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REVIEW

Insight into the liver dysfunction in COVID-19 patients: Molecular mechanisms and possible therapeutic strategies

Naina Khullar, Jasvinder Singh Bhatti, Satwinder Singh, Bhawana Thukral, P Hemachandra Reddy, Gurjit Kaur Bhatti

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Abstract

As of June 2022, more than 530 million people worldwide have become ill with coronavirus disease 2019 (COVID-19). Although COVID-19 is most commonly associated with respiratory distress (severe acute respiratory syndrome), meta-analysis have indicated that liver dysfunction also occurs in patients with severe symptoms. Current studies revealed distinctive patterning in the receptors on the hepatic cells that helps in viral invasion through the expression of angiotensin-converting enzyme receptors. It has also been reported that in some patients with COVID-19, therapeutic strategies, including repurposed drugs (mitifovir, lopinavir/ritonavir, tocilizumab, *etc.*) triggered liver injury and cholestatic toxicity. Several proven indicators support cytokine storm-induced hepatic damage. Because there are 1.5 billion patients with chronic liver disease world-wide, it becomes imperative to critically evaluate the molecular mechanisms concerning hepatotropism of COVID-19 and identify new potential therapeutics. This review also designated a comprehensive outlook of comorbidities and the

impact of lifestyle and genetics in managing patients with COVID-19.

Key Words: COVID-19; Liver damage; Pharmacotherapy; Cytokine storm; Molecular mechanisms

Core Tip: Several review articles have contributed to the pathophysiology, therapeutic strategies, vaccine development, and clinical trials of coronavirus disease 2019 (COVID-19). Since the liver is the primary site of immune protein synthesis, any liver defect may compromise the immune system. Patients with chronic liver disease are at a higher risk of severe COVID-19. This review article demonstrated the pathophysiology and molecular mechanisms responsible for more severe outcomes in patients with hepatic defects. Further, we critically evaluated the molecular mechanisms concerning hepatotropism in patients with COVID-19, which could lead to the development of new therapeutics.

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INTRODUCTION

On December 31, 2019, reports surfaced of patients with an unusual severe acute respiratory syndrome (pneumonia) in Wuhan, China. On January 7, 2020, the causal agent responsible for the mysterious deaths was branded as coronavirus disease 2019 (COVID-19) by the International Classification Committee of Viruses[1]. A severe global pandemic was declared on March 11, 2020. Since then, there have been 623893894 confirmed cases of COVID-19, including 6553936 deaths as of October 21, 2022[2].

The clinical manifestation of COVID-19 is usually interpreted as severe lung infection (acute respiratory distress syndrome), causing turmoil in the patient's respiratory system and even death[3]. Many individuals admitted to intensive care units were known to have hepatic and heart-related complications[4]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in patients with chronic liver disease leads to an elevated and dysregulated immune response[5]. Even healthy individuals infected with COVID-19 displayed liver dysfunction, increasing morbidity and mortality of such patients[6].

Hepatologists have been working to deduce the relationship between COVID-19 and the liver. About 300 million patients with chronic liver diseases in China alone had severe COVID-19 symptoms[7]. It was advocated that SARS-CoV-2 invasion might trigger reactivation of existing liver disorders in the patient, causing hepatotoxicity[8]. In addition, hepatic patients are more prone to COVID-19 infection [9]. Some COVID-19 cases have reported multiorgan failure significantly increasing cytokine levels, including vascular endothelial growth factor, interleukin (IL)-6, macrophage inflammatory protein 1α, and macrophage inflammatory protein 1β[9-11].

Since the liver is a primary site of the synthesis of proteins associated with immunity, it inhibits infectious microbes from entering the bloodstream from the gut. Any liver defect would thus cause a compromised immune system[12]. Increased levels of hepatic biomarkers including alanine transaminase and aspartate transaminase indicate a close pathophysiological association between the liver and COVID-19. Obesity predisposes individuals to metabolic disorders, diabetes, and insulin resistance, which can lead to chronic liver disease and may culminate into cirrhosis, fibrosis, and even hepatocellular carcinoma[13]. This indicates a dire need to investigate the pathophysiology and molecular mechanisms responsible for hindering the immune system, which leads to more severe outcomes in patients with cardiovascular and hepatic defects[14]. In this article, we comprehensively evaluated the invasion and spread of SARS-CoV-2 as a tool to improve therapeutic strategies against liver damage in patients with COVID-19.

ORIGIN OF SARS-COV-2: A BOTTOM-FEEDER OPPORTUNIST

The beta coronavirus isolated from COVID-19 patients was identified using whole genome sequencing and matrix representation with parsimony phylogenetic analysis[15,16]. This virus is the closest relative of Rhinolophus affinis virus (bat CoV RaTG13) with more than 96% similarity[17,18]. Thus, the bat is

Table 1 Summary of severe acute respiratory syndrome coronavirus 2 strains and mutations that enhance viral entry into the host cell

Sr. No.	Strain	Mutation	Host entry	Location	Time of first report	Ref.
1	Alpha (B.1.1.7)	N501Y mutation on the RBD	The affinity between RBD and ACE2 is significantly increased	United Kingdom	December 2020	[40-42]
2	Beta (B.1.351)	N501Y mutation on the RBD N417/K848/Y501	The affinity between RBD and ACE2 is significantly increased	South Africa	December 2020	[41-43]
3	Gamma (P.1)	N501Y mutation on the RBD N417/K848/Y501	The affinity between RBD and ACE2 is significantly increased	Brazil	January 2021	[41,42, 44]
4	Delta (B.1.617.2)	Absence of N501Y mutation	No effect	India	December 2020	[41,42]
5	Omicron (B.1.1.529)	N501Y mutation on the RBD	The affinity between RBD and ACE2 is significantly increased	South Africa	November 2021	[41,42]

RBD: Receptor binding domain; ACE2: Angiotensin-converting enzyme 2.

hypothesized to be its most suitable natural host. The phylogenetic interrelationship is depicted in Figure 1. Whole genome studies revealed the presence of three SARS-CoV-2 strains (A, B, and C) among various human populations[19]. Originally strain A was found in the European population and was regarded as the ancestral strain closest to the bat coronavirus[20,21]. Strain B was primarily found in East Asian populations[22]. Strain C, however, was more common among Americans, thus defining and tracking their outbreak areas[23]. Although the phylogenetic relationship of SARS-CoV-2 is still under investigation, it is well established that the preferred reservoir host of this virus is the bat and was transferred to humans from consumption of wild bats[18,24,25]. These are undoubtedly among the most prevalent RNA viruses (positive sense) that can infect a wide range of hosts[6,26]. Whole genome sequencing demonstrated that SARS-CoV-2 is most closely related to the CoV RaTG13 virus[27,28].

After almost three years since the discovery of SARS-CoV-2, the mechanism that allows SARS-CoV-2 to jump to a human host and invade hepatocytes remains unclear. Upregulated transaminases in patients with COVID-19 indicate a strong link between SARS-CoV-2 and liver injury[29,30]. The genetic algorithm for detecting recombination demonstrated that SARS-CoV-2 possesses a mosaic genome comparable to five coronaviruses (probable donor strains), namely Rhinolophus affinis RaTG13 coronavirus, Rhinolophus pusillus RpYN06, Rhinolophus pusillus BANAL-103, Rhinolophus malayanus RmYN02, and Rhinolophus malayanus BANAL-52[31-33]. The spike protein of SARS-CoV-2 has more similarity with Rhinolophus affinis RaTG13, and human angiotensin-converting enzyme 2 (ACE2) interaction is closely related to Rhinolophus malayanus BANAL-52. Another noteworthy feature is the absence of furin cleavage sites in all of these viruses[34].

SARS-CoV-2 has a deadly combination of disease severity and transmissibility[35,36]. Coronaviruses are remarkable entities (125 nm in size) with one of the heftiest viral RNA genomes, accounting for 30000 nucleotides and equipped with an extraordinary ability to correctly repair drug-induced mutations[37]. The chances of zoonotic transmission or accidental spill-over through exponential proliferation are also possible. In addition, a new host like a human would lack previous immunity to the pathogen, which provides a compatible incapacitating host defense mechanism[38]. Above all, the increasing human-animal interactions due to deforestation, hunting, domestication, wet market, and wild animals as a food source in many countries further increases the chance of viruses to adapt to infect humans[39]. Five SARS-CoV-2 strains are currently prevalent[40-44] (Table 1).

Prognosis and pathophysiology: thwarting effects of SARS-CoV-2 on patients with existing liver diseases

Various factors govern the prognosis of this disease based upon pre-existing health conditions, comorbidity, age, course of treatment adopted, and the response to treatment. The overall fatality rate is above 2%. Like most pathogens, the liver serves as one of the preferred sites of proliferation spots for coronaviruses because it is a common gateway for the blood[45,46]. It has been observed in many studies that one-third of patients with COVID-19 develop liver dysfunction, which was more frequent among elderly, male patients[47]. A survey conducted on 4000 seriously ill COVID-19 patients confirmed an inflated mortality rate after 3 mo, causing death in 31% of the patients[48]. Autopsies and post-mortem biopsies of the liver revealed infection of cholangiocytes, hepatocytes, and endothelial cells with SARS-CoV-2, which led to severe liver damage. Mechanisms of injury include major hepatocyte ballooning, eosinophilic action creating a cytokine storm, hypoxia, and ischemia leading to liver necrosis (Figure 2)[49-51].

Hepatic fat accumulation indicating microvesicular steatosis is a clear consequence of SARS-CoV-2 [52]. Additionally, lobular inflammation and fibrosis cause severe liver cirrhosis. Cirrhosis further reduces blood flow through the liver, thereby increasing blood pressure in the hepatic vein, which is

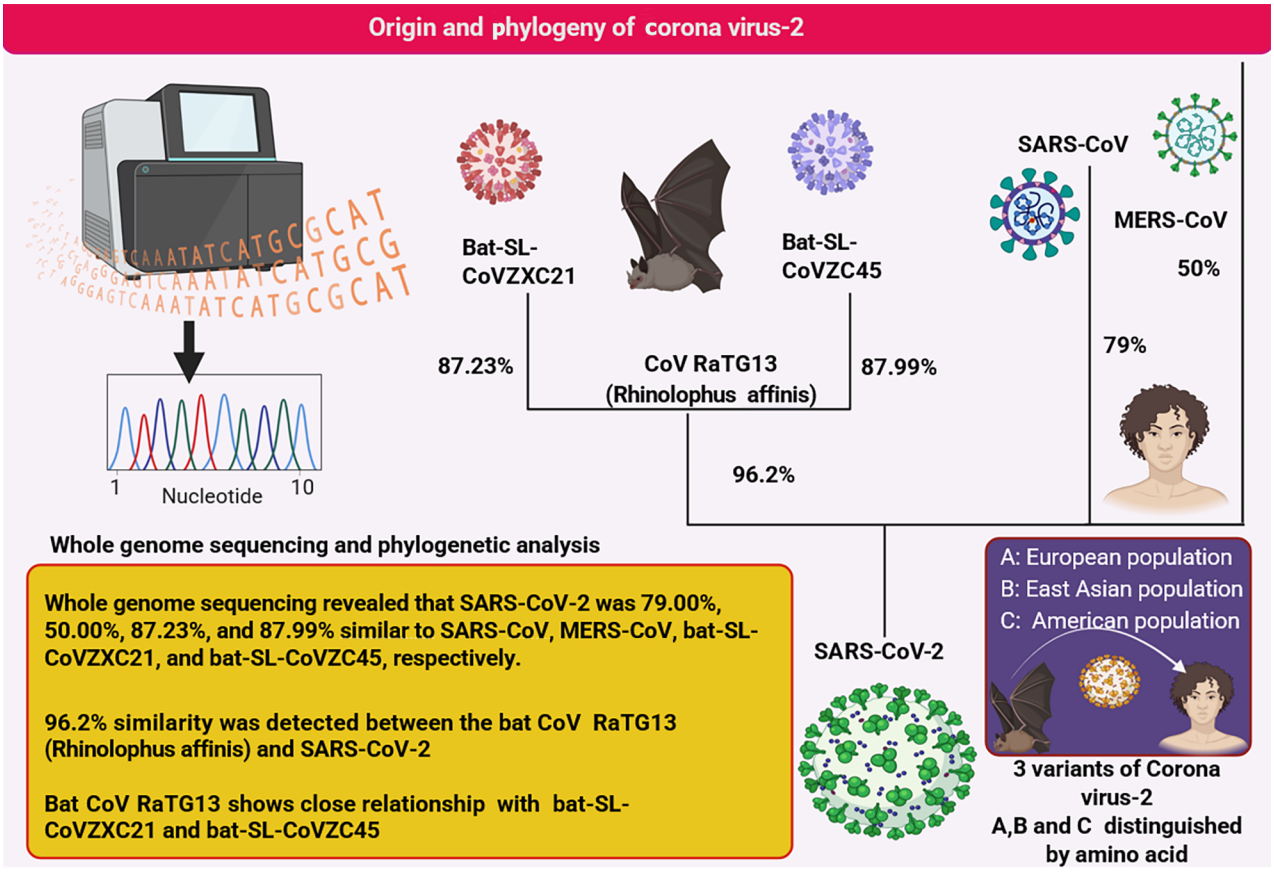

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Figure 1 The origin and ancestral relationship of severe acute respiratory syndrome coronavirus 2 with other related viruses. Whole genome sequencing revealed that severe acute respiratory syndrome coronavirus 2 virus was most closely related to the CoV RaTG13 virus. The variant strains of coronavirus and their biogeographical distribution are illustrated. MERS-CoV: Middle East respiratory syndrome coronavirus; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; R. affinis RaTG13: SARS-like coronaviruses: Bat-SLCoVZX.

supplied by the intestines and spleen. Once SARS-CoV-2 enters the human body, major histocompatibility complexes ensure the release of proinflammatory (acquired and innate)[53-55]. SARS-CoV-2 binds to the ACE2 receptor to enter the target cells[56]. These receptors are typically expressed on the bile duct epithelial cells, sinusoidal and capillary endothelial cells, and hepatocytes[57-59]. The viral antigen epitopes are recognized by antigen-presenting cells (macrophages and dendritic cells). They are presented to CD4+ T helper cells, lymphocytes, and natural killer cells, which activate B cells and CD8+ cytotoxic T cells[60-63].

Several patients with COVID-19 have abnormal biochemistry, displaying fluctuating levels of vital enzymes and biomolecules concerning hepatobiliary manifestations[64]. Patients with COVID-19 have decreased hepatic functions, typically displayed as increased liver enzymes and alanine transaminase and aspartate transaminase levels[5,65-67]. Several case studies observed these findings in more than 50% of critically ill COVID-19 patients[68]. Liver injury, chronic congestion, and nodular proliferation in patients with COVID-19 may occur, with more than 70% of patients developing steatosis[69,70]. The pathogenesis of hepatic damage in patients with COVID-19 is multifactorial, including pre-existing hepatic disease, hypoxia, ACE2 aided viral invasion and damage, ischemia, and drug-induced liver injury[71,72] (Figure 3).

MOLECULAR FEATURES: HEPATOTROPISM OR HEPATIC FRAILTY

The specificity of the virus to preferably invade hepatocytes is considered hepatotropism[65,73-75]. According to current research, SARS-CoV-2 does not specifically display hepatotropism but shows a preference for hepatocytes in patients with a pre-existing liver disease or a compromised immune system[76,77]. Patients with an existing liver disease display severe and prolonged symptoms of SARS-CoV-2 because the immune system dysfunction displays a more pronounced effect[45]. A meta-analysis of 90000 patients with COVID-19 pertaining to 40 case studies in the United States and China provided strong evidence for hepatic deterioration[78]. Hepatic frailty makes liver cells more susceptible and sensitive to COVID-19[79,80]. COVID-19 and comorbidities of hepatic diseases is a global phenomenon

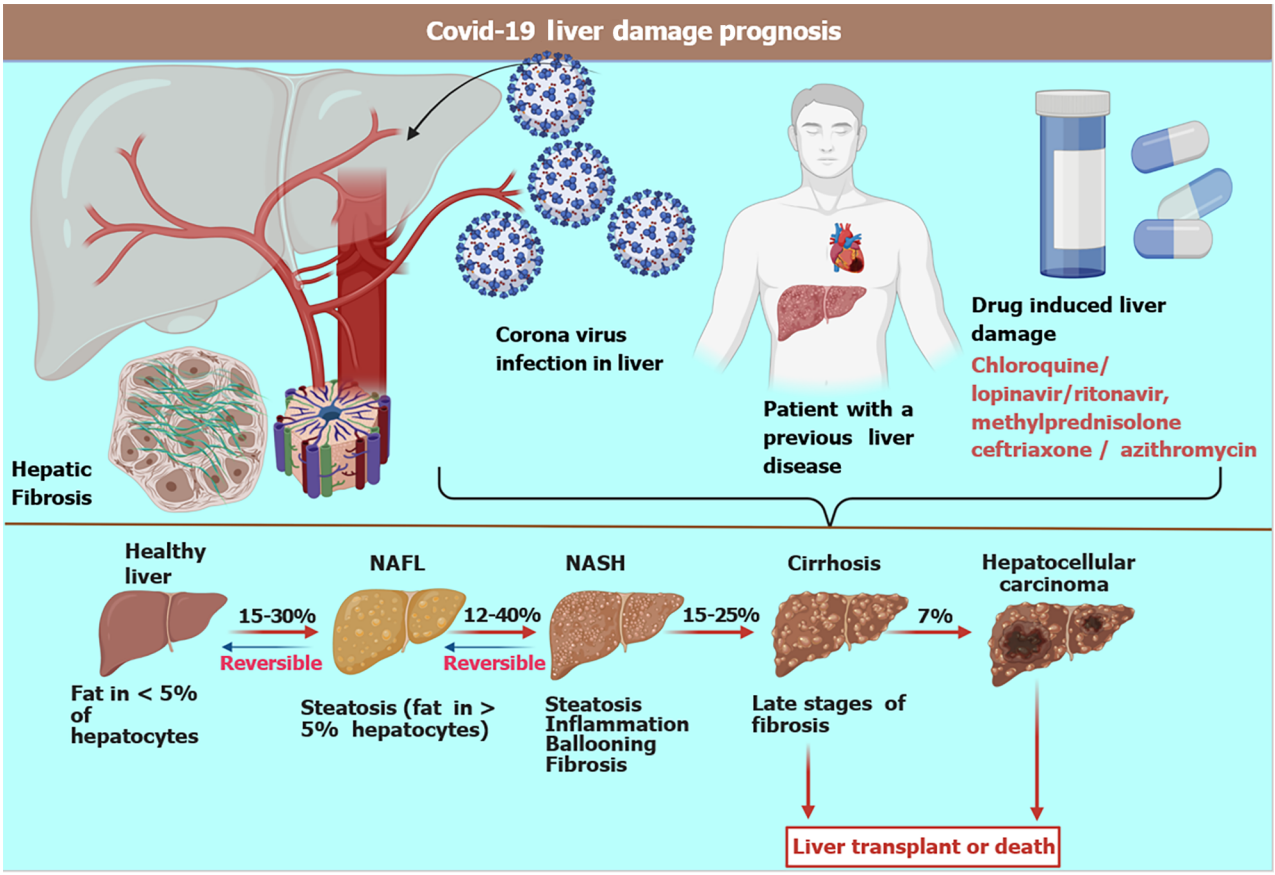

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Figure 2 The schematic representation of the effects of severe acute respiratory syndrome coronavirus 2 on the liver. A patient with a pre-existing liver disorder exhibits higher morbidity than patients with normal liver. Drugs may cause liver damage in some patients, making treatments ineffective. The stages of disease progression are implicated as steatosis (deposition of fat), causing a nonalcoholic fatty liver. The liver deteriorates, indicated by inflammation, ballooning of hepatocytes, and fibrosis. Severe liver cirrhosis is the next stage, which is irreversible. The final stage is hepatic cancer. COVID-19: Coronavirus disease 2019; NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis.

[80,81].

The invasion of SARS-CoV-2 virus in the human body, its genomic single-stranded RNAs, and the replicative double-stranded RNAs are sensed by cytosolic RNA sensors. These are then identified and bound to NOD-like receptors, endosomal toll-like receptors, melanoma differentiation-associated gene 5, and retinoic acid-inducible gene-I-like receptors[82]. These receptors then stimulate the next set of effectors molecules downstream interferon (IFN) regulatory factor 3/7 (IRF3/7); activator protein-1; and nuclear factor-κB (NF-κB). The next step encompasses the synthesis of pro-inflammatory cytokines, namely, interleukin (IL)-2, IL-10, IL-6, IL-8; and IFN-I, by activating their transcription. These IFN-I molecules are thus supposed to be the first line of defense to combat and clear the viral particles from the body; these thus induce signal transducer molecules the Janus kinase 1 (JAK1)/tyrosine kinase 2 and transcription 1/2 (STAT1/2), turning on the JAK1/TYK2-STAT1/2 pathway. This generates STAT1/2/IRF9 complex that additionally triggers the transcription of IFN-stimulated genes. Thus, a cascade of events leads to the massive synthesis of antiviral chemicals: Procalcitonin; IL-6, CCL-5, IL-1, IFN-alpha, CXCL10, and CXCL-8, C-reactive protein[83]. Many studies have hinted at the unconventional triggering of certain supplementary systemic inflammatory responses leading to uncontrolled immune responses signaled by a storm of cytokines produced due to the activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways[82]. This is commonly known as systemic inflammatory response syndrome, where a horde of immune cells (B cells, T-cells, natural killer cells, dendritic cells, neutrophils, and macrophages) bring about a cumulative and exaggerated response[84]. Apoptosis and cell death remain the culminating stage regulated by the MAPK pathway. Pyroptosis is a specialized mechanism induced by coronaviruses to prevent viral spread leading to an inflammatory caspase-1-dependent cell death in patients in response to rapid viral replication within infected cells[85]. In this activation process, the virus secures its persistence through the PI3 kinase/Akt pathway[86].

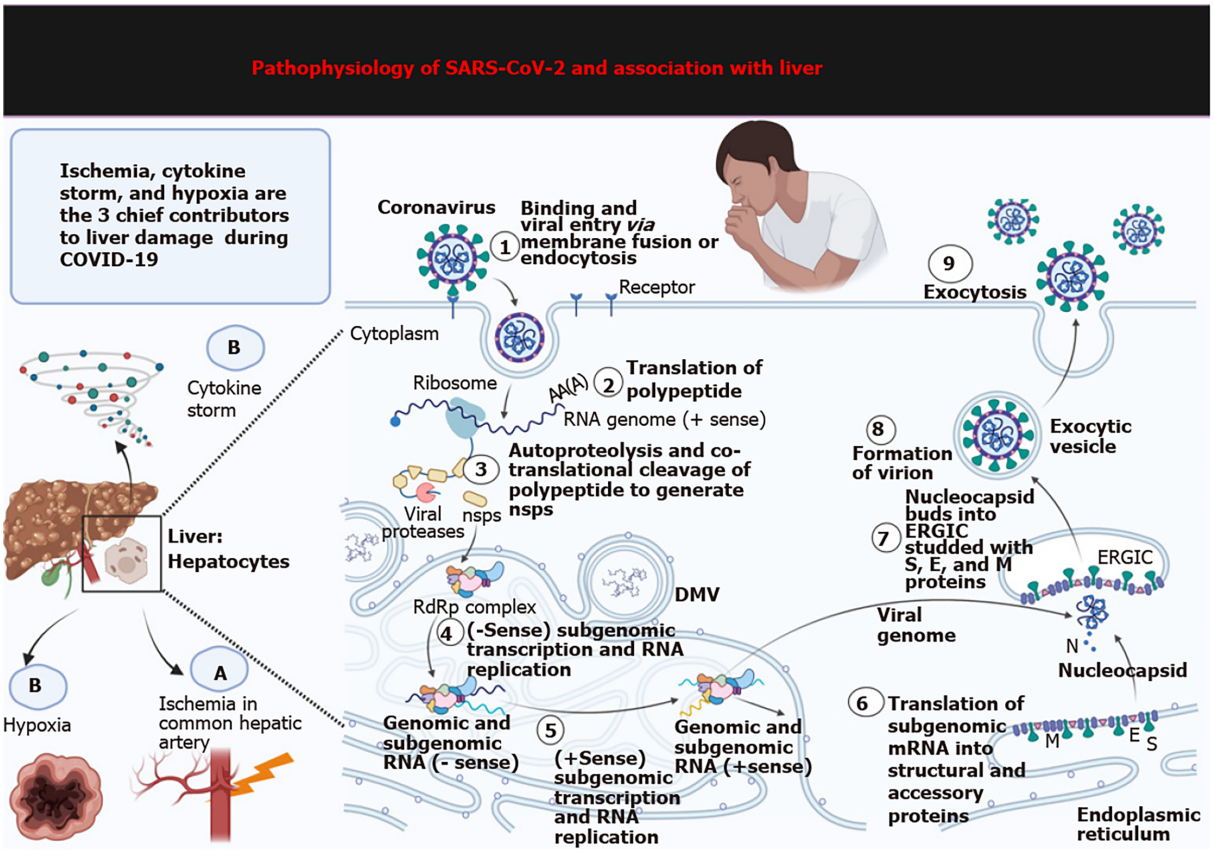

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Figure 3 Molecular mechanisms of liver damage during coronavirus disease 2019 infection. Hypoxia, ischemia, and cytokine storm significantly contribute to liver damage in coronavirus disease 2019-related comorbidities. This figure depicts how severe acute respiratory syndrome coronavirus 2 exponentially proliferates after entering hepatocytes. The steps occur: (1) Binding and viral entry *via* membrane fusion or endocytosis; (2) Translation of polypeptide; (3) Autoproteolysis and cotranslational polypeptide cleavage to generate non-structural proteins (nsps); (4) Sense subgenomic transcription and RNA replication; (5) + Sense subgenomic transcription and RNA replication; (6) Translation of subgenomic mRNA into structural and accessory proteins; (7) Nucleocapsid buds into ER-Golgi intermediate compartment studded with spike, envelope, and membrane proteins; (8) Formation of virion; and (9) Exocytosis. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 2019; ERGIC: ER-Golgi intermediate compartment; DMV: Double-membrane vesicle.

PHARMACOLOGIC THERAPIES SPECIFIC TO CORONA-ASSOCIATED HEPATIC MORBIDITIES

Scientists worldwide could conceive fairly early the devastating effects of SARS-CoV-2, and social distancing was the only way out since impending outcomes were far from the view[87,88]. As an opportunist virus, it intimidated the whole world, shutting down everyday life and hampering the economy and health worldwide. Though we have successfully tamed this dangerous and feral pathogen, the efficacy of existing vaccines and drug therapies in preventing SARS-CoV-2 variants is still a matter of concern[87-89]. Vaccines were tracked on the plan to target spike proteins of the SARS-CoV-2, which the virus variants inventively cons[90-92]. Several novel vaccines, as well as drugs, have ardently helped in tackling these viruses. Since the virus shows high transmissibility and the future modulation in these viruses is erratic and unforeseen[90,92,93], prevention and management strategies should entail a multi-omic, closed-loop follow-up and holistic approach comprising scientists, government authorities, clinicians, pharmacists, and as the general public. Thus, prevention and management, including pharmacologic therapies against COVID-19, have been worked out under different approaches certified under Emergency Use Authorization[94-96]. Several therapeutic strategies are followed depending on the patient's condition as diagnosed by the clinician[97,98].

