL ARTICLE

Retrospective Study

Disease control and failure patterns of unresectable hepatocellular carcinoma following transarterial radioembolization with yttrium-90 microspheres and with/without sorafenib

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Author contributions: Teyateeti A designed the study, collected, analyzed and interpreted the data and wrote the manuscript; Mahvash A, Abdelsalam M, Avritscher R, Odisio B, Ravizzini G, and Surasi D collected data, provided clinical advice and edited the manuscript; Long J supervised and provided advice for statistical analysis and edited the manuscript; Kaseb A and Macapinlac H edited the manuscript; Teyateeti A contributed to study design, provided clinical advice and made critical revision of the manuscrip; and Kappadath SC contributed to

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Abstract

BACKGROUND

Impressive survival outcome of our previous study in unresectable hepatocellular carcinoma (HCC) patients undergoing yttrium-90 glass microspheres transarterial radioembolization (TARE) with/without sorafenib according to individuals' disease burden, *i.e.*, intrahepatic tumor load (IHT) and adverse disease features (ADFs) might partly be confounded by other treatments and underlying hepatic

the design of the study, interpretation of the data, made critical revision of the manuscript and supervised the study.

Institutional review board statement: This study was approved by Institutional review board of The University of Texas MD Anderson Cancer Center, No. DR09-0025.

Informed consent statement: A waiver of informed consent was granted by our Institutional Review Board for this retrospective study. Patient data used complied with all institutional data protection and privacy regulations.

Conflict-of-interest statement: All authors have no any conflicts of interest.

Data sharing statement: Authors are open to data sharing, please email queries.

Country/Territory of origin: United States

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): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

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function. Therefore, a dedicated study focusing on treatment response and assessment of failure patterns might be a way to improve treatment outcome in addition to patient selection based on the disease burden.

AIM

To assess the tumor response, disease control and patterns of disease progression following TARE with/without sorafenib in unresectable HCC patients.

METHODS

This retrospective study was conducted in successful TARE procedures with available pre- and post-treatment imaging studies (n = 169). Three treatment subgroups were (1) TARE only (*TARE_alone*) for IHT ≤ 50% without ADFs, *i.e.*, macrovascular invasion, extrahepatic disease (EHD) and infiltrative/ill-defined HCC (n = 63); (2) TARE with sorafenib (*TARE_sorafenib*) for IHT > 50% and/or presence of ADFs (n = 81); and (3) TARE only for patients who could not receive sorafenib due to contraindication or intolerance (*TARE_no_sorafenib*) (n = 25). Objective response rate (ORR; consisted of complete response (CR) and partial response (PR)), disease control rate (DCR; consisted of CR, PR and stable disease) and failure patterns of treated, intrahepatic and extrahepatic sites were assessed using the modified response evaluation criteria in solid tumors. Time to progression (TTP) was calculated from TARE to the first radiologic progression at any site using Kaplan-Meier method. Identification of prognostic factors for TTP using the univariate Kaplan-Meier method and multivariate Cox proportional hazard model were performed in major population subgroups, *TARE_alone* and *TARE_sorafenib*.

RESULTS

The median radiologic follow-up time was 4.4 mo (range 0.5-48.8). In treated area, ORR was highest in *TARE_sorafenib* (53.1%), followed by *TARE_alone* (41.3%) and *TARE_no_sorafenib* (16%). In intrahepatic area, DCR remained highest in *TARE_sorafenib* (84%), followed by *TARE_alone* (79.4%) and *TARE_no_sorafenib* (44%). The overall DCR was highest in *TARE_alone* (79.4%), followed by *TARE_sorafenib* (71.6%) and *TARE_no_sorafenib* (40%). Dominant failure patterns were intrahepatic for both *TARE_alone* (44.5%) and *TARE_sorafenib* (38.4%). Extrahepatic progression was more common in *TARE_sorafenib* (32%) and *TARE_no_sorafenib* (40%) than in *TARE_alone* (12.7%). TTP was longest in *TARE_alone* (8.6 mo; 95%CI: 3.4-13.8), followed by *TARE_sorafenib* (5.1 mo; 95%CI: 4.0-6.2) and *TARE_no_sorafenib* (2.7 mo; 95%CI: 2.2-3.1). Pre-existing EHD (HR: 0.37, 95%CI: 0.24-0.56, P < 0.001) was a sole prognostic factor for TTP in *TARE_sorafenib* with no prognostic factor for TTP in *TARE_alone*.

CONCLUSION

TARE with/without sorafenib according to individuals' disease burden provided DCR approximately 70% with intrahepatic progression as dominant failure pattern. Extrahepatic progression was more common in procedures with initially high disease burden.

Key Words: Radioembolization; Selective internal radiotherapy; Tumor response; Pattern of progression; Time to progression; Sorafenib

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Core Tip: Hepatocellular carcinoma (HCC) patients treated with yttrium-90 transarterial radioembolization (TARE) alone for intrahepatic tumor load ≤ 50% and TARE with sorafenib for intrahepatic tumor load > 50% and/or present macrovascular invasion, extrahepatic disease or infiltrative HCC yielded acceptable disease control rates of 79.4% and 71.6%, respectively. Between these 2 subgroups, incidence of intrahepatic progression was comparable (about 40%) but extrahepatic progression was much less common with TARE alone (12.7% *vs* 32%). Strategies that improve intrahepatic control for liver-only disease (dosimetry-based TARE) and extrahepatic control for

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Received: March 22, 2021
Peer-review started: March 22, 2021
First decision: June 14, 2021
Revised: July 28, 2021
Accepted: December 8, 2021
Article in press: December 8, 2021
Published online: December 21, 2021

P-Reviewer: Rodriguez-Fraile M
S-Editor: Ma YJ
L-Editor: A
P-Editor: Ma YJ

metastatic disease (additional systemic therapy) could improve TARE outcome for unresectable HCC patients.

Citation: Teyateeti A, Mahvash A, Long J, Abdelsalam M, Avritscher R, Kaseb A, Odisio B, Ravizzini G, Surasi D, Teyateeti A, Macapinlac H, Kappadath SC. Disease control and failure patterns of unresectable hepatocellular carcinoma following transarterial radioembolization with yttrium-90 microspheres and with/without sorafenib. *World J Gastroenterol* 2021; 27(47): 8166-8181
URL: https://www.wjgnet.com/1007-9327/full/v27/i47/8166.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i47.8166

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major health problems worldwide**.** It is the 6th most common malignancy with over 900000 new cases and 830000 deaths per year and the 3rd leading cause of cancer deaths[1]. HCC has a high mortality rate due to the fact that the majority of patients are diagnosed at an advanced stage of disease beyond the curative surgical options. This group of patients, sometimes called unresectable HCC patients, generally have two standard treatment options; local therapies, trans-arterial chemoembolization (TACE) and/or ablation, or systemic therapy[2-5].

Trans-arterial radioembolization (TARE) with yttrium-90 (Y-90) microspheres is an alternative local therapy option for unresectable HCC patients[2,5,6]. Currently, TARE is not an established treatment in most HCC treatment guidelines outside the United State. Consequently, each institution has its own algorithm for selecting TARE candidates. As a result, there exists marked variations in reported treatment outcome for TARE, depending on disease characteristics of enrolled patients[7-10].

At our institution, we offer TARE as monotherapy to patients with intrahepatic tumor (IHT) involvement less than or equal to 50% of total liver parenchyma (IHT ≤ 50%). Patients with IHT greater than 50% (IHT > 50%) or with advanced disease features (ADFs), defined as macrovascular invasion (MVI), extrahepatic disease (EHD) and infiltrative/ill-defined HCC, are candidates for TARE with systemic therapy which historically first line was sorafenib[11].

The clinical outcomes in terms of overall survival (OS) and progression-free survival (PFS) following TARE at our institution has been previously reported[12]. We reported median OS and PFS durations of 21.6 mo (95%CI: 6.1-37.1) and 9.1 mo (95%CI: 5.2-13.0), respectively, for HCC patients with IHT ≤ 50% treated with TARE only; while those for HCC patients treated with TARE in combination with sorafenib were 12.4 mo (95%CI: 9.1-15.6) and 5.1 mo (95%CI: 2.6-7.5), respectively. Better OS for HCC patients treated with TARE in combination with sorafenib was associated with patients with lower disease burden [IHT ≤ 50%, hazard ratios (HR) = 0.39, P = 0.004 and alpha-fetoprotein (AFP) < 400, HR = 0.5, P = 0.027]. Unilobar involvement (HR = 0.43, P = 0.029) correlated with better PFS in HCC patients with IHT ≤ 50% treated with TARE only. However, the OS and PFS survival outcomes reported was affected by several treatment combinations and not solely due to the effect of TARE itself. The objective of this study was to quantify and characterize the benefits of TARE as a local therapy. More specifically, we investigated the objective response rate (ORR), disease control rate (DCR), time to progression (TTP), and, in the case of progression, the pattern and location of disease progression, for HCC patients treated at our institution with TARE, either as monotherapy or in combination with sorafenib.

MATERIALS AND METHODS

Patient selection

This institutional review board approved retrospective study was conducted in unresectable HCC patients who received TARE with Y-90 glass microspheres at The University of Texas MD Anderson Cancer Center (Houston, TX, United States) from November 16, 2010, to October 1, 2018. Inclusion criteria were successful TARE

procedures with available pre-treatment imaging study within 1 mo before TARE and at least one post-treatment imaging study within 2 mo after TARE (n = 176). In case of multiple follow-ups, all of imaging studies were done with the same imaging technique, *i.e.*, all contrast-enhanced computed tomography (CT) or all magnetic resonance imaging (MRI). Exclusion criteria were TARE procedures with restrictions on imaging interpretation and/or comparison, *i.e.*, non-contrast enhanced studies (n = 5), poor quality imaging study (n = 1) and hypo-vascular HCC (n = 1). A total of 169 procedures from 151 patients were finally included for analysis in this study.

Pretreatment evaluation

Pretreatment clinical histories and laboratory tests, including AFP and liver function tests were reviewed retrospectively in procedure-based fashion. Staging of disease and performance status were assessed by the Barcelona Clinic Liver Cancer (BCLC) staging system and Eastern Cooperative Oncology Group (ECOG) performance status, respectively[3,13]. Contrast-enhanced CT or MRI was used to evaluate cirrhosis, infiltrative tumor, MVI, EHD (consisted of lymph node and distant metastasis), number of tumors, lobar involvement and IHT.

Treatment

The technetium-99m macro aggregated albumin (Tc-99m MAA) pre-treatment scan was done to assess vascular anatomies and simulate Y-90 microspheres distribution in all procedures. TARE was usually performed within 1 mo after the Tc-99m MAA pre-treatment evaluation. Administration of Y-90 glass microspheres and sorafenib followed the manufacturer's instructions for use and per the direction of the treating oncologist[14,15]. Dose of sorafenib was adjusted on the basis of patients' tolerability and was reduced or withdrawn due to toxicity. Other treatments *e.g.*, TACE, chemotherapy, immunotherapy, *etc.* were given at the time of progression at the discretion of the treating oncologist.

Treatment strategies were classified into 3 subgroups according to patients' disease burden as assessed by IHT (≤ 50% *vs* > 50%) and ADFs (absence *vs* presence) and patients' general conditions as considered by ECOG and underlying conditions at time of procedures: (1) *TARE_alone* was referred to TARE as a sole treatment in patients with IHT ≤ 50% and absence of ADFs; (2) *TARE_sorafenib* was a combination of TARE and sorafenib in patients with IHT > 50% and/or presence of ADFs; and (3) *TARE_no_sorafenib* was TARE only treatment in patients who could not receive *TARE_sorafenib* due to contraindication or intolerance. All combined treatments were given concurrently or within a 1-month interval.

Post-treatment evaluation

Contrast-enhanced CT or MRI was obtained, usually within 2 mo after TARE and every 2-3 mo thereafter. All imaging studies were reinterpreted by a team, consisting of 4 interventional radiologists and 2 nuclear medicine physicians with diagnostic radiology training. All equivocal findings were determined by a consensus of 2 or more members in a team. The modified response evaluation criteria in solid tumors (mRECIST) was applied for response assessment[16].

Evaluation were performed in both intrahepatic and extrahepatic areas. Intrahepatic area was composed of treated area, referred to target lesions according to mRECIST and untreated area, referred to intrahepatic area outside treated area. Extrahepatic area was elsewhere outside the liver. Radiologic assessment was performed until initiation of new systemic treatment, last radiological follow-up, or patient's death, whichever came first. If patients received additional treatment for residual tumor in treated area (*e.g.*, TACE, radiofrequency ablation (RFA), surgical resection *etc.*), response assessment was not performed after these treatments.

Treatment response and failure patterns

Treatment response was referred to the best radiologic response at any time point during the evaluation period. Responses were categorized as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD) following the mRECIST. ORR was a sum of CR and PR. DCR was a sum of CR, PR and SD. These responses were reported according to the assessed areas which were (1) Treated area; (2) Intrahepatic area, composed of treated and untreated areas; and (3) Overall, composed of intrahepatic and extrahepatic areas.

In case of progression, failure patterns were evaluated. The failure patterns in patients that progressed were classified into 3 categories according to the site of first progression: (1) Treated area; (2) Untreated area; and (3) Extrahepatic area. The first

instance of progression in treated area at any time during the follow-up period was classified into 5 categorized: (1) Development of new HCC; (2) Recurrence/increased enhancement of previously treated HCC; (3) Development of new MVI; (4) Progressive MVI; and (5) Mixed patterns. Progression in untreated area was defined as appearance of new lesion or progression of pre-existing untreated lesion.

Estimation of TTP was performed for: (1) Treated area; (2) Untreated area; and (3) Overall. TTP was defined as the time from TARE to the first unequivocal radiologic progression at pre-specified sites (treated and untreated areas) or at any site (overall). Deaths or loss follow-up were censored at time of last follow-up without radiologic evidence of progression. Analysis on prognostic factors for TTP of overall disease was performed only in major population subgroups, *TARE_alone* and *TARE_sorafenib*.

Statistical analysis

Baseline characteristics, response rate and patterns of disease progression were analyzed by using descriptive statistics. TTP and its 95% confidence interval (95%CI) were estimated by using Kaplan-Meier method and comparison between subgroups were done with log-rank test. The univariate analysis was performed using Kaplan-Meier method. The HR were calculated by using a Cox proportional hazard regression. Factors in the univariate analysis with $P < 0.1$ were further analyzed in a multivariate analysis using Cox proportional hazard model. In the multivariate analysis, statistically significant P value was set at 0.05. All statistical analyses were conducted using IBM SPSS Statistics software for Windows, version 21.0 (Armonk, NY: IBM Corp.).

RESULTS

Baseline patient characteristics

The median age at time of diagnosis was 66 years (range 17-85) with most patients being male (76.2%). In our study cohort, 80/151 (53%) patients received TARE as their first treatment, 46/151 (30.5%) patients received local treatments prior to TARE, and 25/151 (16.6%) patients received systemic treatments prior to TARE. Local treatments included surgical resection, trans-arterial embolization, TACE, TARE with Y-90 resin microspheres, RFA, and microwave ablation; while systemic treatment included targeted therapy, immunotherapy, and chemotherapy. Most patients (133/151, 88.1%) received single TARE treatments only, with 16/151 (10.6%) and 2/151 (1.3%) patients receiving two and three separate TARE procedures, respectively.

Patient and tumor characteristics at time of TARE

Patient and tumor characteristics at time of TARE procedure (n = 169) stratified by treatments are shown on Table 1; the two most common treatments were *TARE_alone* (37.3%) and *TARE_sorafenib* (47.9%). Majority of patients had ECOG status either 0 (48.5%) or 1 (48.5%). While the *TARE_alone* subgroup had similar proportions of BCLC B and C (41.3% *vs* 49.2%), the remaining subgroups predominantly consisted of BCLC C patients (> 80%). Most patients were Child-Pugh A (92.9%) and presented with cirrhosis (69.2%) and multiple tumors (83.4%). While the *TARE_alone* subgroup had similar proportions of unilobar and bilobar disease (52.4% *vs* 47.6%), the remaining subgroups predominantly consisted of (≈ 70%) patients with bilobar disease.

TARE characteristics

TARE characteristic stratified by treatment are displayed on Table 2. *TARE_alone* procedures had the lowest median lung shunt fraction (4.6%), median lung dose (4.7 Gy) and median administered activity (1.7 GBq). In all subgroups, lobar treatment was the most common TARE approach (39.1%), followed by whole liver treatment (27.8%).

Best radiologic response

The median radiologic follow-up time was 4.4 mo (range 0.5-48.8). The best radiologic mRECIST response categorized by treatment are shown on Table 3. In the treated area, *TARE_sorafenib* subgroup had the highest ORR (53.1%), DCR (87.7%), and CR rate (11.1%), with *TARE_alone* subgroup having slightly lower ORR (41.3%) but similar DCR (85.7%). In the treated and intrahepatic areas, the two dominant response categories for *TARE_alone* and *TARE_sorafenib* were PR and SD, accounting for over 70% of all responses; the two dominant response categories for *TARE_no_sorafenib* were SD and PD, accounting for over 80%. The two highest overall DCRs were

Table 1 Patient and tumor characteristics at time of transarterial radioembolization procedures

Parameters	All procedures (*n* = 169)	*TARE_alone* (*n* = 63)	*TARE_sorafenib* (*n* = 81)	*TARE_no_sorafenib* (*n* = 25)
ECOG				
0	82 (48.5)	32 (50.8)	40 (49.4)	10 (40)
1	82 (48.5)	28 (44.4)	40 (49.4)	14 (56)
2	4 (2.4)	2 (3.2)	1 (1.2)	1 (4)
3	1 (0.6)	1 (1.6)	0	0
BCLC				
A	5 (3)	5 (7.9)	0	0
B	45 (26.6)	26 (41.3)	16 (19.8)	3 (12)
C	118 (69.8)	31 (49.2)	65 (80.2)	22 (88)
D	1 (0.6)	1 (1.6)	0	0
Child-pugh class				
A	157 (92.9)	57 (90.5)	78 (96.3)	22 (88.0)
B	12 (7.1)	6 (9.5)	3 (3.7)	3 (12.0)
AFP[1]				
< 400 ng/mL	118 (70.2)	54 (85.7)	52 (64.2)	12 (50)
≥ 400 ng/mL	50 (29.8)	9 (14.3)	29 (35.8)	12 (50)
Cirrhosis				
Absence	52 (30.8)	15 (23.8)	29 (35.8)	8 (32)
Presence	117 (69.2)	48 (76.2)	52 (64.2)	17 (68)
Infiltrative tumor				
Absence	133 (78.7)	63 (100)	58 (71.6)	12 (48)
Presence	36 (21.3)	0	23 (28.4)	13 (52)
Vascular invasion[2]				
Absence	131 (78)	63 (100)	56 (70)	12 (48)
Presence	37 (22)	0	24 (30)	13 (52)
Extrahepatic disease				
Absence	137 (81.1)	63 (100)	60 (74.1)	14 (56)
Presence	32 (18.9)	0	21 (25.9)	11 (44)
Number of tumors				
Single	28 (16.6)	12 (19)	9 (11.1)	7 (28)
Multiple	141 (83.4)	51 (81)	72 (88.9)	18 (72)
Lobar involvement				
Unilobar	63 (37.3)	33 (52.4)	22 (27.2)	8 (32)
Bilobar	106 (62.7)	30 (47.6)	59 (72.8)	17 (68)
Intrahepatic tumor				
≤ 50%	116 (68.6)	63 (100)	37 (45.7)	16 (64)
> 50%	53 (31.4)	0	44 (54.3)	9 (36)
TARE procedures				
1	151 (89.3)	56 (88.9)	71 (87.7)	24 (96)
2	16 (9.5)	7 (11.1)	8 (9.9)	1 (4)
3	2 (1.2)	0	2 (2.5)	0

[1]Unavailable AFP in one *TARE_no_sorafenib* patient.
[2]Unavailable vascular invasion in one *TARE_sorafenib* patient.
Values represent number of procedures (%). TARE: Transarterial radioembolization; ECOG: Eastern cooperative oncology group; BCLC: Barcelona clinic liver cancer; AFP: Alpha-fetoprotein.

Table 2 Characteristics of transarterial radioembolization procedures

	All procedures (*n* = 169)	*TARE_alone* (*n* = 63)	*TARE_sorafenib* (*n* = 81)	*TARE_no_sorafenib* (*n* = 25)
LSF, %	6.0 (0.8-30.4)	4.6 (1.0-26.4)	6.1 (0.8-30.4)	6.3 (2.0-13.6)
Lung mean dose, Gy[1]	8.2 (0.3-29.7)	4.7 (0.3-29.2)	10.1 (0.5-29.7)	11.2 (2.0-29.2)
Mean dose to treated liver volume, Gy[1]	110 (80-135)	110 (80-135)	110 (80-135)	110 (80-135)
Interval between Tc-99m MAA and TARE, d[1]	20 (0-125)	21 (0-125[2])	17 (0-44)	21 (10-34)
Administered activity, GBq	2.5 (0.3-8.1)	1.7 (0.3-6.3)	2.9 (0.6-8.1)	2.7 (0.8-5.9)
TARE approach, *n* (%)				
Whole liver[3]	47 (27.8)	16 (25)	25 (31)	6 (24)
Lobar + segment	22 (13)	4 (6)	15 (18)	3 (12)
Lobar	66 (39.1)	23 (37)	30 (37)	13 (52)
Multiple segments	23 (13.6)	13 (21)	7 (9)	3 (12)
Single segment	11 (6.5)	7 (11)	4 (5)	0

[1]Mean absorbed doses values for each treatment session.
[2]The outliner interval of 125 d was from a single patient whose initial treatment plan was a whole liver treatment with sequential lobar infusion three weeks apart. His subsequent left lobar treatment was delayed for months because of his medical conditions. Administered activity of left lobar approach was calculated using the original Tc-99m MAA plan and re-evaluation CT scan performed prior to left lobar treatment.
[3]Consisted of single infusion (*n* = 19), separate infusion (*n* =24) and sequential infusion (*n* = 4).
Values represent median (range) unless otherwise stated. TARE: Transarterial radioembolization; Tc-99m MAA: The technetium-99m macro aggregated albumin.

observed in *TARE_alone* (79.4%) followed by *TARE_sorafenib* (71.6%) subgroups.

Overall failure patterns

Table 4 shows the overall failure patterns categorized by treatment. Disease progression were observed in 65.7% of all procedures. The lowest and highest rates of progression were noted in *TARE_alone* (57.1%) and *TARE_no_sorafenib* (72%) subgroups, respectively. The most common site of first disease progression was intrahepatic area for both *TARE_alone* (44.5%) and *TARE_sorafenib* procedures (38.4%). Extrahepatic progression (including both extrahepatic only and intrahepatic with extrahepatic) contributed to more than 30% cases in *TARE_sorafenib* (32%) and *TARE_no_sorafenib* (40%) subgroups, much higher than *TARE_alone* (12.7%) subgroup.

Intrahepatic failure patterns

Of total 169 procedures, intrahepatic progression was observed in 100 procedures (59.2%) with 75 procedures being progression in treated area (44.4%). Table 5 stratifies intrahepatic failure patterns of disease progression when intrahepatic progression was observed by treatment subgroups (*n* = 100). The progression rates in treated area of *TARE_alone* (67.6%) subgroup was lower than that of *TARE_sorafenib* (81.6%) and *TARE_no_sorafenib* (70.6%) subgroups. The two most common cause of disease progression in treated area across all subgroups were the development of new HCC (34%), followed by the recurrence/increased enhancement of previously treated HCC (20%). The progression rate of untreated area was highest (32.4%) and lowest (18.4%) in *TARE_alone* and *TARE_sorafenib* subgroup, respectively.

TTP

Median overall TTP of all procedures was 4.9 mo (95%CI: 3.9-5.9). TTP of treated area,

Table 3 Summary of the best radiologic response (modified response evaluation criteria in solid tumors) following transarterial radioembolization

	All procedures (*n* = 169)	*TARE_alone* (*n* = 63)	*TARE_sorafenib* (*n* = 81)	*TARE_no_sorafenib* (*n* = 25)
Treated area				
CR	12 (7.1)	2 (3.2)	9 (11.1)	1 (4)
PR	61 (36.1)	24 (38.1)	34 (42)	3 (12)
SD	66 (39.1)	28 (44.4)	28 (34.6)	10 (40)
PD	30 (17.8)	9 (14.3)	10 (12.3)	11 (44)
OR	73 (43.2)	26 (41.3)	43 (53.1)	4 (16)
DC	139 (82.2)	54 (85.7)	71 (87.7)	14 (56)
Intrahepatic area				
CR	12 (7.1)	2 (3.2)	9 (11.1)	1 (4)
PR	58 (34.3)	22 (34.9)	33 (40.7)	3 (12)
SD	59 (34.9)	26 (41.3)	26 (32.1)	7 (28)
PD	40 (23.7)	13 (20.6)	13 (16)	14 (56)
OR	70 (41.4)	24 (38.1)	42 (51.9)	4 (16)
DC	129 (76.3)	50 (79.4)	68 (84)	11 (44)
Overall				
CR	10 (5.9)	2 (3.2)	7 (8.6)	1 (4)
PR	52 (30.8)	22 (34.9)	28 (34.6)	2 (8)
SD	56 (33.1)	26 (41.3)	23 (28.4)	7 (28)
PD	51 (30.2)	13 (20.6)	23 (28.4)	15 (60)
OR	62 (36.7)	24 (38.1)	35 (43.2)	3 (12)
DC	107 (69.8)	50 (79.4)	58 (71.6)	10 (40)

Values represent number of procedures (%). Objective response consisted of complete response (CR) and partial response (PR). Disease control consisted of CR, PR and stable disease. CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; OR: Objective response; DC: Disease control; TARE: Transarterial radioembolization.

untreated area and overall stratified by treatment subgroups are provided in Table 6. Amongst the 3 subgroups, median overall TTP for *TARE_alone* was highest at 8.6 mo followed by *TARE_sorafenib* at 5.1 mo and *TARE_no_sorafenib* at 2.7 mo.

Prognostic factors of TTP

The result of univariate and multivariate analysis of TTP of *TARE_alone* and *TARE_sorafenib* are provided in Table 7. None of the variables explored were found to be statistically significant prognostic factors for TTP in *TARE_alone* subgroup. Both child-pugh class and lobar involvement with $P < 0.1$, in both univariate and multivariate analysis, could be considered marginally significant factors. For *TARE_sorafenib* subgroups, univariate analysis showed ECOG, EHD, and IHT to be statistically significant prognostic factors for TTP, that compressed to a single factor of EHD in multivariate analysis with a $P < 0.001$ (Table 8).