Immuno-modulators and anti-inflammatory agents

More appropriate to provide in the later stage of COVID-19 infection. Clinical trials conducted on 113 COVID-19 patients critically suffering from this disease, with both Baricitinib (inhibitor of Janus kinase) and Anakinra (IL-1 antagonists on 52 COVID-19 patients), have shown promising results in the case of COVID-19 patients facing hyperinflammation (cytokine storm). These offer a dual inhibitory effect by preventing both entries of SARS-CoV-2 and preventing an exaggerated cytokine response[99-102]. Such trials have attested to the efficacy of critical-stage COVID-19 patients, especially those with hepatic

Table 2 Summary of the effective vaccines developed so far against coronavirus disease 2019

Sr. No.	Name of vaccine	FDA approval	Type of vaccine	Manufactured by	Efficacy	No. of doses	Safety profile	Ref.
1	NVX-CoV2373 vaccine	December 20, 2021	Recombinant SARS-CoV-2 nanoparticle	Novavax	92.6%	2	Safe till date	[105]
2	BNT162b2 vaccine	FDA issued a EUA on December 11, 2020	mRNA-based	BioNTech/Pfizer	95%	2	Safe till date	[106]
3	mRNA-1273 vaccine	FDA issued a EUA on December 18, 2020	mRNA-1273 based	Moderna	94.1%	2	Safe till date	[107]
4	ChAdOx1 nCoV-19 vaccine	Not yet received a EUA or approval from the FDA	Recombinant spike protein vaccine	Serum institute of India, private limited	70.4%	2	Vaccine-induced immune thrombotic thrombocytopenia	[108]
5	Ad26.COV2	EUA by the FDA on February 27, 2021	Recombinant vaccine	Janssen-Cilag International, NV Belgium	73.1%	1	Vaccine-induced immune thrombotic thrombocytopenia	[109]
6	Covaxin	EUA for adults	Whole inactivated virus-based	Bharat Biotech in collaboration with ICMR and NIV, India	64%	2	Safe till date	[110]
7	Sputnik V	EUA qualified	Human adenovirus vector	Russian direct investment fund	97.2%	2	Safe till date	[111]
8	CoronaVac	FDA issued under EUA	Inactivated virus alum-adjuvanted candidate vaccine	Sinovac biotech, China	51% against symptomatic SARS-CoV-2 infection, 100% against severe COVID-19	2	Safe till date	[112]

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 2019; FDA: Food and Drug Administration; EUA: Emergency Use Authorization; ICMR: Indian Council of Medical Research; NIV: National Institute of Virology.

complaints[103,104]. Table 2 shows the currently used effective vaccines developed and successfully reduced morbidity and mortality across the world[105-112].

Antiviral drugs

These are more suitable during the early phase of Corona infection. Molnupiravir, a very effective drug that reduces both morbidity and mortality; paxlovid: Reduced 89% mortality (trial conducted on 1219 patients); remdesivir, hydroxychloroquine, lopinavir/ritonavir, ivermectin, and chloroquine are all Food and Drug Administration approved, but show little or no effect over Coronavirus; it is even not effective against corona variants[113,114]. Therefore, they are not recommended in case of patients with hepatic trouble during COVID-19. Also, some of these drugs (lopinavir/ritonavir, mitifovir, and tocilizumab) are not recommended and prescribed to patients with pre-existing liver diseases as they are known to cause cholestatic toxicity and hepatic injury[115-117].

Neutralizing antibodies against SARS-CoV-2

Antibodies naturally produced by the body of recovering patients or stimulated through vaccination can block the attachment and hence the entry of an enveloped viral pathogen inside the cell, conferring lifelong immunity[118-121]. Convalescent plasma transfusion therapy with a high anti-SARS-CoV-2 immunoglobulin G (IgG) titer effectively lowered the mortality of critical COVID-19 patients[122-124]. The bamlanivimab and etesevimab antibody combination has been found to be super effective in COVID-19 patients with 87% lower death rate[125]. Another antibody cocktail, REGN-COV2, constitutes a group of two IgG1 antibodies (casirivimab and imdevimab) that target the receptor binding domain of SARS-CoV-2 and thereby reduce both morbidity and mortality of COVID-19 patients by 70%[126].

CONCLUSION

Extraordinary efforts have reaped fruits. As per the World Health Organization report on COVID-19 Vaccine Implementation Analysis & Insights, 63.4% of the World's population today stands vaccinated against COVID-19 in September 2022 (https://www.who.int/publications/m/item/covid-19-vaccine-implementation-analysis-insights-2-september-2022), and India alone proudly puts up 68% of its population in the list of a fully vaccinated cluster. This was made possible due to the untiring efforts of clinicians and researchers braced and heavily funded by the government and private agencies to curb this callous pandemic. It is anticipated that once 100% global vaccination is achieved, the virus will no longer be felonious. However, there are reasons to negate this notion. One explanation is that despite marshy governmental efforts, many people are vaccine-hesitant for inexplicable motives which may hamper virus block[127,128]. Even if this temper is somehow overcome, the dynamics of remodelling human immunity to ongoing viral mutations and evolution is worth consideration. The co-evolution may equip the virus with new immune strategies to escape the human immune defense mechanism and maintain its virulence. According to the United States Centres for Disease Control and Prevention, viruses with new mutations are specifically a matter of concern and shall not be considered lightly[128]. Lifestyle, assess to wet markets, climate change, and increased animal-human interactions offer preferred gateways and richer niches to these evolving viruses[129]. The armchair experts in virology, immunology, and genetics contribute substantially to future mitigation strategies. It can no longer be one bug, one drug approach. Herd immunity is expectantly looked upon but is short-term and modulates the virus with new attacking feats. What is good to know is that though the future is unseen, this COVID-19 pandemic has taught us valuable lessons and equipped health agencies, clinical experts, and the general public to face the subsequent pandemic terror.

FOOTNOTES

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REVIEW

Role of prebiotics, probiotics, and synbiotics in management of inflammatory bowel disease: Current perspectives

Supriya Roy, Suneela Dhaneshwar

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Abstract

Experimental evidence supports the fact that changes in the bowel microflora due to environmental or dietary factors have been investigated as implicating factors in the etiopathogenesis of inflammatory bowel disease (IBD). The amassing knowledge that the inhabited microbiome regulates the gut physiology and immune functions in IBD, has led researchers to explore the effectiveness of prebiotics, probiotics, and synbiotics in treating IBD. This therapeutic approach focuses on restoring the dynamic balance between the microflora and host defense mechanisms in the intestinal mucosa to prevent the onset and persistence of intestinal inflammation. Numerous microbial strains and carbohydrate blends, along with their combinations have been examined in experimental colitis models and clinical trials, and the results indicated that it can be an attractive therapeutic strategy for the suppression of inflammation, remission induction, and relapse prevention in IBD with minimal side effects. Several mechanisms of action of probiotics (for *e.g.*, *Lactobacillus* species, and *Bifidobacterium* species) have been reported such as suppression of pathogen growth by releasing certain antimicrobial mediators (lactic and hydrogen peroxide, acetic acid, and bacteriocins), immunomodulation and initiation of an immune response, enhancement of barrier activity, and suppression of human T-cell proliferation. Prebiotics such as lactulose, lactosucrose, oligofructose, and inulin have been found to induce the growth of certain types of host microflora, resulting in an enriched enteric function. These non-digestible food dietary components have been reported to exert anti-inflammatory effects by inhibiting the expression of tumor necrosis factor-α-related cytokines while augmenting interleukin-10 levels. Although pro- and prebiotics has established their efficacy in healthy subjects, a better understanding of the luminal ecosystem is required to determine which specific bacterial strain or combination of probiotics and prebiotics would prove to be the

ideal treatment for IBD. Clinical trials, however, have given some conflicting results, requiring the necessity to cite the more profound clinical effect of these treatments on IBD remission and prevention. The purpose of this review article is to provide the most comprehensive and updated review on the utility of prebiotics, probiotics, and synbiotics in the management of active Crohn's disease and ulcerative colitis/pouchitis.

Key Words: Ulcerative colitis; Crohn's disease; Pouchitis; Dysbiosis; Microbiota; Inflammation

Core Tip: Current treatments for inflammatory bowel disease (IBD), such as corticosteroids and immunosuppressants, have potential adverse effects, and a significant proportion of patients dependent on these treatments are exposed to these associated long-term side effects. The discovery of novel and efficacious therapeutic strategies is a worldwide goal of IBD research, and probiotics, prebiotics, and synbiotics can offer viable solutions. These products offer a novel strategy to deliver beneficial components into the gut and emerge as promising new treatments for IBD, as intestinal dysbiosis has been reported as a major cause of his IBD. The review highlights the current state and action mechanism of these microbial therapies along with various studies that have reported their effectiveness in restoring balance in the gastrointestinal microbiota and thus eventually reducing intestinal inflammation.

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INTRODUCTION

Inflammatory bowel disease (IBD) is an idiopathic disease resulting from a debilitating immunological response of the body to the host's gastrointestinal (GI) microflora (mainly colon and duodenum). IBD classified as Crohn's disease (CD) and ulcerative disease (UC), was previously anticipated to be triggered by adaptive immune responses, however, the recent research findings suggest the prominent role of the innate immune system in instigating an imbalance and disparity between the beneficial microbiome and commensal microflora harboring in the human gut. This imbalance, known as dysbiosis (Figure 1), leads to an aggravated inflammatory response, causing IBD (Figure 2). Alterations in the microbiome affect host homeostatic systems and interactions with luminal stimuli, which can ultimately lead to uncontrolled inflammation in the intestinal mucosa, leading to IBD (Figure 3). This suggests that the human gut microbiome is beginning to be recognized for its important role and potential therapeutic solution for IBD. A better comprehensive understanding of the synergy between host genetics, external environmental factors and gut microbiome has opened new paradigms for seeking alternative effective therapies[1].

PROBIOTICS, PREBIOTICS, AND SYNBIOTICS AS THERAPEUTIC STRATEGY

Standard clinical treatment of IBD consists of agents that modulate the inflammatory pattern of the GI tract (GIT), including mesalamine, azathioprine, anti-tumor necrosis factors (TNFs), and glucocorticoids. However, these drugs often appear to have serious side effects, and some patients require higher doses throughout the course of treatment. A significant proportion of patients with IBD either initially do not respond to treatment or lose response over time. Gut microbiota modulation has emerged as an attractive new therapeutic approach for IBD, and gut microbiota-targeted/based therapies have been intensively investigated with varying degrees of success. Although the exact etiology of IBD remains unknown, the critical role of the gut microbiota in the development and persistence of IBD highlights the importance of microbiota-host interactions in health and disease. Recent advances in assessing the therapeutic potential of microbiota in the treatment of IBD support the reconstitution of microbial resident populations by administration of appropriate microbes. The gut microbiota influences the host by modulating physiological, pathophysiological, and immunological processes. Experimental animal studies along with clinical data have confirmed the influence of the gut microbiome in ameliorating inflammation, highlighting its potential as a therapeutic strategy for treating inflammatory diseases. Numerous therapeutic strategies have been developed to modify and remodel the gut microbiome for

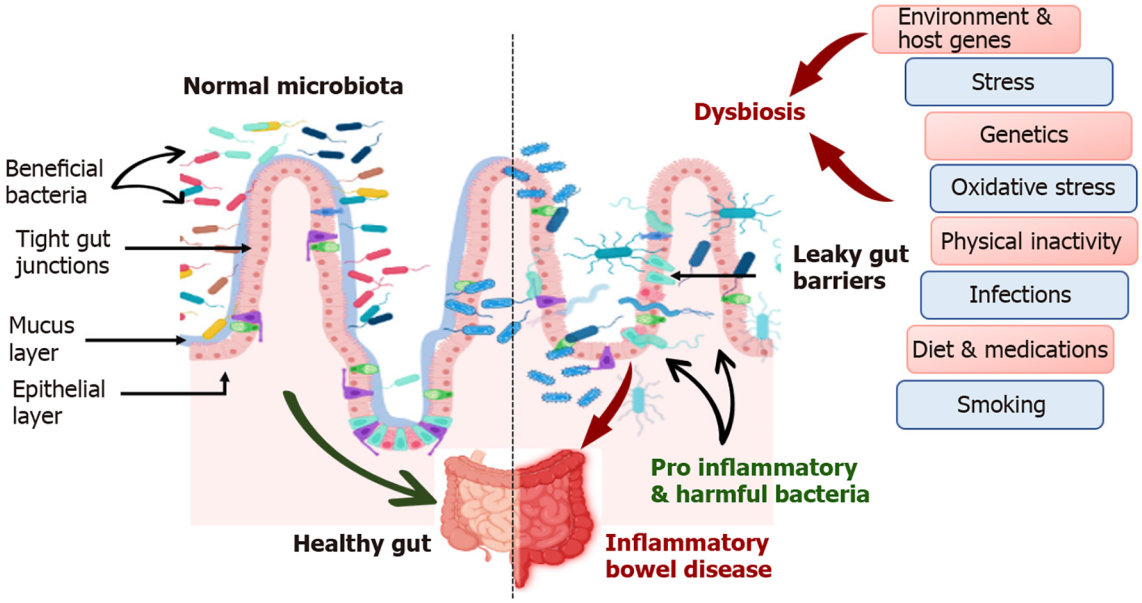

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Figure 1 Comparison between normal microbiome and dysbiosis.

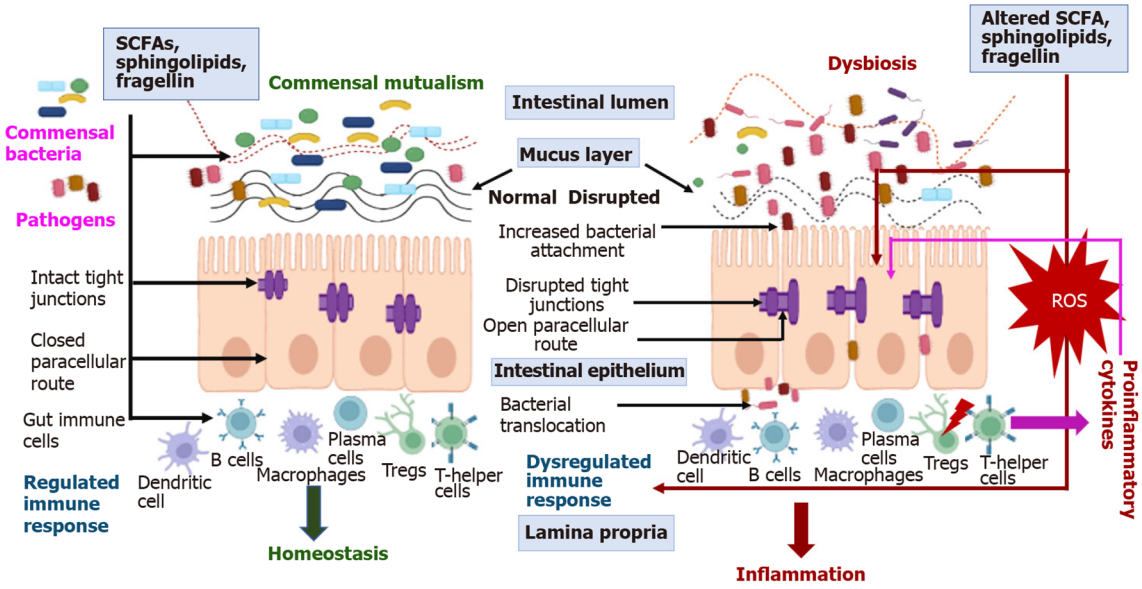

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Figure 2 Role of dysbiosis in inflammatory bowel disease pathophysiology. SCFA: Short chain fatty acids; Tregs: Regulatory T cells; ROS: Reactive oxygen species.

the treatment of other GI diseases, including IBD. Prebiotics, probiotics, synbiotics, and fecal microbiota transplantation (FMT) are currently considered to be the most common treatments[2].

PROBIOTICS

Probiotics are specific live microorganisms, when consumed in appropriate amount, are beneficial to the health of the host. Probiotic therapy involves the targeted introduction of beneficial microorganisms into the intestinal flora. This causes many beneficial bacteria to compete for nutrients and starve harmful bacteria. Probiotics participate in many positive health-promoting activities in human physiology, including the maintenance of a healthy gut. The most common strains currently available as probiotics and possessing beneficial health effects are *Enterococcus faecium, Bifidobacterium, Bacillus, Saccharomyces boulardii* (*S. boulardii*), *Lactobacillus strains,* and *Pediococcus*. Molecular mechanisms for the

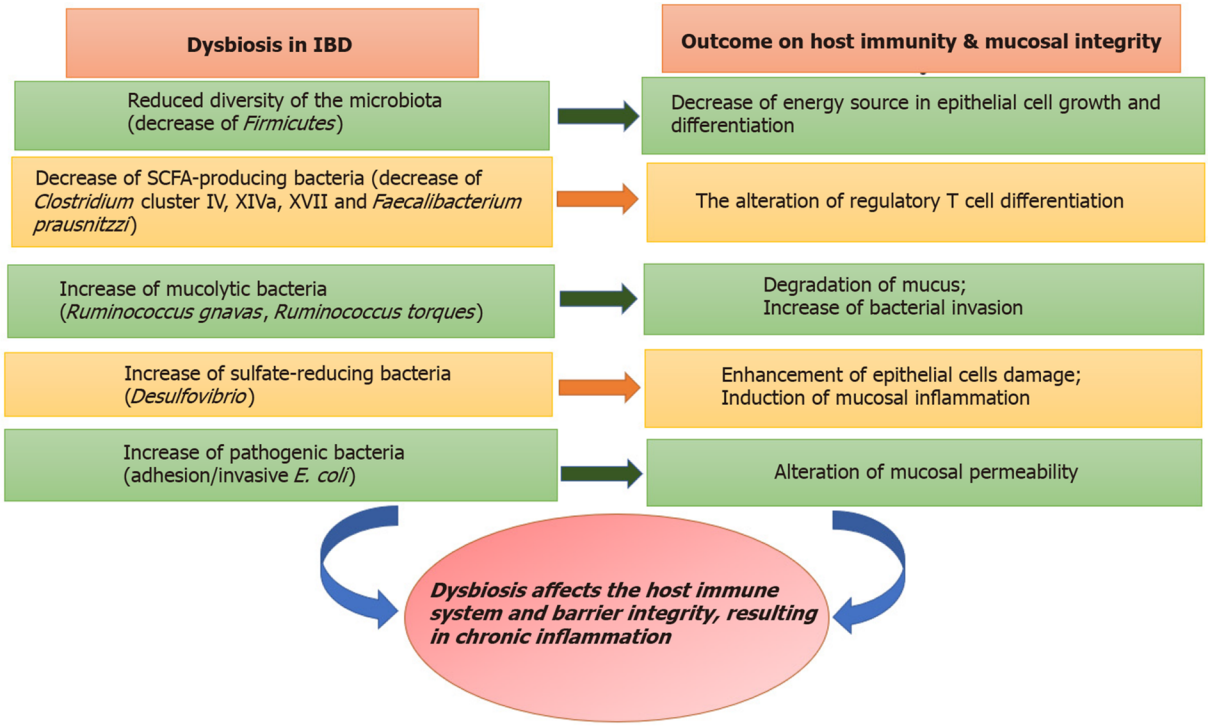

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Figure 3 Dysbiosis in inflammatory bowel disease and its pathological outcomes. IBD: Inflammatory bowel disease; SCFA: Short chain fatty acids.

beneficial effects of these probiotics include (Figure 4): (1) Production of butyrate, immunoglobulin A (IgA), and short-chain fatty acid (SCFA) formation and stimulatory signaling proteins; (2) Reduced secretion of pro-inflammatory cytokines; (3) Increased mucin-2 expression; (4) Increased autophagy; and (5) Augmented upregulation of defensins. Although probiotics have shown promise both preclinically and clinically, the theoretical risks have been explained in several case reports, clinical trial results, and experimental models. These include systemic infections, adverse metabolic activity, overstimulation of the immune system in susceptible individuals, gene transfer, and GI side effects[3].

PREBIOTICS

Prebiotics are non-digestible food ingredients that selectively stimulate the growth of beneficial bacteria or promote the activity of a limited number of health-promoting bacteria. However, prebiotics can also help to improve the existence and effectiveness of ingested PRO bacteria. According to the most recent definition, "a prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the GI microbiota that confers benefits upon host well-being and health". Now, the International Scientific Association of probiotics and Prebiotics has introduced a new definition of prebiotics as "a substrate which is selectively fermented by the gut microflora and bestows health benefits to the host". This new definition states that non-carbohydrate constituents are also considered prebiotics and their applications are not constrained to the GIT only[4].

SYNBIOTICS

Combinations of probiotics and prebiotics are viewed as promising new approaches and provide an opportunity to explore their potential and efficacy in human IBD. When probiotics and prebiotics are combined in a product to achieve synergistic actions, they are commonly referred to as synbiotics. Many examples have demonstrated that prebiotics appears to be more efficacious when used along with a probiotic as a part of the synbiotic combination. The term synbiotic refers to synergism where the prebiotic component is selectively favoured by the live probiotic organism. The synbiotic combination is intended to enhance the *in vivo* survival and activity of proven probiotics to promote or enhance the beneficial properties of both products. However, recently the term 'synbiotics' has been re-defined as preparations favoring synergism, where the probiotics metabolize the complemented prebiotics to induce specific rebalancing of the dysbiotic gut and host health. Synergistic probiotics and prebiotics stimulate selective microbial growth or activate specific metabolism *via* gut flora. The presence of the

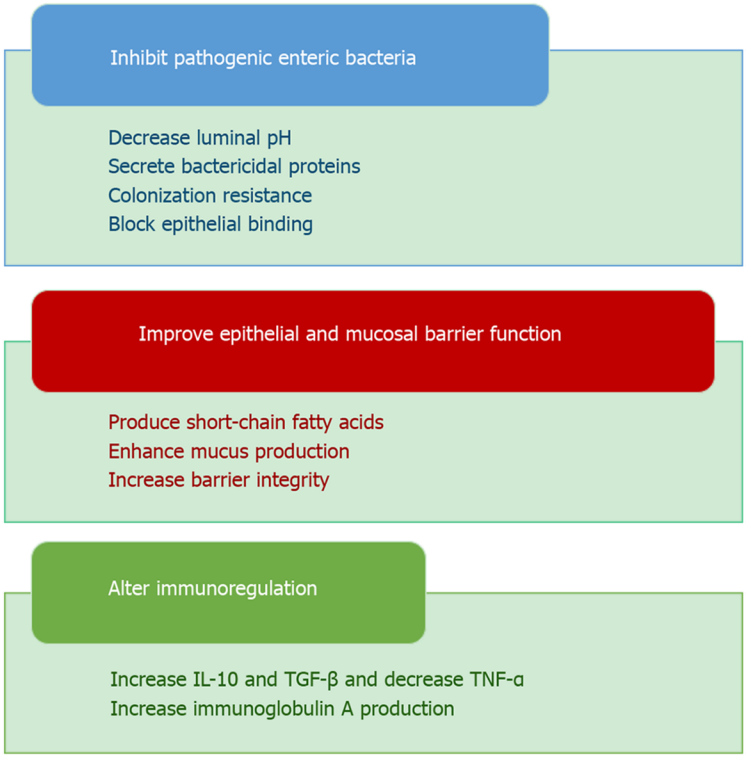

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Figure 4 Various actions of probiotics. IL-10: Interleukin-10; TGF-β: Transforming growth factor-β; TNF-α: Tumor necrosis factor-alpha.

readily fermentable substrate should enhance the survival of the probiotic. The prebiotic component should also protect the probiotic from gastric acidity and proteolysis, possibly through steric hindrance and coating of the probiotic. Therefore, it is important to select specific substrate and microbial combinations in synbiotic products that can enhance beneficial effects compared to products containing probiotics or prebiotics alone[5].

MECHANISM OF ACTION OF PROBIOTICS, PREBIOTICS, AND SYNBIOTICS

Probiotics

Probiotics, prebiotics, or synbiotics can achieve therapeutic effects in IBD through various mechanisms. The mechanisms of action of probiotics include competitive actions with commensal and pathogenic bacteria and effects on epithelial function and immune responses. By augmenting the production of SCFA, they can lower the pH of the intestinal environment, thereby inhibiting the growth of potentially pathogenic microorganisms. Some probiotics enhance the integrity of the mucosal barrier thereby normalizing intestinal permeability[6]. The effects of probiotics vary and depend on type and dose as well as on their interaction with the host in different ways. Some exhibit direct antibacterial action *via* the production of substances such as bacteriocins, hydroperoxides, lactic acid, and defensins. Others exhibit non-immunological action such as competing with pathogens for nutrients, increasing mucus production, changing intestinal pH, by promoting the formation of tight junctions (TJs), or enhancing tissue repair processes, thereby reducing intestinal mucosal permeability. Finally, probiotics can also modulate the immunological response (immunoglobulin production, pro-inflammatory cytokine production) by releasing cell wall fragments or DNA in the intestinal lumen (Figure 5). They also regulate the overactivation of the nuclear factor kappa light chain enhancer of activated B cells (NFκB) pathway, reduce the production and secretion of pro-inflammatory cytokines [such as interleukin (IL)-8, TNF-α, interferon gamma (IFN-γ)], and induce the production of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β[7].