Lobar involvement marginally stratified TTP duration (unilobar 11.0 mo; 95%CI: 5.0-17.0 *vs* bilobar 5.6 mo; 95%CI: 2.4-8.8, $P = 0.058$) for *TARE_alone* patients. Statistically significant differences in TTP duration of *TARE_sorafenib* procedures were noted when stratified by EHD (absent 7.5 mo; 95%CI: 4.9-10.0 *vs* present 2.8 mo; 95%CI: 2.6-3.1, $P = < 0.001$) and IHT (≤ 50% 7.7 mo; 95%CI: 5.1-10.3 *vs* > 50% 5.1 mo; 95%CI: 4.0-6.2, $P = 0.024$).

Table 4 Site of first progression in all cases of progression

	All procedures (*n* = 169)	*TARE_alone* (*n* = 63)	*TARE_sorafenib* (*n* = 81)	*TARE_no_sorafenib* (*n* = 25)
No progression	58 (34.3)	27 (42.9)	24 (29.6)	7 (28)
Progression	111 (65.7)	36 (57.1)	57 (70.4)	18 (72)
Intrahepatic only	67 (39.6)	28 (44.5)	31 (38.4)	8 (32)
Treated area only	36 (21.3)	16 (25.4)	17 (21)	3 (12)
Untreated area only	20 (11.8)	10 (15.9)	6 (7.4)	4 (16)
Both treated and untreated areas	11 (6.5)	2 (3.2)	8 (9.9)	1 (4)
Extrahepatic only	17 (10.1)	3 (4.8)	13 (16)	1 (4)
Intra- and extrahepatic	27 (16)	5 (7.9)	13 (16)	9 (36)

Values represent number of procedures (%). TARE: Transarterial radioembolization.

Table 5 First pattern of intrahepatic progression in cases with intrahepatic progression

	All procedures (*n* = 100)	*TARE_alone* (*n* = 34)	*TARE_sorafenib* (*n* = 49)	*TARE_no_sorafenib* (*n* = 17)
Progression in treated area	75 (75)	23 (67.6)	40 (81.6)	12 (70.6)
New HCC	34 (34)	12 (35.3)	19 (38.8)	3 (17.6)
Recurrence/increased enhancement of previously treated HCC	20 (20)	5 (14.7)	9 (26.5)	6 (35.3)
With new MVI	8 (8)	2 (5.9)	6 (12.2)	0
With progressive MVI	3 (3)	0	3 (6.1)	0
With mixed patterns[1]	10 (10)	4 (11.8)	3 (6.1)	3 (17.6)
Progression in untreated area	25 (25)	11 (32.4)	9 (18.4)	5 (29.4)

[1]New HCC with one or more other patterns (*n* = 11) and new MVI with increased enhancement of previously treated HCC (*n* = 1). Values represent number of procedures (%). TARE: Transarterial radioembolization; HCC: Hepatocellular carcinoma; MVI: Macrovascular invasion.

DISCUSSION

TARE has been an increasing treatment option for unresectable HCC patients[2,5,6]. Treatment outcomes of TARE in the literatures varied considerably, depending on several factors such as the characteristics and stage of enrolled patients, and the experience and preferences of investigators with TARE[7-10,17-20]. In this study, we reported disease control and objective response with TARE for unresectable HCC per our institutional treatment algorithm which may include combination treatment with sorafenib based on two unique features: disease burden assessment by IHT and presence of ADFs. Pertinent findings in our study included development of new HCC tumors as a major intrahepatic failure pattern, disease progression in treated area and extrahepatic area as the most common overall disease failure patterns in *TARE_alone* and *TARE_sorafenib* procedures, respectively.

Our finding that 70% of treated lesions could achieve PR or SD was consistent with the previous studies[9,21]. Interestingly, the *TARE_sorafenib* subgroup provided the highest response rate (ORR 53.1% and CR 11.1%) followed by the *TARE_alone* subgroup (ORR 41.3% and CR 3.2%) which consisted of patients without ADFs or lower IHT. When comparing between subgroups with similar disease burden, DCR of *TARE_sorafenib* was much higher than *TARE_no_sorafenib* (87.7% *vs* 56%). Furthermore, median TTP duration of treated area for *TARE_sorafenib* was much longer than *TARE_no_sorafenib* (7.5 *vs* 3.6 mo). Acknowledging that antiangiogenic effect of sorafenib could promote oxygenation to the core of tumor and thereby increase tumor sensitivity to radiation[19,22,23], we postulated that better disease control observed for *TARE_sorafenib* might be attributed to the beneficial effect of sorafenib.

Table 6 Time to progression

	All procedures (*n* = 169)	*TARE_alone* (*n* = 63)	*TARE_sorafenib* (*n* = 81)	*TARE_no_sorafenib* (*n* = 25)
Treated area				
Censored patients	87	39	37	11
TTP, mo	7.8 (6.4-9.3)	12.3 (10.4-14.1)	7.5 (6.2-8.8)	3.6 (0.8-6.4)
Untreated area				
Censored patients	119	47	59	13
TTP, mo	12.8 (4.3-21.3)	22.9 (10.2-35.7)	11.9 (8.0-15.8)	3.6 (2.1-5.1)
Overall				
Censored patients	58	27	24	7
TTP, mo	4.9 (3.9-5.9)	8.6 (3.4-13.8)	5.1 (4.0-6.2)	2.7 (2.2-3.1)

TTP values represent median (95%CI) in months. TTP: Time to progression; TARE: Transarterial radioembolization.

It is noteworthy that in subgroups without sorafenib, *TARE_alone* and *TARE_no_sorafenib*, decrease of DCRs of treated area and intrahepatic area were 6.3 percentage points (from 85.7% to 79.4%) and 12 percentage points (from 56% to 44%), respectively. In the meantime, decrease of DCR of *TARE_sorafenib* was only 3.7 percentage points (from 87.7% to 84%). Given that intrahepatic area consisted of treated and untreated areas, disease progression in untreated area should make DCR of intrahepatic area lower than DCR of treated area. Thus, a less prominent change of DCR of *TARE_sorafenib* subgroup, compared to others might suggest that addition of sorafenib to TARE could reduce disease progression in untreated area and thereby provided a better intrahepatic control.

The most common intrahepatic failure patterns according to several studies, including this work was the development of new HCC, both in treated or untreated areas[9,21,24]. This might be explained by the hypothesis that newly detected HCC during follow-up might be pre-existing undetectable microscopic HCC. These lesions have generally less developed arterial blood supply compared to the macroscopic ones, and therefore, they do not achieve the tumoricidal dose from TARE. These small tumors might subsequently progress giving the impression of new HCC following TARE[21,24,25].

Regarding the patterns of disease progression, *TARE_alone* which had lowest disease burden was the only subgroup that the most common site of first disease progression was treated area (*n* = 16/36, 44.4%). Additionally, disease progression of *TARE_alone* were mostly limited in intrahepatic area (*n* = 28/36, 77.8%). Therefore, aggressive TARE based on advanced and personalized dosimetry with radiation dose to tumor exceeding tumoricidal threshold, around 200 Gy as claimed by several studies, might increase response of treated area[26,27]. We acknowledge that tumor specific dose estimates may further stratify tumor response status, but the retrospective calculation of tumor doses are beyond the scope of this work. Furthermore, cone-beam CT (CBCT) has been proven to demonstrate additional tumors overlooked by angiography and Tc-99m MAA scan[28]. Consequently, incorporating CBCT to treatment planning might be another way to improve intrahepatic control with TARE.

Rates of first disease progression in extrahepatic area of *TARE_sorafenib* (32%) and *TARE_no_sorafenib* (40%) subgroups were obviously higher than that of *TARE_alone* subgroup (12.7%). We hypothesized that this might be a result of higher baseline disease burden of these subgroups compared with *TARE_alone* (IHT > 50% and/or ADFs *vs* IHT ≤ 50% without ADFs). *TARE_alone* was also the only cohort without EHD whereas *TARE_sorafenib* and *TARE_no_sorafenib* had EHD in 25.9% and 44.4% cases, respectively. Considering *TARE_sorafenib* subgroup as an example, given that overall disease control was a consequence of both intrahepatic and extrahepatic control, a decrease of DCR, from 84% of intrahepatic area to 71.6% of overall could contemplate that extrahepatic progression occurred in a considerable number of *TARE_sorafenib* procedures (12.4%). Hence, enhancement of extrahepatic control by introducing a more potent systemic therapy might be a key of more effective treatment in this group of patients.

Table 7 Univariate analysis of time to progression using Kaplan-Meier method

Prognostic factors	*TARE_alone* (*n* = 63)				*TARE_sorafenib* (*n* = 81)			
	n[1]	*c*[1]	HR (95%CI)	*P* value	*n*[1]	*c*[1]	HR (95%CI)	*P* value
ECOG[2]								
0	32	13	0.57 (0.27-1.19)	0.131	40	8	1.96 (1.11-3.45)	0.018
1	28	13			40	16		
BCLC stage[3]								
B	26	9	0.62 (0.30-1.28)	0.193	16	5	1.02 (0.53-1.99)	0.947
C	31	14			65	19		
Child-pugh class								
A	57	24	0.35 (0.10-1.22)	0.083	78	23	0.75 (0.18-3.13)	0.694
B	6	3			3	1		
AFP								
< 400 ng/mL	54	22	0.73 (0.25-2.10)	0.556	52	15	0.78 (0.45-1.35)	0.378
≥ 400 ng/mL	9	5			29	9		
Cirrhosis								
Absence	15	6	1.08 (0.51-2.32)	0.835	29	7	1.39 (0.82-2.38)	0.222
Presence	48	21			52	17		
Number of tumors								
Single	12	6	0.71 (0.29-1.73)	0.449	9	5	0.59 (0.21-1.65)	0.308
Multiple	51	21			72	19		
Lobar involvement								
Unilobar	33	16	0.52 (0.27-1.04)	0.058	22	7	0.95 (0.52-1.74)	0.870
Bilobar	30	11			59	17		
Infiltrative tumor[4]								
Absence					58	15	1.14 (0.62-2.09)	0.673
Presence					23	9		
MVI[4]								
Absence					56	12	1.76 (0.92-3.35)	0.081
Presence					24	11		
EHD[4]								
Absence					60	23	0.37 (0.21-0.65)	< 0.001
Presence					21	1		
IHT[4]								
≤ 50%					37	15	0.54 (0.32-0.93)	0.024
> 50%					44	9		

[1]Total patients (*n*) and censored patients (*c*).
[2]ECOG 2 (*n* = 2 *TARE_alone*, *n* = 1 *TARE_sorafenib*) and ECOG 3 (*n* = 1 *TARE_alone*, *n* = 0 *TARE_sorafenib*) excluded.
[3]BCLC A (*n* = 5 *TARE_alone*, *n* = 0 *TARE_sorafenib*) and BCLC D (*n* = 1 *TARE_alone*, *n* = 0 *TARE_sorafenib*) excluded.
[4]Absent in all *TARE_alone* procedures according to institutional treatment algorithm.
Hazard ratios (HR) with Cox proportional hazard regression and *P* value with log-rank test. TARE: Transarterial radioembolization; ECOG: Eastern cooperative oncology group; BCLC: Barcelona clinic liver cancer; AFP: Alpha-fetoprotein; MVI: Macrovascular invasion; EHD: Extrahepatic disease; IHT: Intrahepatic tumor.

Table 8 Multivariate analysis of time to progression using Cox proportional hazard model

Population	Prognostic factors	*n*[1]	*c*[1]	HR (95%CI)	*P* value
TARE_alone (*n* = 63)	Child-pugh class				
	A	57	24	0.32 (0.09-1.10)	0.073
	B	6	3		
	Lobar involvement				
	Unilobar	33	16	0.51 (0.26-1.00)	0.051
	Bilobar	30	11		
TARE_sorafenib (*n* = 81)	ECOG[2]				
	0	40	8	0.85 (0.59-1.22)	0.370
	1	40	16		
	MVI				
	Absence	56	12	1.15 (0.74-1.80)	0.532
	Presence	24	11		
	EHD				
	Absence	60	23	0.37 (0.24-0.56)	< 0.001
	Presence	21	1		
	IHT				
	≤ 50%	37	15	0.72 (0.49-1.06)	0.096
	> 50%	44	9		

[1]Total patients (*n*) and censored patients (*c*).
[2]ECOG 2 (*n* = 1) and ECOG 3 (*n* = 0) excluded.
TARE: Transarterial radioembolization; ECOG: Eastern cooperative oncology group; MVI: Macrovascular invasion; EHD: Extrahepatic disease; IHT: Intrahepatic tumor.

In a prospective study on efficacy of TARE in unresectable HCC patients with IHT ≤ 50%, variables affecting TTP were tumor diameter (> 6 cm *vs* ≤ 6 cm., HR 3.65; 95%CI: 1.39-9.59, *P* = 0.0087) and treatment response according to European Association for the study of the liver (PD *vs* CR + PR + SD, HR 22.48; 95%CI: 4.53-111.61, *P* = 0.0001) [7]. Nevertheless, there was no prognostic factor of TTP for *TARE_alone* subgroup in our study. We presumed that our institutional selection criteria for *TARE_alone*, IHT ≤ 50% and absence of EHD, MVI and infiltrative/ill-defined HCC probably made this group of patients had relatively low disease burden. *TARE_alone* was rather effective for all, therefore, minor differences in baseline characteristics might not affect the duration of TTP.

EHD at time of procedure (absence *vs* presence; HR 0.37; 95%CI: 0.24-0.56, *P* < 0.001) was a sole prognostic factor of TTP for *TARE_sorafenib*. A quarter of *TARE_sorafenib* procedures also had EHD before treatment (25.9%) and 32% of progression of *TARE_sorafenib* subgroup was extrahepatic area first. All of these findings supported a significance of pre-existing EHD on disease control. Taking all of these findings together, *TARE_sorafenib* for patient with pre-existing EHD might be inadequate. More aggressive treatment such as TARE with other novel agents should be considered for future clinical trial.

In this study, we demonstrated a correlation between disease burden, given treatment and disease control. Moreover, we successfully identified some unique failure patterns which could guide possible ways to provide a better disease control. To the best of our knowledge, the current study was one of very few studies addressing this kind of issues. Our outcome measurements, TTP and tumor response were both direct parameters reflecting efficacy of treatment[8,29]. Additionally, treatment response of all procedures were re-assessed by using the mRECIST. Anti-tumor effects of TARE and sorafenib might not result in tumor shrinkage but they could produce tumor necrosis[30,31]. As a result, assessment on the basis of enhancement like mRECIST was more appropriate to our study than size-based

evaluation of response evaluation criteria in solid tumors.

Several limitations of our study related to natures of retrospective study. First, we acknowledged that mixed imaging techniques for evaluation of treatment response (CT n = 102, 60.4% and MRI n = 67, 39.6%) might produce some heterogeneities in diagnostic performance. Second, median radiologic follow-up duration was only 4.4 mo. This period was rather short because many patients that were referred for TARE at our institution had only 1 imaging follow-up study at our institution. Furthermore, all of 3 post-treatment imaging studies done within the first month after TARE showed rapid disease progression, either in treated area (*TARE_sorafenib* n = 1) or extrahepatic area (*TARE_no_sorafenib* n = 2). Lastly, number of patients in *TARE_no_sorafenib* subgroup was too limited to be statistically meaningful.

In the present study, we found that disease progression in *TARE_alone* subgroup usually limited to intrahepatic area and majority of progression originated in treated area. Therefore, either local or systemic treatment which promotes disease control at treated area might lead to better overall disease control. In contrast, disease progression in *TARE_sorafenib* subgroup tends to be extrahepatic and pre-existing EHD could worsen disease control. Study on using of TARE in combination with novel systemic therapy that is more potent than sorafenib might be required to improve treatment outcome.

CONCLUSION

TARE_alone for procedures with IHT ≤ 50% and absence of ADFs and *TARE_sorafenib* for procedures with IHD > 50% and/or presence of ADFs could provide acceptable disease control of approximately 70% in unresectable HCC patients. Intrahepatic progression was the most common failure pattern in both subgroups but extrahepatic progression was far more common in *TARE_sorafenib*. Strategies that improve intrahepatic control for liver-only disease (dosimetry-based TARE) and extrahepatic control for metastatic disease (additional systemic therapy) could improve TARE outcome for HCC patients.

ARTICLE HIGHLIGHTS

Research background

Survival outcome of unresectable hepatocellular carcinoma (HCC) patients post yttrium-90 (Y-90) glass microspheres transarterial radioembolization (TARE) with/without sorafenib according to individual's disease burden might partly be confounded by subsequent treatments. Therefore, a study on tumor response might better represent effectiveness of TARE with/without sorafenib.

Research motivation

Disease control and failure patterns following TARE with/without sorafenib might suggest how to intensify treatment to improve treatment outcome.

Research objectives

This study describes the disease control and failure patterns of unresectable HCC patients who underwent Y-90 microspheres TARE with/without sorafenib according to individuals' disease burden, *i.e.*, intrahepatic tumor (IHT) and adverse disease features (ADFs), consisting of macrovascular invasion, extrahepatic disease (EHD) and infiltrative/ill-defined HCC.

Research methods

Y-90 microspheres TARE procedures with available pre and post-treatment imaging studies (n = 169) were retrospectively reviewed and categorized into 3 subgroups on the basis of treatment given and individuals' disease conditions: (1) *TARE_alone*, referred to TARE only for IHT ≤ 50% without ADFs (n = 63); (2) *TARE_sorafenib*, referred to TARE with sorafenib for IHT > 50% and/or presence of ADFs (n = 81); and (3) *TARE_no_sorafenib*, referred to TARE only for patients with contraindication to sorafenib or side effect intolerance (n = 25). Disease control rate (DCR; consisted of complete response, partial response and stable disease) and failure patterns of treated, intrahepatic and extrahepatic sites were assessed using mRECIST.

Research results

The key findings were that *TARE_alone* for procedures with IHT ≤ 50% and absence of ADFs and *TARE_sorafenib* for procedures with IHT > 50% and/or presence of ADFs could provide comparable DCR (79% *vs* 72%) with similar incidence of intrahepatic progression (44.5% *vs* 38.5%). However, extrahepatic progression was much more common in *TARE_sorafenib* procedures (13% *vs* 32%).

Research conclusions

DCR of *TARE_alone* and *TARE_sorafenib* procedures were similar (about 70%). Intrahepatic progression was dominant failure pattern for both (about 40%) but extrahepatic progression was far more common in *TARE_sorafenib* procedures.

Research perspectives

On the basis of findings in the present study, we suggested further investigations on additional treatment to enhance disease control. Disease progression in *TARE_alone* subgroup usually originated in treated area and mostly limited to intrahepatic area. Thus, local or systemic treatment which potentiates disease control at treated lesion might result in better overall disease control. In *TARE_sorafenib* subgroup, extrahepatic progression was common and pre-existing EHD could worsen disease control. Study on novel systemic therapy that is more potent than sorafenib might be required to improve treatment outcome in this group of patients.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 21; 27(47): 8182-8193

DOI: 10.3748/wjg.v27.i47.8182

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

ORIGINAL ARTICLE

Observational Study

Real-world local recurrence rate after cold polypectomy in colorectal polyps less than 10 mm using propensity score matching

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Abstract

BACKGROUND

Cold polypectomy (CP) is a simple and safe procedure for polyps less than 10 mm in size; however, there is concern about local recurrence following CP because of unidentified margins of excised specimens and the lack of tumor suppression effect by coagulation. Some clinical trials have evaluated local persistent recurrence; their results suggest that a higher rate of local recurrence has not been documented so far. There were few reports that observed the course over long periods of time after CP in clinical practice.

AIM

To evaluate the presence of local recurrence following CP and hot polypectomy (HP) using propensity score matching.

METHODS

We analyzed 275 patients who underwent polypectomy for non-pedunculated colorectal polyps less than 10 mm (959 Lesions) between October 2016 and 2017 and underwent follow-up endoscopy subsequently. We divided them into the CP group (706 Lesions), wherein CP was performed, and the HP group (253 Lesions), wherein HP was performed. Using propensity score matching, we extracted 215 Lesions in each group and evaluated the local recurrence and content of CP in the real clinic and adverse events using medical records.

Institutional review board statement: This real-world retrospective study was approved by the Ethical Review Board of the Nagoya University Hospital (2015-0449).

Informed consent statement: Informed consent was obtained in the form of opt-out on the website.

Conflict-of-interest statement: The authors declare no conflicts of interest for this article.

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

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Received: June 17, 2021
Peer-review started: June 17, 2021
First decision: July 2, 2021
Revised: July 15, 2021
Accepted: December 7, 2021
Article in press: December 7, 2021
Published online: December 21, 2021

RESULTS

After propensity score matching, there were no significant differences in the patients' and their endoscopic background (age, use of antithrombotics, indications, size, morphology, location of polyps, and polypectomy device) between the groups. The mean duration between colorectal polypectomy and the next follow-up colonoscopy was 17.5 ± 7.1 (range, 6-39) mo in the CP group and 15.7 ± 6.0 (range, 6-35) mo in the HP group, which was significantly longer in the CP group (P = 0.005). The local recurrence rate was 0.93% in the CP group and 0.93% in the HP group, without a significant difference (P = 0.688). Additionally, no differences were observed in the macroscopic *en bloc* resection rate, histopathological complete resection rate, and pathological results between the groups. Adverse events did not occur in either group.

CONCLUSION

Local recurrence after CP was equivalent to that following HP in clinical practice. CP is useful and safe in the treatment of non-pedunculated polyps of less than 10 mm.

Key Words: Cold polypectomy; Colorectal polyp; Hot polypectomy; Local recurrence; Safety; Propensity score matching

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Core Tip: In this study, the recurrence rate after cold polypectomy (CP) was evaluated with colonoscopy at long intervals in real clinical practice for non-pedunculated colorectal polyps smaller than 10 mm, and compared with hot polypectomy (HP). Although it is a retrospective study, we used propensity score matching to correct the bias of both groups and compared them. The recurrence rates of both procedures were similar, and it was considered that CP, which is easier in clinical practice, is more useful for small polyps than HP.

Citation: Saito M, Yamamura T, Nakamura M, Maeda K, Sawada T, Ishikawa E, Mizutani Y, Ishikawa T, Kakushima N, Furukawa K, Ohno E, Kawashima H, Ishigami M, Fujishiro M. Real-world local recurrence rate after cold polypectomy in colorectal polyps less than 10 mm using propensity score matching. *World J Gastroenterol* 2021; 27(47): 8182-8193
URL: https://www.wjgnet.com/1007-9327/full/v27/i47/8182.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i47.8182

INTRODUCTION

It has been reported that the mortality of patients with colorectal cancer decreases with the resection of all adenomatous polyps (clean colon)[1]; therefore, it has become desirable to resect even diminutive polyps. There are reports which state that ≥ 90% of polyps are less than 10 mm on colonoscopic examination, with 70%-80% being less than 5 mm[2-4]. According to these reports, it is important to decide how to efficiently and safely remove such diminutive polyps for a clean colon. Cold polypectomy (CP) has been used in the treatment of small colorectal polyps because of the suitable efficiency and safe outcomes[5-8]. It has been reported that CP is an easy-to-perform technique. The frequency of adverse events such as delayed bleeding and perforation is lower with CP than those with hot polypectomy (HP) because it avoids electrocoagulation; furthermore, it has been considered useful in the endoscopic resection of sub-centimeter polyps. However, the presence of the lesions with unknown margins in resection specimens, has been reported in as high as 40% of the cases in cold snare polypectomy (CSP); therefore, there is concern about local recurrence after the treatment[9]. Another concern is the increased risk of local recurrence after CP compared to that after HP because the tumor suppression effect may not be expected by electrocoagulation in the resection sites following CP. One randomized trial reported that the ratio of local recurrence after treatment was equivalent between CSP and hot snare polypectomy (HSP)[10]. Another report indicated that the ratio

P-Reviewer: Chen Z
S-Editor: Chang KL
L-Editor: A
P-Editor: Chang KL

immediately after CSP is significantly higher than that after endoscopic mucosal resection (EMR)[11]. However, these reports were all evaluations of local recurrence just after endoscopic resection and did not confirm it after a certain period of time. Therefore, in this study, we assessed the presence of local recurrence following CP and HP after a long period following polypectomy in real clinical practice.

MATERIALS AND METHODS

Study design

This real-world retrospective study was conducted at the Nagoya University Hospital. The inclusion criteria of the CP procedure in Nagoya University Hospital is for non-pedunculated polyps only, less than 10 mm, and diagnosed as Type 2A in the Japan Narrow band imaging Expert Team (JNET) classification[12] using imaged-enhanced endoscopy with magnification, in short, suspected adenomatous lesions. As the size of the polyp increases, the rate of advanced neoplasia (with villous or tubulovillous adenoma components, size ≥ 10 mm, and high-grade dysplasia) also increases[3]. Therefore, a polyp > 10 mm is an indication for EMR. In pedunculated lesions, a large blood vessel is often found in the stem, which may be difficult to resect using CP; this might result in a very high risk of bleeding after resection. Therefore, we excluded them in the inclusion criteria of CP[13-15].

Using clinical records, we extracted data of 612 patients (2619 Lesions) who had undergone polypectomy at the Nagoya University Hospital between October 2016 and October 2017. Of them, data from 313 patients (1449 Lesions) who underwent follow-up colonoscopy more than half a year after the first polypectomy were extracted. We excluded data from 16 patients (303 Lesions) who were diagnosed with polyposis (familial adenomatous polyposis, Peutz–Jeghers syndrome, or other hereditary polyposis syndromes) or inflammatory bowel disease and 187 Lesions (22 patients) which were diagnosed with JNET Type 2B and lesions out of the inclusion criteria for CP such as size ≥ 10 mm and pedunculated or depressed lesions. Finally, we identified 959 Lesions (275 patients), which were divided into the HP (253 Lesions) and CP groups (706 Lesions).

The mean size of the lesions was significantly smaller in the CP group (CP group: 3.82 ± 1.49 mm; HP group: 5.35 ± 1.77 mm; $P < 0.001$), the ratio of flat lesions was significantly higher in the CP group (CP group: 62.0%; HP group: 43.5%; $P < 0.001$), and the resection ratio with the snare was significantly higher in the HP group (CP group: 86.5%; HP group: 99.2%; $P < 0.001$) (Table 1). It has been reported that the recurrence rate after polypectomy increases as the size of the lesion increases. Regarding the morphology of the lesion, the morphological difference might affect the treatment method (CP or HP). For polypectomy devices, the biopsy forceps has been reported to have a lower complete resection rate than the snare, which may affect recurrence rates. Therefore, to adjust for the bias between both groups, we performed propensity score matching based on the size and morphology (sessile or flat) of the lesions and the polypectomy device (biopsy forceps or snare), which could have an influence on the local recurrence. The CP (215 Lesions) and HP groups (215 Lesions) (total 206 patients) were compared after propensity score matching (Figure 1).