Prebiotics

Prebiotics exert beneficial effects on IBD through multiple mechanisms of action. Primarily they accelerate the selective proliferation of native bacteria of the gut microbiota. These ingredients provide a better breeding space for beneficial microorganisms due to their loose structure and large surface area and at the same time inhibit the growth of pathogens. Secondly, they increase the production of SCFAs

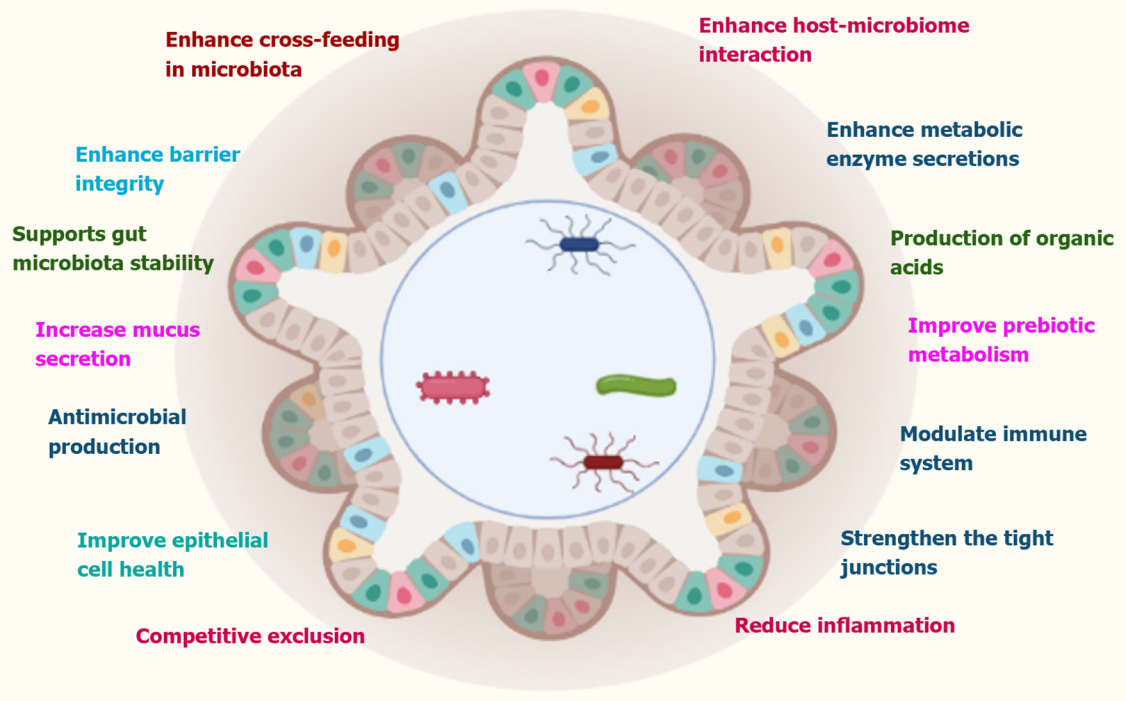

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Figure 5 The mechanism of actions of probiotics in combating inflammatory bowel disease symptoms. IBD: Inflammatory bowel disease.

such as acetate, butyrate, and propionate. These SCFAs are formed during the fermentation of prebiotics and play a very vital role in the proper functioning of the intestine. They accelerate the regeneration and healing process of the intestinal epithelial cell; augment mucus production; maintain the correct pH in the intestine. They also inhibit the attachment of pathogenic microbes to enterocytes. Acetate is commonly used as a cellular fuel for building muscle and colonic tissue. Butyrate exhibit various beneficial effects on the host, such as improving metabolism, modulating the host's immune system, and promoting anti-inflammatory actions, therefore receives special attention[8].

Therefore, prebiotic consumption has been shown to boost host immune function, reduce infection rates, enhance colonic integrity, and downregulate allergic reactions. However, these effects are not directly imposed by ingesting prebiotics. It was suggested that the benefits of prebiotics are achieved indirectly. Prebiotics improves the mucosal barrier *via* encouraging the probiotics growth that can upregulate epithelial defense mechanisms[6].

Synbiotics

In vitro studies specify that synbiotics exert primarily anti-inflammatory actions along with some antiproliferative activities. The literature on synbiotics is difficult to interpret, as it is often impossible to distinguish whether the desired therapeutic benefits are attributable to prebiotics, probiotics, or synergistic interactions between them. Various studies have provided robust preliminary evidence that synbiotic administration to IBD patients results in beneficial therapeutic effects. In one study, prebiotic Synergy 1 in combination with *Bifidobacterium longum* improved sigmoidoscopy scores and reduced b-defensins, TNF-α, and IL-1α in biopsy samples from UC patients. In another study, patients who received *Bifidobacterium longum* and prebiotic Synergy 1 [with Fructooligosaccharides (FOS)/inulin blend] combination revealed a significant histological improvement in comparison to the placebo group. Synbiotics significantly reduced the TNF-α expression and thereby reported the potential beneficial effect of synbiotics in the management of IBD (Figure 6). Combinations of synbiotics may exert beneficial impacts on the intestinal mucosa. Therefore, evaluating the role of synbiotics as an alternative form of IBD treatment should be considered[9].

PROBIOTICS IN IBD

Research on the use of probiotics in the treatment of IBD has been conducted since 1997. A 50% increase in probiotic use has been reported in IBD patients. This is due to the belief that probiotics are safe and beneficial as adjunctive therapy for IBD patients in both exacerbation and remission. Despite the moderately huge data reports on the use of probiotics in IBD, the possibility to draw firm conclusions is significantly limited. This may be due to the small number of patients in the study groups, large

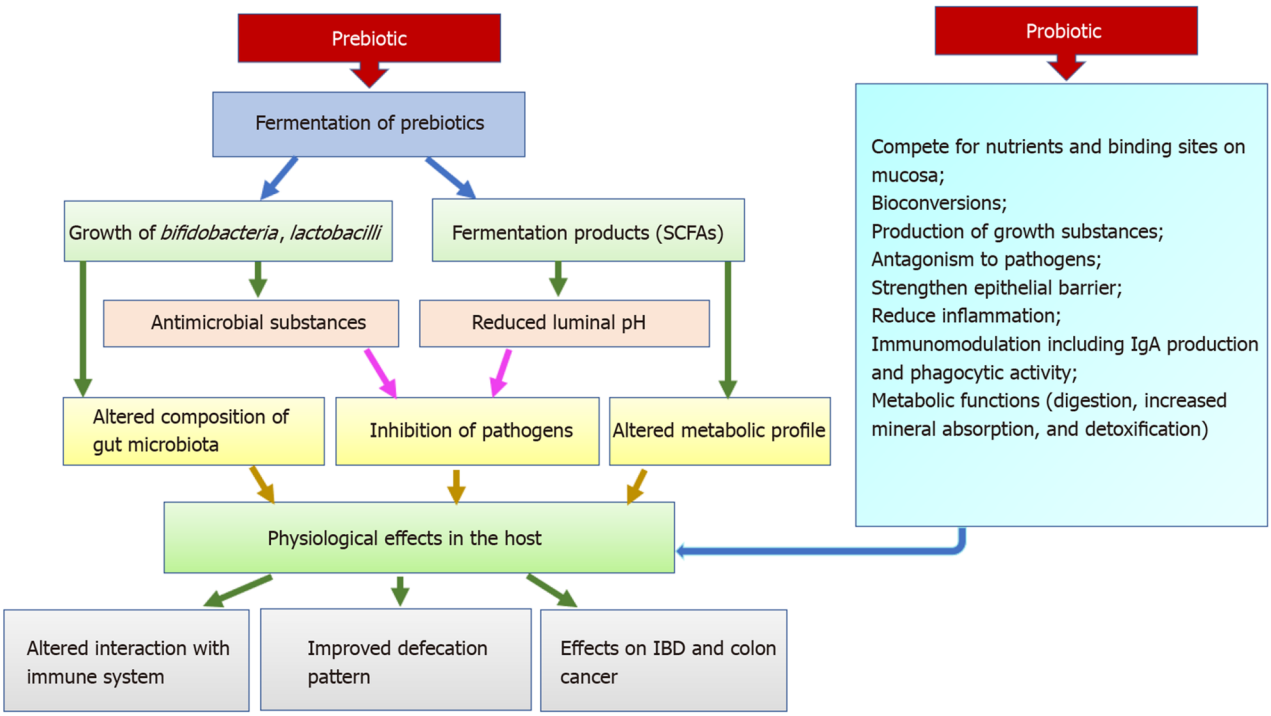

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Figure 6 Mechanisms of action of probiotics, prebiotics, and synbiotics. SCFA: Short chain fatty acids; IBD: Inflammatory bowel disease.

differences in intervention types, or lack of standardization in study methods. There are also few published clinical studies on the effects of probiotics on inflammatory changes examined by GI endoscopy in IBD patients. However, the potential use of well-selected commensal microbial species with protective effects on the intestinal mucosa and modulation of immune responses offers hope for new treatment options for patients with IBD[9]. Various preclinical studies have been conducted to explore the efficiency of probiotics in the IBD are summarized in Table 1 and the clinical interventions are summarized in Table 2.

Effectiveness of probiotics in the animal models of colitis

Eradication of reactive oxygen species (ROS) by antioxidant enzymes such as catalase from the inflammatory site may efficiently restrain IBD pathogenesis. A genetically engineered probiotic *E. coli* Nissle 1917 (EcN), which acts by overexpressing the catalase and superoxide dismutase, was evaluated for the treatment of intestinal inflammation in a mouse IBD model induced by dextran sodium sulfate (DSS), 2,4,6-trinitrobenzene sulfonic acid (TNBS) and oxazolone. The probiotic bioavailability in the GIT was increased by the application of chitosan and sodium alginate effective biofilms. It effectively relieved inflammation and repaired epithelial barriers in the colon and restored the expression of TJ-associated proteins. It also regulated the gut microbial flora and augmented the abundance of vital microbes that helped in the maintenance of intestinal homeostasis such as *Lachnospiraceae*_NK4A136 and *Odoribacter* [10].

With remarkable advances in genetic engineering, scientists have recently developed bacterial/probiotic strains that are genetically engineered to function as 'gut biosensors' that can help to detect inflammatory markers or 'resident cell factories' of therapeutic molecules that will act as biotherapeutic drugs to improve the drug delivery at mucosal surfaces[10].

Wang *et al*[11] showed that an engineered EcN discharging the immunoregulatory protein Sj16 isolated from the helminth *Schistosoma japonicum* alleviated the DSS-induced colitis in mice by modifying the gut microbiota. The immunoregulatory protein exhibited a protective effect against colitis through its action on the peroxisome proliferator-activated receptor-alpha (PPAR-α) receptor, reestablishing populations of the *Ruminococcaceae* family, thereby augmenting intestinal butyrate levels.

Zhang *et al*[12] established a constitutively expressing IL-35 *E. coli* as a novel oral delivery system with immunosuppressive actions, facilitated by regulatory T cells and B cells. The IL-35-producing *E. coli* demonstrated a decrease in the inflammatory response in a mice model of colitis by downregulating Th17 cells.

Lactobacillus paracasei has been genetically engineered with the human N-acylphosphatidylethanolamine-specific phospholipase D gene and, when potentiated with an ultra-low exogenous dose of exogenous palmitate, it selectively induced palmitoylethanolamide in the GIT. PEA exerted potent anti-inflammatory effects, and it had showed improvement in inflammation in animal models of colitis[13].

Table 1 Summary of preclinical studies of probiotics

Ref.	Model	Treatment	Dose and duration	Parameters analyzed	Conclusion
Yoo *et al* [26], 2022	DSS-induced colitis in male C57BL/6 mice	*Lactobacillus plantarum*, *Bifidobacterium longum*, and *Bifidobacterium bifidum*	1 × 10^9 CFU once daily for 5 d	Cytokines and corticosterone, colonic MPO activity, fecal LPS level	Suppressed colonic inflammation, and fatigue by the suppression of the IL-1β or IL-6 to IL-10 expression ratio and gut bacterial LPS production
Qin *et al* [27], 2022	DSS-induced colitis in mice	*Lactobacillus (Pediococcus pentosaceus*, *Lactobacillus plantarum*, and *Weissella cibaria)*	-	DAI, colon length, pathological score, cytokine secretion	Showed the potential to treat IBD
Khan *et al*[28], 2022	DSS-induced colitis in mice (C57BL/6)	*Lactobacillus plantarum*	-	Colitis indexes, IL-17A, IL-17F, IL-6, IL-22, and TNF-α and anti-inflammatory cytokines, *i.e.*, TGF-β, IL-10	Restored gut microbiota balance and modulated the resident gut microbiota and immune response
Fei *et al* [29], 2022	DSS-induced colitis in mice (C57/BL6)	*Ligilactobacillus salivarius* Li01 and RSV	Li01 (10^9 CFU/d) and RSV (1.5 g/kg/d)	IL-1β and IL-6, TGF-β and IL-17A	An improved synergistic anti-inflammatory effect from the RSV and Li01 combination treatment
Liu *et al* [30], 2021	DSS-induced colitis in mice (C57/BL6)	Goji juice fermented by *Lactobacillus plantarum*, *Lactobacillus reuteri* and *Streptococcus thermophilus*	20 mL/kg/d for 30 d	Pro-inflammatory cytokines and total superoxide dismutase in serum and colon, MPO and glutathione peroxidase	Probiotics-fermentation enhanced the anti-ulcerative colitis function of goji berry juice and modulated gut microbiota
Kim *et al* [31], 2020	DSS-induced colitis in male C57BL/6 mice	*Lactobacillus plantarum* CBT LP3 (KCTC 10782BP)	1 × 10^8 bacteria in 0.1 mL PBS once daily for 16 d	DAI, analysis of macrophages and T cell subsets gene expression and cytokine profiles	Effective anti-inflammatory effects, with increased induction of Treg and restoration of goblet cells, suppression of proinflammatory cytokines
Chen *et al*[32], 2020	DSS-induced colitis in C57BL/6J mice	*Bifidobacterium breve*, CCFM683	0.2 mL (10^{10} CFU/d CCFM683, once daily for 2 wk	Weight loss, stool consistency and fecal blood	Improved intestinal epithelial barriers, restored gut microbiota
Chen *et al*[33], 2020	DSS-induced colitis in C57BL/6 mice	*Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Enterococcus faecalis* with (quadruple probiotics, Pqua) or without (triple probiotics, P-tri) aerobic *Bacillus cereus*	*B. infantis*, *L. acidophilus*, and *E. faecalis* (1.5 × 10^9 CFU respectively) in 200 uL PBS and *B. cereus* (0.5 × 10^8 CFU) in 200 uL once daily for 45 d	Intestinal inflammation and functions of multiple barriers, including the mucus barrier, and epithelial barrier	Effective (Aerobe-contained Pqua was a powerful adjuvant therapy for chronic colitis, *via* restoring the intestinal microflora and recovering the multi-barriers in the inflamed gut)
Komaki *et al*[34], 2020	Mice	*Lactococcus lactis subsp. lactic JCM5805*	1 mg, 5 mg, 10 mg, 15 mg, or 20 mg, once daily for 1 wk	The survival rate, length, histopathological parameters of the colon, and concentrations of inflammatory cytokines in serum	Effective (high-dose administration deteriorates intestinal inflammation)
Silveira *et al*[35], 2020	C57BL/6 mice	*Lactobacillus bulgaricus*	1 × 10^9 CFU was diluted in 200 mL of PBS, 3 times per week for 12 wk	Intestinal inflammation, cytokines levels were determined from colon and/or tumor	Regulates the inflammatory response and prevents Colitis-associated cancer

DSS: Dextran sodium sulfate; CFU: Colony forming unit; LPS: Lipopolysaccharide; DAI: Disease activity Index; IBD: Inflammatory bowel disease; RSV: Resveratrol; MPO: Myeloperoxidase; IL: Interleukin; TNF-α: Tumor necrosis factor-α; TGF-β: Transforming growth factor-β; PBS: Phosphate buffer solution; Tregs: Regulatory T cells.

Liu *et al*[14] designed an engineered *Bifidobacterium longum R0175* (*B. longum*) that expresses the antioxidant enzyme manganese superoxide dismutase. The probiotic helped to improve colitis symptoms by attenuating the ROS-mediated oxidative stress and constraining endothelial cell activation. Following the treatment, attenuation in TNF-α, IL levels, as well as a complete improvement in the macroscopic and microscopic inflammatory markers was observed. Yet another study reported the beneficial effect of genetically modified *B. longum* that expresses αmelanocyte-stimulating hormone in a DSS model of colitis. The probiotic exhibited significant anti-inflammatory properties by suppressing the release of proinflammatory cytokines such as ILs, TNF-α, and NO, while increasing the anti-inflammatory cytokine (IL-10) release[15].

Feng *et al*[16] explored the ameliorating effects of pasteurized probiotic fermented milk on DSS-induced IBD in rats and found that intragastric gavage of milk prominently declined the disease activity index (DAI) scores and alleviated the colon tissue damage. The improvement was ascribed to the anti-inflammatory effect of the probiotic by decreasing TNF-α and IL-6 levels. The pasteurized probiotic fermented milk alleviated IBD by reducing the inflammatory response and restoring the gut microbiota.

Table 2 Summary of clinical studies of using probiotics in inflammatory bowel disease patients

Ref.	Treatment	Dose and duration	Parameters analyzed	Conclusion
Agraib *et al* [43], 2022	*Lactobacillus* and *Bifidobacterium* species	3×10^{10} probiotic capsules	Partial mayo score, CRP, IgA, IL-10, hemoglobin, hematocrit, and RBC levels	Significantly induced remission in UC patients
Ojetti *et al* [44], 2022	*Limosilactobacillus reuteri* ATCC PTA 4659	Two times a day for 10 d	Inflammatory markers CRP and calprotectin	Significantly reduced both blood and fecal inflammatory marker
Wu *et al* [45], 2021	Live *bacillus licheniformis* capsules and live combined *Bifidobacterium, lactobacillus,* and *enterococcus* capsules followed by Chamomile capsules	*Bacillus licheniformis* capsules two capsules, three times a day and live combined *Bifidobacterium, lactobacillus,* and *enterococcus* capsules two capsules twice a day. Probiotics consumed at a level of 10^7 CFU/mL	Leiden Index of Depression Sensitivity, Beck Depression Inventory questionnaires	Probiotics tend to improve cognitive reactivity to the sad mood in CD patients
Bamola *et al* [46], 2021	*Bacillus coagulans* Unique IS-2	2 billion-CFU/capsule twice in day 4 wk	Presence of beneficial gut bacteria, serum cytokines, symptoms of the disease	Showed beneficial effect when administered along with standard medical treatment
Waal M van der *et al* [47], 2019	Nine bacterial strains (*Bifidobacterium bifidum* W23; *Bifidobacterium lactis* W51; *Bifidobacterium lactis* W52; *Lactobacillus acidophilus* W22; *Lactobacillus casei* W56; *Lactobacillus paracasei* W20; *Lactobacillus plantarum* W62; *Lactobacillus salivarius* W24; *Lactobacillus lactis* W19)	7.5×10^9 CFU per 3 g in powder form, once daily for 6 wk	Quality of life from a patient perspective (semi-structured interviews)	Effective
Kamarli Altun *et al* [48], 2022	*Enterococcus faecium, Lactobacillus Plantarum, Streptococcus thermophilus, Bifidobacterium lactis, Lactobacillus acidophilus, Bifidobacterium long*, and FOS	Probiotic strains (3×10^9 CFU) and FOS (225 mg/ tablet), 1 tablet twice a day for 8 wk	Hemoglobin, leukocyte, neutrophil-to-lymphocyte ratio, sedimentation, and CRP, clinical and endoscopic activity indices	Effective
Sun *et al* [49], 2018	*Clostridium butyricum*	420 mg per capsule, 1.5×10^7 CFU/g, 3 capsules 3 times a day for 4 wk	Change from baseline in IBD symptoms, quality of life, stool consistency, and frequency	Effective
Matsuoka *et al*[50], 2018	*Bifidobacterium breve* strain Yakult and *Lactobacillus acidophilus*	10 billion bacteria of Bifidobacterium breve and 1 billion bacteria of *Lactobacillus acidophilus*, one pack per day for 48 wk	Clinical procedure (change in abdominal symptom score from baseline. Sutherland DAI subscore, change in abdominal symptom scores (passage of flatus and bloating), and intestinal microbiota	Less significant effect

CRP: C-reactive protein; UC: Ulcerative colitis; CD: Crohn's disease; CFU: Colony forming unit; FOS: Fructooligosaccharides; DAI: Disease activity index; IBD: Inflammatory bowel disease; IgA: Immunoglobulin A; RBC: Red blood cell; IL: Interleukin.

Javed *et al*[17] showed that *Bifidobacterium infantis* had beneficial effects in alleviating TNBS-induced colitis. Supplementation with *Bifidobacterium infantis* demonstrated significantly less damage to mucosal cyto-structures and reduced the colitis symptoms. This demonstrates the importance of probiotics in protecting the goblet cells and epithelial cell layers[17]. Based on another research group, *Bifidobacterium bifidum* supplementation significantly increased IL-10 levels and decreased IL-1β levels in colonic sections, confirming anti-inflammatory effects. These results seem to support the regulatory properties of *Bifidobacterium infantis* and *Bifidobacterium bifidum* to reduce inflammation and clinical signs of colitis [18].

In IBD, a decrease in *Firmicutes* (F) abundance and an increase in the *Bacteroidetes* (B) bacteria are found to be associated with the disease progression. Early administration of *L. reuteri* DSM 17938 to C57BL/6J mice improved the abundance of *F* and diminished the abundance of B comparatively, thereby altering gut microbial homeostasis[19].

Live and dead *L. plantarum* AN1 administered to an IBD mouse model *via* drinking water exhibited intestinal regulatory and anti-inflammatory properties. A combination of two diverse probiotics (*Bifidobacterium bifidum* WBIN03 and *L. plantarum* ZDY2013) diminished the UC in mice by altering the microbiota and reducing oxidative stress as well as inflammation. The combination upregulated antioxidant factors and downregulated TNF-α in UC mice. A probiotic blend augmented the frequency of *F* and reduced the frequency of B[20].

The co-administration of the *L. fermentum* KBL374 and KBL375 amended gut dysbiosis and ameliorated colitis by decreasing the pro-inflammatory cytokine levels and augmented anti-inflammatory cytokines. These probiotics mechanism of action includes balancing the F/B ratio, epithelial cell

barrier improvement and altering cytokine secretion[21].

Numerous data reports the utilization of multi-strain probiotic preparations. The VSL#3 is well known for its efficiency in IBD. VSL#3 is a commercial probiotic blend composed of 8 bacterial strains. Four lactobacillus strains (*Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus delbrueckii subsp. bulgaricus*), three Bifidobacterium strains (*B. breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*), and a streptococcal strain (*Streptococcus salivarius subsp. thermophilus*). Several studies have demonstrated the effect of VSL#3 on DSS-induced colitis[22]. One study showed that VSL#3 (0.5 mL/d) reduced gut bacterial diversity associated with tissue injury. Conjugated linoleic acid was locally produced by VSL#3, which suppressed colitis by targeting myeloid cell PPARγ in the colon. Moreover, the anti-inflammatory effect remains the most important therapeutic mechanism of VSL#3 in DSS-induced rat colitis[23]. Both live and heat-killed VSL#3 decreased the expression of IL-23, IL-6, STAT3, and phosphorylated STAT3 (P-STAT3) in colonic tissue, thus reducing DSS-induced colitis in rats. The expression of inflammation-related mediators such as iNOS, NFκB was also inhibited by VSL#3[22].

Various experimental studies have concluded that VSL#3 normally acts on four components of the intestinal barrier: Biological, chemical, mechanical, and immune barriers. Regarding biological barriers, VSL#3 can increase the abundance of commensal gut bacteria and reduce the abundance of fungi. Regarding chemical barriers, VSL#3 can increase MUC2, MUC3, and MUC5AC gene expression and regulate mucus secretion. Regarding the mechanical barrier, VSL#3 can augment ZO-1 and occludin while attenuating claudin-2 to improve the function of TJs proteins. About the immune barrier, VSL#3 can inhibit the pro-inflammatory NFκB pathway while upregulating PPARα signalling[24].

A research group led by Hrdý *et al*[25] demonstrated that probiotic strains affect host cells in different ways. The mechanism of action of *Bifidobacterium animalis* species *lactis* B15764 and *Lactobacillus reuteri* Lr5454 were determined in mouse models of TNBS-induced colitis. Both strains exert beneficial effects on the host as expressed by body weight, gross indices of inflammation (Wallace score) and histopathological analysis (Ameho score), and lipocalin-2 levels in feces[25].

All of the above studies led to the inference that even a simplified preclinical model of colitis, which skips the genetic and external environmental influences, requires a broad and multidisciplinary approach.

Clinical study of probiotics among IBD patients

Several trials have reported the therapeutic action of the most common probiotic cocktail of proven efficacy, VSL#3 in adults with mild-to-moderate UC. In two clinical studies, VSL#3 was able to reduce DAI scores and significantly reduce clinical UC symptoms compared to the placebo. One study showed that 42.9% of patients treated with VSL#3 achieved remission in comparison to the placebo patients (15.7% remission only)[24]. Furthermore, VSL#3 and conventional drugs appear to have a synergistic effect. Although the mechanism is unknown, it is suggested that VSL#3 could enhance the anti-inflammatory effects of 5-aminosalicylic acid (5-ASA), inhibit free radical production, and suppress leukotriene and IL-1 production. A study also showed that combination therapy with VSL#3 and low-dose balsalazide was more efficacious than mesalazine or balsalazide alone in achieving remission of UC. Longer treatment with VSL#3 may result in greater improvement. Furthermore, in an open-label study, treatment with VSL#3 resulted in remission and improvement in 77% of patients with active mild-to-moderate disease UC. Two bacterial components *B. infantis* VSL#3 and *S. salivarius* subspecies *thermophilus* contributed a significant role in inducing remission by reaching the intestinal site of the disease. The effect of alkaline sphingomyelinase was also examined in 15 UC patients treated with VSL#3 for 5 wk and the outcome showed that VSL#3 upregulated mucosal alkaline sphingomyelinase activity and improved UC[25].

VSL#3 has also demonstrated valuable effects in children having IBD. A study showed that VSL#3 was operative in maintaining remission and reducing relapses in children with active UC. Apart from its role in maintaining remission, VSL#3 therapy also resulted in disease remission in children with mild to moderate acute UC as reported by another pilot study[36].

Jia *et al*[37] performed a meta-analysis of remission, relapse, and complication rates between EcN 1917 and mesalazine. The results demonstrated that there were no significant differences in the EcN1917 group or mesalazine-treated patients and were safe and well-tolerated. In summary, EcN 1917 has a comparable efficacy to mesalazine in terms of remission induction. This probiotic could be considered as an alternative for patients with IBD. Tamaki *et al*[38] reported that treatment with *Bifidobacterium longum* in patients with mild to moderate UC, prominently reduced DAI score and decreased rectal bleeding, as well as showed that they also achieved clinical remission. Treatment with probiotics and commonly used anti-inflammatory drugs together appears to be a more effective solution than treatment with probiotics alone. Palumbo *et al*[39] combined mesalazine with a probiotic mixture (*Lactobacillus salivarius, Lactobacillus acidophilus*, and *Bifidobacterium bifidum* strain). The combination showed beneficial effects among UC patients. The dual-treatment group demonstrated shorter recovery time, lower disease activity, and showed better endoscopic images. Another research group found that oral administration of *Bifidobacterium infantis* suppressed the CRP and TNF-α levels in both GI inflammatory diseases but did not specifically affect UC disease[40].

Also in one study, *S. boulardii* and VSL#3 in combination with conventional therapy in mild to moderate UC (involving 244 patients) showed no improvement in remission rates. However, only modest benefits were obtained in terms of reduction in disease activity[41]. EcN, *S. boulardii*, *B. breve,* and *Bifidobacterium bifidum* strains Yakult has demonstrated efficacy and safety similar to standard 5-ASA in maintaining remission in patients with mild to moderate UC based on histology, endoscopy, or quality of life. Presently, only four clinical trials have reported the use of *S. boulardii* as a probiotic therapy for IBD, with three reports of efficacy. Probiotic therapy using probiotic yeast such as *S. boulardii* could be a probable treatment for clinical trials, but the validation of reported efficacy in animal models requires multiple placebo trials[42].

An randomized controlled trial (RCT) evaluated the ability of VSL#3 to prevent the endoscopic recurrence of CD in humans after surgery. In this study, 10% of patients in the early VSL#3 group (VSL#3 administered throughout 365 d) had no severe lesions on day 90, but severe lesions developed on day 365 compared to the 26% of patients in the late VSL#3 group (administration of VSL#3 from day 90 to day 365). The finding suggested that VSL#3 exposure time was closely related to its therapeutic efficacy. However, DAI and IBD questionnaire (IBDQ) scores were similar in the two groups.

Few clinical studies have also shown that VSL#3 can prevent or maintain remission in chronic pouchitis. It was stated that after ileal pouch-anal anastomosis for UC, in the VSL#3 group 10% of patients had an onset of acute pouchitis when compared to the 40% of patients in placebo group. VSL#3-treated patients (17 patients, 85%) maintained antibiotic-induced pouchitis remission compared to placebo-treated patients (1 patient, 6%)[42].

PREBIOTICS IN IBD

Numerous studies have demonstrated the role of prebiotics on the intestinal flora and demonstrated that the use of prebiotics can enhance the metabolic function of the intestinal flora. Various studies reported that prebiotics diminishes the inflammatory cytokines, such as IL-1α, IL-1β, IL-6, IL-12, TNF-α, IFN-γ, and improve the natural intestinal barrier by increasing the mucinous layer and TJs between epithelial cells. Their ability to decrease pathological bacteria in the gut and provide commensal bacteria with substrates capable of being metabolized into substances that contribute to the production and secretion of anti-inflammatory cytokines make them interesting candidates for various researchers working in IBD management. Surprisingly, more research has been done preclinically and a low number of significant prebiotic-associated human clinical trials are reported. The restricted research studies number is a foremost drawback and not adequately sufficient to support the use of prebiotics to treat IBD. Most prebiotics used in animal studies were polysaccharides derived from grapes, mushrooms such as *Ganoderma lucidum*, and herbs. In contrast, most human clinical trials described the usage of FOS for prebiotic treatment of IBD[34].

Prebiotic effectiveness in animal model of colitis

The use of prebiotics has shown promise in the management of colitis and is also widely used in animal models (Table 3). Various prebiotic preparations have been tested in animal models of colitis. The prebiotic effects of inulin have been studied in DSS-induced distal colitis in a rat model histologically resembling human UC. Daily administration of inulin by the oral route increases the number of natural *lactobacilli* in the lumen of the cecum and also lowers colonic pH. In rats with DSS-induced colitis, mucosal inflammation and histological injury scores are reduced by oral administration of inulin. Furthermore, inulin-fed rats showed a lower degree of mucosal damage and less severe crypt damage compared to controls. Treatment with orally administered inulin showed similarly beneficial effects, regardless of whether treatment was provided before or during the DSS exposure[51].

A nutritional combination of inulin and oligofructose at 5 g/kg body weight reduces intestinal inflammation in transgenic rats. An increase in *Bifidobacteria* and *Lactobacilli* was observed in the gut along with a decrease in pro-inflammatory cytokines and an increase in the growth factor-β that alters immune regulation. Taken together, these results suggest that combination therapy with different prebiotics may be more effective than monotherapy due to the fact that each drug has specific biological properties[52].

Pectic polysaccharides (PPS) are thought to be essential carbohydrates available to the gut microbiota, playing a dominant role in maintaining intestinal balance and are more potent than some conventional prebiotics. PPS can stimulate the growth of beneficial bacteria, such as *Lactobacillus*, *Bacteroides*, and *Bifidobacterium*, fully meeting the condition of a "stimulating probiotic". Some aberrant PPS have shown specific immunological capabilities compared to the PPS on the market. For example, sweet cherry PPS significantly induces the expression of NO and some immune proteins such as IL-6 and IL-10. Moreover, PPS from silver linden flowers enhances immunity in mice by inducing ROS and NO and suppressing iNOS. One study shows that PPS from *Gentiana crassicaulis* can enhance host immunity in terms of immune complement fixation[53].

Lactulose is reported to reduce inflammation and instigate the growth of lactic acid bacteria in IL-10 knockout mice while administration of inulin and germinated barley foodstuff (GBF) reduced DSS-

Table 3 Summary of preclinical studies of prebiotics

Ref.	Model	Type of treatment	Composition	Dose	Parameters analyzed	Conclusion
Cui *et al* [57], 2021	DSS-induced colitis in C57BL/6J mice	Polysaccharide from *Scutellaria baicalensis Georgi*	Mannose, ribose, rhamnose, glucuronic acid, glucose, xylose, arabinose, fucose	50 and 200 mg/kg once daily for 10 d	Body weight, loose stools, morbidity, hematochezia, and the DAI	Effective (attenuated body weight loss, reduced DAI, ameliorated colonic pathological damage, and decreased MPO activity)
Tolonen *et al*[58], 2022	DSS-induced colitis in male C57Bl/6 mice	Synthetic glycans	FOS, GOS, XOS, pullulan, and lactulose	1% (v/v) glycans (days 7-14)	Weight loss, scores of diarrhea, endoscopy, and colonic histology	Synthetic glycans increase survival, reduce weight loss, and improve clinical scores in mouse models of colitis
Qian *et al* [59], 2022	DSS-induced colitis in male C57Bl/6 mice	GOS, FOS along with FMT	FMT alone or combined with various ratios of GOS, and FOS	-	DAI scores, histology, protein or mRNA expression levels of FFAR3 and ZO-1, a tight junction protein	Treatment with FMT plus a prebiotic blend restores thestructure of the intestinal flora and increased the levels of acetic acid, butyric acid, FFAR3, and ZO-1
Liu *et al* [60], 2016	DSS-induced colitis in male C57BL/6 mice	Alpha D-glucan from marine fungus *Phoma herbarum* YS4108	Glucopyranose	40 mg/kg/d once daily for 1 wk	DAI scores, histology immunohistochemistry analysis, evaluation of SOD and MDA activities, and determinations of inflammatory cytokines	Effective (significantly increased butyrate, isovaleric acid levels, and prominent alterations on specific microbiota)
He *et al* [61], 2020	DSS-induced colitis in male C57BL/6 mice	Stachyose	Stachyose	1.5 g/kg/d for 28 d	Inflammatory cytokines including IL-6, IL-10, IL-17a, and TNF-α	Increased beneficial microbiota and bacterial diversity to alleviate acute colitis in mice
Kanwal *et al*[62], 2019		Dictyophora indusiate polysaccharide	Glucose 59.84%, mannose 23.55%, and galactose 12.95%	Low dose 10 mg/kg and (high dose 33 mg/kg once daily for 2 wk	Assessment of DAI, histological, analysis of goblet cells and mucus layer thickness, cytokines	Effective
Li *et al* [63], 2020	Male C57BL/6 mice	FMG or dealcoholized muscadine wine	FMG: Fructose 34.7% glucose 31%, sucrose 9.9%. DMW: Fructose, sucrose, and glucose not detected	FMG (7%, w/w) or DMW (5.5%, v/ w) for 3 wk	Bodyweight, stool consistency and bleeding, DAI, short-chain fatty acids in feces, and Mucin 2 and IgA in feces	Effective (reduced dysbiosis in the colon)
K-da *et al* [64], 2020	Male C57BL/6 mice	GFO	Monosaccharide composition in the GFO was D-galactose	100, 500, or 1000 mg/kg once daily for 2 wk	GI transit time, *ex vivo* propulsive motility, *in vitro* colonic smooth muscle contractility, the composition of colonic microbiota, and production of SCFAs	Effective (prevented and attenuated colitis symptoms and GI dysmotility, reducing populations of harmful bacteria and increasing SCFAs)

DSS: Dextran sodium sulfate; DAI: Disease activity Index; MPO: Myeloperoxidase; FOS: Fructooligosaccharides; GOS: Galactooligosaccharides; XOS: Xylooligosaccharides; FMT: Fecal microbiota transplantation; ZO-1: Zona occludens; SOD: Superoxide dismutase; MDA: Malondialdehyde; SCFAs: Short-chain fatty acids; GI: Gastrointestinal; IL: Interleukin; TNF: Tumor necrosis factor; FMG: Freeze-dried muscadine grapes; DMW: Dealcoholized muscadine wine; FFAR3: Free fatty acid receptor 3; GFO: *Gracilaria fisheri* oligosaccharides.

induced colitis in rats. It has been shown to increase the luminal concentration of SCFA, as well as increase the density of *Lactobacillus* and *Bifidobacterium*[54].

Mushrooms consist of diverse polysaccharides with prebiotic potential, including α-glucans, chitin, mannans, xylans, and galatians. Xie *et al*[55] stated that the prebiotics *Ganoderma lucidum* polysaccharide enhance SCFA-producing bacteria and augments SCFA production and suppresses DAI prominently. The study also described a decrease in infective microbiota such as *Shigella* and *Escherichia* in the rat model[55].

The effect of neoagarotetraose (NT), a hydrolytic product of agar by β-agarase, was evaluated in the DSS-induced murine model. The data show that NT intake improved intestinal integrity and inflammation scores. NT reversed the density of *Proteobacteria* from the DSS-induced increased levels[56].

Reduced clinical signs and increased MUC-3 expression were observed in rats that were nourished with goat's milk oligosaccharides as compared to the DSS-induced colitis rats. In trinitrobenzene sulfonates that provoke colitis rats, the colonic inflammation and necrotic lesions are also reduced by goat's milk oligosaccharides as compared with control rats[56].

Clinical study of prebiotic among IBD patients

Although there are few human studies using prebiotics, some new evidence suggests that the prebiotic treatment holds promise (Table 4). After colectomy for UC inulin showed a positive effect in the management of chronic pouchitis. A small, open-label study in 10 patients with active CD showed that the 21-d oral administration of 15 g of oligofructose and inulin significantly reduced the disease status. In UC patients, *Plantago ovata* (psyllium) outperformed placebo in reducing symptom severity and significantly increasing fecal concentrations of *Bifidobacteria*. Psyllium seeds formerly stimulated SCFA production, when tested as maintenance therapy for the UC patient in remission in an open randomized study. In this, patients received mesalamine (500 mg/d thrice daily for 1 year), psyllium seed alone (10 g twice daily), or a combination of both at the same doses. Remission were comparable in all groups, and a substantial augmentation in fecal butyrate concentration was detected after the administration of *Plantago ovata* seeds[65].

GBF consists of an extract rich in glutamine and hemicellulose. In small pilot and placebo-controlled studies, its use was evaluated in UC patients having mild to moderate disease severity. GBF significantly increased fecal levels of *Bifidobacteria* and reduced clinical and endoscopic activities at a dose of 25-30 mg/d. Comparable outcomes were described by 24-wk open-label study. A combination of 15 g/d oligofructoses and inulin was investigated on ten patients with active CD in a small open-label study. A substantial decrease in DAI accompanied by a substantial increase in mucosal *Bifidobacteria* was observed. Interestingly, prebiotics amplified colonic dendritic cells expressing IL-10, toll-like receptors-2, and 4, signifying the mechanism of the prebiotics on the mucosal innate immune response.

Wilson *et al*[66] explored the effects of prebiotic Galactooligosaccharides (GOS) supplementation on colonic inflammation in 17 patients with active UC. Patients reported improved stool consistency, decreased incidence and severity of loose stools, and decreased urgency of defecation after administration of GOS at 2.8 g/d for 6 wk. The proportion of *Bifidobacterium* and *Christensenelaceae* increased only in patients with low disease activity, suggesting that prebiotic effects may depend on disease activity. A controlled study is required to validate these observations to essentially determine if the GOS prebiotic is a useful adjunct therapy in active UC[66].

The effect of enteral inulin on ileal pouch function was assessed by examining epithelial gene expression, cell turnover, and mucosal morphology. The authors found that enteric supplementation with 24 g/d inulin increased butyrate production, reduced inflammation-related factors, decreased secondary bile acids, and significantly reduced endoscopic and histological DAI scores[67].

SYNBIOTICS IN IBD

Synbiotics not only improve the survival of beneficial microorganisms added to food and feed but are also used to stimulate the growth of certain natural bacterial strains present in the GIT. Given a large number of possible combinations, the application of synbiotics to modulate the human gut microbiota appears promising.

Effectiveness of synbiotic in animal models of colitis

Recently, several preclinical studies have shown that the use of probiotics and prebiotics as a synbiotic combination alleviates intestinal inflammation more than either probiotics or prebiotics alone (Table 5). The effects of formulated prebiotic mixtures, probiotic mixtures, and synbiotics were investigated in the colitis model induced by DSS in mice. Results in Synbiotic-treated colitis mice showed the preservation of colonic histological architecture and mucin production, upregulation of occludin expression, and diminished cell infiltration. A significant decrease in plasma IL-6 levels was observed after treatment. Treatment also modified gut microbiome, improved colonic integrity, upregulated anti-inflammatory cytokines, and suppressed inflammation markers, possibly through inhibition of IL-6/STAT3 signaling. In addition, synbiotic-treated mice displayed the highest levels of anti-inflammatory mediator IL-10 among the treatment groups in colitis mice. Among the treatments, synbiotics showed the most pronounced effect, indicating the highest potential for prevention and treatment of IBD[73].

Kangwan *et al*[74] demonstrated a protective effect of *L. pentosus* A14-6, CMY46 against DSS-induced intestinal inflammation. A14-6 and CMY46 are the novel strain of *L. pentosus* isolated from tea leaves (Miang) in Northern Thailand. The anti-inflammatory actions of *L. pentosus* CMY46 combined with GOS and *L. pentosus* A14-6 combined with XOS and were explored in C57BL/6 mice for 21 d. Synbiotics ameliorated DSS-induced colitis by preserving weight loss, reducing DAI, restoring colon length, and suppressing histopathological damage. Moreover, synbiotics enhanced intestinal barrier integrity and reduced colonic inflammation. Further, synbiotics possessed excellent anti-inflammatory and

Table 4 Summary of clinical studies of using prebiotics in inflammatory bowel disease patients

Ref.	Type of treatment	Dose	Parameters analyzed	Conclusion
Valcheva *et al*[68], 2022	β-fructans (oligofructose and inulin)	15 g/d for 6 mo	Mayo score. FCP, along with stool metabolites	Did not prevent symptomatic relapses in UC patients but reduced the severity of biochemical relapse and increased anti-inflammatory metabolites
Pietrzak *et al*[69], 2022	Sodium butyrate	150 mg sodium butyrate twice a day for 12-wk	DAI, FCP	As adjunctive therapy, it did not show efficacy in newly diagnosed children and adolescents with IBD
Vernero *et al*[70], 2020	Oral microencapsulated sodium butyrate (BLM)	Dose of two capsules/day for 12 mo (500 mg of BLM for each capsule)	DAI, FCP, CRP	BLM supplementation appears to be a valid add-on therapy to maintain remission in patients with UC
Valcheva *et al*[71], 2019	Oligofructose-enriched inulin	7.5 g (n = 12) or 15 g (n = 13) daily oral oligofructose-enriched inulin for 9 wk	Mayo score, endoscopic activity and FCP	15 g/d dose inulin-type fructans produced functional but not compositional shifts of the gut microbiota. Controlled studies for the use of β-fructans as an adjunct therapy in patients with active UC are required
Azpiroz *et al*[72], 2017	scFOS	5 g per sachet, twice daily for 4 wk	Rectal sensitivity, anxiety/depression, quality of life scores, and composition of fecal microbiota	Less significant (scFOS on rectal sensitivity may require higher doses and may depend on the subgroup)

FCP: Fecal calprotectin; DAI: Disease activity index; CRP: C-reactive protein; UC: Ulcerative colitis; scFOS: Short chain fructooligosaccharides.

immunomodulatory activities, as evidenced by decreased inflammatory mediator expressions of TNF-α, IL-1β, IL-6, and cyclooxygenase-2 (COX-2) in the colon. Symbiotic CMY46 in combination with GOS markedly increased IL-10 expression. These results suggest that synbiotics isolated from Mian are more effective than sulfasalazine[74].

The efficacy of *Bacillus amyloliquefaciens* enriched camel milk (BEY) was evaluated in TNBS-induced colitis mice models. Results showed that BEY treatment attenuated the proinflammatory cytokines (IL-1 β, IL-6, IL-8, and TNF-α) and myeloperoxidase levels. In addition, the protein markers such as phosphatase and tensin homolog, NFκB, COX-2, proliferation nuclear antigen, and occludin were substantially downregulated by BEY treatment. The BEY alleviated the colitis symptoms[75].

Bacillus coagulans FCYS01 spores in combination with chitooligosaccharides (COS) were evaluated for the possible ameliorating effects on DSS-induced colitis in mice. In comparison to the DSS group, the supplement significantly modulated the levels of CPR and cytokines IL-4, IL-6, IL-8, and IL-10. It significantly restored the TJ proteins and mucin protein expressions, thereby promoting the recovery of the intestinal barrier. Additionally, these dietary supplements improve SCFA production by modulating the composition of the gut microbiota and enhancing SCFA-producing bacteria. In conclusion, synbiotics mitigated the inflammatory status of the experimental UC model and showed better therapeutic efficacy than individual *B. coagulans* or COSs[76].

In another study, supplementation with synbiotics could substantially ameliorate the disease activity in DSS-induced acute colitis mice. The synbiotic significantly preserved the epithelial TJ proteins at colon, signifying the shielding of the intestinal barrier. The pro-inflammatory cytokines were reduced while augmentation of anti-inflammatory cytokines was mediated by the symbiotic treatment. The synbiotic used in the study was composed of 8 probiotic strains, including *Bifidobacterium animal*, *Lactobacillus paracasei*, *Bifidobacterium lactis*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *B. breve*, *Lactobacillus fermentum*, and *Streptococcus thermophilus* along with FOS[77].

In another study, a synbiotic consisting of *Lactobacillus fermentum* HFY06 and arabinoxylan showed that the synbiotic can prevent and treat DSS-induced colitis. The results exhibited their synergistic effect by inhibiting the activation of the NFκB signaling pathway, upregulating the mRNA expression of NFκB inhibitor-α, downregulating mRNA expressions of NFκB-p65, inhibiting the cytokines TNF-α, inducible NOS, and COX-2, and exerted anti- colitis effects[78].

Synbiotic supplement with probiotic *Bacillus coagulans* spores and prebiotic green banana resistant starch ameliorated intestinal inflammation in the murine IBD model induced by DSS. A considerable efficacy of synbiotic supplementation was highlighted as it reduced the colitic manifestations and its severity. A significant anti-inflammatory effect was produced by suppressing abnormal immunological responses and colonic damage induced by DSS. Synbiotic accounted for about 29% increase in IL-10 levels and about 37% suppression of CPR along with about 40% IL-1β suppression compared to that of the DSS-control. The combination also improved SCFA production. The synbiotic supplementation amended the complete inflammatory condition *via* synergistic actions[79].

Table 5 Summary of preclinical studies of synbiotics

Ref.	Model	Probiotics	Prebiotics	Dose	Parameters analyzed	Conclusion
Xue *et al* [80], 2023	DSS-induced colitis in mice	*Lactobacillus plantarum* LP90	Soluble dietary fiber obtained from *Lentinula edodes* by products	1×10^9 CFU/kg	DAI, histological studies, IL-10, IL-17, IgA levels, TNF-α	Alleviated colitis
Ivanovska *et al*[81], 2017	DSS-induced colitis in C57BL/6J mice	*Bifidobacterium infantis* and *Bifodobacterium longum*	Equal parts FOS, GOS and XOS	0.5×10^9 CFU Probiotics and 2.5 g of prebiotics for 1 mo	Gut microbiome, cecal and fecal SCFAs	Increased the diversity of the microbiome and be associated with more SCFAs, and less gut inflammation
Seong *et al* [82], 2020	Chronic restraint stress in male Wistar rats	*L. paracasei*	Opuntia humifusa extract (mucilage + pectin)	*L. paracasei* (1×10^{10} CFU/g) & (10.0/30.0 mg%, w/w) of *Opuntia humifusa* extract once daily for 4 wk	Fecal microbial analysis, serum corticosterone levels, TNF-α levels in the colon tissue	Effective (greater abundance of *L. paracasei* in fecal microbial analysis, lower serum corticosterone levels, lower TNF-α levels in the colon tissue
dos Santos Cruz *et al* [83], 2020	IL 10-knockout mice	VSL#3	Yacon (6% FOS + inulin)	VSL#3 (10^9 CFU/d) + PBY (6% FOS and inulin) for 13 wk	Manifestations of colitis, colon histology, expression of antioxidant enzymes, production of organic acids, and intestinal microbiota	Preservation of intestinal architecture, improve intestinal integrity, increased expression of antioxidant enzymes and concentration of organic acids
Wang *et al* [84], 2019	DSS-induced colitis in C57BL/6J mice	*Lactobacillus acidophilus*, *L. Rhamnosus*, and *Bifidobacterium lactis*	Inulin	Probiotics: 1.0×10^9 CFU per day per mice, and prebiotic 5×10^8 CFU/d	Pathologic scores, mucosal flora	Increased the proportion of helpful bacteria and regulated the balance of intestinal microbiota, reduced the degree of inflammation in acute colitis mice
Son *et al* [85], 2019	DSS-induced colitis in female BALB/c mice	LGG	Tagatose	10^9 CFU/mL of LGG and 25 mg of tagatose once in 2 d for 3 wk	Body weight, food intake, rectal bleeding, stool conditions, blood in stool, expression of proinflammatory cytokines	Effective (gut microbiota composition recovered from the dysbiosis caused by DSS treatment)
Ivanovska *et al*[81], 2017	TNBS-induced colitis in Wistar rats	*L. Casey 01*	Oligofructose-enriched inulin	1 mL containing non-encapsulated probiotic/prebiotics once daily for 2 wk	Assessment of colonic damage, inflammation scoring, MPO and microbiological studies	Effective

DAI: Disease activity index; IL: Interleukin; TNF: Tumor necrosis factor; FOS: Fructooligosaccharides; GOS: Galactooligosaccharides; XOS: Xylooligosaccharides; CFU: Colony forming unit; DSS: Dextran sodium sulfate; MPO: Myeloperoxidase; LGG: *L. rhamnosus strain*; SCFAs: Short-chain fatty acids.

Clinical study of synbiotic among IBD patients

The effects of daily supplementation of total gut repair (TGR) on microbial community composition and activity were investigated in the short-term-Quad-M-SHIME model inoculated with gut microbiota from two individual IBD donors. TGR comprises of probiotics, prebiotics along with combination of amino acids, immunoglobins and flavonoids. TGR supplementation increased SCFA production, increased beneficial bacterial density, decreased inflammation, and damage to the intestinal barrier from endotoxin exposure. Intestinal barrier function was improved compared to controls, and levels of the anti-inflammatory molecules IL-6 and IL-10 were elevated. TGR supplementation daily promoted changes in the gut microbiota of IBD patients.

The efficacy of synbiotic therapy has been evaluated in UC patients in a clinical study (Table 6). Patients received a synbiotic formulation consisting of prebiotic FOS along with 6 probiotic strains. The results showed a significant decrease in inflammation and improved disease status. The double-blind randomized, placebo clinical trial study by Liang *et al*[88] confirmed the efficacy of a synbiotic formulation in suppressing IBD symptoms. The synbiotic consisted of FOS and *L. acidophilus*, *B. bifidum*; *B. longum*; *B. lactis*; and *L. rhamnosus*. Rectal pain, bloating, incomplete bowel movements and diarrhea sensations were significantly improved in patients compared to placebo[88].

A recent randomized, double-blind, controlled trial observed synbiotic use in 18 subjects with functional UC. The treatment included the prebiotics inulin and oligofructose. Sigmoidoscopy inflammation scores were reduced in the synbiotic group compared to the placebo group. The TNF and IL-1α levels in the intestine were also reduced. Furthermore, rectal cultures showed greater epithelial

Table 6 Summary of clinical studies of using synbiotics in inflammatory bowel disease patients

Ref.	Probiotics	Prebiotics	Dose	Parameters analyzed	Conclusion
Amiriani *et al*[86], 2020	*Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Streptococcus thermophiles*	FOS	Lactocare capsule twice daily for 8 wk	DAI	Mitigated symptoms in patients with UC and suggested to use pre-probiotics in the standard treatment, particularly in those with more than five years of the disease
Kamarlı Altun *et al* [87], 2019	*Enterococcus faecium*, *Lactobacillus plantarum*, *Streptococcus thermophilus* , *Bifidobacterium lactis*, *Lactobacillus acidophilus*, *Bifidobacterium longum*	FOS	3×10^9 CFU probiotic and 225 mg/tablet prebiotic for 8 wk	Hemoglobin, leukocyte, neutrophil-to-lymphocyte ratio, sedimentation, and CRP and clinical and endoscopic activity indices	Improvement in clinical activity

DAI: Disease activity index; FOS: Fructooligosaccharides; UC: Ulcerative colitis; CRP: C-reactive protein; CFU: Colony forming unit.

regeneration and reduced inflammation in synbiotic-treated subjects. A tiny, open-labeled trial of 10 active CD subjects, 21 d of 15 g oligofructose and inulin oral administration also showed a substantial lowering of the disease symptoms[89].

Since it has been shown that obesity-induced gut microbiota aggravation can exacerbate IBD symptoms, BG from the Schizophyllum commune, a probiotic, and a synbiotics containing both BG and probiotic (SYN) may improve symptoms of obesity-related colitis. BG and probiotic protected intestinal TJs, but did not modulated the inflammatory markers (*i.e.*, IL-6 and TNF-α) infiltration. In contrast, SYN displayed more prominent actions in attenuating colonic inflammation. SYN treatment group supported the growth of both indigenous and supplemented bacteria while maintaining bacterial diversity, thereby improving the obesity-associated colitis symptoms[90].

SAFETY ASPECTS OF PRO, PREBIOTIC, AND SYNBIOTIC PRODUCTS

The Centers for Disease Control and Prevention advises that over-the-counter prebiotics and probiotics are generally safe for use by healthy people. The ignoring the importance of dose and strain specificity is a concern today. Probiotics manufactured as dietary supplements rather than pharmaceuticals are not subject to regulatory review because they are not required to support claims about the safety or efficacy of food or dietary supplements. This is one of the main reasons for the insufficient or nonexistent information on the efficacy and safety of most marketed products. Probiotic is characterized by a generally safe profile but should be used with caution in certain population groups such as pregnant women, neonates born prematurely, or with immune deficiency. A World Health Organization working group proposed several criteria that are to be considered in order to define strains with GRAS status: Resistance of probioticstrains to antibiotics; evaluation of metabolic properties (lactate production, bile deconjugation) monitoring of side effects in clinical trials, epidemiological studies on the occurrence of side effects after commercial approval, identification of all substances excreted by strains that are toxic to mammals, and determination of the hemolytic capacity of strains[91].

Risks and side effects

No serious side effects of probiotic interventions in IBD patients have been reported. Inadequate immunological stimulation, genes transfer, systemic infections, and fatal metabolic activities have been detected in certain individuals receiving probiotic supplementation. A mild dry cough has been reported in one UC patient with *B. longum* 536 supplementations. Septicemia, and certain cased of endocarditis, have been associated with certain probiotic strains, like *L. acidophilus, L. rhamnosus*, *Bacillus subtilis, S. boulardii, L. Casey,* and *B. breve.* Administration of *Lactobacillus rhamnosus* in 64-year-old UC patient who was treated with prednisone, caused bacteremia due to bacterial transfer from intestinal lumen to the blood. Probiotic interventions have been reported to induce an inflammatory response in the small intestinal region, leading to D-lactic acidosis. The complications in certain patient populations, especially those with compromised immune systems are accelerated by *S. boulardii* and *Lactobacillus GG* administration. Pregnant women, new-borns, and the elderly are at increased risk of potential probiotic infections due to their weakened immune systems[92].