The evaluation items included local recurrence, histological complete resection rate, delayed bleeding, and perforation. We defined local recurrence as a polyp on the post-polypectomy scar and delayed bleeding as bleeding requiring endoscopic hemostasis treatment within two weeks of the polypectomy. For identification of the resected lesion, we referred to the post-polypectomy scar and the scope insertion length from the anal verge to the lesion as described in the patient's previous colonoscopy report.

Procedures

The instruments used in this study included XL-4450/LL-4450 (light source), VP-4450HD (processor), EC-L590ZW/EC-600ZP (scope) (Fujifilm Co., Tokyo, Japan) and CLV-290SL (light source), CV-290 (processor), and CF-H260AZI/CF-HQ290I (scope) (Olympus Co., Tokyo, Japan). The participating physicians were 22 expert endoscopists who had each performed > 1000 colonoscopies, including polypectomies. The snare used in this study included Snare Master 15 mm (Olympus Co., Tokyo, Japan), Profile 11 mm/13 mm and Captivator II 10 mm (Boston Scientific Co., Boston, MA, United States) in both groups. Additionally, as the biopsy forceps, Radial Jaw 4 JUMBO in the CP group and Radial Jaw 4 in the HP group (Boston Scientific Co., Boston, MA, United States) were used. In principle, the biopsy forceps were used for lesions < 4 mm, while the snare was used for lesions ≥ 4 mm because the histological

Table 1 Characteristics of excised polyps before propensity score matching

	CP, mean ± SD or *n* (%)	HP, mean ± SD or *n* (%)	*P*-value
Number of polyps resected	706	253	
Size, mm	3.82 ± 1.49	5.35 ± 1.77	< 0.001[a]
Morphology			< 0.001[b]
Sessile	268 (38.0)	143 (56.5)	
Flat	438 (62.0)	110 (43.5)	
Location			0.439[b]
Cecum	46 (6.5)	15 (5.9)	
Ascending colon	182 (25.8)	59 (23.3)	
Transverse colon	220 (31.2)	58 (22.9)	
Descending colon	92 (13.0)	33 (13.0)	
Sigmoid colon	128 (18.1)	67 (26.5)	
Rectum	38 (5.4)	21 (8.3)	
Polypectomy device			< 0.001[b]
Snare	611 (86.5)	251 (99.2)	
Biopsy forceps	95 (13.5)	2 (0.8)	

[a]Welch test.
[b]Chi-Square test.
CP: Cold polypectomy; HP: Hot polypectomy.

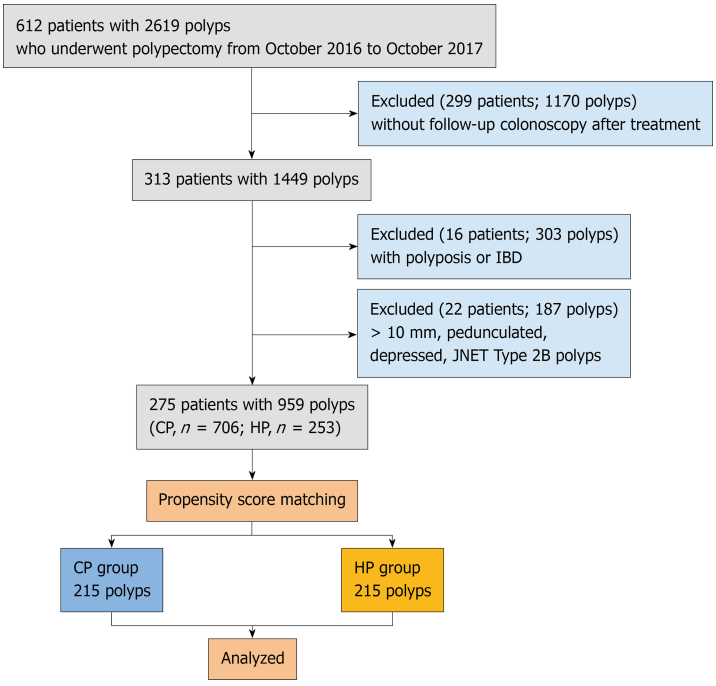


Figure 1 Flowchart of the study.

complete resection rate for lesions ≥ 4 mm is lower with cold forceps polypectomy (CFP)[16]. The physicians decided whether to use the biopsy forceps or the snare. We determined the size of the lesion based on the outer diameter of the tip cup diameter of the biopsy forceps or the snare.

Statistical analysis

JMP v15 (SAS Institute, Cary NC, United States) was used for propensity score matching, and SPSS v24.0 (IBM, Armonk, New York, United States) was used for statistical analysis in this study. We used the chi-square test to compare the morphology, location of lesions, polypectomy device (before propensity score matching), pathological diagnosis, histopathology results, and histological complete resection rate between the groups. Student's *t*-test was used to compare the mean size of lesions (after propensity score matching) and the mean follow-up period. Welch test was used to compare the mean size of lesions (before propensity score matching), and Fisher's exact test was used to compare the polypectomy device (after propensity score matching), tissue retrieval rate, macroscopic *en bloc* resection rate, acute bleeding rate, and local recurrence. Statistical significance was set at $P < 0.05$.

RESULTS

Characteristics of patients and lesions after propensity score matching

In the 206 patients (139 men and 67 women) included in the study, the mean age was 68.7 ± 8.63 years, the use of antithrombotics was 19.4%, and the indications for colonoscopy included screening ($n = 186$), constipation ($n = 9$), abdominal pain ($n = 6$), anemia ($n = 2$), and bloody stools ($n = 2$) (Table 2).

Regarding the excised polyps, the mean size was 4.95 ± 1.60 mm in the CP group and 4.94 ± 1.58 mm in the HP group. The morphology included 111 sessile lesions (51.6%) and 104 flat lesions (48.4%) in the CP group, and 113 sessile lesions (52.6%) and 102 flat lesions (47.4%) in the HP group. The lesion locations in the CP and HP groups included the cecum (6.0% and 7.0%, respectively), ascending colon (25.1% and 23.7%, respectively), transverse colon (33.0% and 22.3%, respectively), descending colon (12.6% and 12.1%, respectively), sigmoid colon (19.1% and 25.6%, respectively), and the rectum (4.2% and 9.3%, respectively). In both groups, 99.1% of the procedures were performed using a snare and 0.9% using biopsy forceps. There were no significant differences in the mean size, morphology, location, or polypectomy device between the groups.

Regarding the subsequent pathological diagnosis of the lesions, the difference was not statistically significant between the groups ($P = 0.117$): 186 Lesions with low-grade adenoma (88.6%), seven lesions with advanced neoplasia (high-grade dysplasia or lesions including villous or tubulovillous adenoma components) (3.3%) in the CP group *vs* 168 Lesions with low-grade adenoma (79.2%), 14 Lesions with advanced neoplasia (6.6%) in the HP group (Table 3).

Treatment outcomes and complications

The macroscopic *en bloc* resection rate was 99.1% (213 Lesions) in the CP group and 98.1% (211 Lesions) in the HP group; however, the difference was not significant ($P = 0.343$). The number of lesions with low-grade adenoma and advanced neoplasia whose margins were evaluated pathologically was 183 in the CP group and 181 in the HP group. The rate of histological complete resection was 82.5% (151 Lesions) in the CP group and 84.0% (152 Lesions) in the HP group, and no significant difference was identified between the groups ($P = 0.708$). Acute bleeding was observed in six (2.8%) lesions in the CP group and three (1.4%) lesions in the HP group; it tended to be more common in the CP group, but the difference was not statistically significant ($P = 0.252$). Delayed bleeding or perforation was not observed in either group (Table 4).

Follow-up colonoscopy after polypectomy

The mean duration between colorectal polypectomy and the next follow-up colonoscopy was 17.5 ± 7.1 (range, 6-39) mo in the CP group and 15.7 ± 6.0 (range, 6-35) mo in the HP group, which was significantly longer in the CP group ($P = 0.005$). Local recurrence was observed in two (0.93%) lesions in both groups with no significant difference between them ($P = 0.688$) (Table 5; Figures 2 and 3).

DISCUSSION

CP is a safe treatment with a simple procedure and few complications. However, compared to HP, it may be difficult to evaluate whether or not complete resection is possible pathologically, and there is a concern that the risk of local recurrence may

Table 2 Characteristics of patients after propensity score matching

	mean ± SD, (%)
	n = 206
Male/female	139 (67.5)/67 (32.5)
Age, yr	68.7 ± 8.6
Range	46-85
Antithrombotic agent users	40 (19.4)
Indication for colonoscopy	
Screening	186 (90.3)
Constipation	9 (4.3)
Abdominal pain	6 (2.9)
Bloody stools	2 (1.0)
Anemia	2 (1.0)
Others	1 (0.5)

increase because there is no tumor suppression effect by electrocoagulation. Some facilities are cautious about its adaptation. In this study, we focused on the local recurrence rate and retrospectively analyzed lesions that could be followed up with an endoscope for a relatively long period of time in real clinical practice. From the results of this study, it was considered that there is no difference in recurrence rate between CP and HP in non-pedunculated colorectal polyps smaller than 10 mm, and CP can be selected as one of the useful treatment methods for small colorectal polyps.

Endoscopic resection of colorectal polyps is one of the common treatments in digestive endoscopy, and resection of all adenomatous polyps, including diminutive lesions, is expected to become increasingly important to decrease the morbidity and mortality of colorectal cancer. Delayed bleeding or perforation, as complications of polypectomy, may require re-admission to the hospital along with additional endoscopy to stop the bleeding, blood transfusion, or surgery if necessary, which is not only a burden to the patient but also to the medical staff and the economy. The incidence of delayed bleeding and perforation in conventional HP has been reported to be 0.26%-1.4% and 0.017%-0.091%[17]. However, polypectomy is routinely performed in many patients, and the adverse events should never be ignored, even if their frequency is low. It has been reported that CP has a lower risk of complications compared with HP[6,16,18,19]. However, the long-term risk of residual recurrence after CP in clinical practice has not yet been sufficiently investigated.

It is important to visually confirm that there are no residual lesions following polypectomy; however, since CP does not have a burn effect like HP does, there is a risk of recurrence if there are residual lesions that cannot be detected visually after the treatment. There are three methods for examining the presence of remnants after polypectomy. First, histopathological evaluation of the resected specimen is performed to confirm whether complete resection was achieved. In CP, specimen damage due to aspiration and collection of specimens is more likely to occur than in HP; it has been reported that pathological resection margins are more frequently unknown in CP[18, 19]. Second, biopsy of the resected ulcer margins or mucosal resection is performed to confirm any remnants histologically. It has been reported that the resected region is resected again with a snare or biopsied with forceps immediately after polypectomy and histopathologically evaluated for the presence of remnants. The residual rate has been reported to be 3.4% in CSP and 17.4% in CFP by Kim *et al*[20], 10% in CSP and 11% in CFP by Gómez *et al*[21], 3.9% in CSP by Matsuura *et al*[22], and 1.8% in CSP and 2.6% in HSP by Kawamura *et al*[10]. The rates varied slightly between these reports. However, lesions left at the margins immediately after resection may fall off later (especially in HP, due to the effects of electrical coagulation). It remains unclear whether they will eventually become residual recurrent lesions. Third, endoscopic confirmation of the polyp resection site is repeated after a certain duration. This is a reliable assessment of residual recurrence but includes some hurdles. First, the patient must undergo a follow-up colonoscopy, which can be physically burdensome. Additionally, because of the sufficient follow-up period, the resection sites become scars, and the scar after CP is more obscured than that after HP, which may make it

Table 3 Characteristics of excised polyps after propensity score matching

	CP, mean ± SD or *n* (%)	HP, mean ± SD or *n* (%)	*P*-value
Number of polyps excised	215	215	
Size, mm	4.95 ± 1.60	4.94 ± 1.58	0.952[a]
Morphology			0.847[b]
Sessile	111 (51.6)	113 (52.6)	
Flat	104 (48.4)	102 (47.4)	
Location			0.736[b]
Cecum	13 (6.0)	15 (7.0)	
Ascending colon	54 (25.1)	51 (23.7)	
Transverse colon	71 (33.0)	48 (22.3)	
Descending colon	27 (12.6)	26 (12.1)	
Sigmoid colon	41 (19.1)	55 (25.6)	
Rectum	9 (4.2)	20 (9.3)	
Polypectomy device			0.688[c]
Snare	213 (99.1)	213 (99.1)	
Biopsy forceps	2 (0.9)	2 (0.9)	
Pathologic diagnosis[1]	210 lesions	212 lesions	0.117[b]
Low-grade adenoma	186 (88.6)	168 (79.2)	
Advanced neoplasia[2]	7 (3.3)	14 (6.6)	
Hyperplastic polyp and SSL	14 (6.7)	28 (13.2)	
Others	3 (1.4)	2 (0.9)	
Failure of tissue retrieval	5 (2.3)	3 (1.4)	0.362[c]

[1]Indicates polyps that were retrieved successfully and evaluated pathologically.
[2]Defined as high-grade dysplasia or the lesions including villous or tubulovillous adenoma component.
[a]Student's *t*-test.
[b]Chi-Square test.
[c]Fisher's Exact test.
CP: Cold polypectomy; HP: Hot polypectomy; SSL: Sessile serrated lesion.

difficult to identify the regions of post-polypectomy. Lee *et al*[23] reported that the overall recurrence over 59.7 mo was 17% (4% definite recurrence and 13% probable recurrence) after CFP in 1111 diminutive polyps. This recurrence rate is much higher than that reported in other studies. Probable recurrence was defined as recurrence at a similar distance from the anal verge (± 3 cm) in the same colorectal segment as a previous polyp and accounted for the majority of all recurrences. These lesions may be indistinguishable from newly formed polyps or previously overlooked polyps.

Murakami *et al*[24] reported that recurrence was observed in 1.4% of lesions less than 10 mm and 5.4% of lesions of 10-14 mm in follow-up colonoscopy more than 10 mo after CSP. If the scar was unclear, they were observed by going back and forth multiple times across segments estimated by the distance from the anal verge. When there were no new polyps after such cautious colonoscopy, the patient was considered to have no recurrence. The frequency of detection of scars was not reported; however, it appears to be an acceptable method in actual clinical practice. Maruoka *et al*[25] reported that clipping was performed in the vicinity of the ulcer after CSP, and colonoscopy was repeated three weeks later. After the scar was identified using the clip as a guide, the scar area was biopsied to evaluate the remnants. They indicated that the recurrence rate using the above method was 0.98%. Since the clip would naturally drop off after a certain period, it appeared to be the limit of the period that can be evaluated using this method.

In this study, follow-up colonoscopy was performed after 6-39 mo (average of 17.5 mo in the CP group and 16.2 mo in the HP group) after the treatment. We adopted an

Table 4 Treatment outcomes and complications

	CP, *n* (%)	HP, *n* (%)	*P* value
	n = 215	*n* = 215	
Macroscopic *en bloc* resection	213 (99.1)	211 (98.1)	0.343[a]
Tissue retrieval successfully	210 (97.7)	212 (98.6)	0.362[a]
Snare polypectomy	208/213 (97.7)	210/213 (98.6)	
Biopsy forceps polypectomy	2/2 (100)	2/2 (100)	
Lesions diagnosed with low-grade adenoma or advanced lesion and evaluated for histological margin	*n* = 183	*n* = 181	
Histological complete resection	151 (82.5)	152 (84.0)	0.708[b]
Complications (%)			
Acute bleeding[1]	6 (2.8)	3 (1.4)	0.252[a]
Delayed bleeding[2]	0 (0)	0 (0)	-
Perforation	0 (0)	0 (0)	-

[1]Indicates bleeding continued for 30 seconds immediately after polypectomy.
[2]Indicates bleeding requiring endoscopic hemostasis within two weeks after polypectomy.
[a]Fisher's Exact test.
[b]Chi-Square test.
CP: cold polypectomy; HP: hot polypectomy.

Table 5 Results of follow-up colonoscopy after polypectomy

	CP, mean ± SD or *n* (%)	HP, mean ± SD or *n* (%)	*P* value
Resected polyps	215	215	
Follow-up period, mo	17.5 ± 7.1	15.7 ± 6.0	0.005[a]
Range	6-39	6-35	
Local recurrence	2 (0.93)	2 (0.93)	0.688[b]

[a]Student's *t*-test.
[b]Fisher's Exact test.
CP: Cold polypectomy; HP: Hot polypectomy.

evaluation method similar to that used by Murakami *et al*[24] to assess the presence of residual recurrence. That is, we first looked for a scar after treatment, and if it was unclear, observed the excision site estimated from the distance from the anal margin multiple times, and judged that there was no recurrence if there was no new polyp. The number of lesions that led to recurrence was 2 (0.93%) in both groups, which was not significantly different (P = 0.688). The local recurrence rate in the CP group in this study was 0.93%. It was similar to 1.4% in CSP for < 10-mm colorectal polyps that Murakami *et al*[24] reported or 0.98% in CSP that Maruoka *et al*[25] reported, and it was expected to be lower than the residual rates reported by the second method of confirming the presence of remnants after polypectomy, that is, pathologically evaluated by biopsy or snare immediately after polypectomy[10,20-22]. The result may be due to the fall-off of small residual lesions at the margins of the excision and overlooking recurrent lesions in actual clinical practice. Additionally, no significant difference was observed in the residual rate compared with the HP group, as previously reported.

Regarding the safety, the acute bleeding rate immediately after the procedure was 2.8% in the CP group and 1.4% in the HP group (P = 0.252), with no significant difference between the two groups. In all cases, the bleeding was stopped by clipping hemostasis. Acute bleeding in CP often stops spontaneously. In contrast, delayed bleeding and perforation were not observed in either group, thus, confirming the safety of CP as reported previously[5-8].

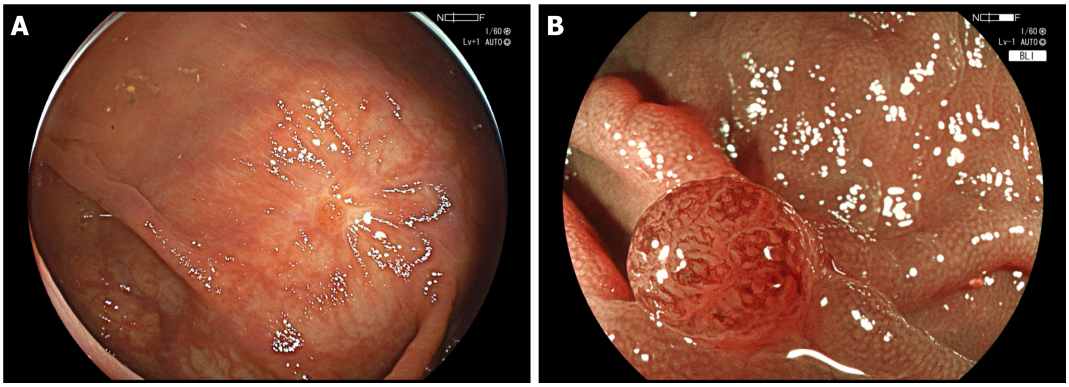


Figure 2 A case of local recurrence following cold snare polypectomy. A: A diminutive polyp was detected on the scar during follow-up colonoscopy 16 mo after CSP with a 6-mm sessile polyp in the transverse colon; B: Magnified imaging using blue laser imaging.

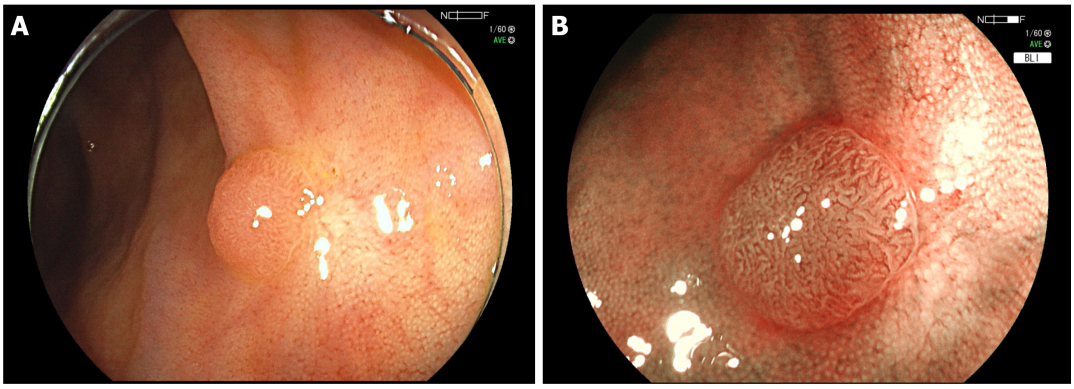


Figure 3 A case of local recurrence following endoscopic mucosal resection. A: A diminutive polyp was detected on the scar during follow-up colonoscopy 12 mo after EMR with a 6-mm sessile polyp in the cecum; B: Magnified imaging using blue laser imaging.

It has been reported that CP has a higher rate of pathologically positive or unknown resection margins than HP[18,19]. In this study, the histopathological complete resection rate was 82.5% in the CP group *vs* 84.0% in the HP group (P = 0.708), with no significant difference between the two groups.

Limitations

This study was a retrospective examination at a single institution, and the sample size was not very large. The follow-up period was not long and averaged a little over a year. The mean follow-up period was significantly longer in the CP group than that in the HP group. However, the results emphasize that the local recurrence rate in the CP group did not become higher compared with the HP group because the local recurrence rate was equivalent in both groups.

Colonoscopy was performed by several different endoscopists who might not have detected all recurrences because of differences in individual skills and the possibility of missing residual or recurrent lesions. Although the endoscopists in this study were experts, a new study should be conducted, including colonoscopy trainees.

Of the lesions selected in this study using propensity score matching, only two lesions were resected using forceps in each of the groups, and biopsy polypectomy was not fully evaluated because of the small sample size. Future prospective studies with a larger number of patients and longer follow-up periods are needed.

CONCLUSION

CP for non-pedunculated polyps of less than 10 mm is equivalent to HP in terms of the local recurrence rate. There were no complications of delayed bleeding or perforation, and CP was considered a safe and useful procedure for the treatment of non-pedunculated colorectal polyps less than 10 mm.

ARTICLE HIGHLIGHTS

Research background

Cold polypectomy (CP) is widely used as a simple and safe procedure for small colorectal polyps. However, there is concern that recurrence rate following CP may be higher than Hot polypectomy (HP) because of unidentified margins of excised specimens and the lack of tumor suppression effect by coagulation.

Research motivation

There were few reports that observed the course over long periods of time after CP in clinical practice. It is important to compare and evaluate the recurrence rate following CP and HP.

Research objectives

The aim of this study was to evaluate the presence of local recurrence following CP and HP using propensity score matching.

Research methods

We analyzed 275 patients who underwent polypectomy for non-pedunculated colorectal polyps less than 10 mm (959 Lesions) and follow-up endoscopy subsequently. We divided them into the CP group (706 Lesions) and the HP group (253 Lesions). Using propensity score matching, we extracted 215 Lesions in each group and evaluated the local recurrence of CP in the real clinic using medical records.

Research results

The local recurrence rate was 0.93% in the CP group and 0.93% in the HP group, without a significant difference (P = 0.688).

Research conclusions

Local recurrence after CP was equivalent to that following HP in clinical practice. CP is useful and safe in the treatment of non-pedunculated polyps of less than 10 mm.

Research perspectives

Future prospective studies with a larger number of patients and longer follow-up periods are needed in clinical practice.

ACKNOWLEDGEMENTS

The authors would like to thank Nagura A, Yoshimura T, Nakano A, Oshima H, Sato J, Ueno Y, Matsuura R, and Mizutani Y for performing the colonoscopies and polypectomies.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 21; 27(47): 8194-8198

DOI: 10.3748/wjg.v27.i47.8194

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

LETTER TO THE EDITOR

Microarray analysis to explore the effect of *CXCL12* isoforms in a pancreatic pre-tumor cell model

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Author contributions: Mi DH and Miao YD designed the research; Miao YD wrote this comment; Wang JT and Tang XL made academic advice; Mi DH reviewed this manuscript; all authors approved the final manuscript.

Conflict-of-interest statement: No conflict of interest associated with any of the senior authors or other coauthors contributed their efforts in this manuscript.

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

Open-Access: This article is an open-access article that was

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Abstract

CXCL12 expression was significantly lower in tumor samples than in corresponding normal samples. *CXCL12* expression was significantly positively related to the infiltration levels of T cells, dendritic cells (DCs), immature DCs, cytotoxic cells, Tfh cells, mast cells, B cells, Th1 cells, natural killer (NK) cells, pDCs, neutrophils, and T helper cells (Spearman correlation coefficient > 0.5, $P < 0.001$) and negatively correlated with the infiltration level of NK CD56bright cells. In addition, pancreatic hTERT-HPNE cells treated with three diverse *CXCL12* isoforms exhibited changes mainly in the regulation of the epithelial-mesenchymal transition activation pathway.

Key Words: *CXCL12*; Pancreatic cancer; Splicing isoforms; Bioinformatics analysis; Tumor microenvironment; Pathway

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Core Tip: *CXCL12* expression was significantly lower in tumor samples than in normal samples. *CXCL12* expression was significantly positively associated with the infiltration levels of 12 immune cells, especially T cells, which may encourage further exploration of the effect of *CXCL12* in pancreatic ductal adenocarcinoma immunotherapy. In addition, treating pancreatic hTERT-HPNE cells with three diverse *CXCL12* isoforms mainly affected the regulation of the epithelial-mesenchymal transition activation pathway.

Citation: Miao YD, Wang JT, Tang XL, Mi DH. Microarray analysis to explore the effect of

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Received: June 22, 2021
Peer-review started: June 22, 2021
First decision: July 4, 2021
Revised: July 6, 2021
Accepted: December 2, 2021
Article in press: December 2, 2021
Published online: December 21, 2021

P-Reviewer: Nagaraju GP
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR



CXCL12 isoforms in a pancreatic pre-tumor cell model. *World J Gastroenterol* 2021; 27(47): 8194-8198
URL: https://www.wjgnet.com/1007-9327/full/v27/i47/8194.htm
DOI: https://dx.doi.org/10.3748/wjg.v27.i47.8194

TO THE EDITOR

We read with interest the article by Cecati *et al*[1]. They investigated the specific roles of α, β, and γ *CXCL12* isoforms in pancreatic ductal adenocarcinoma (PDCA) onset by microarray analysis of hTERT-HPNE cells cured by three diverse isoforms of *CXCL12*, which indicated that *CXCL12* isoforms have different roles in PDAC pathogenesis.

We appreciate the unique perspective provided by the authors' exploration of the roles of the different isomers of *CXCL12* in PDAC. However, the results might be made more meaningful if the authors built on this by presenting the differential expression of *CXCL12* in normal and tumor tissues of PDCA as a whole, such as through a bioinformatics analysis of PDCA cases in The Cancer Genome Atlas (TCGA) database or their own data. We discovered that the *CXCL12* expression was significantly lower in tumor samples than in normal samples (Figure 1A). Detailed statistical results are described in Table 1.