STUDY LIMITATIONS

Significant heterogeneity between studies jeopardizes the interpretation of the current literature on probiotics and prebiotics in IBD. Choice of prebiotic or probiotic studied, the trial design, their doses,

and the outcome were also reported to vary. Study populations varied, with some studies included active disease patients while others working on the remission maintenance persuaded by conventional therapy, antibiotics, or surgery. Most studies enrolled small numbers of patients, limiting their statistical power, which is especially important given the high placebo response rates observed in IBD clinical trials. Finally, no studies provided information on patient diet, which may have a significant impact on the effectiveness of microbial therapy. The exact mechanisms of action have not yet been elucidated. The understanding of the mechanisms responsible for the beneficial effects of probiotics, prebiotics, and synbiotics is rather superficial.

Inadequate evidence on probiotic dosages essential for specific clinical effects has amplified the necessity for molecular description of probiotics to establish health claims. Evidence for immunological mechanisms of probiotics is still limited. Evaluation of interactions between cocktail of probiotic strains in formulations such as #VSL-3 have not been considered and yet to be investigated. Clinical trials and validation studies planned with larger sample sizes require an understanding of the interactions between the microbiota, host, and prebiotic components. Due to the very limited published literature in the field of manufacturing processes and subsequent formulation, much needs to be done to improve strain viability during formulation and storage[93].

OBSTACLES, CHALLENGES, AND FUTURE PROSPECTS

The main barrier with the pre-, pro-and synbiotics is the "difficulty in demonstrating clinical efficacy". The situation is complicated by the different levels of evidence essential to support health claims from country to country. The Food and Drug Administration now states that active ingredients, including probiotics, taken to cure, alleviate, treat, diagnose, or prevent disease must be classified as pharmaceuticals and go through the same approval process as new drugs. Eventually, high-quality human intervention studies are needed to substantiate health claims on products.

Probiotic supplements vary widely in composition, dosage, as well as in host interactions, and these should be specifically considered before recommending their use. Remarkably, several prebiotic and synbiotic products contain a slight amount of prebiotic ingredients (per serving), that may be too small to produce any health benefits. Lower doses are used in part to avoid unwanted GI discomfort, but possibly also for cost reasons. Developing clinically effective synbiotic combinations is a major challenge and must meet several requirements. It is generally expected that the minimum effective dose of each component must be determined.

Maintaining probiotic bacteria viability is a foremost marketing and technical challenge in probiotic applications. A basic prerequisite for probiotics is that the product comprises an adequate number of microorganisms by the expiration date. Therefore, probiotics should cover precise strains and maintain a specific number of viable cells to provide a health benefit to the host. Many viable cultures die during final product manufacturing, storage, transportation, and passage through the gut. As a result, the majority die before consumers can reap the health benefits. Market research has also shown that even before the expiry date, product show much lower count. Therefore, the shelf life of probiotics cannot be accurately predicted. As a result, the industry has to struggle alot to substantiate the label's claims[94].

Also, for optimal effectiveness, probiotics must remain viable after contact with stomach acid, bile, and digestive enzymes to cross the upper GIT. This is a basic property that many products have not tested. Microorganisms may die while passing through the upper intestinal tract to the colon and thus may not be able to colonize the colon. Therefore, they must withstand the gastric acid and bile salts encountered during transit through the GIT.

Even when screening of synbiotic combinations was performed *in vitro* or *in situ*, the methods ignored the environmental factors that influence probiotic strains *in vivo.* Also, competition for the prebiotic substrate between the probiotic strain and members of the gut microbiota was not considered. Identifying prebiotics that specifically and selectively boost the probiotic strain of interest can be challenging.

Clinically effecacious synbiotic development remains a challenge and must meet numerous requirements. It is generally expected that the minimum effective dose of each component must be determined. Including adequate controls in synbiotic studies is particularly challenging. Prebiotic-only and probiotic-only controls must be included, in addition to standard control, for checking the synergistic or additive actions. Justification on how the probiotics and prebiotics were selected and combined should be included[95,96].

FMT IN IBD

For patients with metabolic syndromes linked with gut dysbiosis, FMT is an evolving microbial therapy. The technique involves the transfer of healthy fecal microbe population to patients with metabolic conditions. FMT's technical approach involves oral capsules, nasogastric or nasojejunal tubes, and enemas that are utilized for restoring a healthy GI microbiome. FMT samples are carefully chosen from

healthy donors who have undergone a standard screening procedure for avoiding the risk of transmission of unknown pathogens from donor to recipient. Donors are usually evaluated for their historical backgrounds such as health profiles, family history of autoimmune reactions, metabolic disorders, transfusion details, or any previous surgery. Other donor data include travel history, food intake, particularly alcohol and drugs, and sexual behavior. After selecting an appropriate donor, their stool and blood samples are tested for the presence of pathogens. Extensive support and education are provided to the patients undergoing FMT prior to the treatment. No fecal substances such be present in the colon. Patients may even consume loperamide prior to infusion to make sure that transplanted feces stay there for at least 4 h. The recipient is not allowed to consume antibiotics 48 h prior to infusion[97].

There are several methods of transferring fecal material to the recipients. Presently, fecal material is administered *via* the upper GIT or lower GIT route, or as oral capsules. For patients who are suffering from an inflamed colon, FMT is performed *via* nasogastric tube, esophagogastroduodenoscopy, nasojejunal tube, or upper GI route *via* nasoduodenal tube. Lower GI route FMT can be accomplished by retention enema or by colonoscopy. While colonoscopy aids in the successful recolonization of all the parts of the colon with favorable microflora, retention enemas are only restricted to the distal colon. Retention enemas are however much cheaper and less invasive than colonoscopy. For most reported treatments, FMT patients receive an average relative dose of 25 g *via* the upper GI route compared to an average of 90 g *via* colonoscopy. In an RCT, colonoscopy using a 152 g stool sample reported a 90% success rate in preventing recurrent infections. Another research group reported that consuming 17 g of frozen and thawed or fresh FMT showed 60% efficacy using the retention enema method. The fecal capsules can also aid in restoring ecosystem integrity and overcoming microbial loss in the GI environment[98].

Recently, a study reported that FMT in IBD patients showed a response rate of 53.8% and a complete response rate of 37%. Furthermore, it has been reported that FMT is a more practical treatment with safe and beneficial results for the treatment of active UC patients. Pooled results exhibited that FMT treatment might improve clinical and endoscopic rates of active UC. FMT also significantly alters the microbiota composition of UC patients as compared with control groups[98]. In UC patients, Tian *et al* [99] assessed the B proportion that exhibited a steady rising trend after FMT. *Prevotella* and *Proteus* were also prominently augmented as compared to healthy control. On other hand, it was found that the populations of *Klebsiella* and *Streptococcus*, which are pathogenic bacteria decreased significantly after FMT treatment. Reducing the abundance of *Prevotella* while increasing the proportion of *Klebsiella* and *Streptococcus* was a key factor in the development of UC. Unfortunately, several studies have stated conflicting results, with FMT therapy failing to ameliorate the disease severity and restore the gut microbiota.

In this regard, several studies have found that the efficacy of FMT for treating IBD is unpredictable. Therefore, it is still unclear whether FMT fits into the therapeutic paradigm. Despite reports of significant positive taxonomic changes in the GIT in patients diagnosed with FMT, observations remain conflicting and its functional and metabolic effects are not well documented. For UC, FMT may be a promising treatment, but for CD or pouchitis, very limited information have been available to draw good conclusions[100].

CONCLUSION

Although probiotics and prebiotics have been studied in many animal models and clinical trials of intestinal inflammation and offer health benefits, the individual efficacy of each probiotic strain and its administration remains uncertain. Large, rigorously designed, high-quality human studies need to be evaluated to examine dosage, duration of use, formulations containing one or more strains, and probiotics, prebiotics, antibiotics as well as simultaneous use of substances. For a thorough knowledge of the structure and function of the microbiome with regards to probiotics and prebiotics, modern approaches based on bioengineering, genetic engineering, system biology, molecular biology, multiomics, nanotechnology, and immunology must be employed. These investigations will aid in the comprehension of the relationship between human physiological processes and the microbiome. A potential area for future developments includes implementation of a personalized therapy for IBD, based on a detailed assessment of the gut microbiota and immune system profile in the individuals. Such personalized holistic therapy, that combines biotics with dietary and pharmaceutical therapy, would improve therapeutic efficacy while decreasing adverse effects. This approach will also enable a more thorough understanding of the pathophysiology of IBD and the adoption of targeted therapies for the preservation of the gut microbiome and rectification of bacterial metabolic activities, as well as the restoration of the regulatory immune system. This will enable the use of innovative treatment approaches to manage IBD patients in a safer and more effective manner.

FOOTNOTES

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REVIEW

Changing trends in the minimally invasive surgery for chronic pancreatitis

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Abstract

Chronic pancreatitis is a debilitating pancreatic inflammatory disease characterized by intractable pain resulting in poor quality of life. Conventional management of pancreatic pain consists of a step-up approach with medications and lifestyle modifications followed by endoscopic intervention. Traditionally surgery is reserved for patients who do not improve with other interventions. However, recent studies suggest that early surgical intervention is more beneficial as it can mitigate the progression of the pathological process and prevent loss of pancreatic function. Despite the widespread adoption of minimally invasive approaches in various gastrointestinal surgical disorders, minimally invasive surgery for chronic pancreatitis is slow to evolve. Technical difficulty due to severe inflammatory changes has been the major impediment to the widespread usage of minimally invasive surgery in chronic pancreatitis. With this background, the present review aimed to critically analyze the available evidence on the minimally invasive treatment of chronic pancreatitis. A Pub Med search of all relevant articles was performed using the appropriate keywords, parentheses, and Boolean operators. Most initial laparoscopic series have reported the feasibility of lateral pancreaticojejunostomy, considered an adequate procedure only in a small proportion of patients. The pancreatic head is the pacemaker of pain, so adequate decompression is critical for long-term pain relief. Recent studies have documented the feasibility of minimally invasive duodenum-preserving pancreatic head resection. With improvements in laparoscopic instrumentation and technological advances, minimally invasive surgery for chronic pancreatitis is gaining momentum. However, more high-quality evidence is required to document the superiority of minimally invasive surgery for chronic pancreatitis.

Key Words: Robotics; Laparoscopy; Surgery; Chronic pancreatitis; Pancreas; Pancreatitis

Core Tip: Pain in chronic pancreatitis is a significant symptom that demands utmost attention as it compromises the quality of life and inherently risks narcotic addiction. Surgical management for chronic pancreatitis began with various open surgical drainage and resection procedures. Since pain is the primary indication for intervention, a minimally invasive approach is an attractive proposition in chronic pancreatitis. Despite the slow adoption of laparoscopic and robotic surgery in chronic pancreatitis, safety, and feasibility have been documented in recent studies. The challenges and limitations highlighted in the present review could guide future research on minimally invasive surgery in chronic pancreatitis.

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INTRODUCTION

Chronic pancreatitis is a progressive pancreatic inflammatory disease that leads to fibrosis and parenchymal tissue loss resulting in impaired endocrine and exocrine function. While consumption of alcohol is the leading cause of chronic pancreatitis worldwide, idiopathic pancreatitis remains common in India and China[1,2]. The most common and dominant symptom of chronic pancreatitis is pain, which can be persistent, severe, or recurrent episodes with pain-free intervals significantly impacting the quality of life[2,3].

Traditionally, pain in chronic pancreatitis is managed initially with analgesics, pancreatic enzyme replacement therapy, and lifestyle modifications followed by endoscopic or surgical intervention. Almost half of the patients who do not respond to medical or endoscopic management are referred for surgical intervention[4]. The most common indication for surgery is intractable pain. Studies have reported that early surgical management has better outcomes than intervention in the advanced disease stage[3,5]. While the minimally invasive approach is widely used for various gastrointestinal and pancreatobiliary disorders, its application in chronic pancreatitis is disproportionately low. As chronic pancreatitis is a benign disorder with pain as the primary indication for intervention, a minimally invasive approach is an attractive proposition. The technical difficulty, combined with the potential for vascular injury and bleeding associated with pancreatic inflammation and fibrosis, is the primary reason for the slow adoption of minimally invasive techniques in chronic pancreatitis. However, recent studies have shown the feasibility of laparoscopic and robotic surgery for chronic pancreatitis. Also, with advancements in endoscopic treatment, there is a trend towards the less invasive treatment of chronic pancreatitis. The present review focuses on the challenges, evolution, and changing trends in the minimally invasive management of chronic pancreatitis.

SEARCH STRATEGY

All the authors did a PubMed search of relevant articles. Further, the articles' reference lists were also searched for additional appropriate studies. The keywords and combinations included in the search were: "Pancreatitis"; "chronic pancreatitis"; "idiopathic pancreatitis"; endoscopic management" and "chronic pancreatitis"; "Frey's procedure" and "Laparoscopic"; "Frey's procedure" and "robotic"; "Puestow procedure" and "Laparoscopic"; "Puestow procedure" and " robotic"; "Beger procedure" and "Laparoscopic"; "Beger procedure" and "robotic"; "chronic pancreatitis" and "total pancreatectomy" and " laparoscopic"; "chronic pancreatitis" and "total pancreatectomy", and "robotic". The search was limited to publications in English literature. All the authors agreed that the articles selected for review were relevant.

ENDOSCOPIC MANAGEMENT OF CHRONIC PANCREATITIS

Endoscopic intervention is recommended as a minimally invasive alternative to surgery in patients who do not improve with medical management. The journey of stone removal from the pancreatic duct dates back to 1891 when Alfred Pearce Gould retrieved calculi from the Wirsung duct in a London hospital [6]. Berkeley G Moynihan performed transduodenal removal of pancreatic stones in 1902, followed by transpancreatic stone removal in 1908 by Mayo Robson. The development of the fiberoptic endoscope for diagnosis in 1958 by Basil Isaac Hirschowitz changed the trends of endoscopic management[7]. Watson *et al*[8] succeeded in developing the technique for papillotomy with an energy source leading to

the endoscopic extraction of calculi and subsequent stent placement. Pathogenesis of pain in chronic pancreatitis is multifactorial and includes anatomical and neuropathic factors. Anatomical alterations include ductal hypertension, raised pancreatic parenchymal pressure, acute inflammation, and pancreatic ischemia[9-11]. The lack of correlation between pain severity and anatomical changes suggests neurological factors' role. Neuropathophysiology of pain in chronic pancreatitis includes peripheral sensitization-induced pain, neuropathic remodeling, and central sensitization of pancreatic pain[10,11]. Endoscopic therapy aims to relieve pain by clearance of intraductal stones, thereby decompressing the pancreatic duct. Proponents of endoscopic therapy suggest that patients with complete ductal clearance by endoscopic approach have shown similar pain relief compared to the surgical group[12]. At the same time, the ability to modify the disease progression and prevent loss of pancreatic function with early surgical intervention was proposed in favor of surgical management[13,14].

Traditionally chronic pancreatitis patients were managed initially with analgesics and pancreatic enzyme replacement therapy, followed by endoscopic intervention with or without extracorporeal shockwave therapy[15,16]. Patients with an inadequate clinical response following endoscopic treatment should be discussed in the multidisciplinary meeting and considered for surgical management[10,16]. The potential benefit of this approach is that surgery could be avoided in some patients with successful endoscopic management. However, various studies comparing the efficacy of endoscopic management with surgery have shown better pain control with the early surgical intervention[17-19]. Randomized trials comparing the endoscopic and surgical approaches have shown a better quality of life and pain relief in the surgical group, especially with early surgery[12,20,21] (Table 1). Also, patients with inflammatory pancreatic head mass, distal pancreatic duct stricture, and extensive parenchyma calcifications of the pancreatic head might be difficult to treat by endoscopy. Despite the available evidence favoring surgical treatment, advancements in endoscopic lithotripsy techniques and extracorporeal shockwave lithotripsy (ESWL) are continuously improving the ductal clearance rate, and the endoscopic approach is helpful in a subset of chronic pancreatitis patients[16]. Patients with dominant stricture in the pancreatic head with upstream dilatation and those with intraductal calculi in the pancreatic head or proximal body are ideal candidates for endoscopic intervention.

Pancreatic lithotripsy

Worldwide, ESWL is commonly used for pancreatic lithotripsy, especially in Asia and Europe. While recent advancements like intraductal endoscopic laser or electrohydraulic lithotripsy might improve ductal clearance, they are not widely available. Hence, most of the available data are for ESWL[22]. A meta-analysis of various studies has reported a 70% stone clearance rate with ESWL[23]. With ESWL, stone clearance is more favorable for solitary calculus in the head of the pancreas. However, recurrence of stones after ESWL was seen in 14% to 23% of patients mandating further intervention[24]. ESWL is combined with pancreatic duct stenting in patients with associated pancreatic duct stricture[25]. European Society of Gastrointestinal Endoscopy (ESGE) recommends ESWL for the removal of radiopaque obstructive main pancreatic duct calculi greater than 5 mm found in the head or body of the pancreas and endoscopic retrograde cholangiopancreatography (ERCP) for main pancreatic duct calculi that are radiolucent or smaller than 5 mm[26].

Endoscopic stenting

Main pancreatic duct strictures are often seen in half of the chronic pancreatitis patients and are usually located in the pancreatic head region. The standard management of these strictures is balloon dilatation and placement of a temporary stent for at least a year[27]. Pain relief after a long-term follow-up of 5 years has been seen in almost half of patients after stent withdrawal[28,29]. However, stricture recurrence has been reported in up to 38% of patients after two years[28,30]. Complications related to stenting include stent migration and occlusion. Distal stent migration toward the pancreatic tail and proximal stent migration to the duodenum was reported in 7.5% and 5.2% of patients, respectively[31]. Stent migration can be prevented with large-winged or pigtail catheters[31,32]. The ESGE recommends managing painful pain pancreatic duct strictures with the help of a single 10 Fr stent for one uninterrupted year if symptoms improve after placement[26]. The stent should be exchanged based on symptoms or signs of stent dysfunction on imaging at least six months intervals.

Endoscopic ultrasound-guided intervention

Endoscopic ultrasound (EUS) guided drainage of the main pancreatic duct is used as a second-line procedure after the failure of ERCP. Despite high success rates of 68% to 75%, the complications like perforation, bleeding, and pancreatitis reported in 5% to 43% of patients were key drawbacks for EUS-guided drainage[33,34]. Another EUS-guided procedure is the celiac plexus block, whereby a steroid with a local anesthetic agent is injected at the celiac plexus to block the pancreatic nerve fibers. EUS-guided celiac plexus block is preferred over the traditional percutaneous method. However, despite the high technical success and short-term pain relief in 55%-70% of patients, long-term outcomes are discouraging[35-37].

Table 1 Studies comparing endoscopic and surgical management of chronic pancreatitis

Ref.	Country	No. of patients (*n*), surgery/endoscopy	Complete and partial pain relief (%), surgery/endoscopy	Complication (%), surgery/endoscopy	Mortality (*n*), surgery/endoscopy	Follow up (mo), surgery/endoscopy
Díte *et al* [12], 2003	Czech Republic	36/36	86/61	Not reported	0/0	60
Cahen *et al* [20], 2011	Netherlands	16/15	80/38	0/25	0/1	79
Hong *et al* [19], 2011	China	27/35	77/47	14/22	1/0	60
Kawashima *et al*[17], 2018	Japan	41/10	100/100	20/27	0/0	-
Jiang *et al* [18], 2018	China	40/46	83/80	26/8	0/0	63.5/57.3
Issa *et al* [21], 2020	Netherlands	44/44	58/39	27/25	0/0	18

SURGICAL MANAGEMENT OF CHRONIC PANCREATITIS

Evolution of open surgical procedures

The systemic reviews and meta-analyses of the available studies comparing surgical management with endoscopic intervention continue to support the superiority of surgical treatment in chronic pancreatitis [38-40]. The intriguing voyage of surgical management of chronic pancreatitis has witnessed a huge transformation from surgical decompression of the obstructed duct and denervation of the pancreas to pancreatic head resection, total pancreatectomy, and various other hybrid procedures. In 1911, Link[41], a gynecologist from Indiana, was the first to describe external tube drainage of the pancreatic duct for ductal calculi or stricture with good long-term pain relief. Desjardins and Coffey, around similar times, proposed drainage of the pancreas using the intestine after conducting animal studies[42,43]. This empiricism was materialized by Duval[44] and separately by Zollinger *et al*[45] in 1954 by describing the first caudal end-to-end pancreaticojejunostomy using a Roux-en-Y jejunal loop for chronic pancreatitis. Further, modification in the decompressive procedure was done by Puestow and Gillesby[46], in 1958, by invaginating caudal pancreaticojejunostomy after longitudinally opening the pancreatic duct from the body to the tail region of the pancreas. Their procedure was further modified by Partington and Rochelle[47], in 1960, by creating side-to-side Roux-en-Y pancreaticojejunostomy, commonly called the Puestow procedure. Though the Puestow procedure was considered standard drainage procedure for almost 40 years, the long-term benefits were not befitting. Despite short-term pain relief in 80% of patients, the pain recurred in 30% on long-term follow-up[48-51]. The foremost reason for recurrent pain was undealt nidus of inflammation and persistent ductal disease in the head of the pancreas. To tackle the head disease, German surgeon Hans Beger performed the first duodenum-preserving pancreatic head resection in 1971 and reported postoperative outcomes of 52 patients in 1980[52]. In 1984, Warren described splenopancreatic flap, a denervation procedure for chronic pancreatitis[53]. However, the long-term results of this complex procedure were never published or replicated by other surgeons[9,53].

In 1987, Frey and Smith[54] described a hybrid operation consisting of pancreatic head resection and longitudinal pancreaticojejunostomy, also known as Frey's procedure. Izbicki *et al*[55] modified Frey's procedure by doing a more extensive excavation of the pancreatic head and duct and named it Hamburg modification. Similarly, Gloor *et al*[56] from Berne modified the duodenum-preserving pancreatic head resection by omitting the challenging step of pancreatic neck transection. To minimize the risk of penetrating the posterior pancreatic capsule, Ho and Frey[57], and Frey and Amikura[58] recommended limiting the posterior extent of head coring to the back wall of opened Wirsung and the uncinate duct, also known as modified Frey's procedure. Sakata *et al*[59] described the minimum Frey procedure in which a small spindle-shaped anterior resection of the pancreatic head was performed and reported an equivalent outcome. However, a retrospective study by Tan *et al*[60] reported superior long-term pain relief and quality of life with the original Frey's procedure compared to the modified Frey's. A systematic review and meta-analysis of trials comparing various resectional and hybrid procedures reported similar postoperative pain relief. However, quality of life and other perioperative outcomes favor duodenum-preserving pancreatic head resection procedures[61].

Era of minimally invasive surgery

There is a trend towards minimally invasive procedures for various surgical disorders, and the change is inevitable for chronic pancreatitis[62-64]. Though the surgical procedures described for chronic

pancreatitis are complex and challenging due to inflamed gland they can be accomplished in selected patients[63-65]. In high-volume centers with expertise in advanced laparoscopic procedures, complex pancreatic procedures can be safely performed with comparable postoperative outcomes[63-69]. Also, with its ergonomic advantages, robotic surgery could overcome some of the technical limitations of laparoscopic surgery and potentially widen the use of a minimally invasive approach in chronic pancreatitis[70,71].

Minimally invasive Puestow procedure

Like open surgical procedures, minimally invasive surgery for chronic pancreatitis began with a modified Puestow procedure. Kurian and Gagner[72], in 1999, reported the first series of five patients who underwent a laparoscopic Puestow procedure. Subsequently, two series with 17 and 12 patients were published from India[73,74]. The first small case series of 5 patients from the United Kingdom was published by Khaled *et al*[75] in 2014. The feasibility and favorable short-term outcomes of the laparoscopic Puestow procedure were documented in multiple case series[76-81] (Table 2). In most series, the procedure was performed with five laparoscopic trocars. The initial entry to the lesser sac and exposure of the anterior surface of the pancreas can be technically challenging in patients with recent or recurrent acute episodes of pancreatitis. Hence, those patients should be avoided during the early phase of the minimally invasive Puestow procedure. In most laparoscopic series, two to three gastric retraction sutures are used to lift the stomach away from the pancreas and improve exposure. Needle aspiration is commonly used to identify the pancreatic duct, and intraoperative ultrasound is helpful in patients with undilated duct[74]. Extraction of all intraductal calculi, especially those in the head and tail region, is critical for long-term pain relief. Sahoo *et al*[76] reported the usefulness of cystoscope and endoscopic basket in clearing residual intraductal stones. Proficient intracorporal suturing skill is critical to accomplish safe pancreaticojejunostomy. Kim *et al*[77] reported the benefits of using barbed sutures for laparoscopic pancreaticojejunostomy. Bhandarwar *et al*[78] used endostaplers for laparoscopic pancreaticojejunostomy anastomosis with the anvil part placed within the pancreatic duct. However, as highlighted by the authors, the technique was feasible only in seven out of 17 patients with pancreatic duct diameter of more than 10 mm[78]. Alternatively, a robotic platform can minimize the challenges associated with intracorporeal suturing. After the initial case reports of the robotic Puestow procedure documented its usefulness in a series of seven patients[82-87]. However, with the emerging evidence supporting some form of head resection to achieve long-term pain relief, the minimally invasive Puestow procedure is recommended only in a subset of chronic pancreatitis patients with an atrophic pancreas and dilated pancreatic duct with predominant intraductal calculi.

Minimally invasive Frey's procedure

Frey's procedure is one of the most commonly performed surgeries for chronic pancreatitis. Pancreatic head coring is the technically challenging step of this hybrid procedure, especially during minimally invasive surgery. The first series of laparoscopic Frey's procedure was published by Tan *et al*[88] in 2015. Subsequently, a small series of four patients reported the feasibility and short-term outcomes of laparoscopic Frey's procedure[89]. The largest published series to date had 15 patients in the laparoscopic Frey's group[90]. The relatively small number of studies with fewer patients highlight the technical challenges of laparoscopic Frey's procedure (Table 3). In open Frey's procedure, the surgeon's left hand, kept under the posterior surface of the pancreas head, guides the extent of posterior head coring. In the absence of a definite landmark, pancreatic head coring until the level of the posterior pancreatic capsule is challenging during laparoscopic surgery. Hence, in all the laparoscopic series, only modified Frey's procedure was performed using the main pancreatic duct as the landmark and coring to the posterior wall of the duct[88-90]. In the laparoscopic approach, ultrasonic shears and bipolar vessel sealing devices are commonly used for head coring. Lack of articulation and difficulty securing precise hemostatic sutures further increase the difficulty of laparoscopic pancreatic head coring, especially along uncinate ducts[90]. With its articulating instruments, the robotic platform could potentially overcome technical difficulties during head coring and pancreaticojejunostomy. Hamad *et al*[62] highlighted the usefulness of the robotic approach in bleeding control during head coring in their series of four patients. However, similar to the laparoscopic approach, due to the lack of a definite landmark, coring was limited to the posterior wall of the duct in the robotic modified Frey's procedure (Figure 1). The median operative time and blood loss were 372 min and 163 mL, respectively[62]. The parenchyma posterior to the main pancreatic duct in the pancreatic head was preserved to prevent injury to the superior mesenteric vein[62,91].

Shukla *et al*[92] reported the feasibility of robot assisted Frey's procedure in nine patients with chronic pancreatitis. Robotic approach is associated with less blood loss and shorter hospital stay compared to open Frey's procedure. Tile Pro technology in the robotic platform allows the surgeon to view ultrasound images in the console, thereby avoiding damage to the common bile duct and portal vein during dissection[62]. Bleeding is one of the common causes of conversion in minimally invasive Frey's procedure. Inflammatory pancreatic head mass and preoperative acute exacerbation of pancreatitis were identified as significant risk factors for intraoperative blood loss. Hence, minimally invasive Frey's procedure is recommended in patients with dilated pancreatic duct and enlarged pancreatic head on imaging without inflammatory mass, recent acute exacerbation, and pancreatitis-related complic-

Table 2 Studies on laparoscopic Puestow procedure

Ref.	Country	No. of patients (*n*)	Mean operative time (min)	Mean hospital stay (d)	Conversion (*n*)	Mortality	Follow up, (mo)	Complete pain relief (%)
Kurian *et al*[72], 1999	United States	5	240.0	3-7	Nil	Nil	30.0	80.0
Tantia *et al*[73], 2004	India	17	277.0	5.2	4	Nil	12.0	82.3
Palanivelu *et al* [74], 2006	India	12	172.0	5.0	Nil	Nil	52.8	83.3
Khaled *et al*[75], 2014	United Kingdom	6	278.0	7.0	Not reported	Nil	14.2	66.7
Sahoo *et al*[76], 2014	India	12	265.5	5.8	Nil	Nil	16.5	100 (follow up reported for 8 patients)
Kim and Hong [77], 2016	Korea	11	200.0	7.0	Nil	Nil	21.0	100
Bhandarwar, *et al* [78], 2019	India	28	189.7	5.8	4	Nil	12.0	87.5
Rege *et al*[79], 2019	India	32	131.2	5.2	1	Nil	14.2	75.0
Javed *et al*[80], 2020	India	41	180.0	5.0	Excluded	Nil	43.6	91.0
Nag *et al*[81], 2022	India	33	300.0	7.0	Nil	Nil	25.0	71.0

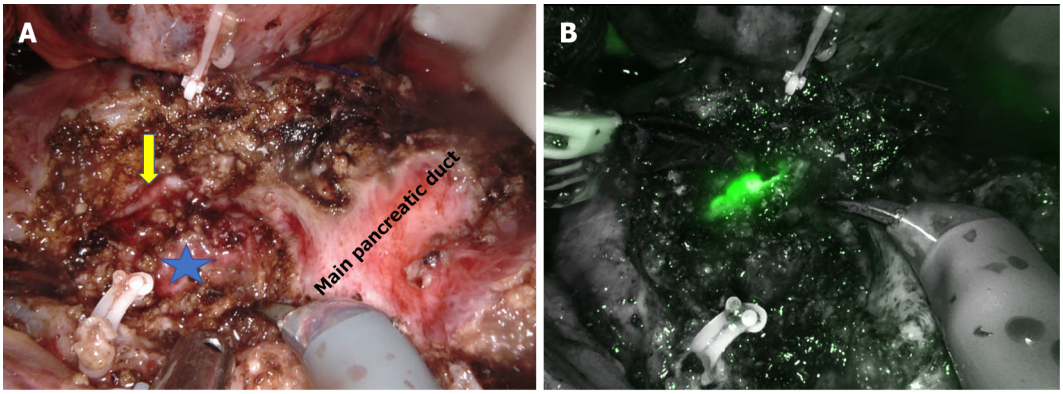
Table 3 Studies on laparoscopic Frey's procedure

Ref.	Country	Patients (*n*)	Mean operative time (min)	Mean hospital stay (d)	Conversion (*n*)	Mortality	Follow up (mo)	Complete and partial pain relief
Tan *et al*[88], 2015	China	9	323	7	2	Nil	3	Not reported
Kilburn *et al*[89], 2017	Australia	4	130	7	Nil	Nil	26	100%
Senthilnathan *et al* [90], 2019	India	15	271	6.4	10 out of 57 patients in different arms	Nil	60	88%

ations.

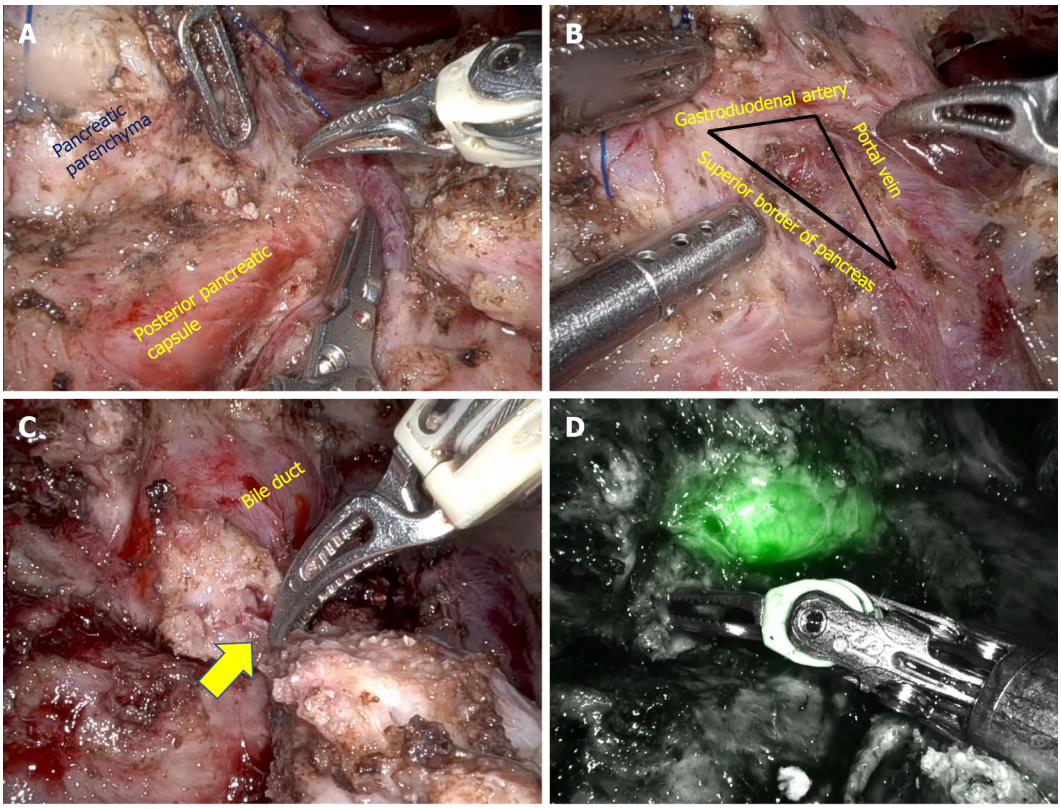
Minimally invasive duodenum preserving pancreatic head resection

In patients with inflammatory head mass, duodenum preserving pancreatic head resection (DPHR) is preferred over pancreatoduodenectomy because of favorable long-term outcomes. As previously highlighted, lack of definite landmarks precludes pancreatic head coring until the posterior pancreatic capsule in minimally invasive Frey's procedure. Minimally invasive duodenum-preserving pancreatic head resection could potentially overcome that limitation. After the initial case reports, it is reported that the feasibility of laparoscopic DPHR procedure in 5 patients with chronic pancreatitis[93-95]. The mean operative time and hospital stay were 275 min and 11 d, respectively. One patient had grade B postoperative pancreatic fistula, and pancreaticojejunostomy anastomotic site bleed in one patient[95]. In laparoscopic DPHR after the pancreatic neck transection, the pancreatic head is retracted to identify the plane between the pancreatic parenchyma and posterior pancreatic capsule. As the dissection proceeds along the superior border of the pancreas, the bile duct should be identified and preserved. Identifying the intrapancreatic duct is a significant technical challenge with minimally invasive DPHR. Indocyanine green (ICG) fluorescence facilitates bile duct identification in the triangle formed by the gastroduodenal artery, portal vein, and superior border of the pancreas (Figure 2). Energy sources should be judiciously used around the bile duct to prevent thermal damage[96-98]. Also, ischemia of the bile duct can be prevented by preserving the posterosuperior pancreaticoduodenal artery, a proximal branch of the gastroduodenal artery, and preserving pancreatic tissue medial to the bile duct. To prevent duodenal ischemia and delayed gastric emptying, the pancreaticoduodenal arcade along the medial border of the duodenum should be preserved. Hong *et al*[95] reported the usefulness of ICG in assessing vascular arcade and identifying common bile duct in a series of 22 patients with different pancreatic pathology. The mean operative time and blood loss of five patients with chronic pancreatitis



DOI: 10.3748/wjg.v29.i14.2101

Figure 1 Robotic modified Frey's procedure. A: Pancreatic head coring is done till the level of the posterior wall of the pancreatic duct (marked with star). The bile duct can be seen on the medial wall of the cored-out tissue (arrow); B: Indocyanine green fluorescence demonstrates the bile duct on the medial wall of the cored-out tissue.



DOI: 10.3748/wjg.v29.i14.2101

Figure 2 Robotic duodenum preserving pancreatic head resection. A: Dissection of the pancreatic parenchyma from the posterior pancreatic capsule; B: Identification of the common bile duct in the triangle formed by the gastroduodenal artery, superior border of the pancreas, and portal vein; C: Pancreatic duct (arrow) divided at its junction with the bile duct; D: Post pancreatic head resection, indocyanine green fluorescence demonstrates bile duct.

included in their series were 264 min and 215 mL, respectively. The mean postoperative hospital stay was 7.5 d, and there was no conversion to open surgery or postoperative mortality[96].

Inflammation and tissue adhesion in chronic pancreatitis can distort the anatomy of pancreaticoduodenal vessels resulting in vascular injury and significant bleeding during DPHR. A 3D reconstruction of preoperative cross-sectional imaging could help to better understand the anatomy of pancreaticoduodenal vessels and the relationship of the intrapancreatic common bile duct. Also, 3D printing technology can be helpful for surgical training and preoperative planning in patients undergoing minimally invasive DPHR[96-98]. As with other minimally invasive procedures for chronic pancreatitis, a robotic platform could minimize the technical challenges associated with DPHR. Peng *et al*[98] first reported the feasibility of robotic DPHR. However, in a recent series of 68 patients undergoing robotic DPHR for various pancreatic diseases, only three patients had chronic pancreatitis

[99]. Different published series on minimally invasive DPHR highlight technical development and challenges of the procedure in chronic pancreatitis. Minimally invasive DPHR is the preferred procedure in patients with inflammatory head mass and those with enlarged pancreatic head and extensive parenchymal calcifications. However, if the inflammatory changes preclude the safe creation of a retropancreatic tunnel over the portal vein alternative surgical procedure should be considered.

Minimal invasive total pancreatectomy with or without islet cell autotransplantation

Total pancreatectomy is primarily indicated in chronic pancreatitis patients with debilitating pain in whom all other measures are unsuccessful and those with recurrent acute pancreatitis[100]. However, total pancreatectomy should be combined with islet cell autotransplantation to minimize the risk of brittle diabetes. Some centers recommend total pancreatectomy early in the disease course before activation of neuropathic pain circuits, especially in patients with small duct disease or genetic etiology [101]. However, selecting suitable patients is critical as, despite islet cell autotransplantation, more than 50% of patients might require lifelong exogenous insulin. Literature on minimally invasive total pancreatectomy is sparse, with variations in technique[102-105]. Blair *et al*[103], in 2016, reported the feasibility and safety of laparoscopic total pancreatectomy with islet cell autotransplantation in 20 patients with chronic pancreatitis. The mean operative time and hospital stay were 430 min and 11 d, respectively, with no postoperative mortality. Similarly, Fan *et al*[101] reported the feasibility of laparoscopic total pancreatectomy with islet cell autotransplantation in 22 patients with two conversions. In both the laparoscopic series, the pancreatic neck was transected, and two-stage retrieval was used, with the pancreatic head and body retrieved separately[102,104]. However, studies have shown the importance of preserving pancreatic arterial and venous flow until retrieval to reduce warm ischemia time during the pancreatic dissection phase and improve islet yield[105]. In the robotic series reported by Galvani *et al*[102] and Zureikat *et al*[104] the feasibility of total pancreatectomy without pancreatic neck transection and preserving vascular flow till the final step to reduce warm ischemia was documented. Another technical challenge is dense retroperitoneal adhesions due to recurrent pancreatic inflammation. Although laparoscopic and robotic total pancreatectomy with islet autotransplantation is safe and feasible, appropriate patient selection is critical for deriving the benefit of a minimally invasive approach.

CHALLENGES AND FUTURE PERSPECTIVES

The available evidence suggests that minimally invasive surgery for chronic pancreatitis is feasible in selected patients. However, the poor quality of available evidence precludes definite conclusions. Also, the surgeon should adhere to surgery principles for chronic pancreatitis, irrespective of the approach. As the pancreatic head is the pacemaker of pain in most patients, adequate resection and decompression of the pancreatic head are critical. However, most of the reported series on minimally invasive approaches for chronic pancreatitis have focused on the feasibility of lateral pancreaticojejunostomy or modified Frey's procedure which may be appropriate only in a minority of chronic pancreatitis patients. Recent series have shown the feasibility of minimally invasive duodenal preserving pancreas head resection, which may be the ideal procedure for most chronic pancreatitis patients. However, a minimally invasive approach is feasible only in patients without extensive inflammatory adhesions or recent acute exacerbation. Preoperative cross-sectional imaging and biochemical parameters like serum amylase and lipase are not sensitive to predict inflammatory changes. Also, studies evaluating the predictive value of markers of systemic inflammation like white blood cell count, IL-6, and C reactive protein yielded disappointing results[88]. Future studies should focus on identifying reliable markers that can accurately predict ongoing pancreatic inflammation, thereby aiding patient selection for a minimally invasive approach. With recent evidence supporting early surgical intervention before the development of extensive fibrosis or local complications, more patients may be suitable for minimally invasive surgery. Also, the main problem with the existing procedures is they are primarily focused on pancreatic ductal and parenchymal decompression. However, it is well-documented that anatomical factors alone do not contribute to pancreatic pain in all patients. In a subgroup of chronic pancreatitis patients, neurological pathways of pain play a dominant role, which is not addressed by the commonly performed surgical procedures. Also, future studies should compare laparoscopic and robotic procedures for chronic pancreatitis to document the advantages of the robotic platform.

CONCLUSION

As pain is the primary indication for intervention in chronic pancreatitis use of a minimally invasive approach is an attractive proposition. However, due to technical challenges, both endoscopic intervention and minimally invasive surgery for chronic pancreatitis have lagged compared to other benign gastrointestinal orders. With improvements in laparoscopic instrumentation and technological

advances like ICG fluorescence, minimally invasive surgery for chronic pancreatitis is gaining momentum. Also, with its distinct advantages, the robotic platform can widen the adoption of minimally invasive surgery in chronic pancreatitis. However, well-designed trials with long-term follow-ups are required to document the superiority of minimally invasive surgery for chronic pancreatitis.

FOOTNOTES

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REVIEW

Challenges in pediatric inherited/metabolic liver disease: Focus on the disease spectrum, diagnosis and management of relatively common disorders

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Abstract

The clinical scenario of pediatric liver disease is becoming more intricate due to changes in the disease spectrum, in which an increasing number of inherited/metabolic liver diseases are reported, while infectious diseases show a decreasing trend. The similar clinical manifestations caused by inherited/metabolic diseases might be under-recognized or misdiagnosed due to nonspecific characteristics. A delayed visit to a doctor due to a lack of symptoms or mild symptoms at an early stage will result in late diagnosis and treatment. Moreover, limited diagnostic approaches, especially liver biopsy, are not easily accepted by pediatric patients, leading to challenges in etiological diagnosis. Liver dysfunction due to inherited/metabolic diseases is often caused by a variety of metabolites, so precision treatment is difficult; symptomatic treatment is a compelling option for inherited disorders.

Key Words: Hepatitis; Genetic; Liver disease; Cholestasis; Challenge; Pediatric

Core Tip: The spectrum of diseases causing pediatric liver dysfunction has been changing, and an increasing number of inherited/metabolic disorders have been increasingly recognized as major contributors to liver disease in children. Etiological diagnosis remains challenging due to the frequent absence of symptoms or nonspecific signs and limited diagnostic approaches, especially liver biopsy, which is not easily accepted by pediatric patients. Consequently, the treatment of pediatric inherited/metabolic liver disease is challenging. In this manuscript, we review here the challenges in pediatric inherited/metabolic liver disease, including epidemiological changes in the disease spectrum and challenges in etiological diagnosis and treatment.

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INTRODUCTION

Pediatric liver disease is a common entity in children resulting from different causes, including hepatocyte injury and cholestasis. Recently, the spectrum of diseases causing pediatric liver disease has been changing. Infectious diseases are decreasing due to improvement in hygienic conditions, while the proportions of metabolic disorders, inherited diseases and genetic defects are increasing. At an early stage, elevated liver transaminase may be a unique nonspecific manifestation[1]. Early diagnosis mainly depends on biochemistry, pathogen identification and imaging findings for patients without distinctive clinical features[2]. While new diagnostic methods have improved the diagnostic capability for liver disease in children, some novel challenges remain for pediatricians. Diet and/or lifestyle modifications are usually the primary treatment for some inherited/metabolic liver diseases. Where indicated, drug therapy, surgery and gene modification are necessary to provide lifelong functional correction of liver-based metabolic defects. We review here the changes in epidemiology and the challenges in etiological diagnosis and treatment of inherited/metabolic liver disease in children. Due to the broad spectrum of diseases, we focus on some of the most common conditions resulting in metabolic/inherited liver disease and easily misdiagnosed diseases without evident malformations.

EPIDEMIOLOGICAL CHANGES IN CAUSES OF PEDIATRIC LIVER DISEASE

The exact prevalence of pediatric liver disease is not clear. However, there have been some changes in the disease spectrum causing pediatric liver disease in the past decade. With the popularization of hepatitis B vaccines and the development of effective antiviral treatments, the global incidence of hepatitis B has declined. By 2019, the incidence of hepatitis B in children younger than 5 had fallen from 5% in the 1980s to less than 1% in the 2000s[3,4]. With the promotion of blood screening, the incidence of hepatitis C also declined slightly. By 2018, the global incidence of hepatitis C in children under 18 years old was 0.13%[5]. While the understanding of cholestatic liver diseases (CLD) has deepened in the field of inherited/metabolic liver diseases, an increasing number of noninfectious factors have been recognized as major contributors to liver dysfunction or cholestasis in children[6-10]. A single-center study in China showed that nonviral liver diseases accounted for 23.3% of pediatric liver diseases in 2011-2017, increasing from 17.53% in 2001-2010[11]. Nonalcoholic fatty liver disease (NAFLD) has rapidly become one of the most common liver diseases in the pediatric population. Approximately 2.6% to 9.6% of the population was affected by NAFLD in the Americas and Asia in 2006. The global prevalence of NAFLD in the pediatric population is approximately 10% currently[12]. Recent genetic diagnosis techniques and their easy accessibility raise the possibility of detecting some metabolic diseases involving Wilson's disease (WD)[13], glycogen storage disease (GSD)[14] and α1-antitrypsin deficiency[15]. The incidence of WD is usually quoted as 1:30000, but recent genetic studies have shown a higher prevalence ranging from 1:2400 to 1:6500[16]. CLD affects approximately 1/2500 infants, and biliary atresia accounts for 1/3[17]; genetic or metabolic factors account for approximately one-third of cholestasis cases causing subsequent liver dysfunction, including citrin deficiency (CD)[18], progressive familial intrahepatic cholestasis (PFIC)[19] and Alagille syndrome (AGS)[20]. In the early 2000s, the incidence of CD was 1 in between 17000 and 34000 births[18], while its incidence increased to 1 in 7000 births in 2020[21]. The incidence of GSD is approximately 1:10,000 Live births[22]. AGS had an incidence of approximately 1 in 70000 Live births in 2020[23], an increase from approximately 1 in 100000 in the 1990s[24]. The estimated overall incidence of PFIC increased from 1/300000-1/500000 Live births in the

1990s[24] to 1/50000-1/100000 Live births in the 2010s[25].

CHALLENGES IN ETIOLOGICAL DIAGNOSIS

It is difficult to determine an etiological diagnosis for liver diseases that are undetectable by newborn screening, especially at an early stage. A diversity of inherited/metabolic causes can result in pediatric liver disease[26]. Fatty liver disease (nonalcoholic and alcoholic), autoimmune hepatitis, WD, disorder of bile acid synthesis, AGS, hereditary hemochromatosis, and α-1 antitrypsin deficiencies are among the causes of pediatric liver disease[27]. Hepatocytes have similar responses to different impairments, while clinical and laboratory signs are frequently monomorphic. In addition, the absence of symptoms and nonspecific signs at an early stage contributes to the difficulty of diagnosis.

Liver disease is usually divided into two types - hepatocellular and cholestatic disease - which sometimes overlap. Alanine transferase (ALT), alkaline phosphatase (ALP), aspartate transferase (AST), γ-glutamyl transferase (GGT), prothrombin time, bilirubin and albumin are the most common biochemical markers for liver function, but they are susceptible to extrahepatic factors. A disproportionate increase in the level of ALT and AST compared with ALP suggests hepatocyte damage or muscle disorders; conversely, a disproportionate increase in ALP level compared to ALT and AST indicates cholestatic injury. Moreover, an elevated serum conjugated bilirubin level suggests hepatocellular or cholestatic disease. These markers can assist in roughly sorting the types of pediatric liver disease, but they are not helpful for accurate etiological diagnosis.

With advances in diagnostic tools for pediatric liver disease over the last few decades, some novel diagnostic modalities, such as ultrasound, magnetic resonance, and genetic testing, have been used to evaluate liver disease; however, percutaneous liver biopsy for histopathological evaluation remains crucial for assessment of the severity of hepatopathies[28]. We focus on the challenges in etiological diagnosis in the following section (Figure 1).

NAFLD

Pediatric NAFLD is frequently free of symptoms, and diagnosis is based on hepatic steatosis on ultrasound or evidence of abnormal liver enzymes and the exclusion of some known causes of hepatosteatosis other than NAFLD. As a standard diagnostic method in NAFLD, liver biopsy has some inherent limitations: the method is invasive; the accuracy depends on the experience of the pathologist; it is difficult to use for NAFLD staging due to the nonuniformity of the small liver specimen; and it may present special challenges in extremely obese children due to difficulty in evaluating the liver location and the depth of subcutaneous fat tissue. Finally, the optimal timing of liver biopsy for diagnosis and follow-up has not been determined[29].

Several noninvasive diagnostic tools have been proposed instead of hepatic histology, including the hepatic steatosis index, NAFLD fatty liver index, liver fat score, and pediatric prediction score, which are widely accepted clinically. However, surrogate markers and scores remain to be validated or are not sufficient to predict the presence of hepatic steatosis[30-32]. Ultrasound technology has been shown to be inaccurate for the diagnosis of hepatosteatosis due to its low specificity and sensitivity[33]. Due to radiation risk, computed tomography is not commonly recommended as a diagnostic tool despite being reasonably specific and sensitive for hepatic steatosis. Despite its accuracy for hepatic steatosis, magnetic resonance spectroscopy and imaging are not widely used due to cost, lack of availability and lack of validated cutoffs to determine NAFLD[34]. As a novel noninvasive tool, transient elastography (TE) has shown promise in the evaluation of steatosis in pediatric patients. TE was reported to perform better than ultrasound for the assessment of hepatic steatosis in children[35]. It is a good diagnostic tool for measuring liver stiffness and can differentiate advanced fibrosis. However, there are some limitations to this diagnostic modality, particularly abdominal adiposity, which increases the distance between the probe and liver; body mass index greater than 30 kg/m^2; and operator experience[36]. Further validation of TE is necessary due to the limited data available for pediatric NAFLD patients. Controlled attenuation parameters are a promising imaging method to detect steatosis based on vibrationally controlled TE. The same probe is used to quantify steatosis by assessing the attenuation of ultrasound in liver fat. Some magnetic resonance-based methods, e.g., magnetic resonance spectroscopy/magnetic resonance proton density fat fraction, have been developed to quantify hepatic steatosis and fibrosis[37]. Its accuracy, cost effectiveness and ability to monitor changes in the level of steatosis in patients with NAFLD remain unknown[38]. Most challenging is that no reliable biomarkers have been identified to detect or predict inflammation in NAFLD. Recently, Chae *et al*[39] successfully developed machine learning-based NAFLD diagnostic models based on metabolome profiles and metabolic pathway changes. It can be utilized as a less-invasive approach for diagnosing the disease.

WD

WD is an autosomal recessive copper metabolic disorder caused by mutations in the ATP7B gene[40]. The clinical manifestations regarding liver involvement range from incidental findings of liver function abnormalities to acute hepatic failure. Symptoms at any age are frequently nonspecific[41]. Early clinical

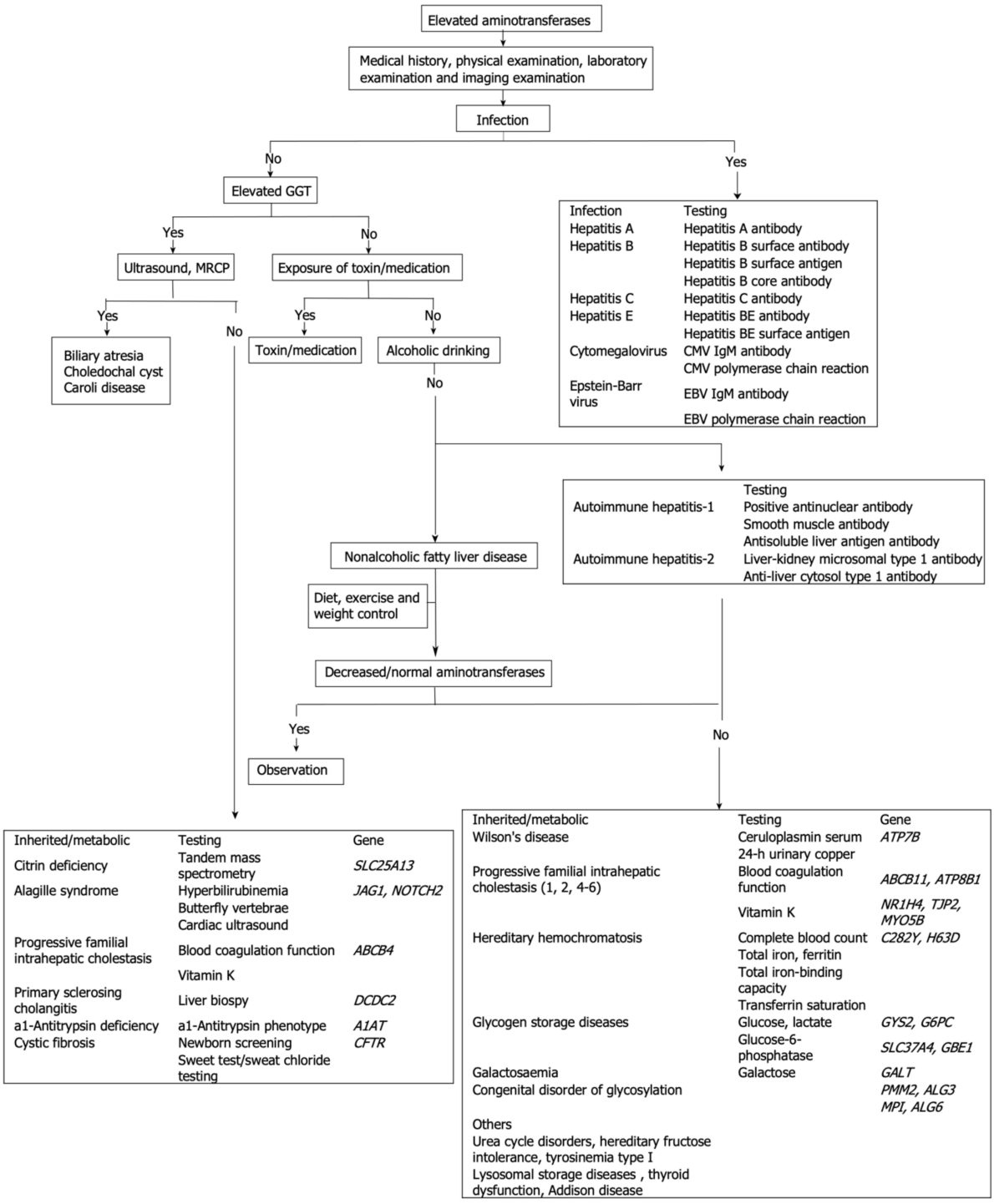

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Figure 1 Diagnostic flow chart of pediatric liver injury. GGT: Gamma-glutamyl transferase; MRCP: Magnetic resonance cholangiopancreatography.

symptoms in WD patients are diverse and atypical, and biochemical tests have false positives and false negatives, making early diagnosis more difficult. In most cases, its diagnosis is based on a combination of laboratory findings and clinical presentations[40]. The diagnosis might be difficult in children with WD, particularly at early stages of liver disease, given the absence of a single distinctive characteristic or available biochemical test to confirm or exclude the diagnosis. Indeed, many affected children do not have elevated liver copper levels, high urinary copper excretion or a Kayser-Fleischer ring[42].