The tumor microenvironment (TME), mediated by interactions between stromal cells and pancreatic epithelial/carcinoma cells, is essential for PDCA progression and has been associated with failure of chemotherapy, radiotherapy, and immunotherapy [2]. The formation of the microenvironment requires interactions between pancreatic cancer cells and stromal cells. A pancreatic cancer microenvironment composition that favors demyelination and immunosuppression is related to poor prognosis[3-5]. Although immunotherapy has transformed cancer therapy, patients with PDCA rarely respond to these regimens, and this failure is attributed to poor infiltration and activation of T cells in the TME. We found that *CXCL12* expression was positively correlated with the level of infiltration of 22 immune cells, especially T cells (Figure 1B and C), which may encourage further exploration of the effect of *CXCL12* in PDCA immunotherapy. Detailed information on the correlation between *CXCL12* expression and immune cell infiltration is shown in Table 2.

We agree with Cecati *et al*[1], who reported that all *CXCL12* isoforms influenced cell migration, adhesion, and cytoskeleton-associated gene expression. In our study, we found that treating pancreatic hTERT-HPNE cells with three diverse *CXCL12* isoforms mainly affects the regulation of the EMT activation pathway (Figure 1D-F), which confirms that the work done by Cecati *et al*[1] is worthy of recognition and that our findings can be a supplement to their study. In the future, we should investigate the role played by *CXCL12* in the PDCA immune microenvironment in depth.

Statistical analysis

Software: R (version 3.6.3) was used to perform statistical analysis and visualization results. Differential expression of *CCXL12* between pancreatic cancer tissues and normal tissues was adopted by the Wilcoxon rank-sum test and visualized results using R-package "ggplot2". Immune cell algorithm: ssGSEA (built-in algorithm of GSVA package[6]). Correlation test using Spearman's correlation coefficient. Pathway analysis was performed by the online tool GSCALite (http://bioinfo.life.hust.edu.cn/web/GSCALite/)[7].

Table 1 Detailed statistical results of *CXCL12* differential expression analysis in pancreatic ductal adenocarcinoma (mean ± SD)

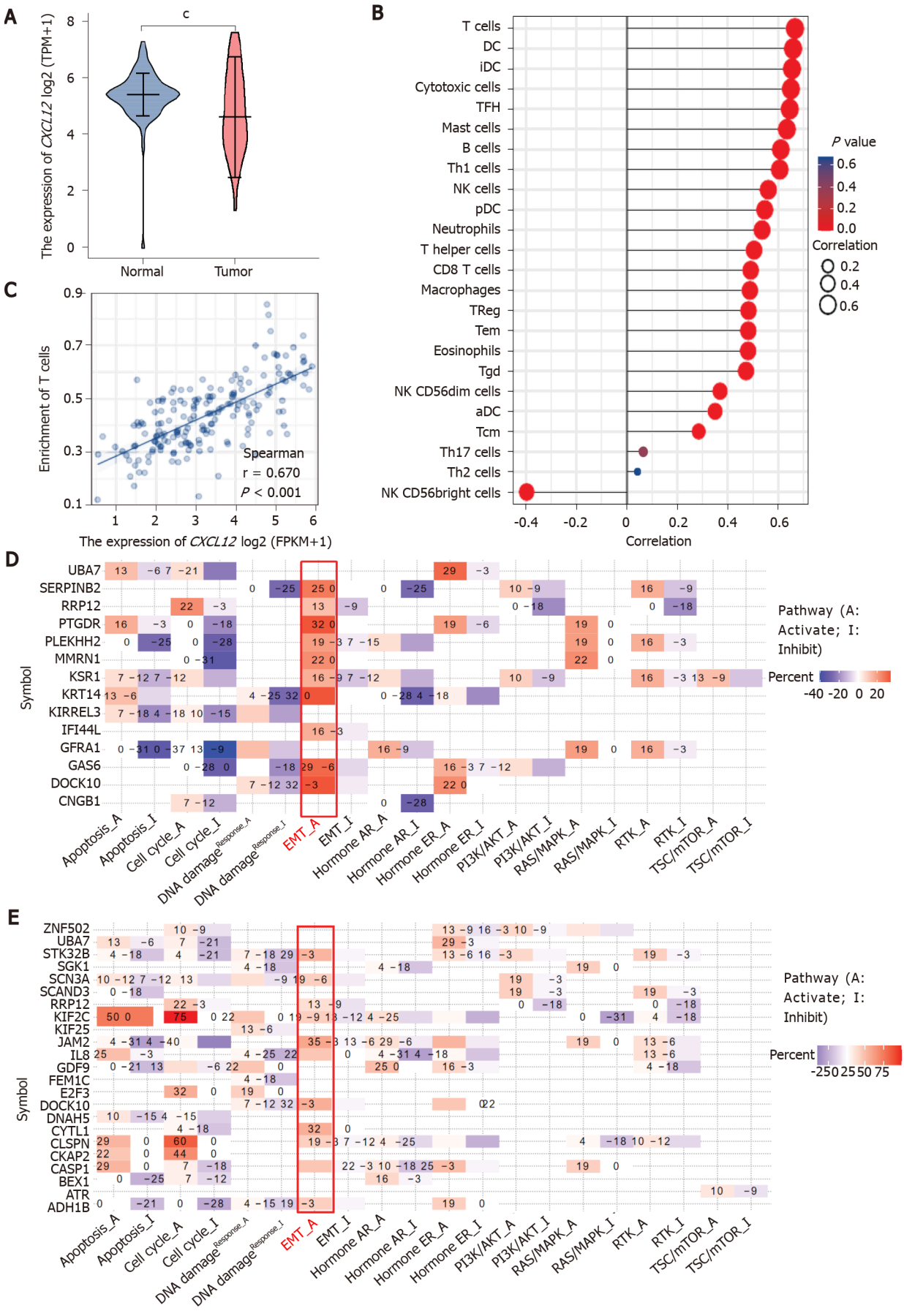
Group	Number	Minimum	Maximum	Median	IQR	Lower quartile	Upper quartile	Mean		SE
Normal	171	0	7.296	5.433	0.756	5.028	5.784	5.403	0.88	0.067
Tumor	179	1.333	7.629	4.632	2.134	3.727	5.861	4.803	1.445	0.108

IQR: Interquartile distance; SE: Standard error.

Table 2 Detailed information on the correlation between *CXCL12* expression and immune cell infiltration

Gene	Cell	Correlation coefficient (Pearson)	*P* value (Pearson)	Correlation coefficient (Spearman)	*P* value (Spearman)
CXCL12	aDC	0.355	< 0.001	0.350	< 0.001
CXCL12	B cells	0.614	< 0.001	0.610	< 0.001
CXCL12	CD8 T cells	0.508	< 0.001	0.491	< 0.001
CXCL12	Cytotoxic cells	0.674	< 0.001	0.650	< 0.001
CXCL12	DC	0.668	< 0.001	0.658	< 0.001
CXCL12	Eosinophils	0.488	< 0.001	0.480	< 0.001
CXCL12	iDC	0.639	< 0.001	0.654	< 0.001
CXCL12	Macrophages	0.488	< 0.001	0.487	< 0.001
CXCL12	Mast cells	0.635	< 0.001	0.634	< 0.001
CXCL12	Neutrophils	0.554	< 0.001	0.535	< 0.001
CXCL12	NK CD56bright cells	-0.411	< 0.001	-0.397	< 0.001
CXCL12	NK CD56dim cells	0.376	< 0.001	0.369	< 0.001
CXCL12	NK cells	0.566	< 0.001	0.560	< 0.001
CXCL12	pDC	0.558	< 0.001	0.546	< 0.001
CXCL12	T cells	0.682	< 0.001	0.666	< 0.001
CXCL12	T helper cells	0.511	< 0.001	0.504	< 0.001
CXCL12	Tcm	0.337	< 0.001	0.285	< 0.001
CXCL12	Tem	0.483	< 0.001	0.481	< 0.001
CXCL12	TFH	0.668	< 0.001	0.645	< 0.001
CXCL12	Tgd	0.364	< 0.001	0.472	< 0.001
CXCL12	Th1 cells	0.594	< 0.001	0.605	< 0.001
CXCL12	Th17 cells	0.057	0.453	0.065	0.387
CXCL12	Th2 cells	0.069	0.357	0.032	0.675
CXCL12	TReg	0.493	< 0.001	0.482	< 0.001

aDC: Activated DC; DC: Dendritic cells; iDC: immature DC; pDC: Plasmacytoid DC; Tfh: T follicular helper; Tgd: T gamma delta; NK: Natural killer.


A
c
The expression of CXCL12 log2 (TPM+1)
Normal
Tumor
B
T cells
DC
iDC
Cytotoxic cells
TFH
Mast cells
B cells
Th1 cells
NK cells
pDC
Neutrophils
T helper cells
CD8 T cells
Macrophages
TReg
Tem
Eosinophils
Tgd
NK CD56dim cells
aDC
Tcm
Th17 cells
Th2 cells
NK CD56bright cells
Correlation
P value
Correlation
C
Enrichment of T cells
Spearman
r = 0.670
P < 0.001
The expression of CXCL12 log2 (FPKM+1)
D
Symbol
Pathway (A: Activate; I: Inhibit)
Percent
Apoptosis_A
Apoptosis_I
Cell cycle_A
Cell cycle_I
DNA damage Response_A
DNA damage Response_I
EMT_A
EMT_I
Hormone AR_A
Hormone AR_I
Hormone ER_A
Hormone ER_I
PI3K/AKT_A
PI3K/AKT_I
RAS/MAPK_A
RAS/MAPK_I
RTK_A
RTK_I
TSC/mTOR_A
TSC/mTOR_I
E
Symbol
Pathway (A: Activate; I: Inhibit)
Percent

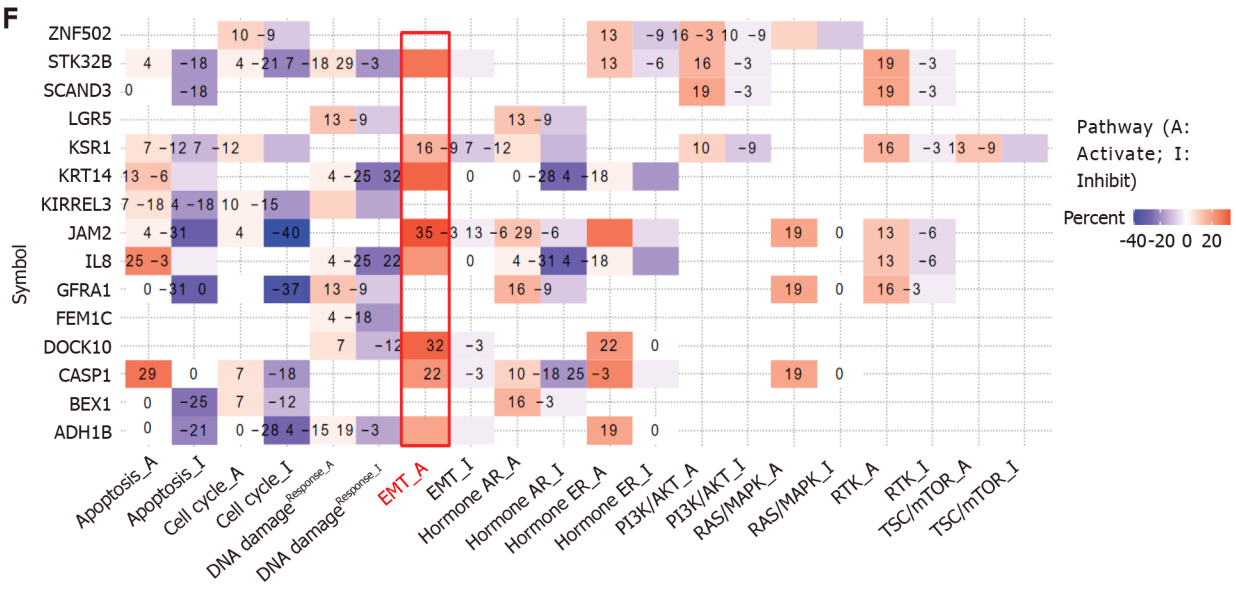


Figure 1 The effect of *CXCL12* in the development of pancreatic ductal adenocarcinoma. A: The differential *CXCL12* expression in pancreatic ductal adenocarcinoma (PDCA) and normal samples. The expression level of *CXCL12* in tumor tissues is indicated in orange, and that in normal tissues is indicated in purple. Data source: UCSC XENA (https://xenabrowser.net/datapages/) RNAseq data in TPM format for The Cancer Genome Atlas (TCGA) and GTEx processed uniformly through the Toil process[4]. PAAD (pancreatic cancer) data were extracted from TCGA, and corresponding normal sample data were from GTEx. Significance markers: NS, $P \geq 0.05$, $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$; B: The expression level of *CXCL12* and its relationship to 24 immune cell infiltration levels in PDCA. Data source: RNAseq data and clinical data in level 3 HTSeq-FPKM format from the TCGA (https://portal.gdc.cancer.gov/) PAAD (pancreatic cancer) project. Data filtering: Removal of paraneoplastic tissue; C: The expression level of *CXCL12* and its relationship to the T cell infiltration level in PDCA; D and E: Pathway analysis of differentially expressed genes under all treatment conditions (α, β, and γ *CXCL12* isoforms); D: *CXCL12* α isoform *vs* control; E: *CXCL12* β isoform *vs* control; F: *CXCL12* γ isoform *vs* control.

ACKNOWLEDGEMENTS

Yan-Dong Miao would like to give particularly grateful to Wu-Xia Quan for her care, patience, and support over the past years and for her contributions to this study.

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World Journal of Gastroenterology

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

World J Gastroenterol 2021 December 21; 27(47): 8199-8200

DOI: 10.3748/wjg.v27.i47.8199

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

LETTER TO THE EDITOR

Progress on global hepatitis elimination targets

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ORCID number: Yasir Waheed 0000-0002-5789-4215.

Author contributions: Waheed Y solely contributed to the article.

Conflict-of-interest statement: The authors declare having no conflicts of interest in relation to this publication.

Country/Territory of origin: Pakistan

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

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Abstract

In 2016, the World Health Assembly adopted a Global Health Sector Strategy on viral hepatitis, with targets set for the years 2020 and 2030 to achieve hepatitis elimination. The main target of hepatitis elimination strategy is to reduce the incidence of hepatitis B virus (HBV) and hepatitis C virus (HCV) by 90% and mortality by 65% in 2030. In last 5 years, the number of people receiving HCV treatment has increased from 1 million to 9.4 million; however, this number is far from the 2030 target of 40 million people receiving HCV treatment. HBV and HCV incidence rates are down from 1.4 million to 1.1 million annual deaths but this is far from the 2030 target of < 0.5 million deaths. The coronavirus disease 2019 pandemic has severely affected the efforts in the fight against hepatitis. No major donor has committed to investing in the fight against hepatitis. Time is running out. There is a need to speed up efforts in the fight against hepatitis to achieve hepatitis elimination by 2030.

Key Words: Hepatitis elimination; Blood donations; Safe injections; Hepatitis B vaccination; Harm reduction

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Core Tip: In 2020, progress was made in improving blood donations, hepatitis B and C treatments, and decreasing the incidence of hepatitis B and C. Some of the 2020 targets for hepatitis elimination were achieved but the 2030 targets are very ambitious and need strong political and financial support.

Citation: Waheed Y. Progress on global hepatitis elimination targets. *World J Gastroenterol* 2021; 27(47): 8199-8200

URL: https://www.wjgnet.com/1007-9327/full/v27/i47/8199.htm

DOI: https://dx.doi.org/10.3748/wjg.v27.i47.8199

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Received: July 13, 2021
Peer-review started: July 13, 2021
First decision: August 19, 2021
Revised: August 24, 2021
Accepted: December 7, 2021
Article in press: December 7, 2021
Published online: December 21, 2021

P-Reviewer: Komatsu H, Poustchi H
S-Editor: Wang LL
L-Editor: Filipodia
P-Editor: Wang LL

TO THE EDITOR

I read articles from Tijera *et al*[1], and Pisano *et al*[2], on viral hepatitis update, progress, challenges, and ways to elimination. Both articles are discussing important points on viral hepatitis but both are missing the actual progress on hepatitis elimination targets set by World Health Organization (WHO).

In this article, I am presenting the latest data on targets set by the WHO to achieve hepatitis elimination by 2030. In last 5 years, the incidence of hepatitis B virus (HBV) and hepatitis C virus (HCV) has decreased from 8 million infections to 3 million infections per year and mortality has decreased from 1.4 million deaths to 1.1 million deaths per year[3]. Only 17 countries had a national hepatitis strategic plans in 2012, but this increased to 124 by 2019[3].

The WHO Global Health Sector Strategy on viral hepatitis has shown five areas in which progress is requested to achieve hepatitis elimination by 2030. These areas are: HBV vaccination, birth dose HBV vaccination, safe injection, harm reduction, and diagnosis and treatment of HBV and HCV[4].

From 2015 to 2020, the worldwide coverage of the third dose of HBV vaccine has increased from 82% to 85% and administration of birth dose of HBV vaccine has increased from 38% to 43%. The number of safe blood donations has increased from 89% to 97% and only 3.9% of injection equipment are still reused. The number of clean syringes given to each person who inject drugs per year has increased from 20 to 33. The HBV diagnosis rate has increased from < 5% to 10% and 22% of diagnosed cases received treatment. The HCV diagnosis rate has increased from < 5% to 21%, and 62% of diagnosed cases received treatment[3,4].

New data shows progress with reference to 2020 hepatitis elimination targets for blood donation screening, HBV and HCV treatments, reduction in drug pricing, and decreasing the incidence of HBV and HCV[3]. However, the 2030 targets of hepatitis elimination are very ambitious and need a strong political and financial commitment [5].

Hepatitis elimination targets needs an investment of US $6 billion per year[3]. No major donor has committed to the fight against hepatitis[3]. Many countries with well-developed hepatitis control programs are lacking financial resources to achieve targets.

The coronavirus disease 2019 (COVID-19) pandemic has severely affected the hepatitis elimination targets. Outpatient departments/liver clinics have remained closed in many countries due to lockdown restrictions. Many countries have spent a major proportional of their health budget on COVID-19 and the nascent viral hepatitis programs are being held back due to a lack of funding[3].

This is the time to put money into the fight against hepatitis and increase HBV and HCV diagnosis and treatment rates[5]. There is a strong need to obtain a cure for HBV and further develop and simplify hepatitis screening tests and make them available in primary health care settings[3]. It is the time to set interim hepatitis elimination targets for 2026 as a milestone towards 2030 targets.

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Telephone: +1-925-3991568
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https://www.wjgnet.com

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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

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Bae JM

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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

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NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORS-IN-CHIEF
Andrzej S Tarnawski

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

PUBLICATION DATE
December 28, 2021

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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

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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

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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

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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

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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

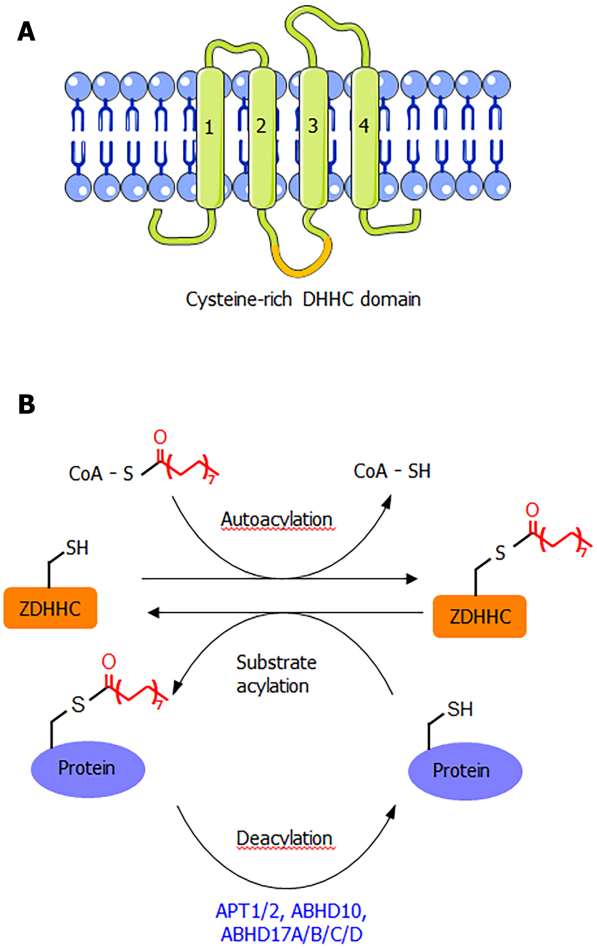


Figure 1 Protein S-palmitoylation. A: The ZDHHC-type palmitoyltransferases are integral membrane proteins with at least four transmembrane helices. The cysteine rich domain containing the DHHC motif is between the second and third transmembrane helices; B: Scheme showing the palmitoylation and depalmitoylation 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.

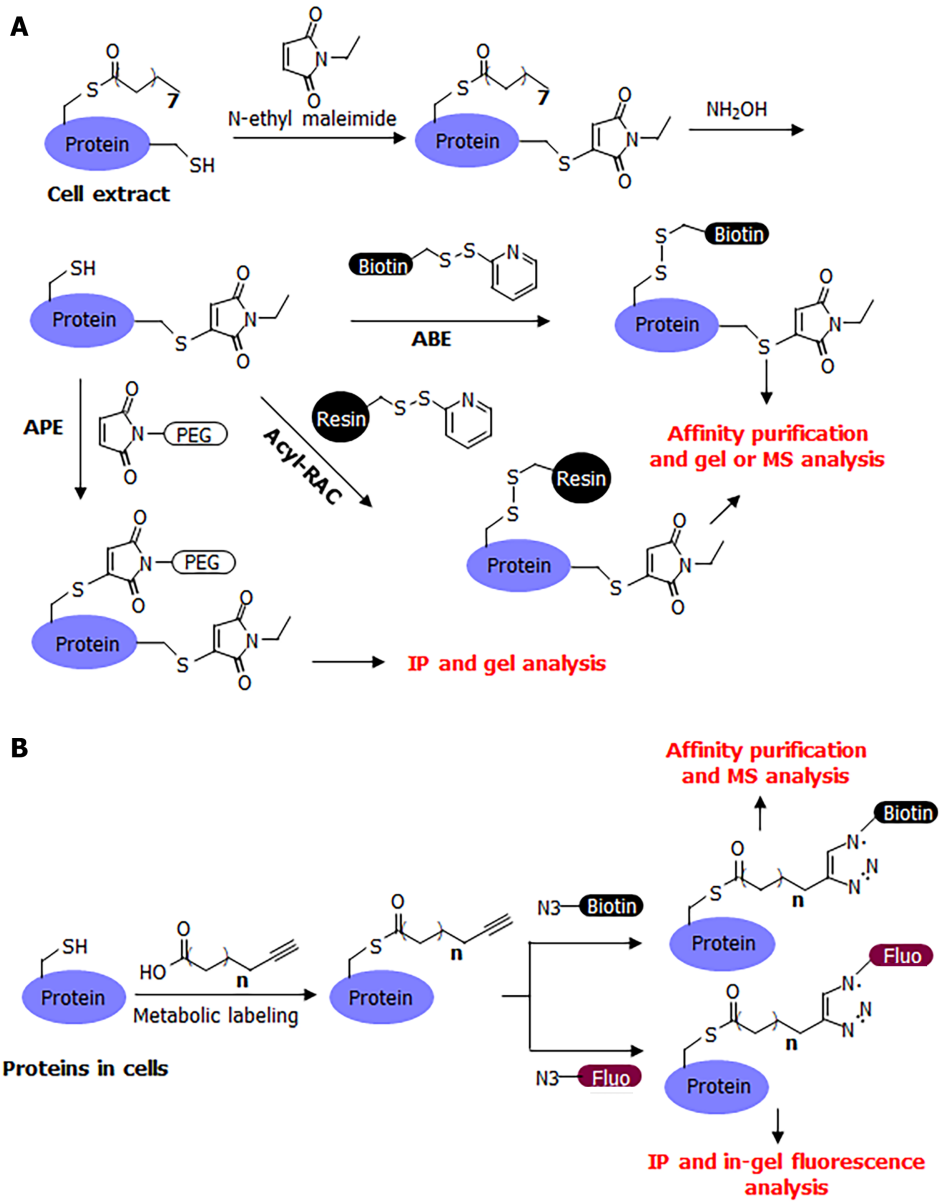


Figure 2 Commonly used methods for detecting S-palmitoylation. A: Scheme showing how acyl-biotin exchange (ABE), acyl-resin assisted capture (acyl-RAC), and acyl-PEG exchange (APE) work; B: Scheme showing how metabolic labeling with alkyne-tagged fatty acid analogs works. ABE: Acyl-biotin exchange; acyl-RAC: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

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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

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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

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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

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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

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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

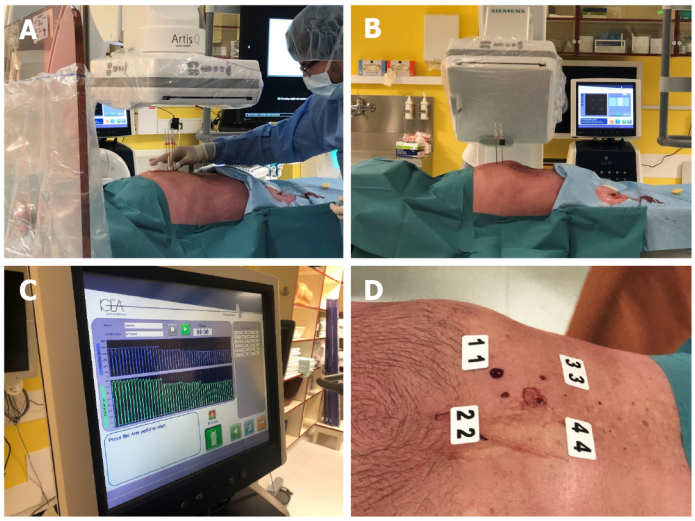


Figure 1 Electrochemotherapy using long needle electrodes in the percutaneous procedure. A: Positioning of the electrodes; B: Verification of the electrode positioning with computerized tomography; C: Treatment of the tumor, electric pulse delivery; D: Posttreatment.

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.