Serum ceruloplasmin is typically decreased in patients with WD, although normal ceruloplasmin levels are also seen in some patients[40]. Notably, the levels can also be affected by other factors[43]. Therefore, WD cannot be diagnosed solely based on ceruloplasmin levels. Exchangeable copper is an experimental technique that might be able to determine bioavailable copper in plasma, but it does not reliably measure no ceruloplasmin-bound copper levels[44]. Twenty four-hour urinary copper excretion is a useful method to diagnose and monitor treatment in WD; however, it can be lower than 100 μg in

some patients with WD, especially in children and their asymptomatic siblings[45]. Moreover, it might be difficult to interpret because of overlapping findings with other hepatopathies, especially acute hepatic damage or liver failure, and the reference limits vary among laboratories[45]. Although copper quantification in liver tissue has high specificity and diagnostic value, the test is invasive and involves risks; the concentration can be underestimated due to sampling errors or increased in cholestatic disorders[46]. These equivocal findings emphasize the importance of genetic testing for mutation identification. ATP7B gene mutational analysis is an important tool for diagnosing WD. However, it may take time to obtain the results of molecular genetic diagnosis; analysis is difficult due to more than 700 possible mutations, and many patients are compound heterozygotes[47]. Another difficulty of genetic testing research is that, except for a few hotspot mutations, most mutations are extremely low in frequency in the population, making it difficult to collect sufficient cases for statistical analysis, accurate analysis of ATP7B genotype-phenotype and clarification of the corresponding pathogenicity to aid diagnosis. Recently, a scoring system based on all available tests developed by the 8th International Meeting on WD and Menke Disease was found to have good diagnostic accuracy in pediatrics[48].

GSD

GSD refers to a group of inherited disorders of glycogen synthesis, degradation, or metabolism regulation. The hepatic forms usually have a wide spectrum (I, III, IV, VI, IX, and 0) but similar cardinal manifestations[49], although they may have variable expressivity. GSD type I is the most common; type Ia is present in 80% of cases, whereas type Ib accounts for the remaining 20%. The enzyme defect in GSD type Ia is primarily expressed in the liver, kidney, and intestine, while Ib is expressed ubiquitously. Consequently, both types Ia and Ib manifest as hepatomegaly, hypoglycemia, and lactic acidosis, but neutropenia and myeloid dysfunctions are unique to GSD type Ib[50]. GSD classification is sometimes difficult due to the common characteristics among different types. Newborn screening is currently not available for GSD. Encouragingly, some specific screening systems using dried blood spots were reported to be applicable for newborn screening for GSDIa in the real world[51]. A detailed clinical history and careful examination along with laboratory findings could suggest the diagnosis. Reduced enzymatic activity in peripheral blood can confirm the diagnosis of GSD types III, IV and IX; however, normal activity in leukocytes does not exclude the other forms[52]. According to related studies, urinary glucose tetrasaccharide (Glc4) excretion, a biomarker for GSD II, is also elevated in patients with hepatic GSD, so Glc4 might be a good biomarker for GSD, but more studies are still needed to confirm this hypothesis. In pediatric GSD III patients, decreasing Glc4 in urine reflects improved fasting tolerance [53]. Traditional invasive liver biopsy for the assessment of enzymatic activity in hepatic cells is no longer the gold standard and is neither cost effective nor convenient. As a robust alternative diagnostic tool that has emerged over the course of the past few years, gene examination has replaced liver biopsy. It is a noninvasive method for confirming the diagnosis and classification of GSDs, allowing for appropriate specific therapy for different types of GSDs[54]. For many years, the conventional Sanger sequencing method has been the gold standard for the detection and screening of mutations, but this laborious, costly and time-consuming method leads to delayed diagnosis which decelerates care and treatment[55]. Combined with clinical signs and biochemical indices, next-generation sequencing (NGS), as the gold standard, provides a high-throughput and accurate method for genetic diagnoses of GSDs. It bridges the difficulties of GSD diagnosis due to broad genetic heterogeneity and clinical manifestations. The much faster and more sensitive NGS makes clinical application more practicable. However, the identification of variants of uncertain significance poses a challenge in diagnosis owing to variable gene coverage, noncoding and structural variants that are unable to be captured and duplications/deletions that are missed[56]. Higher sequencing coverage increases the validation of findings as well as costs. Therefore, methods with the best accuracy, coverage, and cost are expected to be designed. Finally, traditional static biochemical markers might not adequately reflect the dynamic changes in response to metabolic stressors or treatment. Continuous glucose monitoring appears attractive as a highly informative noninvasive technique to monitor the dynamics of hypoglycemia in patients with GSDs, but it might be limited by regional availability[57].

CLD

CLD covers a spectrum of disorders caused by inborn errors of metabolism, primarily manifesting as a cholestatic syndrome. Intrahepatic cholestasis can be divided into hepatocellular and biliary forms[58]. Hepatic cholestatic disease can cause related clinical symptoms and secondary changes resulting from cholestasis. The diagnosis of CLD is challenging due to the nonspecific symptoms and the broad differential diagnosis. Biochemical indicators include increases in serum ALP and GGT in patients without symptoms at an early stage and an increase in conjugated bilirubin at advanced stages. Isolated elevation of serum GGT and/or ALP can result from certain rare disorders, rapid bone growth or bone disease, and drug intake; consequently, it is limited in the diagnosis of cholestasis due to low specificity. As a sensitive and specific, non-invasive, and relatively inexpensive tool, ultrasonography has some disadvantages in that abnormalities of bile ducts may be misdiagnosed. Moreover, the pancreas and the lower common bile duct are frequently not well depicted. Abdominal computed tomography holds the risk of radiation and might not be superior to ultrasound for biliary tree delineation. Routine new-born screening (NBS) using electrospray ionization tandem mass spectrometry technology has been widely

used for inborn errors of metabolism[59]. Nevertheless, this strategy depends on complete clinical information and therefore is associated with false negatives. In addition, the selection of limited known metabolic pathways likely hampers the discovery of novel metabolic defects[60]. In contrast, NGS panels have recently emerged as an appealing tool to diagnose pediatric metabolic liver disease[60].

CD

CD is the most common inherited autosomal recessive metabolic disorder and is caused by mutations in the SLC25 gene family encoding proteins[61]. There are mainly 3 age-dependent phenotypes[21,61], and neonatal intrahepatic cholestasis (NICCD) is the major pediatric CD phenotype. Neonates or infants with CD manifest with intrahepatic cholestasis, citrullinemia, dyslipidemia, hyperammonemia, galactosemia, and hypoglycemia[62]. In early infancy, these symptoms overlap with those of other CLD, e.g., biliary atresia and neonatal hepatitis, contributing to the difficulty in prompt and accurate diagnosis. Patients with adult-onset symptoms, hyperammonaemia and neuropsychosis can be misdiagnosed with other neurological entities. NBS for CD provides the opportunity to initiate early treatment in newborns with NICCD; however, NBS for CD is not performed in certain countries, and the sensitivity and specificity of NBS results are not satisfactory[63]. False negatives at the cutoff value in NBS have been reported in some studies; less than 30% of NICCD patients were detected by NBS using tandem mass spectrometry to measure amino acids[64]. Abnormal biochemical parameters are important clues for CD diagnosis. The increases in serum transaminase, total galactose, alkaline phosphatase, and direct hyperbilirubin levels, accompanied by the prolongation in coagulation parameters, lead to confusion of the disease with galactosemia and causes misdiagnosis. However, galactose-1-phosphate uridyl transferase activity is normal in these patients[65]. AFP elevation might be an important laboratory finding suggesting NICCD[66]. *SLC25A13* genetic analysis is a reliable method for confirmation of NICCD. However, a percentage of *SLC25A13* mutations are not detectable by conventional DNA analysis alone[67]. The verification of these mutations is challenging for the definite diagnosis of NICCD. Moreover, *SLC25A13* mutations worldwide demonstrate remarkable heterogeneity. In such cases, some reliable evidence for molecular diagnostic strategies originated from sophisticated functional, molecular and in silico analysis of the *SLC25A13* gene and its cDNA, although it is usually labor-intensive and expensive. Sanger sequencing, denaturing high-performance liquid chromatography and PCR restriction fragment length polymorphism are alternative tools for the analysis of gene mutations. However, these methods are too complex to be widely used in clinical practice.

CHALLENGES IN TREATMENT

Challenges in treatment of NAFLD

A universal consensus on drug therapy for NAFLD has not been established. A balanced diet and lifestyle interventions based on exercise are the mainstay of NAFLD management[68]. The side effects of drugs, poor compliance of diet and exercise control, and the risks of surgical therapy remain challenges for pediatricians in the treatment of NAFLD. The provision of low-fat diets and hypocaloric, low-carbohydrate diets, *e.g.*, a diet low in free sugar content[69] or a Mediterranean diet rich in fiber, polyunsaturated fats and antioxidants[70], provide reductions in liver fat content as long as weight loss is achieved. Although proven effective, this treatment modality has some inherent barriers. The patient's compliance, the lack of clinical nutritionists, and the therapeutic recipes juggling the nutrition requirements for development restrict the application. Moreover, no specific diet or program is recommended for the treatment of NAFLD in children from different regions and with different ethnicities. Increasing physical activity is important; however, the appropriate type of physical activity is controversial. There is no general consensus on the exercise category (aerobic, resistance exercise or combined) or the volume and intensity of physical activity. Some studies have demonstrated that combined exercise is more effective than aerobic exercise alone in improving NAFLD progression[71]. Additionally, affected children frequently fail to implement prescribed lifestyle changes due to a lack of motivation to alter contributory habits. The antioxidants metformin and ursodeoxycholic acid, initially used in the treatment of NAFLD in adults, have only limited effects in the treatment of pediatric NAFLD[72]. Similarly, omega-3 fatty acid supplementation was not effective in inducing a significant reduction in ALT or improving liver steatosis on ultrasonography[73]. Some randomized controlled trials indicated that probiotic supplementation might be beneficial in children with NAFLD, but the current evidence does not specify the exact beneficial strain of probiotics, requiring further studies[74]. Interestingly, the strategy of targeting the lysosome has seemed to encourage a new direction for future NAFLD treatment, *e.g.*, inhibition of mTOR localized on the lysosome surface and restoration of normal lysosomal function and autophagy[75]. Nevertheless, all of the studies are limited to animal models.

While bariatric surgery has been indicated as a treatment option in severely obese children and those with comorbidities for the improvement of NAFLD-related liver damage[76-78], the unidentified evidence on nutritional deficiency and its impact on growth and development make it controversial in pediatric patients[76]. Intragastric balloons, a promising alternative temporary physical device, could

improve metabolic parameters and liver alterations in pediatric patients with morbid obesity[79], but further clinical observational evidence is needed for validation in pediatric patients.

Challenges in treatment of CD

Nutritional and medicinal therapy regimens are important for CD patients, and the basic therapy is nutrition. The principle of therapy should be based on the specific food intake[61]. The common therapeutic diet for liver diseases is a high carbohydrate and low protein diet, although it is controversial[80]; other options include a low carbohydrate diet or additional high protein and a low carbohydrate-restricted formula with medium-chain triglyceride supplementation[81]. Dietetic treatment with medium-chain triglyceride (MCT) supplementation and lactose-free or low-carbohydrate formulas has been recommended. The clinical manifestations of NICCD often improve spontaneously within the first year, even without treatment for some patients. Most infants can be reintroduced to protein- and lipid-enriched food by 1 year of age. MCT supplements are suggested even after improvement of clinical manifestations based on the biochemical rationale[64]. In addition to dietary treatment, administration of sodium pyruvate might be effective in correcting growth restriction. However, some subjects develop severe hepatic dysfunction. Liver transplantation (LT) is the most effective option to correct metabolic disturbances in patients with an inefficient therapeutic diet[82]. However, LT also has some disadvantages, *e.g.*, the shortage of liver donors, the cost and the possibility of failing to improve the lives of some CD patients due to immunological complications or other causes. It was reported that supplementation with nicotinamide might be worth trying as a supplemental therapy for CD. mRNA therapy was reported to improve metabolic and behavioral abnormalities in an animal model of CD. Further study is needed to develop safer and more effective treatments for CD patients[21].

Challenges in treatment of WD

The focus of drug therapy for children with WD is to remove excessive copper by promoting copper excretion using chelating agents, blocking intestinal copper absorption with zinc salts[13], or both. At the same time, WD patients should avoid copper-rich foods until they have normal liver biochemistry. D-penicillamine is utilized as a first-line therapeutic medication for acute and/or symptomatic patients with WD. Although it is highly effective, it is linked to serious adverse side effects and requires discontinuation of the drug in some patients[83]. As a second-line drug for patients intolerant to penicillamine, trientine dihydrochloride has equal effectiveness and less frequent adverse reactions, but its high costs limit its application. Trientine tetrahydrochloride is a hybrid with similar benefits and risks to trientine dihydrochloride. A good patient response with a lower dose makes it more cost effective. It has been used for the treatment of WD in adolescents and children older than 5 years old who are intolerant to penicillamine in the European Union, but it was reported that trientine was associated with a higher frequency of initial neurological worsening and increased transaminases[42]. Furthermore, chelation therapies that increase copper excretion can result in iatrogenic copper deficiency[84]. Zinc salts are used for asymptomatic children or in combination with penicillamine for the initial management of symptomatic patients, but some patients display progressive aggravation on zinc therapy[85]. The increased circulating Cu as a result of chelating therapy may lead to oxidative stress and disease deterioration. Melatonin, as an antioxidant, has been shown to strongly ameliorate liver and brain damage from oxidative stress[86]. In fact, treatment failure poses another challenge in the treatment of WD, which can occur with any WD medication early during treatment initiation or later while on maintenance therapy[41]. The main difficulty of WD treatment is to address Wilson's disease crises, including hemolytic crisis and acute liver failure. LT corrects the metabolic defect in the livers of patients with WD and restores normal copper metabolism. The indications for LT are chronic liver disease not responsive to medical treatment, advanced liver failure and fulminant liver failure[13,87]. The fact that acute liver failure is unpredictable, coupled with the shortage in liver donors, remains a challenge to liver transplantation for WD patients.

Challenges in treatment of GSDs

The focus of treatment for GSDs is to maintain blood glucose levels and control lipid and uric acid levels. Diet therapy has remained the primary treatment for GSDs. Cornstarch therapy can better control blood sugar, but infants younger than 6 mo who have not yet fully developed amylase are prone to indigestion and abdominal distention. Starch with a slower rate of absorption may extend sleep periods for children with GSDs. The new waxy maize starch (Glycosade) effectively increases safety through avoidance of overnight feeding and improved sleep and quality of life; however, there have been no dosing guidelines published. Tolerance to the volume required to maintain euglycemia through the night and its strong taste, granular texture, and cost are the main problems. Ketogenic diets were shown to have positive outcomes in the management of patients with some GSD types[88], but the optimal β-hydroxybutyrate concentration in ketogenic diets has not been defined. In addition, high ketones may contribute to osteoporosis, delayed growth, and elevated transaminase[89]. Impaired cortisol levels secondary to deregulation were observed in a cohort study, which could constitute a new potential therapeutic target in GSD I patients[90]. Dietary therapy not aimed at the cause of disease might not

prevent long-term complications and might even trigger secondary metabolic manifestations. Hepatic GSDs are associated with microbial dysbiosis; therefore, supplementation with probiotics is recommended[57]. In recent years, adeno-associated virus (AAV)-mediated gene therapy has moved to preclinical studies and clinical trials. However, current limitations involve the immune response to the AAV product, potential genotoxicity, the need for high vector doses to target nonpermissive tissues, the reduced persistence of vector genomes in diseases with underlying liver degeneration and the need to treat a second tissue other than the liver[91]. LP is the most effective option for GSD treatment, while the shortage of liver donors and the control of postoperative bloodstream infection are major challenges[92].

Challenges in treatment of CLD

Therapy currently focuses on reducing damage to the liver and other organs caused by cholestasis and prevention of the progression of liver fibrosis by reducing toxic substances in hepatocytes. Although medications or dietary modifications are potentially effective for many of these conditions if recognized early, there are some challenges in therapy for CLD. Many metabolic and CLD are sufficiently rare such that pediatricians might have never before seen them.

In some instances, the etiology is unknown, and no specific therapy is available; however, in rare cholestatic disorders in which the cause is known, no specific treatments have been developed[93]. Ursodeoxycholic acid (UDCA) is presently the mainstay of therapy for most cholestatic hepatic diseases; however, all patients with primary sclerosing cholangitis and approximately one in three patients with primary biliary cholangitis do not respond to or intolerant to UDCA treatment[94,95]. As a strong farnesoid X nuclear receptor agonist that downregulates the intestinal bile acid transporter, obeticholic acid is currently the only approved second-line therapy for UDCA nonresponders or intolerance to UDCA; however, only half of these patients respond to obeticholic acid. Patients who do not respond to treatment with UDCA and/or obeticholic acid have an increased risk of progression to biliary cirrhosis, end-stage liver disease and death or liver transplantation[96,97]. Oral vancomycin has been reported to improve liver biochemical tests (ALT and γGTP) and symptoms in children with primary sclerosing cholangitis due to its minimal oral absorption, high concentration in the gut and inhibition of cytokine release from T cells[98]. Further clinical studies of vancomycin treatment for CLD are expected. Although liver transplantation (LT) remains the only curative therapy for patients who fail to respond to conservative management, disease recurrence and extrahepatic manifestations significantly affect quality of life, and long-term studies are needed to understand patient history after LT[99,100]. Hepatocyte transplantation, as an alternative to organ transplantation, is a promising treatment for patients with CLD arising from inherited metabolic disorders, *e.g.*, PFIC[101,102]. The problem of low engraftment rates and long-term survival of transplanted hepatocytes is the most important obstacle regarding current techniques. Moreover, a major limitation is the lack of good-quality donor organs, and thus far, there have been no clinically applicable techniques to monitor the function and survival of engrafted cells[101].

CONCLUSION

Liver function injury in children is etiological heterogeneous. Early etiological diagnosis is critically important because it could help to determine the history of the disease and modify of the treatment regimen, but diagnosis is often delayed due to the presence of nonspecific presentations or the frequent absence of symptoms. It is frequently at the stage of progressive disease when typical symptoms appear. Therefore, recognition leading to diagnosis and treatment remains a great challenge for pediatricians. In this paper, we reviewed the changes in epidemiology and the challenges in the diagnosis and treatment of pediatric inherited/metabolic liver disease. We hope to assist pediatricians in understanding this condition better and identifying and treating this condition as early as possible.

FOOTNOTES

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MINIREVIEWS

COVID-19 related biliary injury: A review of recent literature

Sujani Yadlapati, Simone A. Jarrett, Daniel Baik, Adib Chaaya

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Abstract

Since its emergence in 2019, it has become apparent that coronavirus 2019 (COVID-19) infection can result in multi systemic involvement. In addition to pulmonary symptoms, hepatobiliary involvement has been widely reported. Extent of hepatic involvement ranges from minor elevation in liver function tests (LFTs) to significant hepatocellular or cholestatic injury. In majority of cases, resolution of hepatic injury or improvement in LFTs is noted as patients recover from COVID-19 infection. However, severe biliary tract injury progressing to liver failure has been reported in patients requiring prolonged intensive care unit stay or mechanical ventilation. Due to the timing of its presentation, this form of progressive cholestatic injury has been referred to as COVID-19 cholangiopathy or post-COVID-19 cholangiopathy, and can result in devastating consequences for patients. COVID-19 cholangiopathy is recognized by dramatic elevation in serum alkaline phosphatase and bilirubin and radiologic evidence of bile duct injury. Cholangiopathy in COVID-19 occurs weeks to months after the initial infection and during the recovery phase. Imaging findings and pathology often resemble bile duct injury associated with primary or secondary sclerosing cholangitis. Etiology of COVID-19 cholangiopathy is unclear. Several mechanisms have been proposed, including direct cholangiocyte injury, vascular compromise, and cytokine release syndromes. This review summarizes existing data on COVID-19 cholangiopathy, including reported cases in the literature, proposed patho-physiology, diagnostic testing, and long-term implications.

Key Words: COVID-19 cholangiopathy; Post COVID-19 cholangiopathy; Cholestatic injury; Liver transplant

Core Tip: Severe cholangiopathy can develop in critically ill coronavirus 2019 patients during recovery, which is reflected by significant derangements in liver function tests and imaging findings consistent with bile duct injury. This condition may progress to acute liver failure, necessitating liver transplantation, and has emerged as a novel indication for transplantation during the pandemic. There are still uncertainties regarding the long-term survival and clinical outcomes of patients who experience incomplete recovery.

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INTRODUCTION

Coronavirus 2019 (COVID-19) infection, caused by severe acute respiratory distress syndrome coronavirus 2 (SARS-CoV-2), has been a major public health concern in recent years, resulting in significant mortality and morbidity worldwide. Although most patients affected by COVID-19 present with respiratory symptoms and sequelae, extra-pulmonary manifestations, including renal failure, neurological deficits, hepatic injury, and gastrointestinal symptoms, worsening coagulopathy, have been reported. Abnormal liver enzymes are seen in up to 20% of patients and are associated with poor clinical outcomes[1]. In the early stages of infection, aspartate (AST) and alanine aminotransferase levels (ALT) are elevated, followed by cholestatic markers like serum bilirubin, alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT) in later stages. Cholestatic liver injury is particularly associated with worse outcomes[2]. This biphasic pattern was described by Hart and colleagues in a study evaluating 496 hospitalized COVID-19 patients[3]. About 6%-20% of patients may have elevation in ALP and GGT at initial presentation. A rise in ALP more than three times the upper limit of normal (ULN) associated with COVID-19 induced cholestatic injury is reported among 1% of critically ill patients[3].

Cholestatic injury in patients ranges from marginal elevation in liver function tests (LFTs) to secondary sclerosing cholangitis (SSC) and liver failure[4]. SSC has been reported in critically ill patients with prolonged hospital stays, patients recovering from sepsis, burns, trauma, and major cardiothoracic surgery.

Post COVID-19 cholangiopathy is a recently described entity considered to be a secondary complication of COVID-19. Currently, there is no consensus on diagnostic criteria for this rare entity. Faruqui *et al*[5] at American Association for the Study of Liver Diseases 2020 described COVID-19 cholangiopathy as severe biliary tract injury resembling SSC seen in patients recovering from severe COVID-19 infection. Diagnosis is made weeks to months after initial admission for COVID-19 infection hence called "post COVID-19 cholangiopathy". These patients often have abnormal LFTs similar to cholestatic injury and bile duct injury on imaging. This review article highlights existing literature on COVID-19 cholangiopathy, including proposed pathophysiology, epidemiology, clinical presentation, treatment, and long-term outcomes[5].

DEFINITION AND DIAGNOSTIC CRITERIA

Although there is no clear consensus on diagnostic criteria for post COVID-19 cholangiopathy, in most studies, patients with severe COVID-19 cholangiopathy were defined as having ALP greater than 1.5 times the ULN, serum bilirubin greater than 2 times the ULN or GGT greater than 3.0 times the ULN[6-9]. These patients often do not have active sepsis or underlying chronic liver disease that may contribute to cholestasis or liver injury. Bile duct abnormalities are noted on imaging.

Magnetic resonance cholangiopancreatography (MRCP) findings include biliary strictures, beaded appearance of intrahepatic bile ducts, biliary dilation, and irregularities of common bile duct, among others (Table 1). A liver biopsy may be needed in some patients to further corroborate the diagnosis. Biopsy findings include cholangiocyte injury, ductal fibrosis, strictures, intravascular microthrombi, *etc.* (Table 1). Endoscopic retrograde cholangiopancreatography (ERCP) may be indicated in some cases to evaluate the bile ducts further and treat biliary strictures or manage choledocholithiasis which may contribute to cholestasis.

Table 1 Liver biopsy and Imaging findings in few previously reported cases of coronavirus disease 2019 cholangiopathy

Ref.	Pathology findings	MRCP
Faruqui *et al*[5], 2021	Acute and chronic large duct obstruction	Beaded appearance of intrahepatic ducts
	Peri-portal fibrosis	Biliary hyper-enhancement
	Cholestasis	
Roth *et al*[6], 2021	Intra-hepatic ductal beading	Beaded appearance of bile ducts along with segments of strictures and dilation
	Biliary strictures and dilation	
	Peri-portal fibrosis	
	Cholangiocyte regeneration	
	Endothelial swelling of hepatic arteries	
Rojas *et al*[7], 2021	Cholestasis	No biliary obstruction
	Peri-portal inflammation	
Daneshjoo *et al*[22], 2020	Enlarged portal tracts	Prominence of intra and extrahepatic bile ducts
	Bile duct epithelial changes	
	Cholestasis, hepatocyte dropout and biliary metaplasia	
	Focal biliary infarcts	
Tafreshi *et al*[17], 2021	Bridging fibrosis	Beaded appearance of intrahepatic bile ducts. Periductal prominence
	Cholestasis and cholangiocyte injury	Normal liver parenchyma
	Bile duct proliferation	
Lee *et al*[16], 2021	Bridging fibrosis	Mild intrahepatic ductal dilation
	Onion skinning of bile ducts and cytoplasmic vacuolization of epithelium	
	Cholestasis	
	Bile duct loss	
	Lymphoplasmacytic infiltration	
Durazo *et al*[8], 2021	Degenerative cholangiocyte injury and cytoplasmic vacuolization	Beaded appearance of intrahepatic ducts with multiple segmental strictures
	Intrahepatic microangiopathy	
	Hepatic artery endothelial swelling, portal vein phlebitis, sinusoidal obstruction	
Cesar Machado *et al*[23], 2022	Cholangiocyte injury	Multi focal strictures and segmental dilation of intra and extra hepatic bile ducts
	Neutrophilic infiltrate	
	Severe cholestasis and fibrosis	

MRCP: Magnetic resonance cholangiopancreatography.