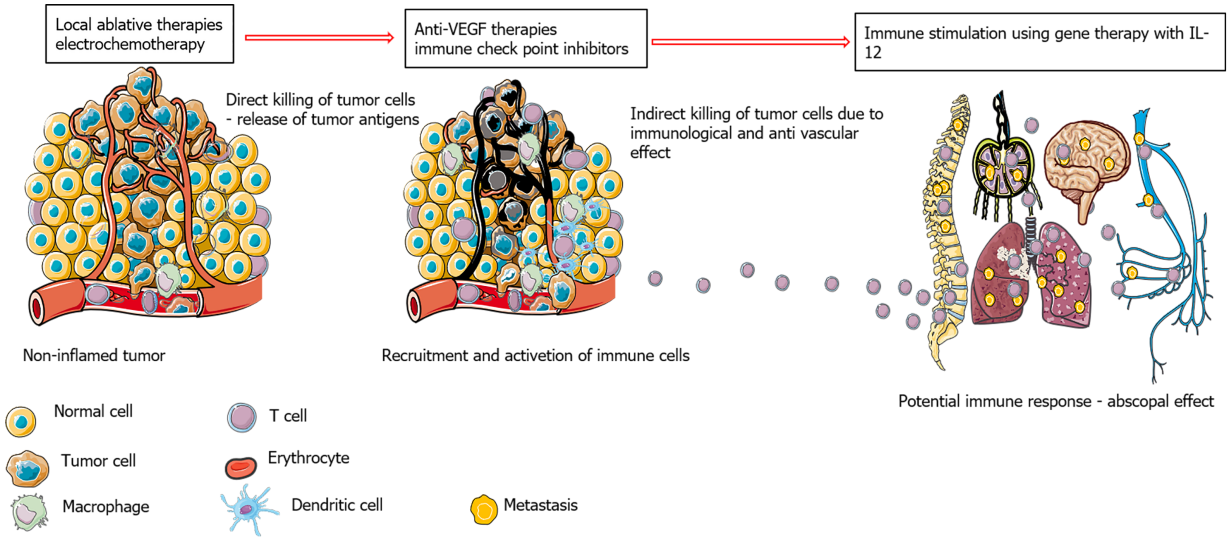


Figure 2 Potential benefits of combining local ablative therapies with immune checkpoint inhibitors and anti-vascular therapies. IL-12: Interleukin-12.

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.

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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

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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

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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

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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

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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.

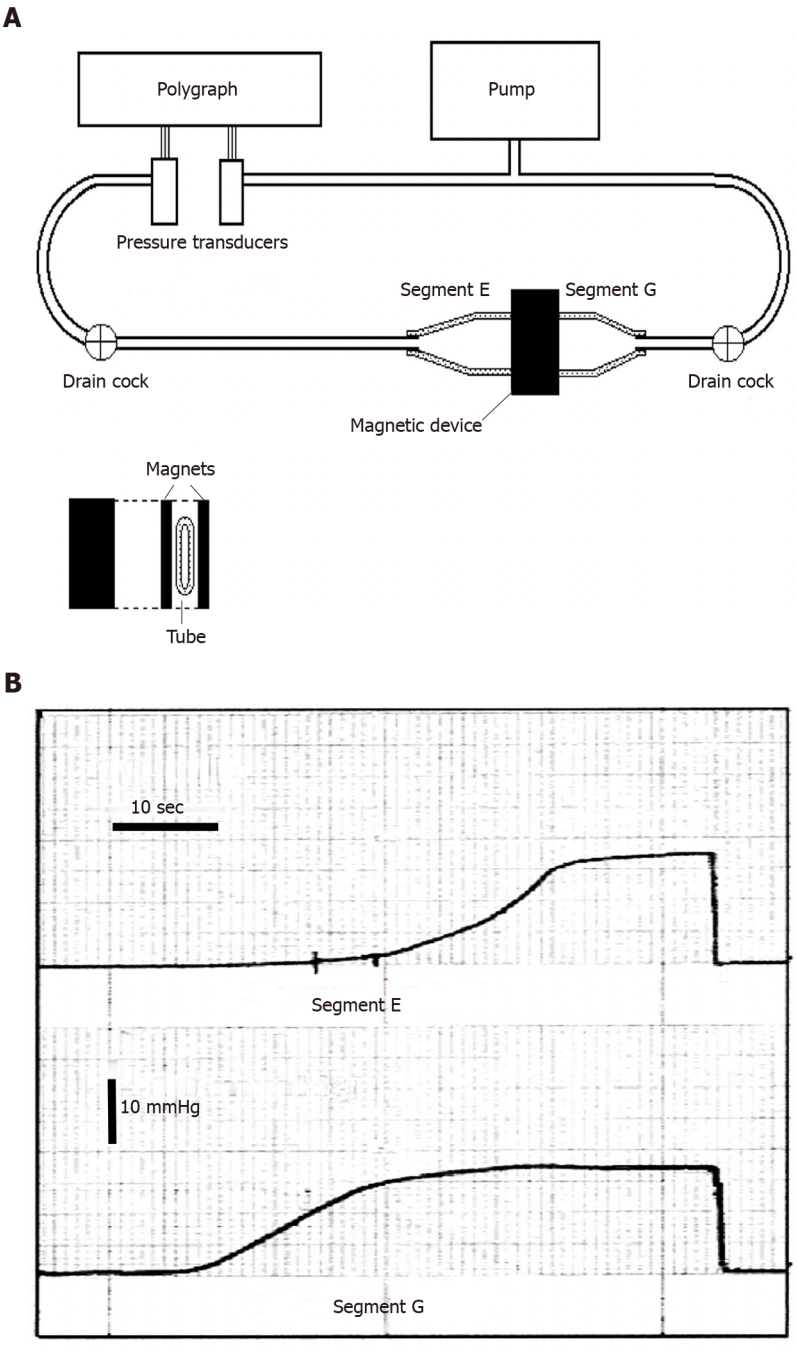


Figure 1 Benchtop experiment to demonstrate the possibility of creating a sphincter with two magnetic plaques. A: Schematic illustration of the bench model used to study the new anti-reflux device based on magnets. On the right there is a flaccid polyethylene tube 2.8 cm in diameter, mimicking the gastro-esophageal 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,

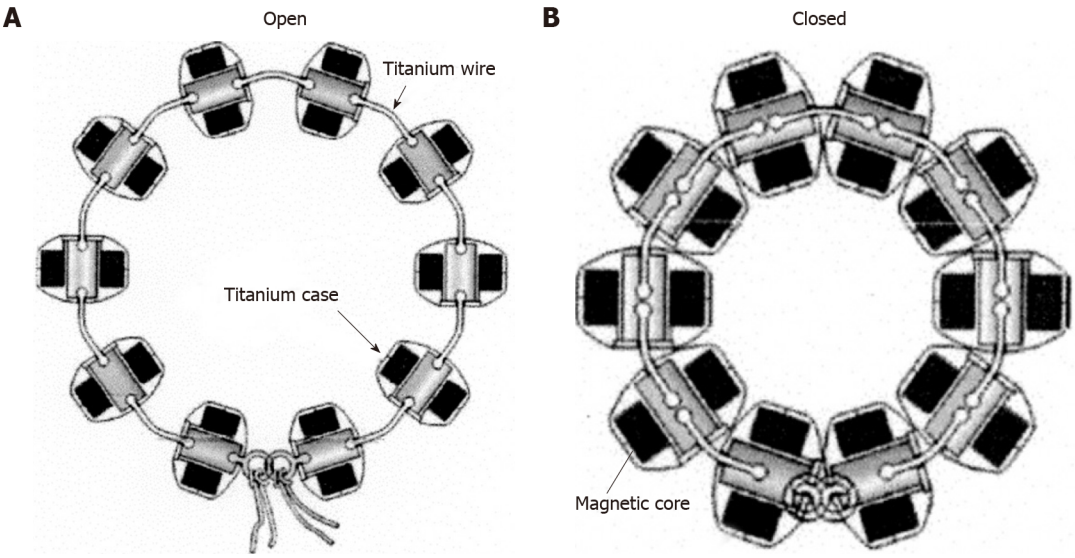


Figure 2 Schematic drawing of the Linx magnetic augmentation device to insert around the abdominal portion of the esophagus in the open and closed position. A: Open position; B: Closed position. A-B: Citation: Bonavina L, Saino GI, Bona D, Lipham J, Ganz RA, Dunn D, DeMeester T. Magnetic augmentation 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

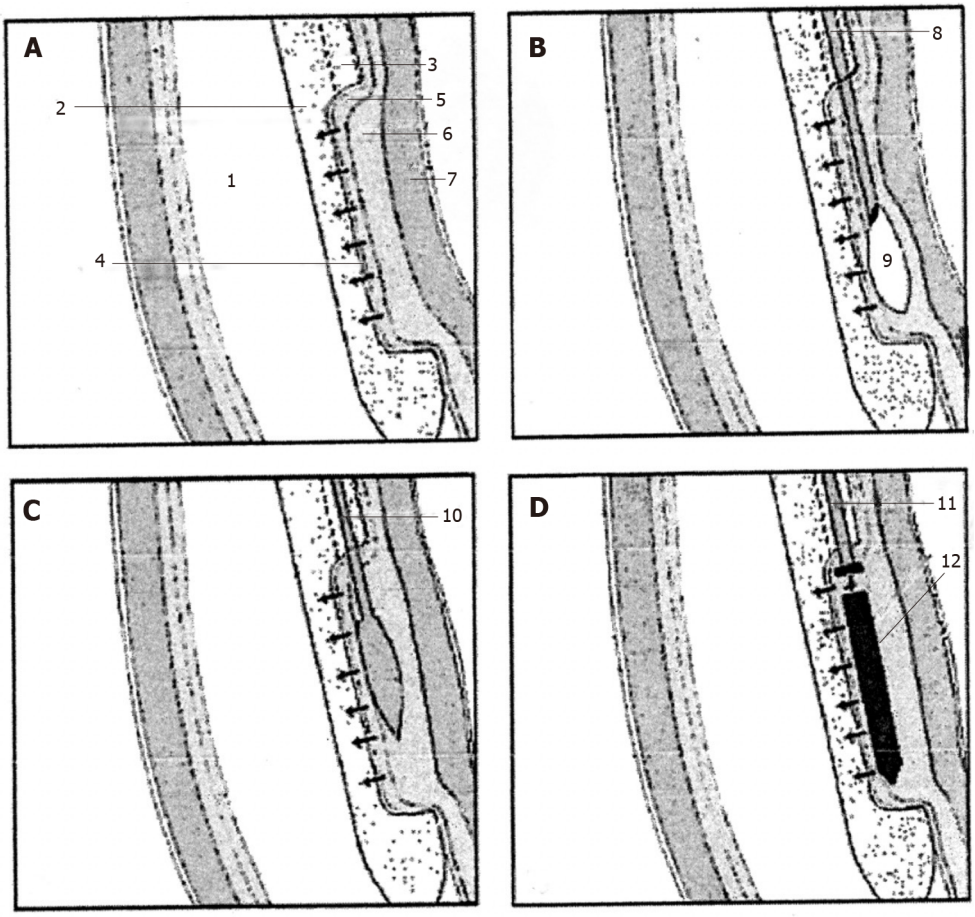


Figure 3 Extremity of the special endoesophageal probe positioned at the LES level in a sequence of operations for the deployment of a magnetic plaque seen in profile. A: The mucosa of the distal esophagus is sucked onto the perforated wall of the operative chamber; B: The needle injects milliliters 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

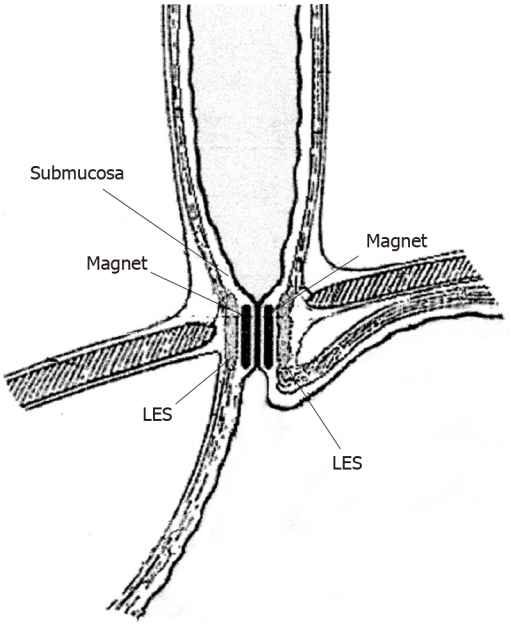


Figure 4 Schematic section following a vertical frontal plane through the lower portion of the esophago-gastric wall showing in profile the two magnetic plaques inserted face to face in the submucosal position at the lower esophageal sphincter level; these attracting each other close the gastro-esophageal 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).

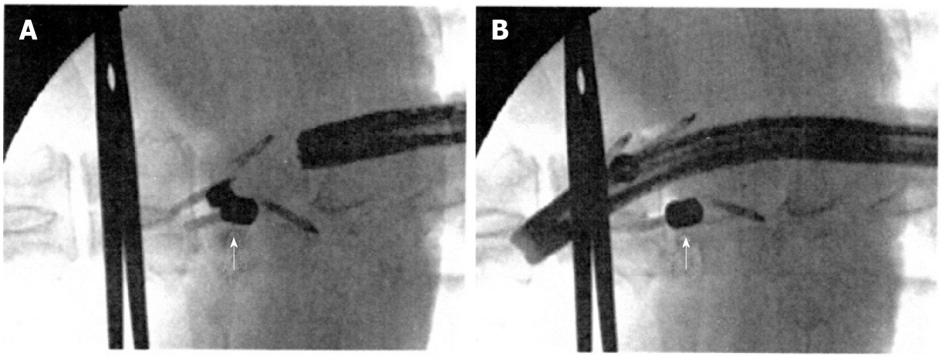


Figure 5 Fluoroscopic view after insertion of the magnets. A: Magnets in the sub-adventitial space opposing the respective esophageal walls. A surgical clamp indicates the level of the esophago-gastric junction and the arrow indicates the magnets attracted to one another and closing the lumen; B: Magnets 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.

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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

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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

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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

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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

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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

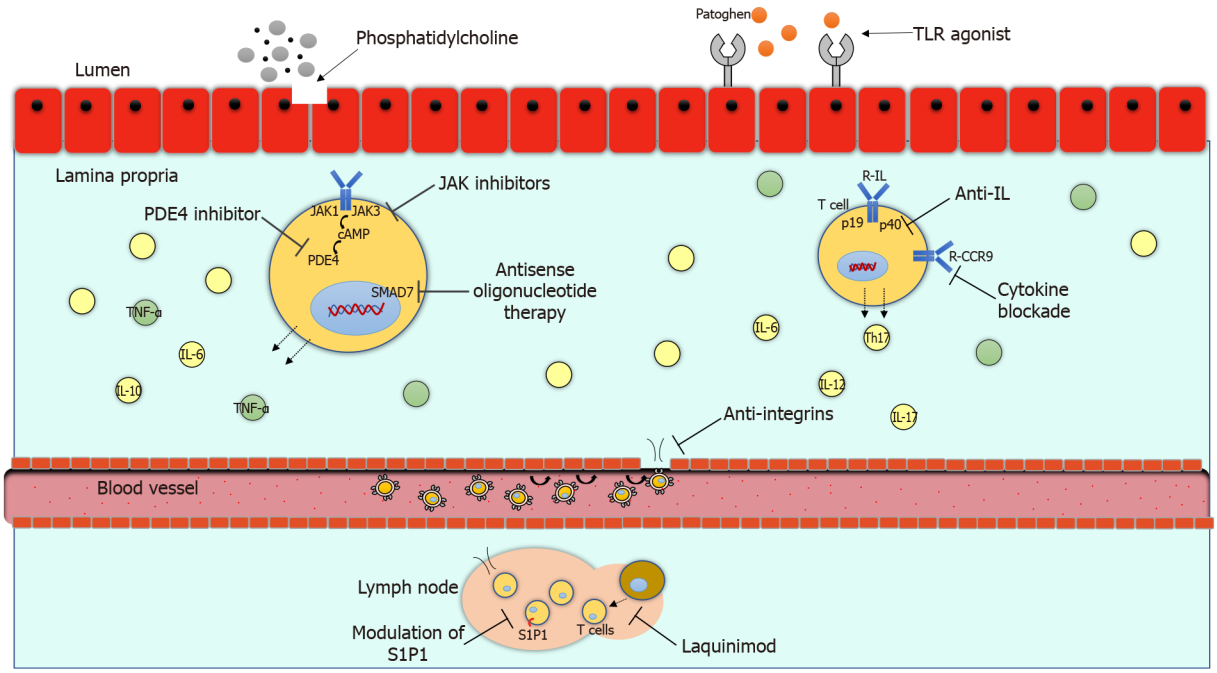


Figure 1 Mechanism of action of new therapies in inflammatory bowel disease. JAK: Janus kinase; TLR: Toll like receptor; IL: Interleukin; S1P1: Sphingosine-1-phosphate; PDE4: Phosphodiesterase-4; TNF: Tumor necrosis factor.

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.

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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

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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

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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

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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

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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

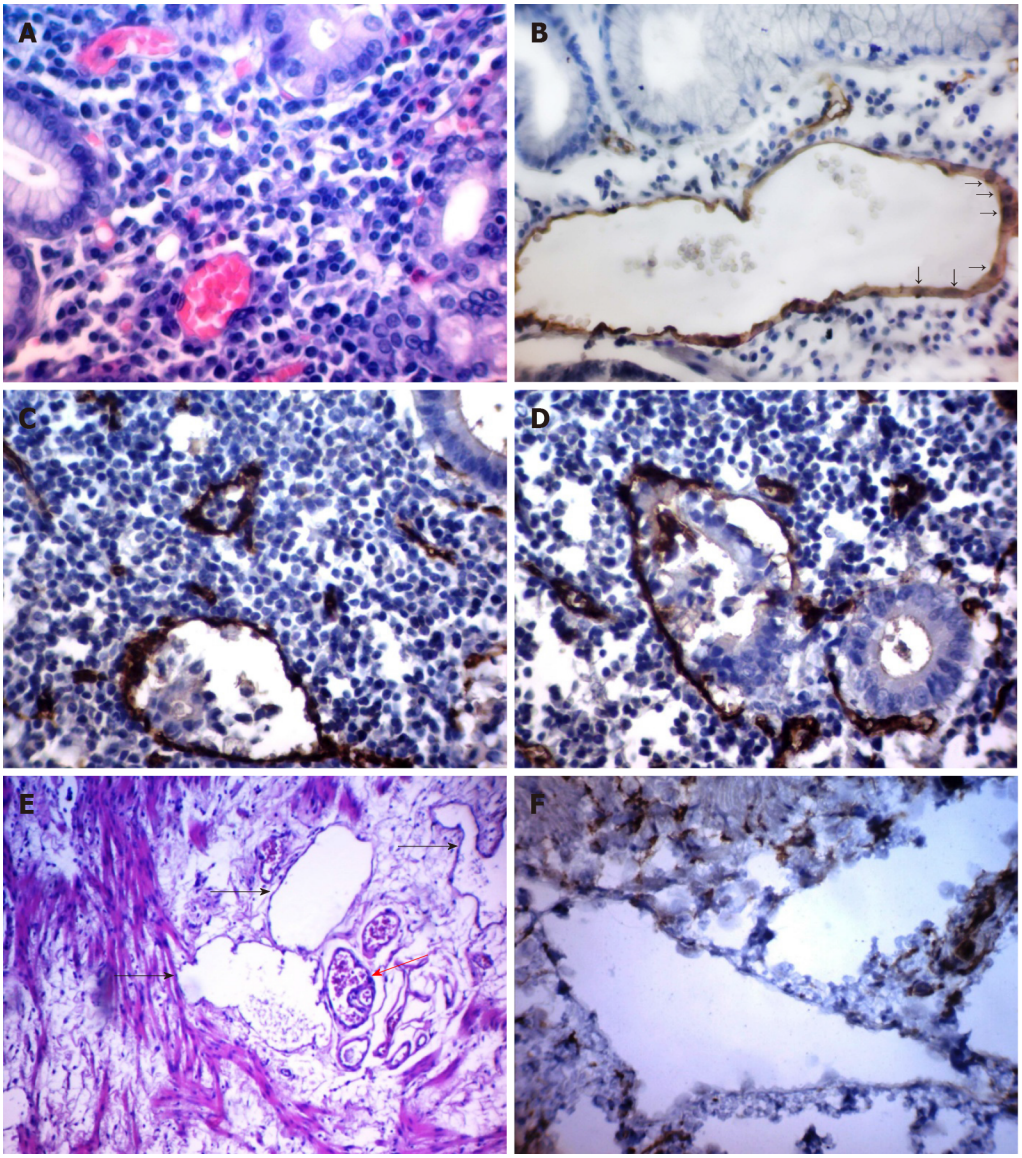


Figure 1 Different types of tumor microvessels in gastric cancer. A: Normal capillaries in the gastric mucosa adjacent to the tumor [hematoxylin and eosin (HE), 600×]; B: Dilated capillary formed by endothelial cells with large, pale nuclei with fine-netted chromatin structure (arrows) in the gastric 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.

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World Journal of Gastroenterology

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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

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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

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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

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

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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.

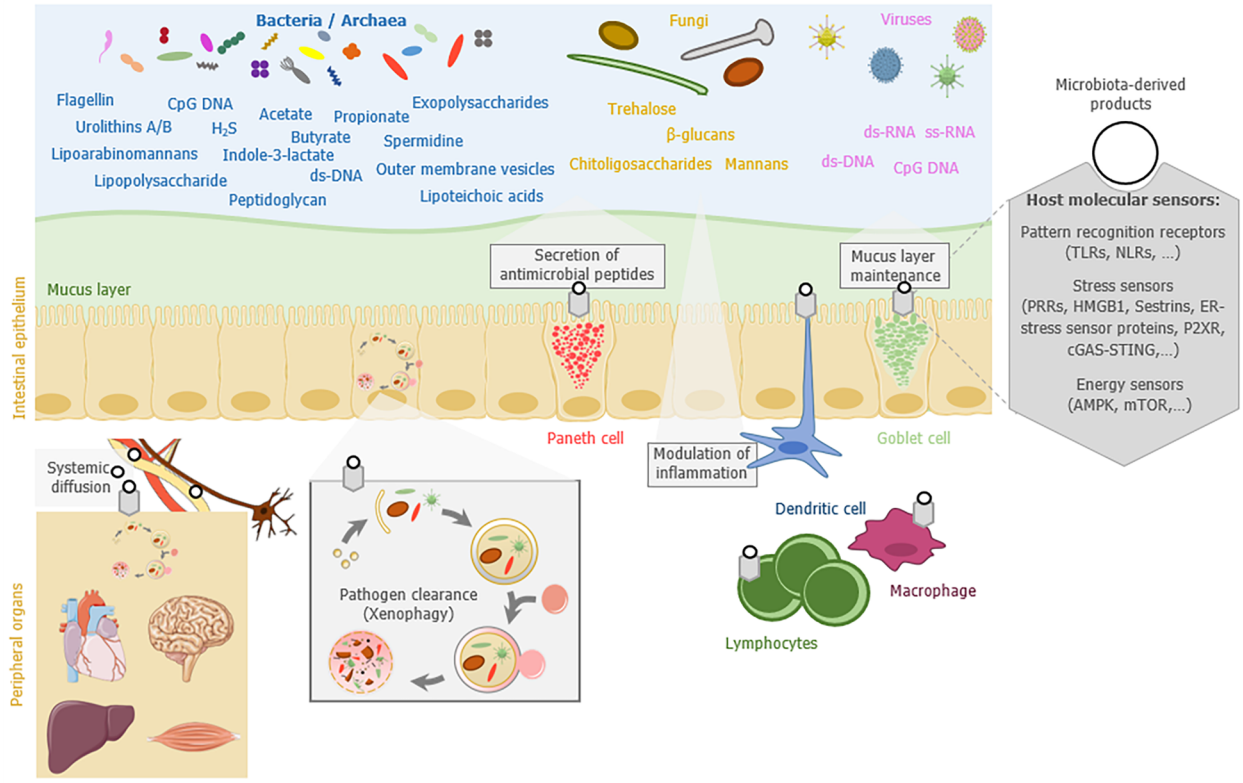


Figure 1 Complex interplay between gut microbiota and autophagy. The interactions between the gut microbiota and autophagy are bidirectional. Autophagy is involved in the regulation of several mechanisms (grey boxes) that shape the gut microbiota. Reciprocally, some bacterial- (blue), fungal- (orange), 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.

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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

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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

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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

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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

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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.

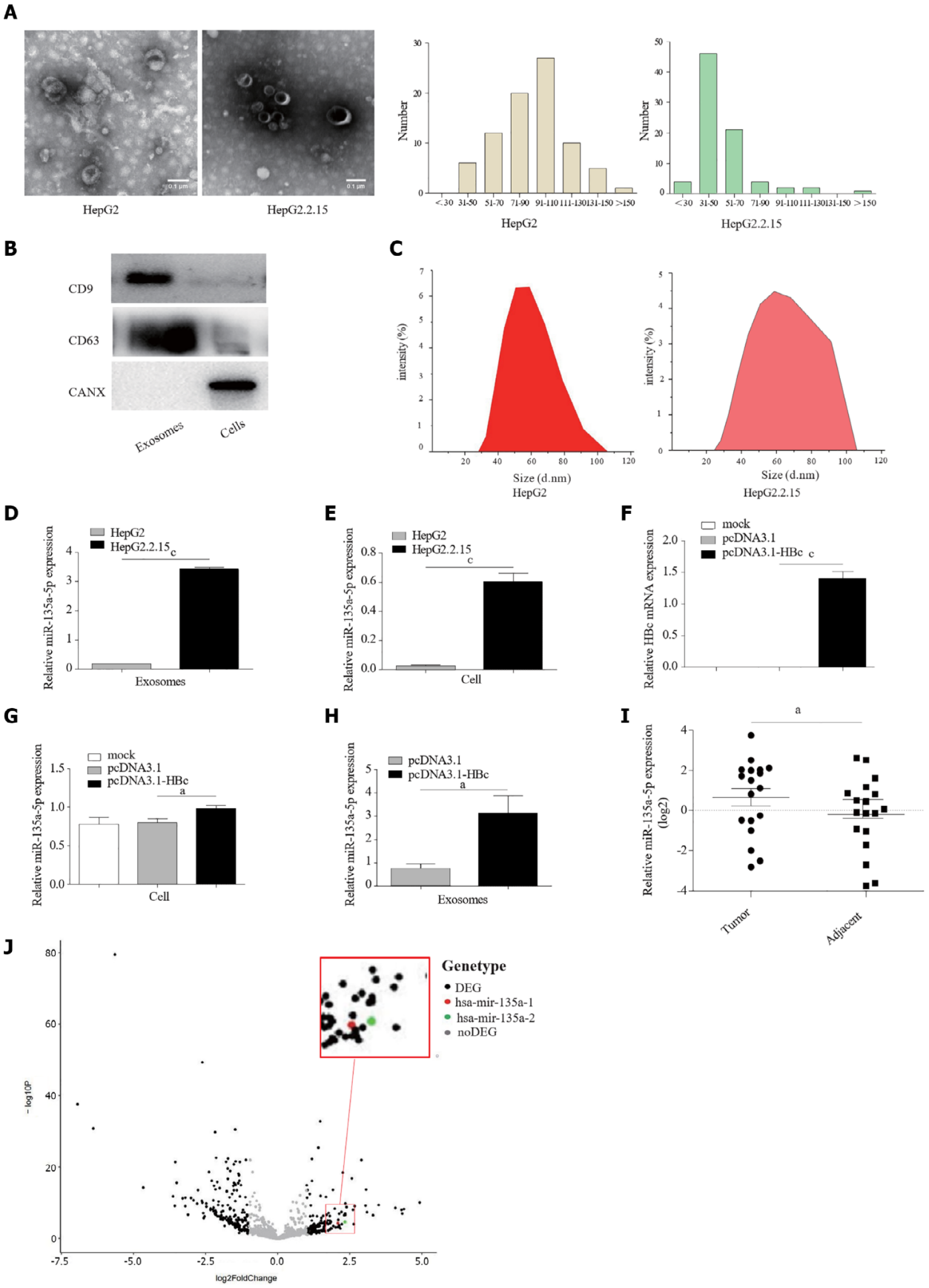


Figure 1 Hepatitis B virus upregulated the expression levels of miR-135a-5p in exosomes. A: Transmission electron microscopy image of exosomes; B: Western blotting indicated proteins in exosomes; C: Analysis of particle size distribution of exosomes; D and E: Quantitative polymerase chain reaction (qPCR) 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

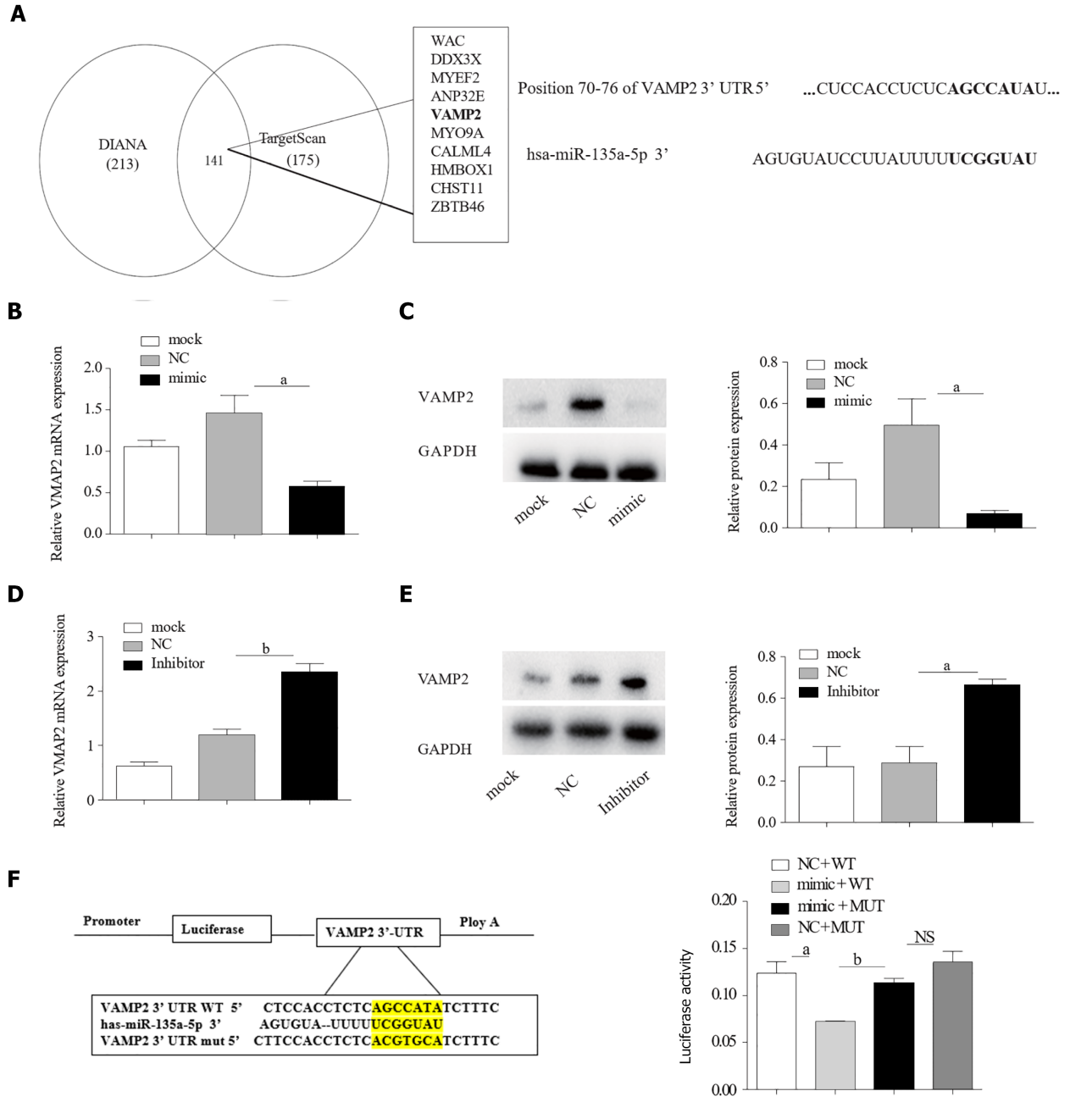


Figure 2 miR-135a-5p targeted vesicle-associated membrane protein 2 in hepatocellular carcinoma cells. A: Prediction results of target gene of miR-135a-5p; B: The expression of miR-135a-5p was measured by quantitative polymerase chain reaction (qPCR) in HepG2 cells transfected with miR-135a-5p mimics; 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.

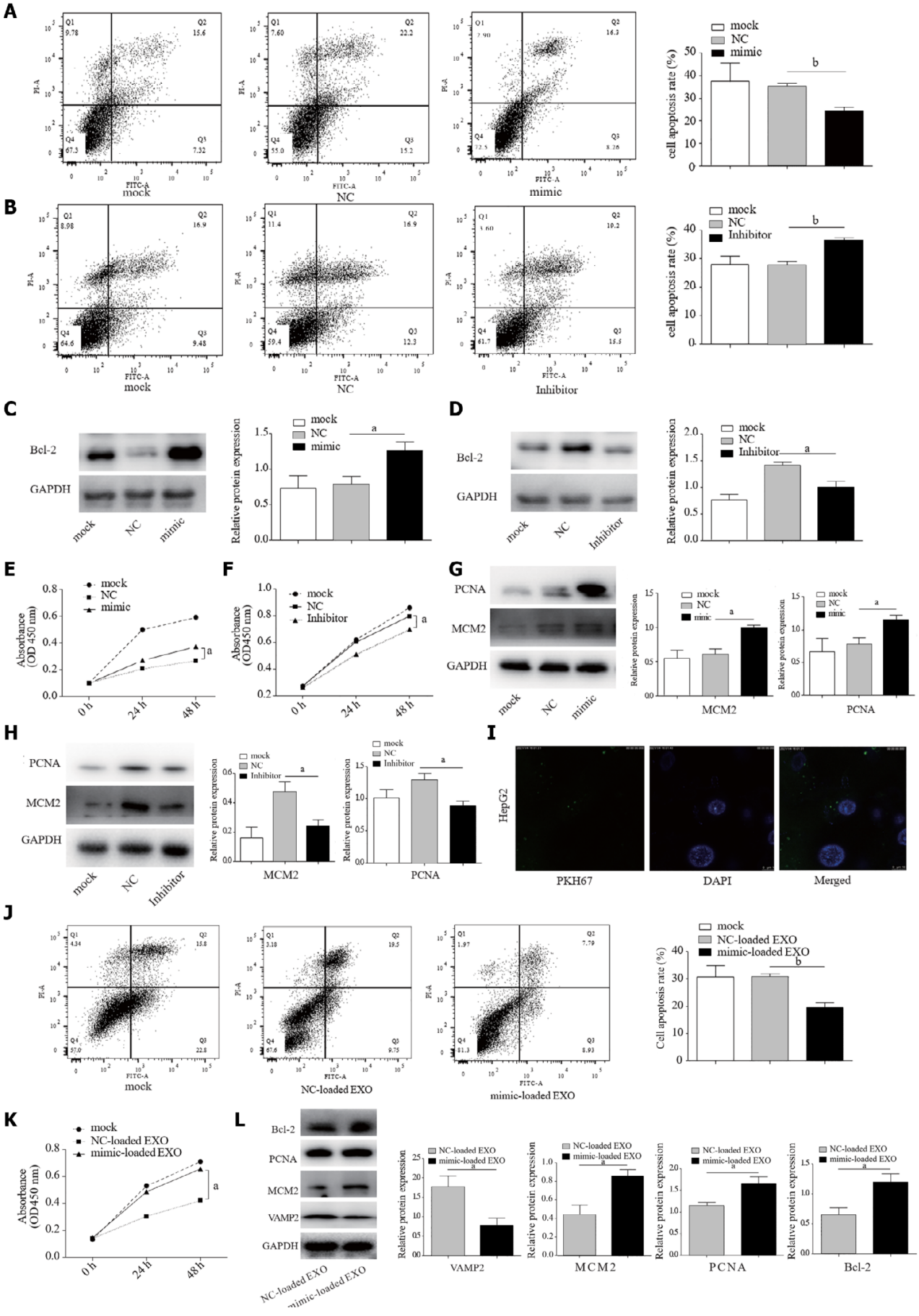


Figure 3 miR-135a-5p suppressed apoptosis and promoted proliferation. A and B: Annexin V-FITC/PI assay for the effect of overexpression or

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

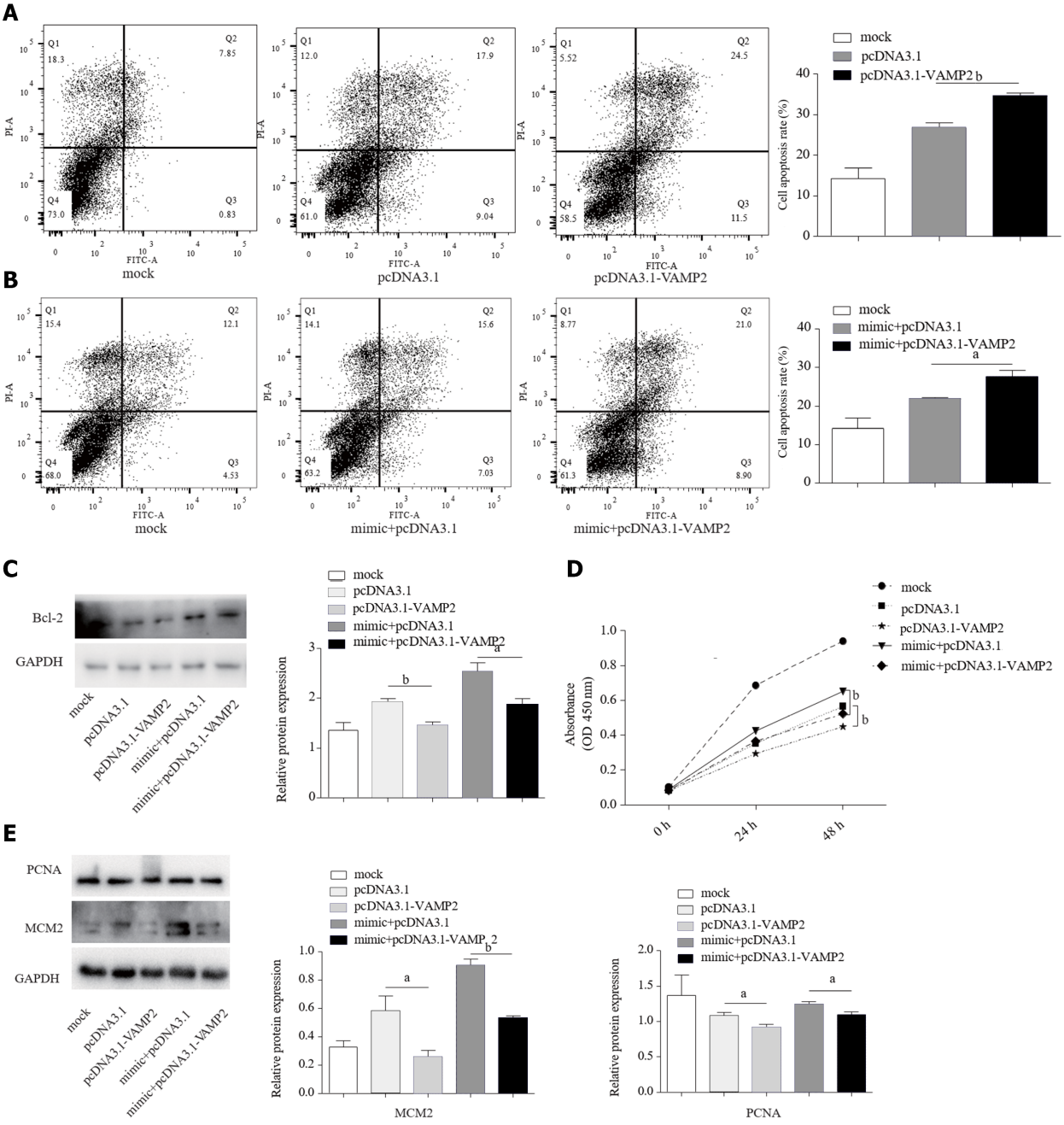


Figure 4 Vesicle-associated membrane protein 2 contributed to anti-apoptosis and proliferation induced by miR-135a-5p. A and B: Cell apoptosis was examined by flow cytometry in HepG2 cells transfected with the specific plasmid combinations; C: B-cell lymphoma-2 expression was detected by Western blot 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

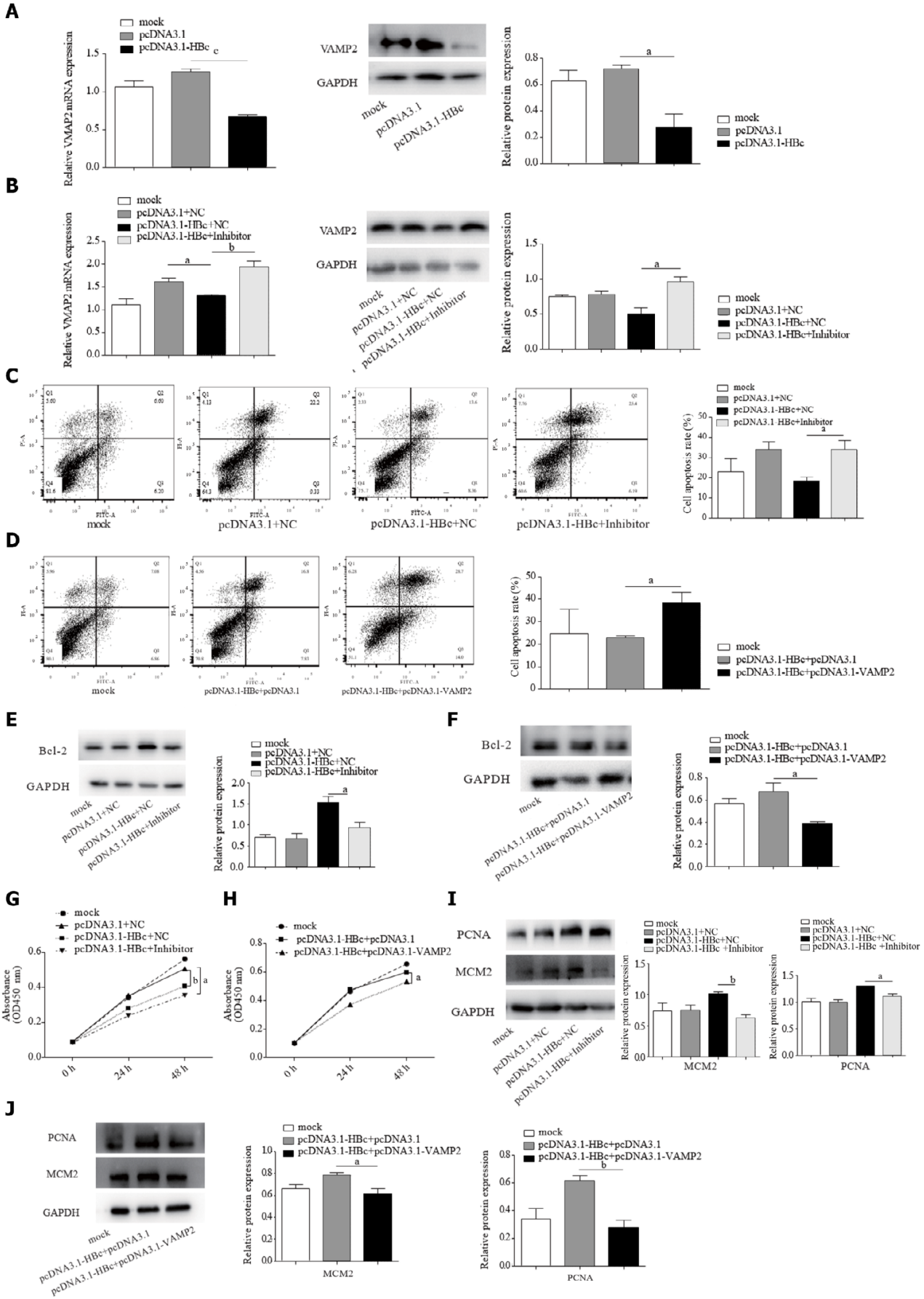


Figure 5 Hepatitis B core antigen induced anti-apoptosis and proliferation *via* miR-135a-5p and its target gene vesicle-associated membrane protein 2. A and B: Quantitative polymerase chain reaction and western blot analyses of the level of vesicle-associated membrane protein 2 in HepG2

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).

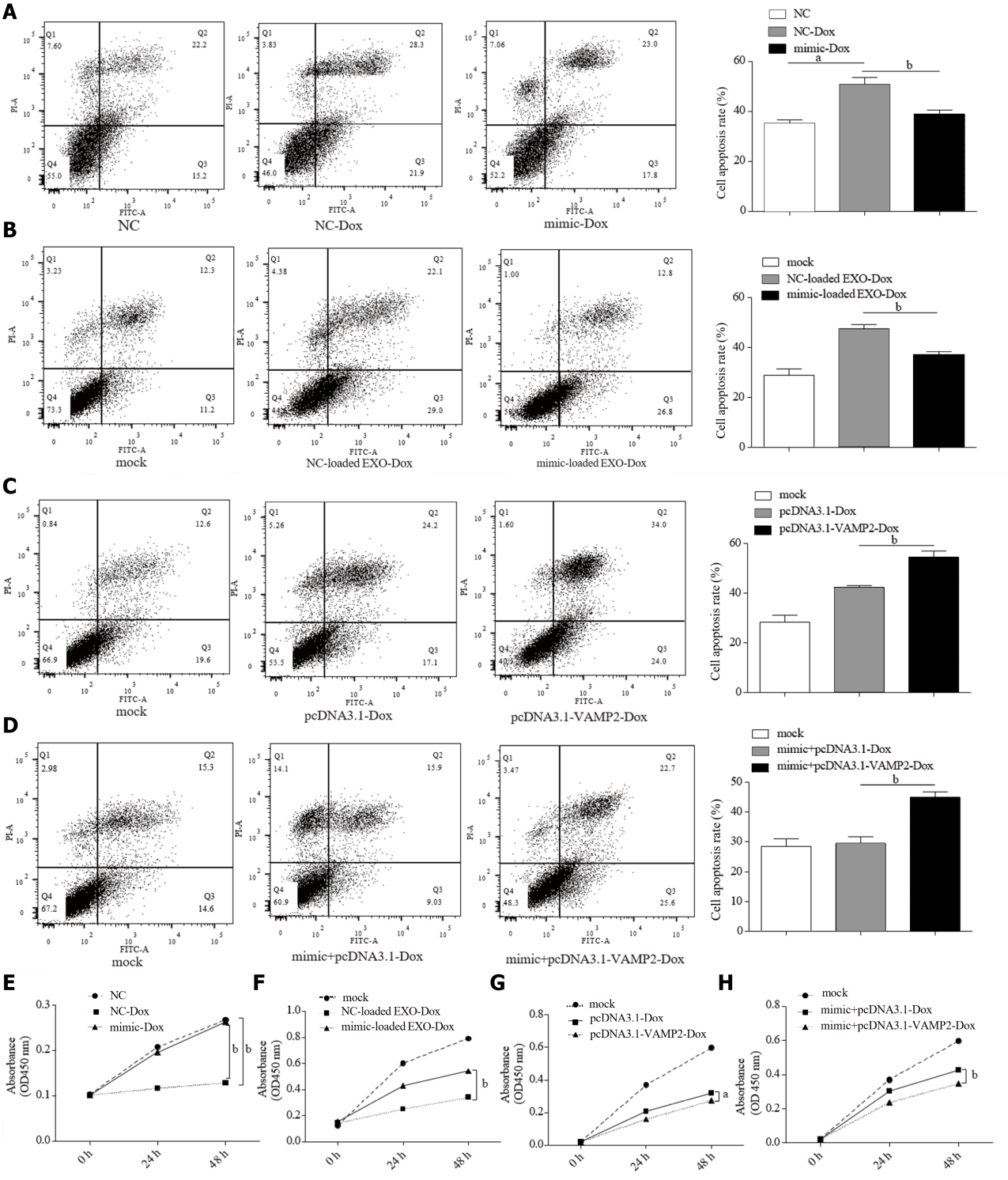


Figure 6 miR-135a-5p enhanced Dox-resistance and reduced cell apoptosis of hepatocellular carcinoma cells by down-regulating vesicle-associated membrane protein 2. A: The apoptosis rate of HepG2 cells after treatment with Doxorubicin hydrochloride (Dox). Flow cytometry was used to detected the effect 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.

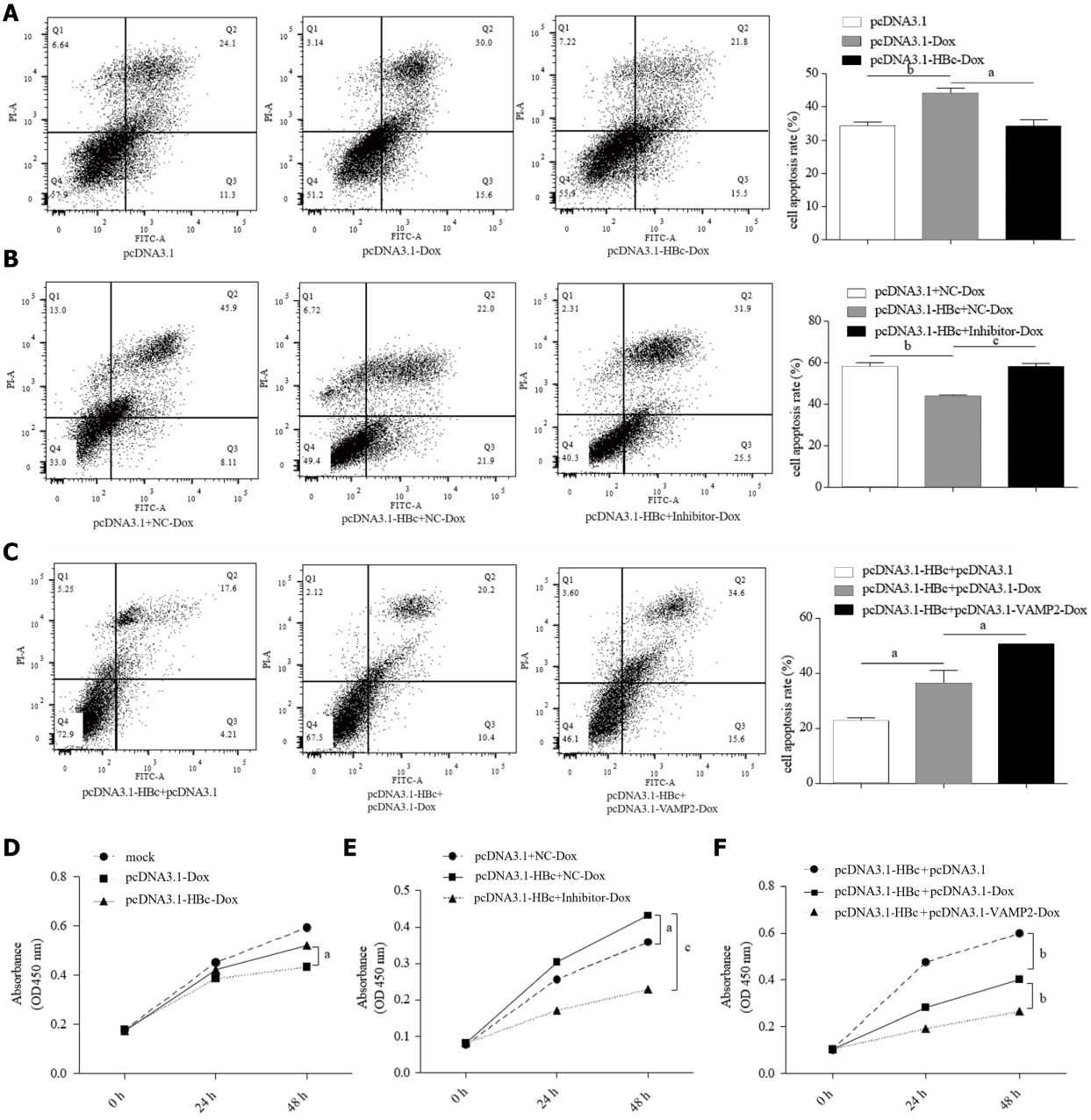


Figure 7 Hepatitis B core antigen mediated resistance of hepatocellular carcinoma cells to Doxorubicin hydrochloride *via* miR-135a-5p/vesicle-associated membrane protein 2. A: HepG2 cells were transfected with pcDNA3.1-hepatitis B core antigen (HBc) plasmids, flow cytometry was used to determine the 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.

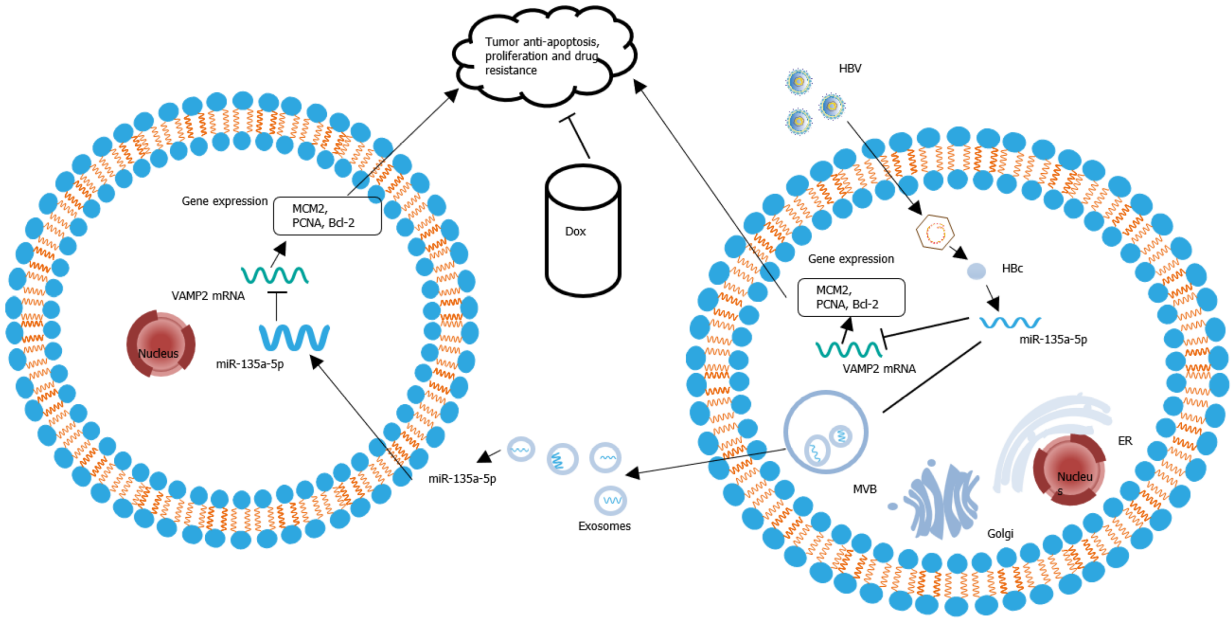


Figure 8 Hepatitis B core antigen promoted tumor anti-apoptosis, proliferation and chemoresistance in hepatocellular carcinoma cells by the miR-135a-5p/vesicle-associated membrane protein 2 axis. HBV: Hepatitis B virus; HBc: Hepatitis B core antigen; Bcl-2: B-cell lymphoma-2; MCM2: Mini-chromosome maintenance 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.

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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

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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).

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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

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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

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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

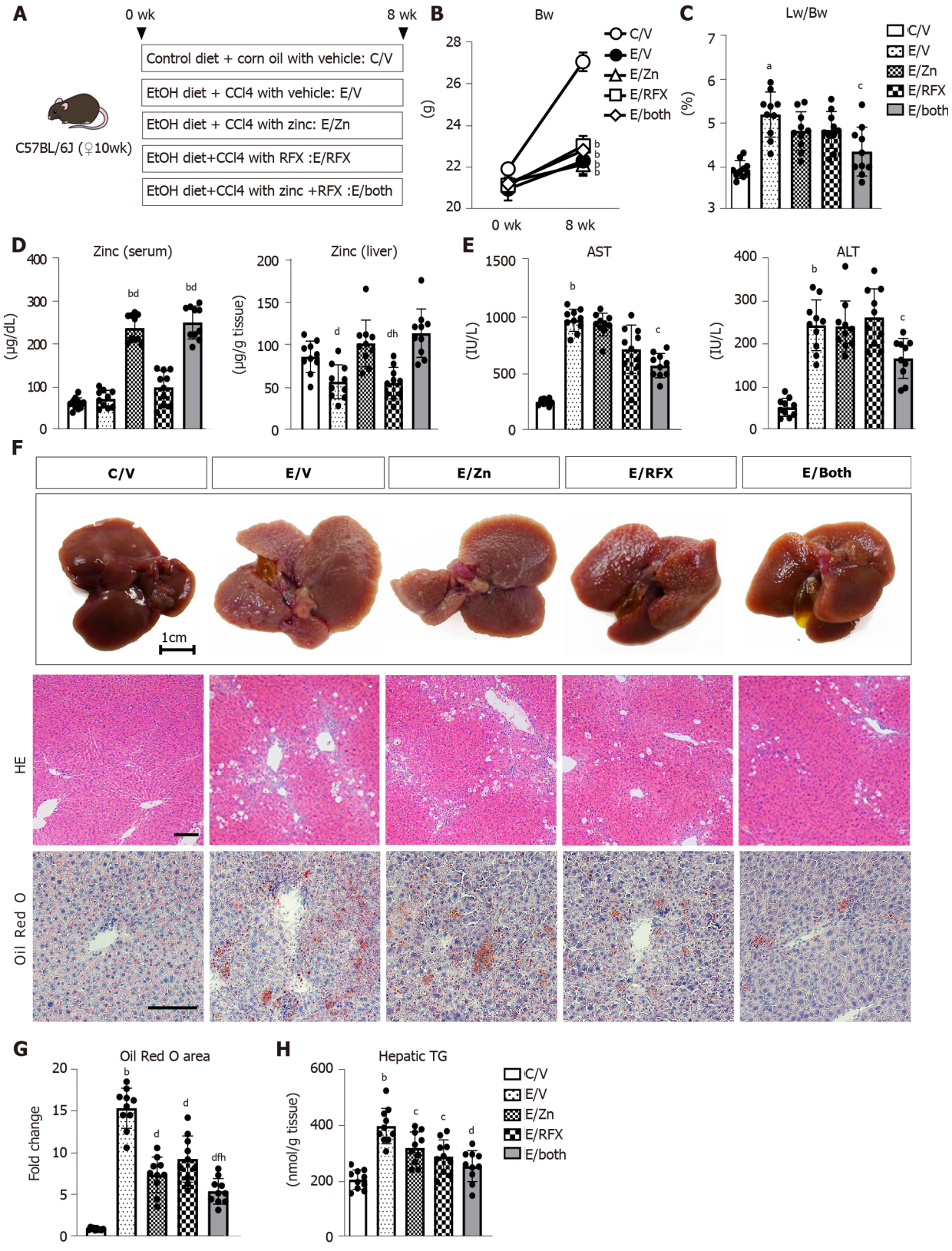


Figure 1 Zinc acetate and rifaximin against hepatic steatosis in alcoholic liver disease mice. A: Experimental protocols; B: Changes in body weights during experimental period; C: Ratio of liver weight to body weight at the end of experiment; D: Zinc concentrations of the serum (left) and the liver (right); 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).

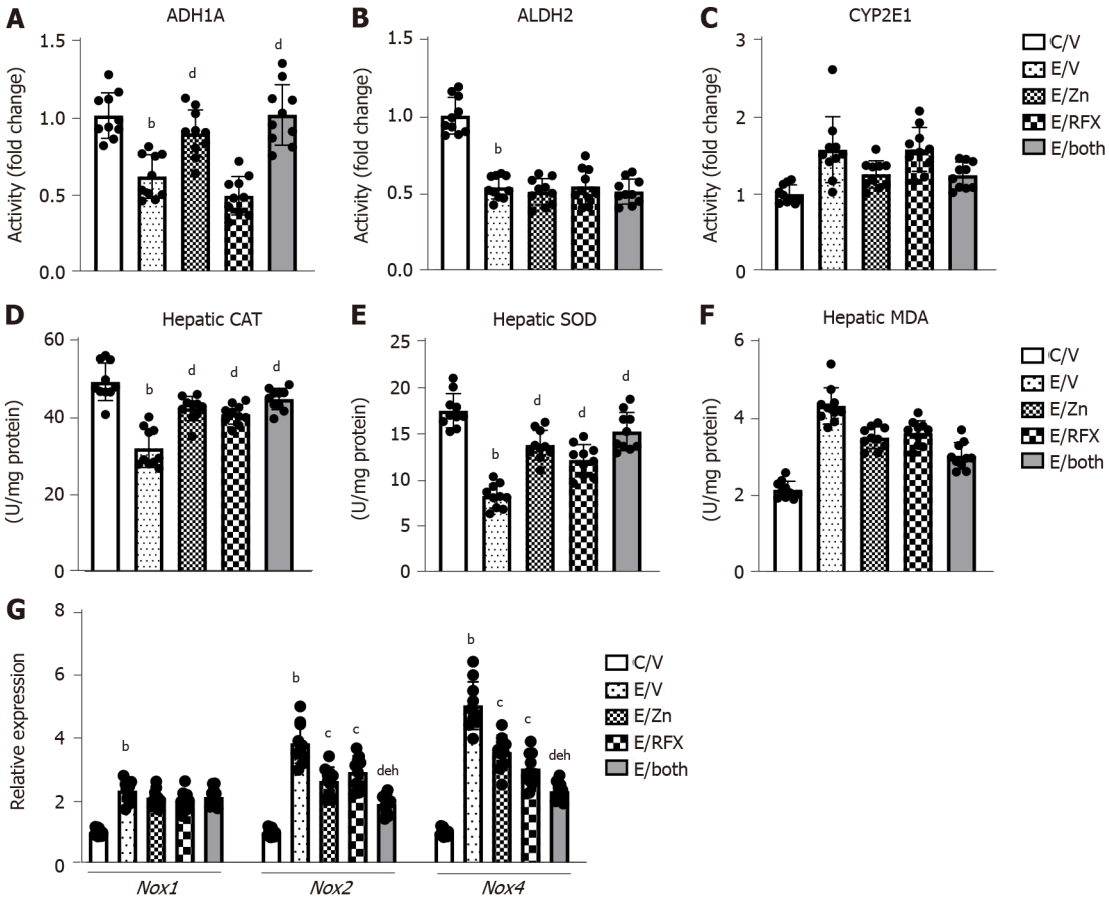


Figure 2 Zinc acetate and rifaximin on ethanol metabolism and accumulation of oxidative stress in alcoholic liver disease mice. A-C: Hepatic activity of alcohol dehydrogenase 1 (A), aldehyde dehydrogenase 2 (B) and cytochrome P450 2E1 (CYP2E1) (C). Quantitative values are indicated as fold changes to 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

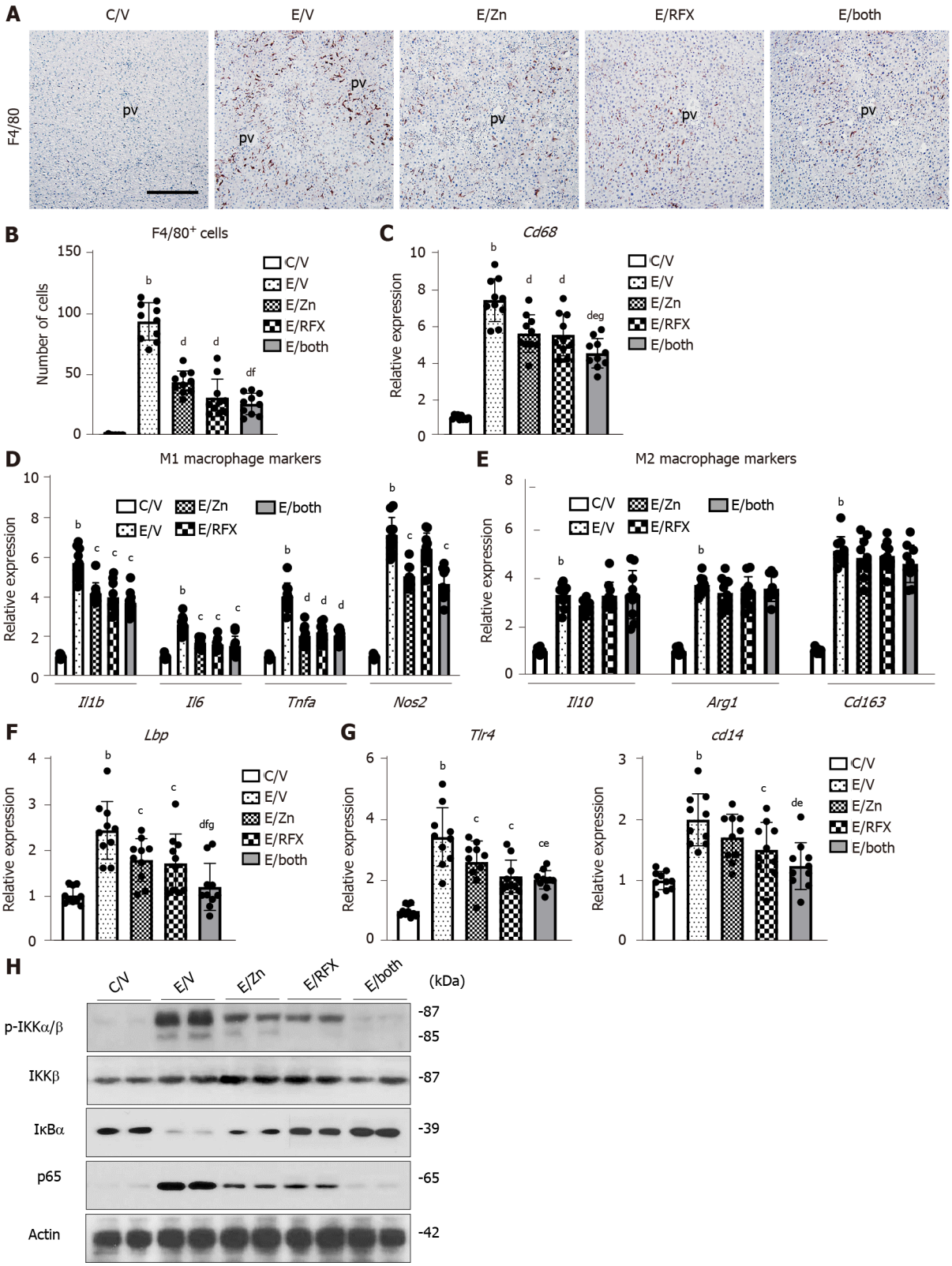


Figure 3 Zinc acetate and rifaximin against toll-like receptor 4-mediated pro-inflammatory response in alcoholic liver disease mice. A: Representative microphotographs of liver sections stained with F4/80. Scale bar: 50 μm. B: Semi-quantitation of F4/80 immuno-positive Kupffer cells in high-power field 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

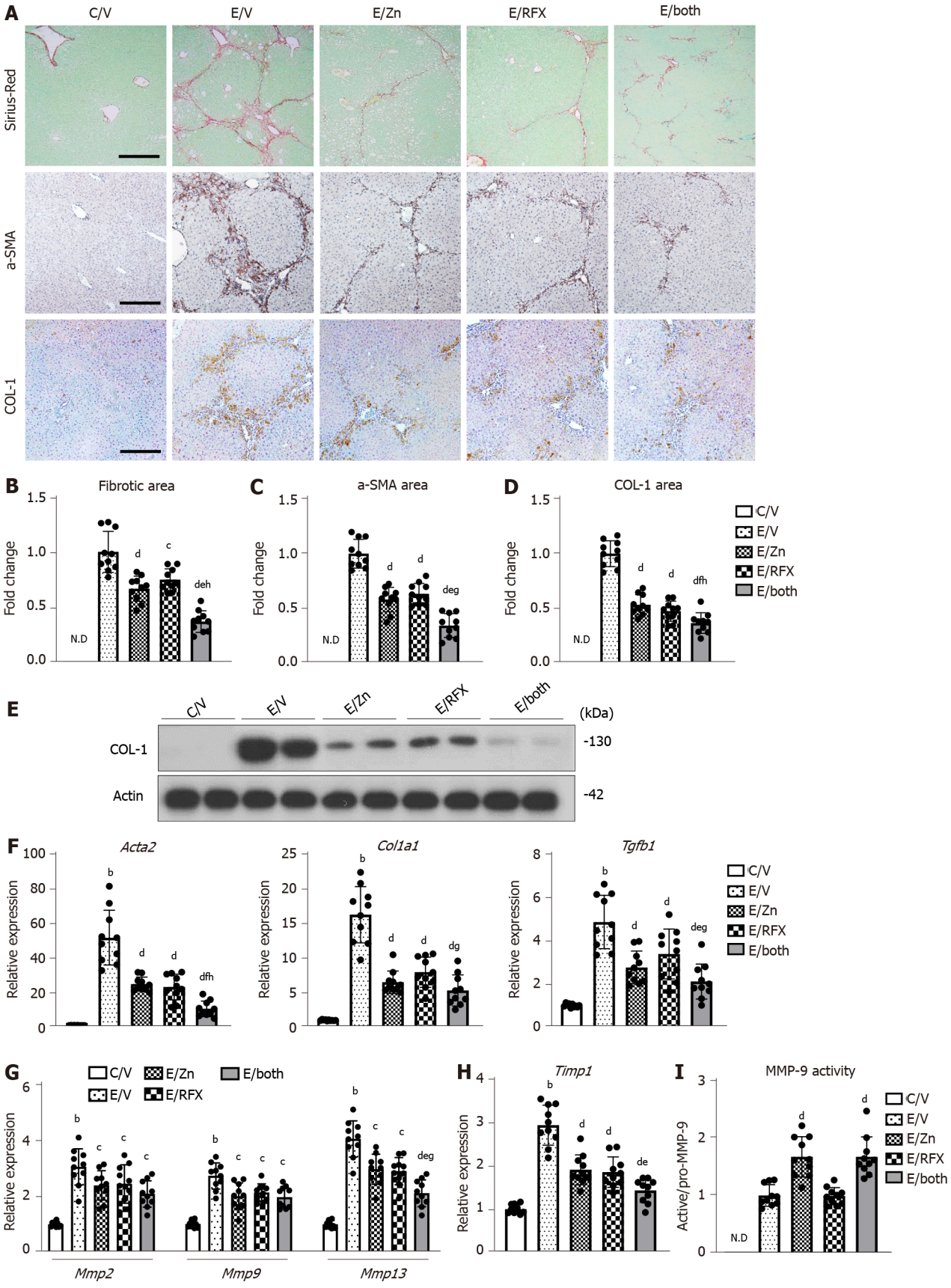


Figure 4 Zinc acetate and rifaximin against liver fibrosis development in alcoholic liver disease mice. A: Representative microphotographs of liver sections stained with Sirius-Red, α-smooth muscle actin (αSMA) and COL-1. Scale bar: 50 μm; B-D: Semi-quantitation of Sirius-Red-stained fibrotic area (B), 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.

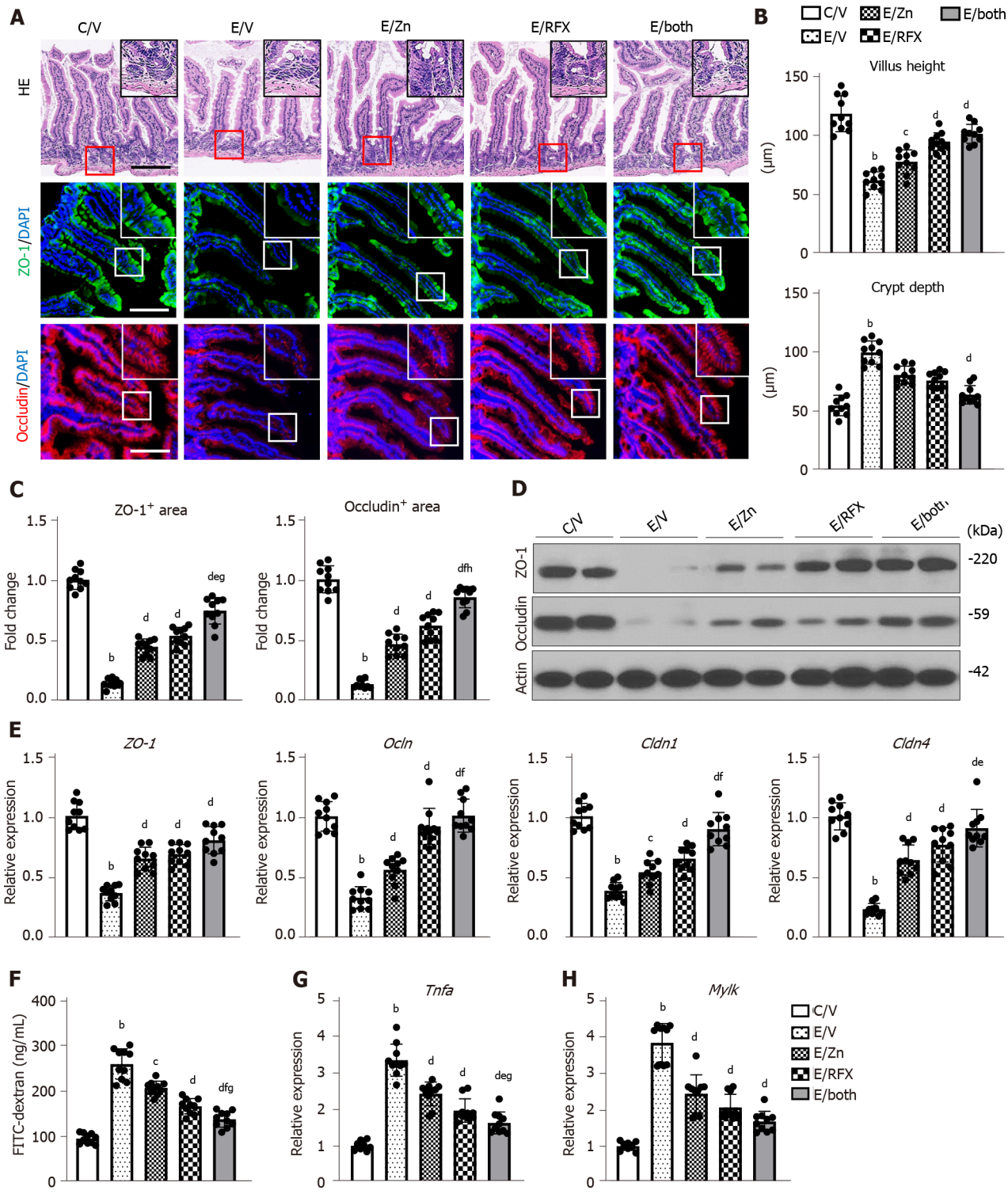


Figure 5 Zinc acetate and rifaximin on intestinal barrier function in alcoholic liver disease mice. A: Representative microphotographs of ileum sections stained with hematoxylin-eosin (upper), zonula occludens-1 (ZO-1) (middle) and Occludin (lower) in the experimental groups. Boxes are selected regions 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.

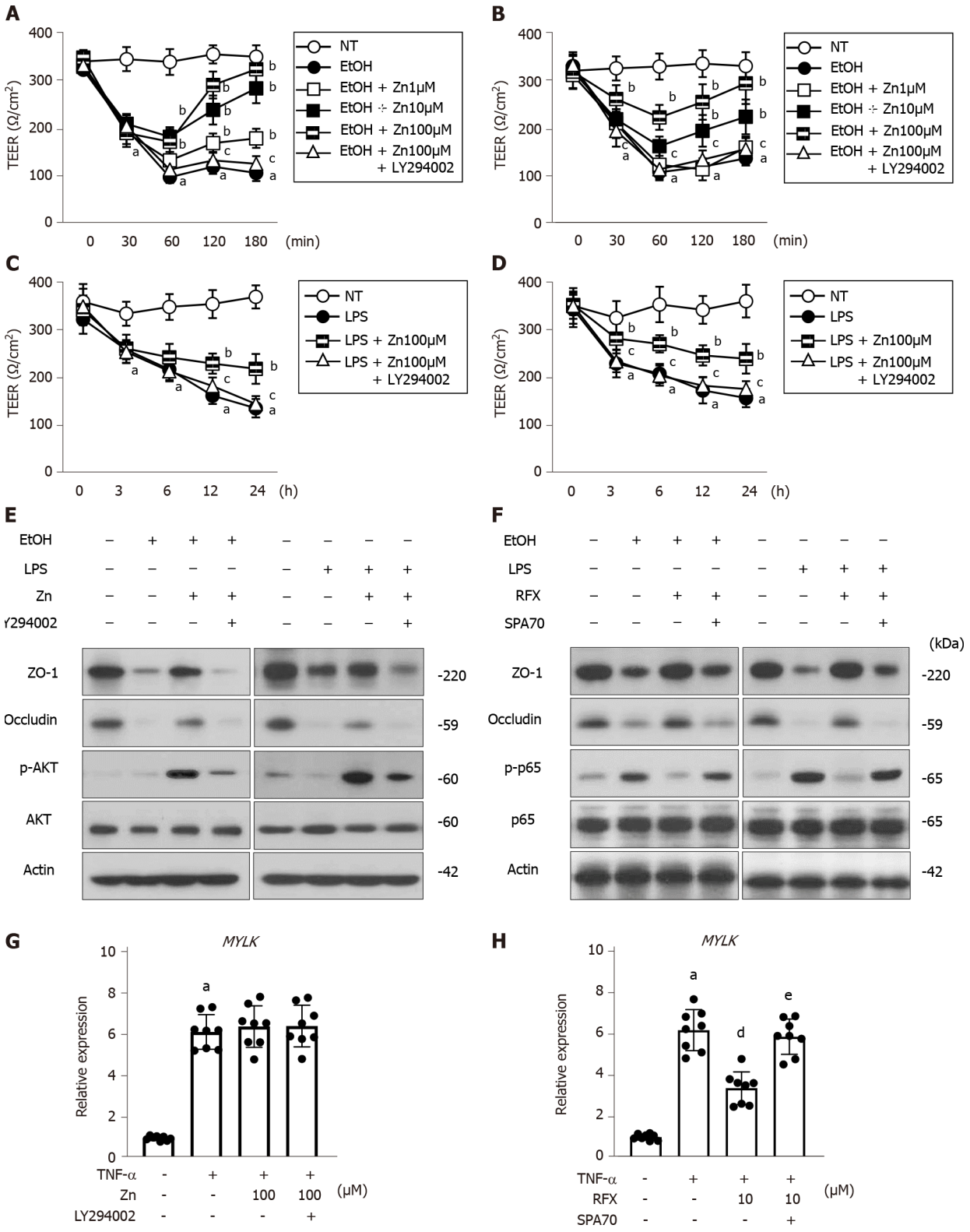


Figure 6 Effects of zinc acetate and rifaximin on *in vitro* EtOH/LPS/TNF-α-stimulated Caco-2 cells. A-D: *In vitro* paracellular permeability in ethanol (EtOH) (A and B)- or lipopolysaccharide (LPS) (C and D)-stimulated Caco-2 cells determined as transepithelial electrical resistance; E and F: Western 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

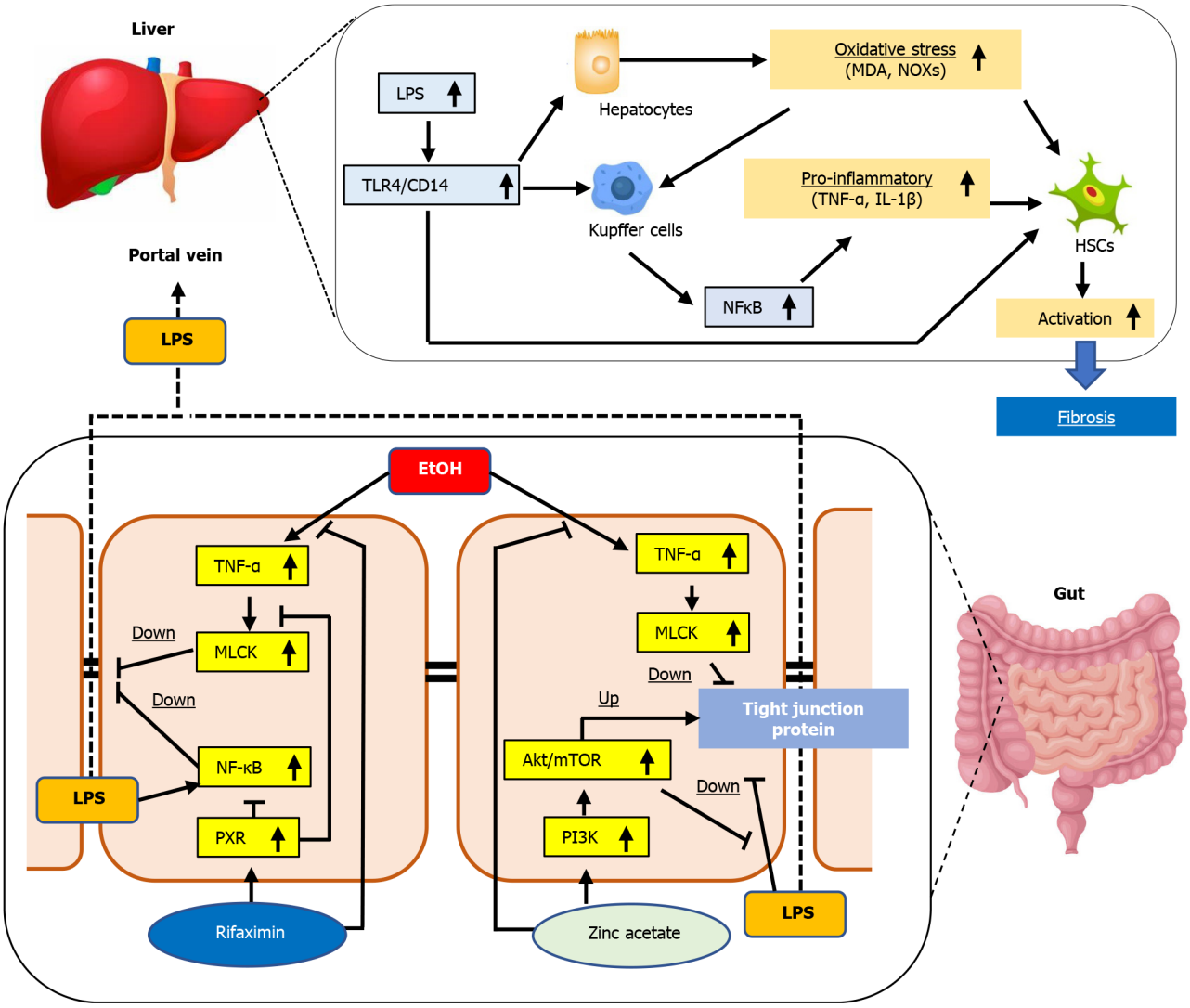


Figure 7 Graphic summary of the effect of zinc acetate and rifaximin on the alcoholic liver disease-related liver fibrosis. ALD: Alcoholic liver disease; TLR4: Toll-like receptor 4; MDA: Malondialdehyde; LPS: Lipopolysaccharide; HSC: Hepatic stellate cell; TNFα: Tumor necrosis factor α; MLCK: Myosin 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.

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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

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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

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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

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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

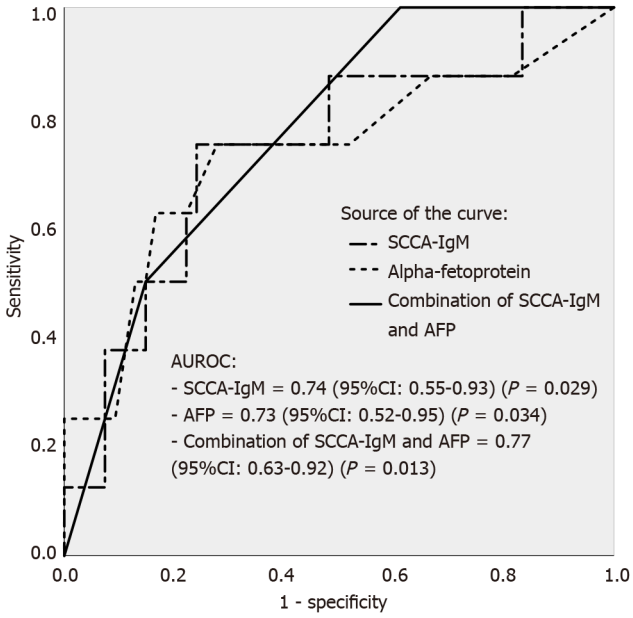


Figure 1 Receiver operating characteristic curves of the combination of squamous cell carcinoma antigen immunocomplex and alpha-fetoprotein as compared to squamous cell carcinoma antigen immunocomplex and alpha-fetoprotein in predicting two-year mortality in the estimation cohort. The clinical relevance 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.

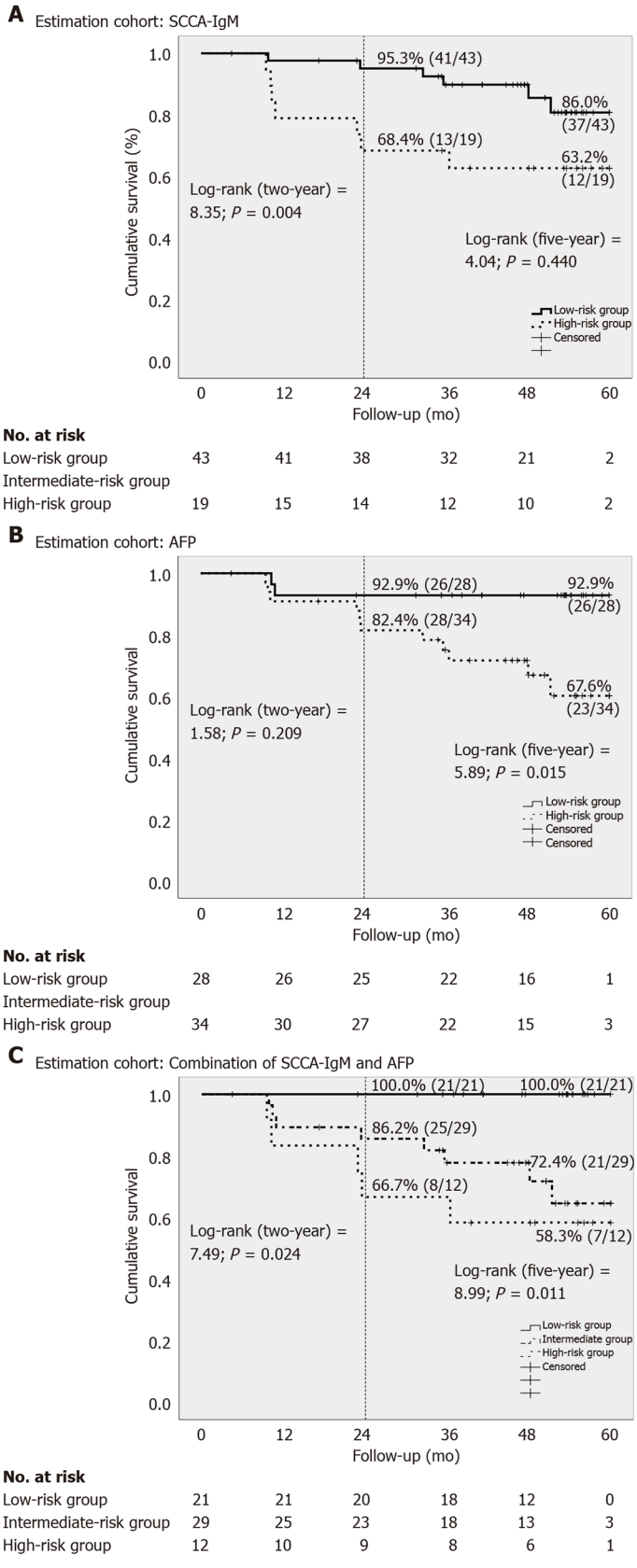


Figure 2 Estimating two- and five-year hepatocellular carcinoma disease-free survival by using Kaplan-Meier method according to the squamous cell carcinoma antigen immunocomplex, alpha-fetoprotein and combination of those in estimation cohort. A: Squamous cell carcinoma antigen immunocomplex (SCCA-IgM); 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.

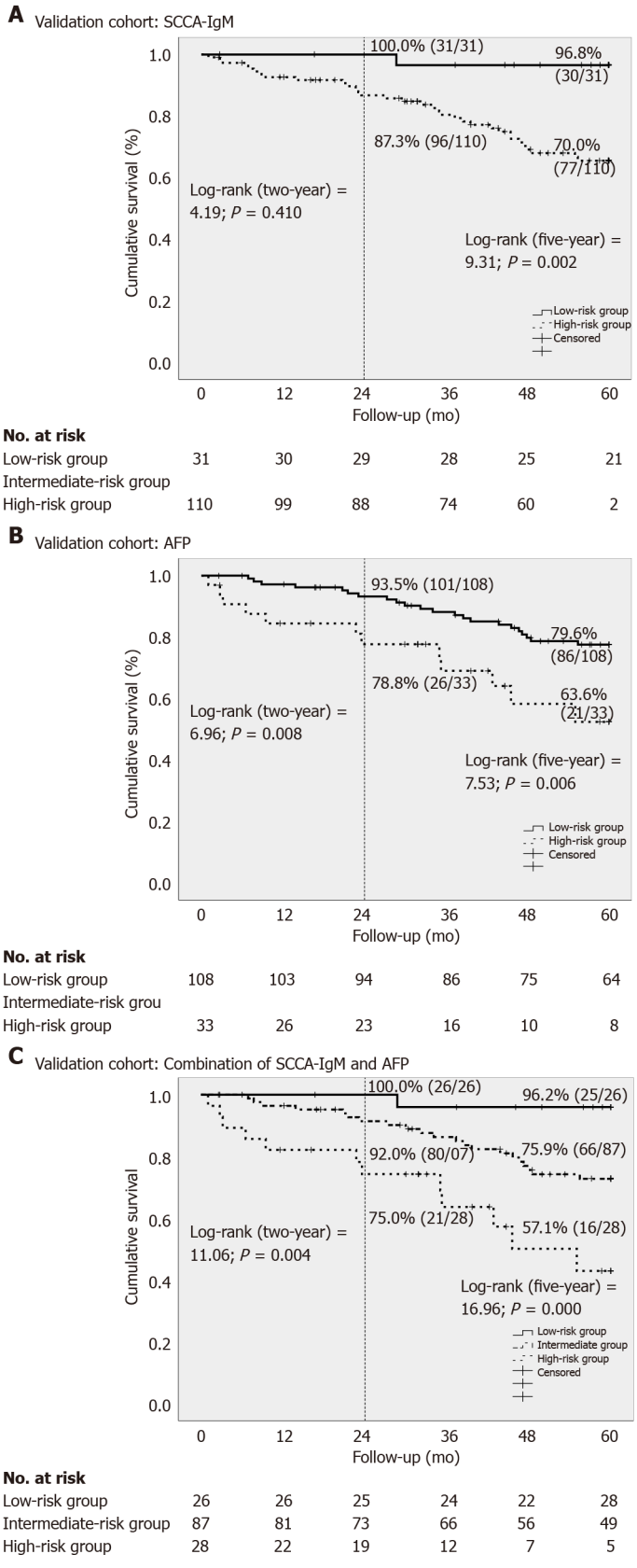


Figure 3 Estimating two- and five-year hepatocellular carcinoma disease-free survival by using Kaplan-Meier method according to the squamous cell carcinoma antigen immunocomplex, alpha-fetoprotein and combination of those both in validation cohort. A: Squamous cell carcinoma antigen immunocomplex (SCCA-IgM); 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.

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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

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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

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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

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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

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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

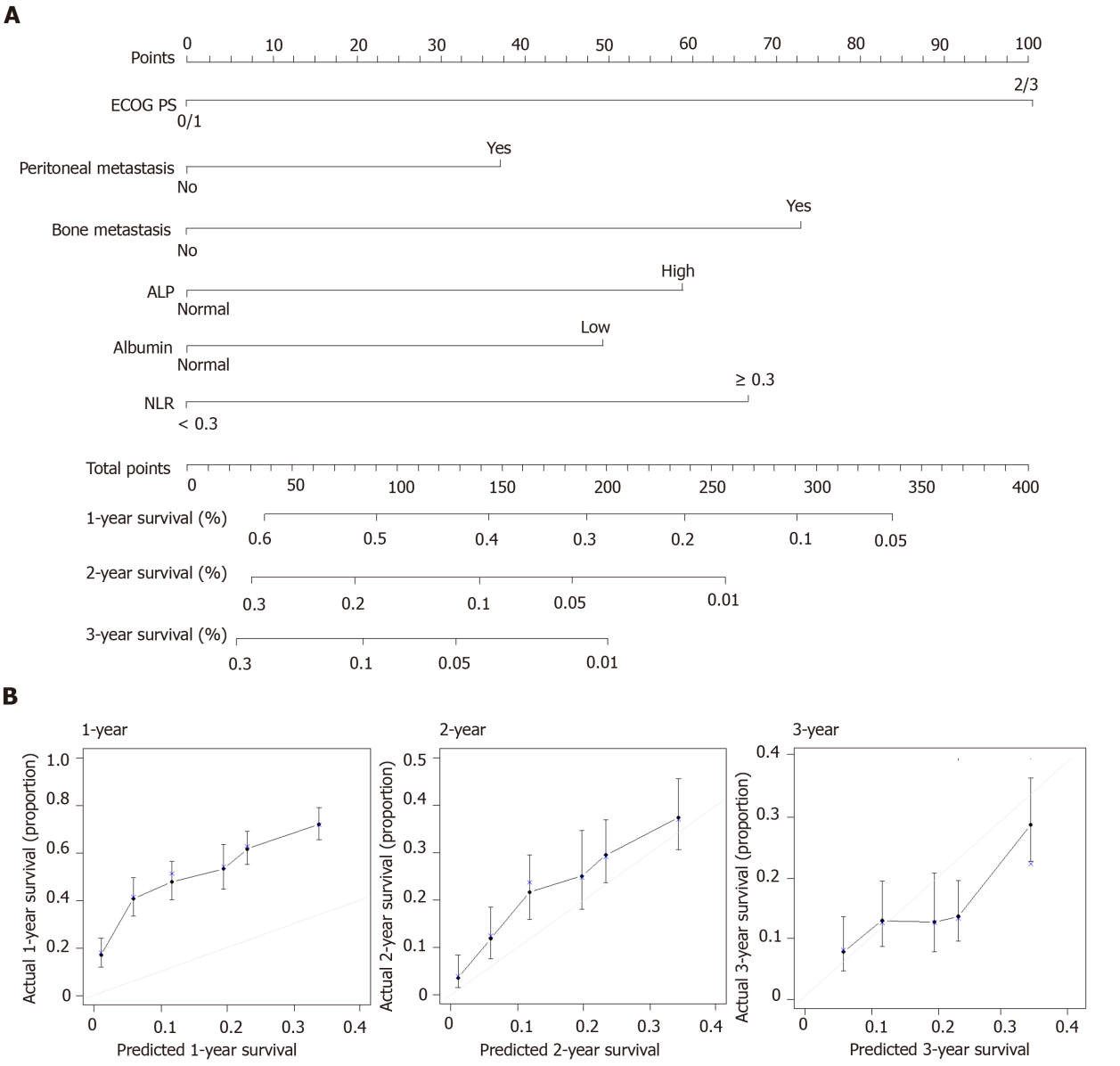


Figure 1 The nomogram using six factors to predict survival rates in the training set. A: The nomogram was applied by summing the scores projected onto the corresponding scale for each factor. The total number of scores projected onto the bottom scale represents the probability of one-, two-, and three-year 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.

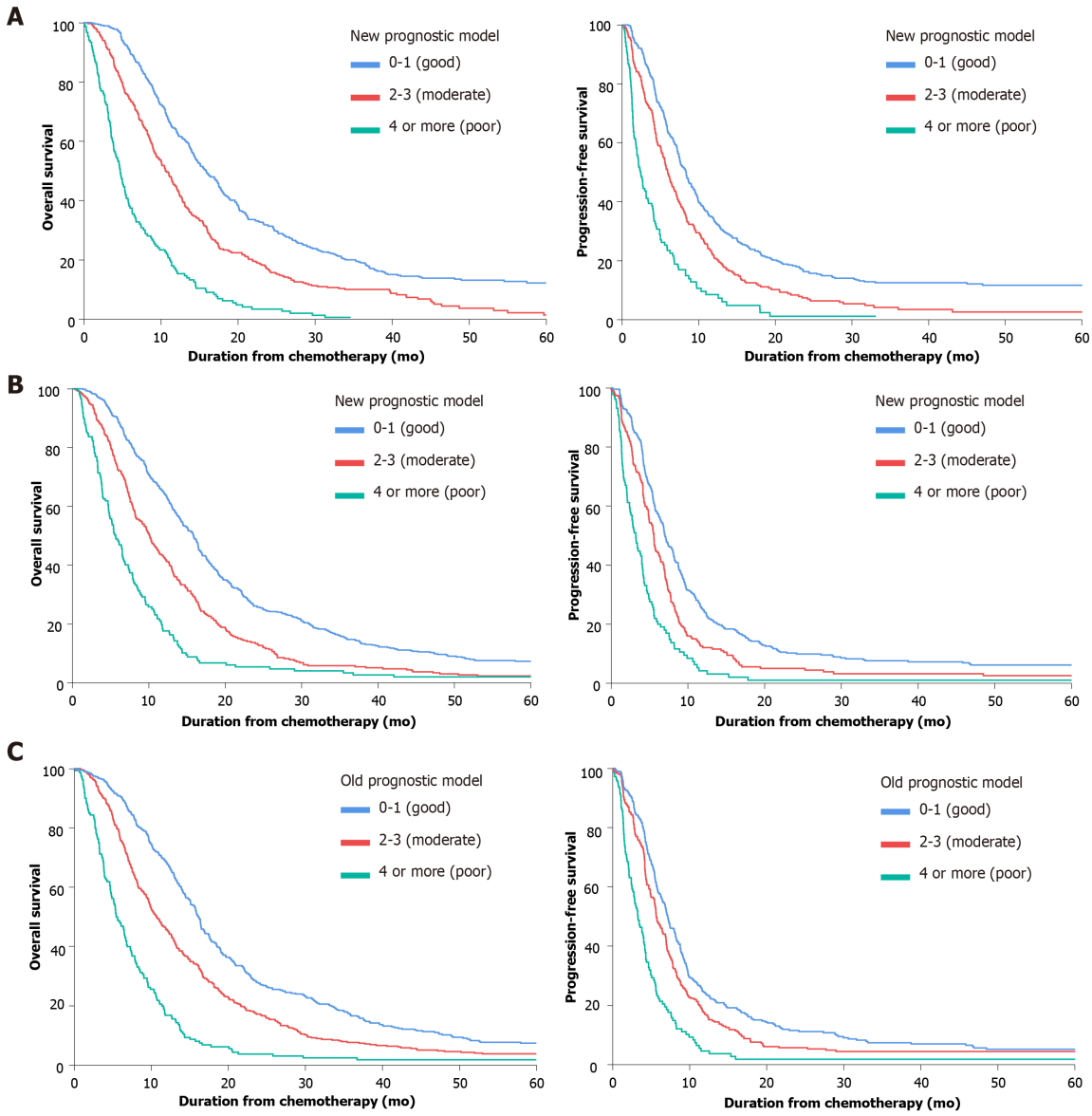


Figure 2 Overall survival and progression-free survival curves according to the new prognostic model. A: In the training set; B: In the validation set; C: According to the old prognostic model in the validation set.

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.

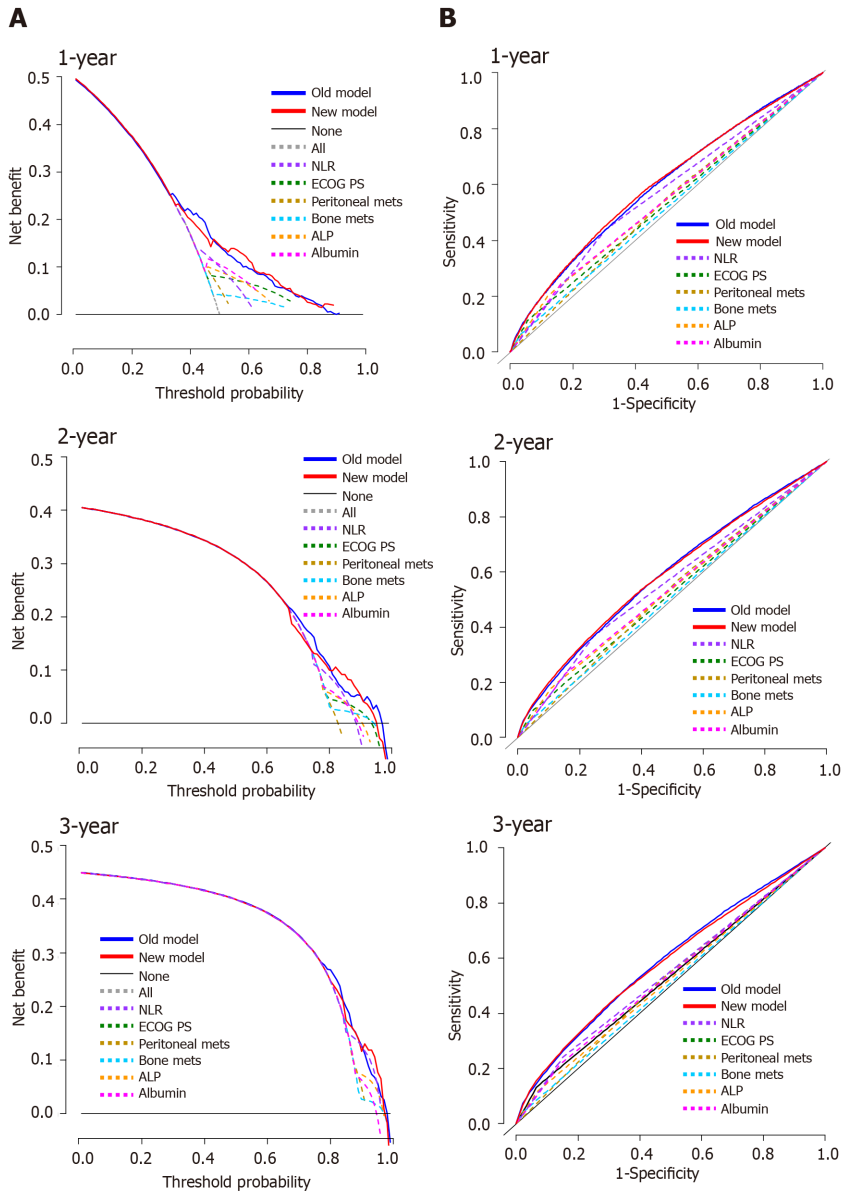


Figure 3 Decision curve analysis curves and time-dependent receiver operating characteristic curves for the nomogram in the validation set. A: The calculated net benefit (Y-axis) corresponds to the threshold probability of survival on the X-axis; B: The time-dependent receiver operating characteristic 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.

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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

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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

Open-Access: This article is an open-access article that was selected by an in-house editor and

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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

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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

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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.

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World Journal of Gastroenterology

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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

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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

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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

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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

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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.

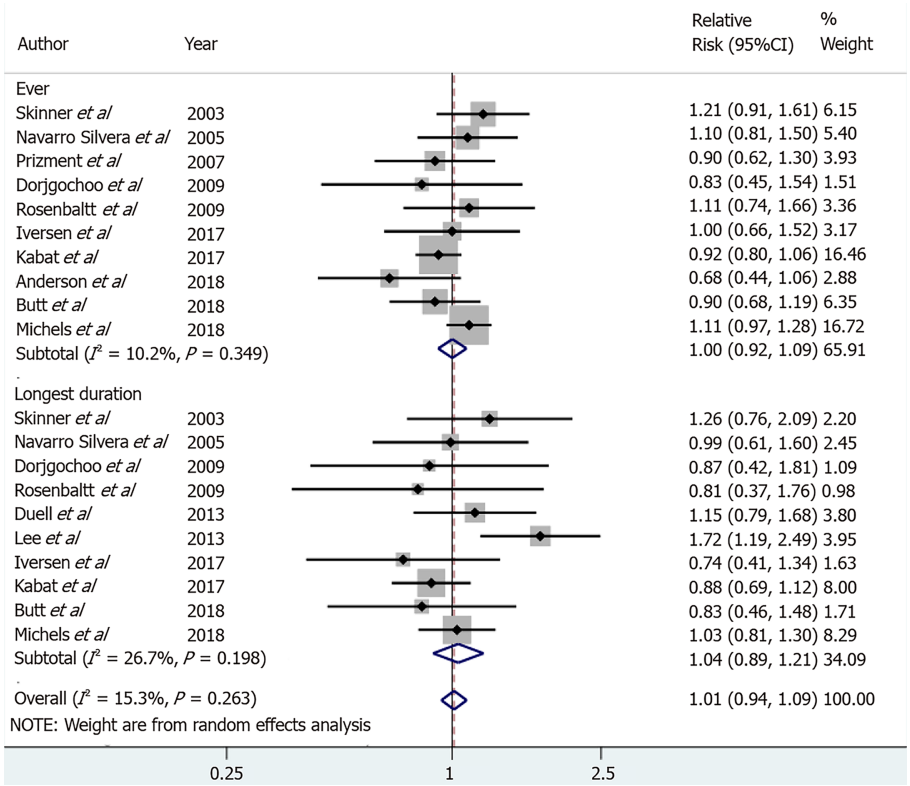


Figure 1 Forest plots in the ever and the longest duration group.

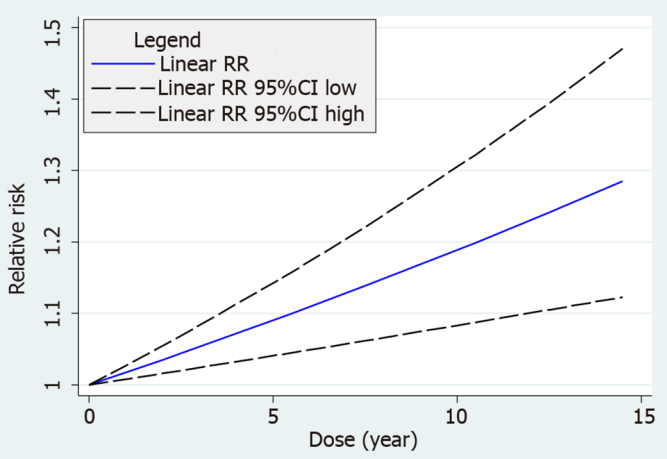


Figure 2 The linear dose-response relationship between duration (year) of oral contraceptive usage and risk of pancreatic cancer in women. RR: Relative risk.

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