PATHOPHYSIOLOGY OF COVID-19 RELATED BILIARY INJURY

Mechanisms by which COVID-19 results in biliary injury are unclear; however, several hypotheses have been put forth from review of liver biopsies and autopsy studies in COVID-19 patients with significant cholestatic injury (Table 2). One such theory highlights the possibility of bile duct ischemia resulting in cholangiocyte necrosis[10]. Intrahepatic biliary epithelium is often more susceptible to ischemia because of its single source of arterial blood supply from hepatic artery compared to common bile duct and hepatocytes supplied by the portal vein and hepatic artery. Autopsy series by Lagana *et al*[11] and Bütikofer *et al*[12] showed sinusoidal microthrombi with mild hepatic steatosis in patients with COVID-19. Some studies noted the presence of platelet aggregation in sinusoids without gross intravascular thrombi[5]. Contrarily others did not report any evidence of thrombotic injury[5,8,13].

Table 2 Proposed mechanisms of coronavirus disease 2019 cholangiopathy

No.	Proposed pathogenesis of COVID-19 cholangiopathy
1	Cholangiocyte necrosis because of bile duct ischemia[24,25]
2	Micro thrombosis of hepatic sinusoids and ischemic injury[6]
3	Inflammation triggered due to cytokine release (cytokine release syndrome)
4	Direct virus mediated damage[11]
5	Drug induced liver injury in setting of severe COVID-19 infection (antibiotics, Remdesivir among others)
6	Ketamine related cholangiopathy in setting of COVID-19[14]

COVID-19: Coronavirus disease 2019.

Other theories include direct viral damage to biliary epithelium and direct inflammation resulting from cytokine release syndrome. Cytokine release syndrome has been described in patients with critical illness from COVID-19. Release of pro- inflammatory cytokines and mediators results in direct cholangiocyte injury and fibrosis. Angiotensin-converting enzyme 2 (ACE2), the host receptor for SARS-CoV-2, is expressed on respiratory epithelium as well as cholangiocytes. Prior studies evaluating ACE2 expression have concluded that this receptor is more frequently expressed in cholangiocyte clusters (59.7%) compared to hepatocytes (2.6%)[11]. This explains the potential for direct cholangiocyte injury in severe cases of COVID-19. Critically ill patients are often ventilator dependent and receive anesthetics such as ketamine in addition to antibiotics and antiviral therapy. Ketamine associated cholangiopathy has been described in several case reports[14]. Drugs such as remdesivir and amoxicillin-clavulanate are implicated in liver injury, although predominantly hepatocellular damage[15].

CASES OF COVID-19 CHOLANGIOPATHY AND LONG-TERM IMPLICATIONS

To date several authors have reported cases of post COVID-19 cholangiopathy. In the initial published case series, Roth NC and colleagues reported three such cases[6]. These patients developed severe cholestasis after prolonged hospitalization. Biochemical markers consistent with cholestatic injury, persistent jaundice, and liver biopsy findings concerning for moderate peri-portal fibrosis, cholangiocyte injury with microvascular changes were noted in these patients. These abnormalities persisted despite recovery from COVID-19 infection in all patients. No cirrhosis was seen, and none of the patients had prior history of chronic liver disease[6]. Liver transplantation was not required in any of these cases. These patients received hydroxychloroquine, tocilizumab in addition to prophylactic antibiotics during hospital course.

Similarly, Faruqui *et al*[5] reported 12 cases of COVID-19 cholangiopathy. Average time to diagnosis of cholangiopathy was over 100 d in these cases. Bilirubin levels as high as 13 mg/dL and ALP with a median around 1900 U/L was reported. All twelve patients required intubation and three patients required extracorporeal membrane oxygenation (ECMO). Abnormal MRCP findings were noted in all patients. These findings include beaded appearance of intrahepatic ducts and hyperenhancement of common bile duct. Liver biopsy findings in these patients include ductal obstruction as well as mild peri- portal fibrosis without definite duct loss. Majority of these patients were treated with ursodiol without any significant improvement. Liver transplantation was considered in five patients however only one patient received a living donor transplant. One patient in the series developed decompensated cirrhosis with liver and kidney failure at one year[5].

Keta-Cov research group[13] studied 34 patients admitted to intensive care unit (ICU) with COVID-19 pneumonia in a single center in Zurich. Four of the patients in this cohort had persistently elevated cholestatic markers and abnormal imaging. MRCP findings in this group include irregular bile ducts with stricturing. Average time to diagnosis of cholangiopathy ranged from two weeks to over nine months. All patients required mechanical ventilation and were treated with hydroxychloroquine. Ultimately, two of the four patients died. One patient was listed for liver transplant and one patient had stable but persistent disease at one year[13].

Linneweber *et al*[9] reported two patients in Germany who developed biliary injury during recovery from COVID-19 infection. Both patients developed biliary strictures and required ERCP and stent placement. MRCP findings were overall consistent with SSC. One patient remained stable but required multiple ERCPs while the other patient had progressive near complete destruction of intrahepatic bile ducts and died 8 mo after initial diagnosis[9]. Similar findings of SSC were reported in case series by Edwards *et al*[15], Bütikofer *et al*[12], Lee *et al*[16], and Rojas *et al*[7].

Rojas *et al*[7] reported described a case of COVID-19 cholangiopathy in a young female who had a prolonged hospital course for COVID-19. Patient developed jaundice and deranged LFTs 3 mo after her index admission. Peak bilirubin was close to 15 mg/dL and ALP more than 6000 U/L. Interestingly this patient did not have bile duct abnormalities on imaging or ERCP. Patient subsequently underwent a liver biopsy which showed severe obstructive cholestatic picture with periportal inflammation. This patient eventually had improvement in her liver function and did not require a transplant[7].

Durazo *et al*[8] reported a case of COVID 19 cholangiopathy in a 47-year-old obese man who required mechanical ventilation and ECMO in setting of severe COVID-19 infection. Patient developed markedly deranged liver tests two and half months after initial hospitalization. A peak bilirubin close to 20 mg/dL and ALP close to 2700 U/L was noted. Imaging in this case was concerning for diffuse intrahepatic biliary strictures and beading. Pt underwent ERCP. Pt continued to have significant involvement and destruction of intrahepatic ducts and was listed for liver transplant. This patient successfully received liver transplant and had an uncomplicated post op course. Similar cases of successful liver transplant for refractory cholangitis have been reported by Lee *et al*[16], Tafreshi *et al*[17], Blondeel *et al*[18], as well as Rela *et al*[19], among others[17-20]. Allografts in these patients have been reported to be disease free. Cases of persistent liver function abnormalities with stable disease have been reported by a few authors [16,21]. Rate of progression of disease appears to vary among cases with poor outcomes more commonly noted among men, obese patients with metabolic syndromes and more severe COVID-19 illness.

CONCLUSION

Post-COVID 19 cholangiopathy has been described in several case reports and case series. This entity is characterized by severe progressive cholestatic liver injury that can result in liver failure and require transplantation. Progression and onset of disease varies among patients and is not well understood. Individuals with metabolic risk factors and comorbidities who require prolonged ICU stay, mechanical ventilation are at the greatest risk of developing COVID-19 cholangiopathy. It is more commonly reported in men in published literature. At present there is no effective treatment. Hydroxychloroquine, azithromycin and ursodiol have been used in treatment of these patients. Based on follow ups reported in published cases majority of the patients have continued elevation in LFTs, while some progress to liver failure and require liver transplant. In a meta-analysis published by Daneshjoo *et al*[22], the authors concluded that 16% of 30 patients described in the study required liver transplant. Although a rare complication of COVID-19 infection, medical personnel must be aware of this clinical entity. High risk patients should be monitored closely during the recovery period particularly if they suffered a severe clinical course and prolonged recovery. Patients suspected to have COVID-19 cholangiopathy should be referred to liver transplant centers promptly. As time progresses, we will continue to learn more about long term outcomes of those patients diagnosed with cholangiopathy including post-transplant survival and clinical course of those without liver failure however with incomplete recovery.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Anti-inflammatory effect and antihepatoma mechanism of carrimycin

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Abstract

BACKGROUND

New drugs are urgently needed for the treatment of liver cancer, a feat that could be feasibly accomplished by finding new therapeutic purposes for marketed drugs to save time and costs. As a new class of national anti-infective drugs, carrimycin (CAM) has strong activity against gram-positive bacteria and no cross resistance with similar drugs. Studies have shown that the components of CAM have anticancer effects.

AIM

To obtain a deeper understanding of CAM, its distribution, metabolism and anti-inflammatory effects were assessed in the organs of mice, and its mechanism of action against liver cancer was predicted by a network pharmacology method.

METHODS

In this paper, the content of isovaleryl spiramycin III was used as an index to assess the distribution and metabolism of CAM and its effect on inflammatory factors in various mouse tissues and organs. Reverse molecular docking technology was utilized to determine the target of CAM, identify each target protein based on disease type, and establish a target protein-disease type network to ascertain the effect of CAM in liver cancer. Then, the key action targets of CAM in liver cancer were screened by a network pharmacology method, and the core targets were verified by molecular docking and visual analyses.

RESULTS

The maximum CAM concentration was reached in the liver, kidney, lung and spleen 2.5 h after intragastric administration. In the intestine, the maximum drug concentration was reached 0.5 h after administration. In addition, CAM significantly reduced the interleukin-4 (IL-4) levels in the lung and kidney and especially the liver and spleen; moreover, CAM significantly reduced the IL-1β levels in the spleen, liver, and kidney and particularly the small intestine and lung. CAM is predicted to regulate related pathways by acting on many targets,

such as albumin, estrogen receptor 1, epidermal growth factor receptor and caspase 3, to treat cancer, inflammation and other diseases.

CONCLUSION

We determined that CAM inhibited inflammation. We also predicted the complex multitargeted effects of CAM that involve multiple pathways and the diversity of these effects in the treatment of liver cancer, which provides a basis and direction for further clinical research.

Key Words: Carrimycin; Reverse molecular docking; Network pharmacology; Liver cancer; Anti-inflammatory; Anti-hepatoma

Core Tip: Although some studies have shown that carrimycin (CAM) has therapeutic effects on inflammation and liver cancer, there are few experimental studies on its component analysis and mechanism of action. The mechanism of action was predicted by reverse molecular docking between the liver cancer target and CAM. By establishing an inflammatory mouse model to assess the state of inflammatory factors after administration, we further proved the therapeutic effect of CAM on inflammation and its possible mechanism in the treatment of liver cancer.

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INTRODUCTION

Carrimycin (CAM), also known as bitespiramycin and shengjimycin, is a new national class I drug that was jointly developed by the Institute of Pharmaceutical Biotechnology of the Chinese Academy of Medical Sciences and Shenyang Tonglian Group Co., Ltd and was approved for listing in June 2019. CAM is a biosynthetic drug produced through genetic engineering technology that was designed according to the characteristics of bacterial drug resistance. CAM catalyzes the formation of isovaleryl side chains by introducing foreign genes, that is, the isovaleryltransferase gene introduced into *Streptomyces spiralis*, which encodes a sixteen-membered ring multicomponent macrolide antibiotic that was obtained by directional acylation of spiramycin (SPM) in microorganisms[1]. Its main components include isovaleryl SPM III, isovaleryl SPM II, isovaleryl SPM I and a certain amount of 9 mixed chemicals, including butyryl-, propionyl-, and acetylspiramycin III and propionyl- and acetylspiramycin II[2]. Structurally, CAM is a macrolide antibiotic. CAM can block the activity of peptidyl transferase in 50S ribosomes to inhibit bacterial protein synthesis to produce antibacterial effects. Moreover, CAM can combine with peroxide scavenging enzymes to induce peroxide generation, destroying biological macromolecules such as DNA and achieving sterilization. In addition, CAM can promote phagocytosis by damaged neutrophils and macrophages in the body. A completed phase III clinical trial showed that CAM is safe for administration, requires a low oral dose, has a low rate of adverse reactions, produces a wide range of antibacterial activities and shows no secondary drug resistance[3]. Thus, CAM has strong medicinal value.

At present, there is a bottleneck in the research and development of new drugs, and using the traditional 'multiple ligands-single target' research and development method makes drug innovation difficult. The cost of discovering a new molecular entity and applying it to the market exceeds $2 billion USD[4]. However, the rate of return is far lower than the investment cost and the time needed. The research strategy of 'multiple targets-single ligand', namely, utilizing reverse molecular docking technology, has effectively solved these problems and become a favorable tool for drug repositioning. Starting from protein receptors and ligands with known chemical structures and using the principles of geometric complementarity and energy complementarity, researchers can calculate the force and affinity of the interaction between small molecule ligands and biological receptor macromolecules and score the results. These scoring results provide the optimal conformation in which a small molecule ligand binds to the biological target macromolecule, leading to prediction of the ideal binding mode between the two molecules. Finally, the corresponding biological targets can be enriched and analyzed to find the most likely disease spectrum. In this study, the distribution and metabolism of CAM in various tissues and organs of mice were assessed by hyphenated to liquid chromatography (HPLC), and the effect of CAM on inflammatory factors in these tissues and organs was detected by enzyme-linked

immunosorbent assay (ELISA). Reverse molecular docking technology was used to screen the targets of CAM, and the predicted target was enriched and analyzed to obtain data on the related diseases. Then, the key targets and associated pathways for the treatment of liver cancer were analyzed by network pharmacology. The molecular mechanism of CAM and the related experimental research in disease treatment are presented in Figure 1. This study provides a reference for relevant research.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade. Chromatographic grade acetonitrile, methanol, and isopropanol, ethyl acetate, ammonium acetate, SPM, roxithromycin, and sodium carbonate were purchased from Aladdin (United States). NaCl, NaOH, and Na_2HPO_4 were purchased from Sinopharm Chemical Reagent Co., Ltd. CAM was provided by Shanghai Tonglian Pharmaceutical Co., Ltd.

Assessment of the distribution and metabolism of CAM in various mouse tissues by HPLC

Preparation of solution: A standard solution of CAM was prepared as follows. CAM was diluted to 1200 μL with acetonitrile to prepare a 500 μg/mL solution. This solution was then diluted to 400 μg/mL, 300 μg/mL, 200 μg/mL, 150 μg/mL, 100 μg/mL, 80 μg/mL, 60 μg/mL, 40 μg/mL, 20 μg/mL and 10 μg/mL with acetonitrile. Preparation of the control sample solution was performed as follows. The appropriate amount of roxithromycin was weighed, and acetonitrile was added to prepare a 10 μg/mL mother liquor solution. Then, a total of 980 μL of acetonitrile was added to 20 μL of the solution to prepare a 200 μg/mL sample solution.

HPLC conditions: HPLC (LC-20AT, Shimadzu, Japan) was used to analyze the chemical composition of the samples using the following parameters: Detector, spd-20a; chromatographic column, Zorbax Eclipse XdB C18 (Agilent, United States); mobile phases, phase A (10 mmol/L ammonium acetate) and phase B (acetonitrile), 50:50 (v/v); flow rate, 1 mL/min; column temperature, 30 °C; and detection wavelength, 232 nm.

Animals and drug administration: Kunming mice were purchased from the Experimental Animal Center of Jiangsu University using animal certificate No. 201927943. A total of 24 mice were used, and they were adapted to the environment in the laboratory before the experiment. The mice were randomly divided into two groups, and a standard curve was constructed. Mice in the administration group were given compound by gavage at one time point, and the second dose was double that of the first dose. The dose administered was 240 mg/kg. Three mice were randomly selected at 0.5, 2.5, 4.5, 12 and 24 h after administration, and their liver, spleen, lung, kidney, intestine and brain were dissected and stored at -80 °C. During the experiment, the mice ate and drank water normally.

Preparation of tissue homogenate: Tissue samples were weighed using LE104E/02 METTLER electronic scales (METTLER TOLEDO Instrument Co., Ltd, China) (lung, spleen, brain, and kidney, 0.3 g), 0.6 mL of acetonitrile was added, and a tissue homogenate instrument (Shanghai Jingxin Industrial Development Co., Ltd, China) was used to prepare the homogenate at a speed of 2800 rpm for 30 s. Pure water (0.3 mL) was added to the homogenate, which was then ultrasonicated (Ningbo Xinzhi Biotechnology Co., Ltd, China) and centrifuged (3500 r/min) for 10 min, and the supernatant was removed for analysis. Tissue samples (liver and intestine, 0.5 g) were used to prepare the homogenate. Pure water (0.5 mL) was added to the homogenate, which was then ultrasonicated and centrifuged (3500 r/min) for 10 min. The supernatant was removed for analysis.

Preparation of the standard curve: First, 100 μL of tissue homogenate was added to 200 μL of water, 50 μL of internal standard (200 μg/mL roxithromycin acetonitrile solution), and 50 μL of standard solution. Two hundred microliters of alkaline reagent (0.05 M sodium carbonate solution) and 2 mL of ethyl acetate-isopropanol (95:5, v/v) extraction solvent were added, and the solution was placed in a vortex shaker (Qilin Bell Instrument Manufacturing Co., Ltd, China). The solution was vortexed for 3 min and shaken at 50 rpm in the dark for 10 min (Changzhou Langyue Instrument Manufacturing Co., Ltd, China), followed by centrifugation (Thermo, United States) for 10 min at 2000 rpm. Two milliliters of the upper organic phase was placed in a 2 mL EP tube (Jiangsu Shitai Experimental Equipment Co., Ltd, China), which was then placed in a metal bath (Scilogex, United States) at 40 °C and dried with nitrogen. The organic solvent was dissolved diluted with 100 μL of mobile phase, vortexed, and injected into the instrument after passing through a 0.45 μm nylon membrane (Jianhu Yadong Glass Instrument Factory, China). The chromatogram was recorded. The standard curve was drawn with the concentration as the abscissa and the peak area/internal standard peak area (AT/AS) as the ordinate.

Sample pretreatment to determine the drug content in tissue: A total of 600 μL of tissue homogenate was placed in a 50 mL centrifuge tube (Corning, United States), 1.2 mL of ultrapure water, 300 μL of internal standard (200 μg/mL roxithromycin acetonitrile solution), and 300 μL of acetonitrile were

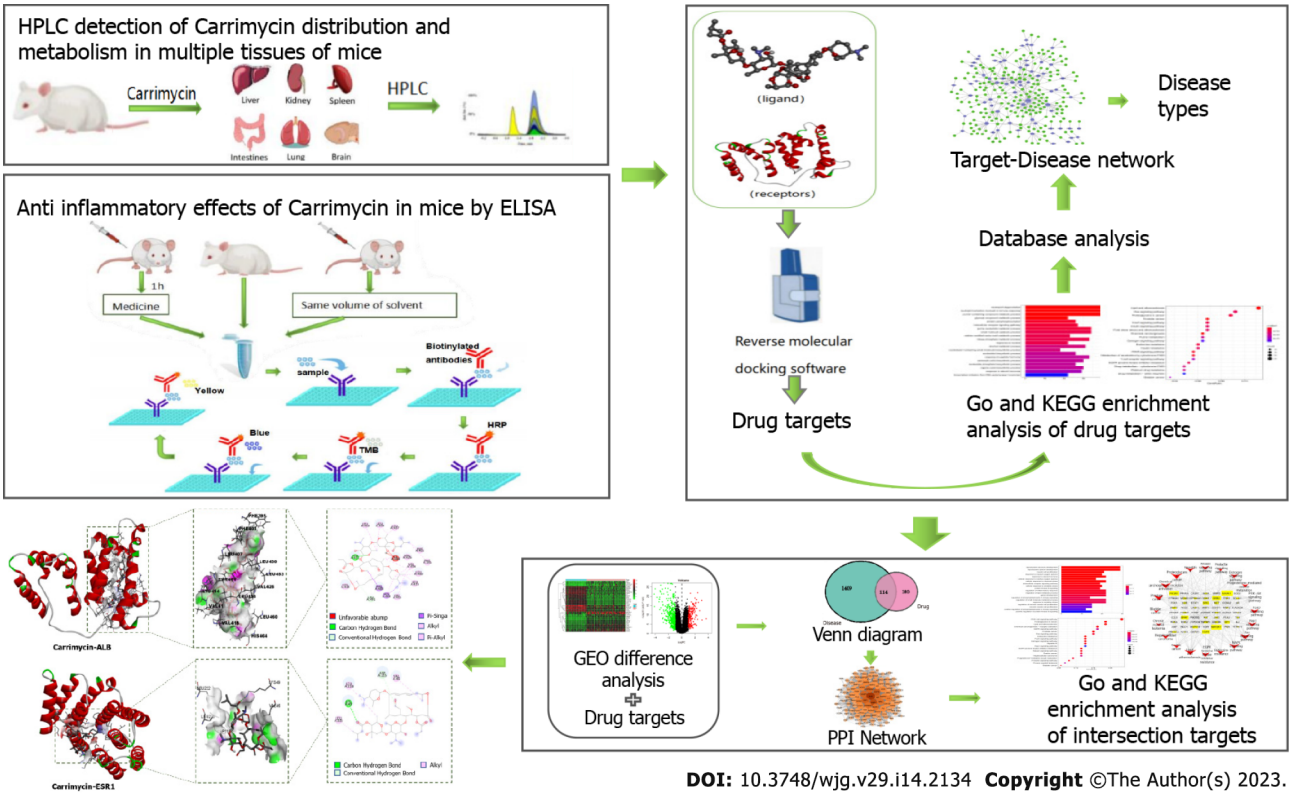

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Figure 1 Experimental flow chart. The distribution and metabolism of carrimycin in various mouse tissues and organs were assessed by hyphenated to liquid chromatography, and the effect of carrimycin on inflammatory factors in these tissues and organs was detected by enzyme-linked immunosorbent assay. Reverse molecular docking technology was used to screen for the targets of carrimycin, and the predicted target was enriched and analyzed to obtain data on the related diseases. Then, the key targets and associated pathways for the treatment of liver cancer were analyzed by network pharmacology. HPLC: Hyphenated to liquid chromatography; ELISA: Enzyme-linked immunosorbent assay; GO: Gene Ontology; KEEG: Kyoto Encyclopedia of Genes and Genomes; PPI: Protein-protein interaction.

added, the solution was mixed, and 1.2 mL of alkaline reagent (0.05 M sodium carbonate solution) was then incorporated. Next, 12 mL of ethyl acetate-isopropyl alcohol (95:5, v/v) was added for extraction, and the solution was vortexed for 3 min and shaken at 50 rpm for 10 min. After centrifugation at 2000 rpm for 10 min, 12 mL of the upper organic phase was taken from 6 centrifuge tubes and combined in a new centrifuge tube. This tube was placed in a metal bath at 40 °C and volatilized with nitrogen for accelerated solvent extraction. When approximately 300-500 μL of liquid remained, the EP tube was vortexed for 10 s, and the liquid was added to a 2 mL EP tube and dried under nitrogen flow. The final sample was dissolved in 100 μL of mobile phase, vortexed, and injected into the instrument after passing through a 0.45 μm nylon membrane, and the chromatogram was recorded.

Data processing: According to the standard curve, we calculated the changes in drug content in various tissues and organs at 0, 0.5, 2.5, 4.5, 12 and 24 h after administration and drew a histogram of these changes in the tissues at different times after administration. Excel 2016 statistical, drawing and calculation software was used.

Anti-inflammatory effect of CAM in mice

Drug preparation: Drug preparation was carried out as follows. Several doses of CAM were added to polyethylene glycol 400 and Tween 80, and the combination was mixed well by oscillation. Then, appropriate amounts of distilled water were added to prepare CAM solutions with high, medium and low concentrations. The model drug was prepared as follows. A 1.82 mg/mL solution of the model drug azithromycin (Aladdin Reagent Co., Ltd) was diluted with a small amount of anhydrous ethanol, and then water was added to produce a solution with a final ethanol content of 10%.

Reagent preparation: PBS (pH 7.35) and Tween 20 were used to prepare a washing solution composed of PBS with 0.05% Tween 20 (if crystals formed in the buffer concentrate, the solution was gently heated until they had completely dissolved). PBS (10 ×) was diluted 1:10 in deionized water to prepare coating buffer (1 ×). The concentrated diluent (5) was diluted 1:5 in deionized water to prepare a 5 × ELISA/ELISPOT diluent.

Standard preparation was carried out as follows. The recombinant mouse interleukin-β (IL-1β) standard was dissolved in distilled water, the volume of which was indicated on the label of the vial. The standard solution was prepared 10-30 min in advance and mixed well to ensure complete and uniform dissolution (concentration of recombinant standard = 1000 pg/mL). Antibody detection was performed by diluting the detection antibody (250 ×) 1:250 in ELISA/ELISPOT diluent (1 ×). The enzyme was prepared by diluting HRP concentrate (100 ×) 1:100 in ELISA/ELISPOT diluent (1 ×).

Establishment of the animal model: Kunming mice were purchased from the Experimental Animal Center of Jiangsu University using animal certificate No. 201929470. A total of 144 mice were used, and they were adapted to the environment in the laboratory before the experiment. Male Kunming mice weighing approximately 24 g were randomly divided into 6 groups, including the normal group, model group, low-dose group (30 mg/kg), middle-dose group (60 mg/kg), high-dose group (120 mg/kg), and azithromycin group (37.9 mg/kg). The effect of each component was assessed at 0 h, 0.5 h, 2.5 h, 4.5 h, 12 h, 24 h, 48 h and 72 h, and three mice were used at each time point. *Staphylococcus aureus* (*S. aureus*) was inoculated into 50 mL of culture medium (washed with PBS and resuspended in 1000 μL of PBS with normal saline. *S. aureus* (3×10^8 CFU·mL^{-1}) was injected into the mice *via* the tail vein at 100 μL *S. aureus*·24 g^{-1} mouse body weight. Drugs were administered one hour later. The mice in the normal group were not administered drugs or injected with bacteria. The mice in the model group were not treated with drugs. The mice in the CAM and azithromycin groups were intragastrically administered 500 μL of the appropriate compound, with a double dose given on the first day. The mice in the model group were given the same volume of solvent. The mice were sacrificed in batches at different time points after administration.

Detection of inflammatory factors: The mice were sacrificed after orbital blood was taken, and each tissue and organ was weighed by an electronic balance. Fifty milligrams of each tissue was placed into a 1.5 mL EP tube, and then 1 mL of precooled PBS and magnetic beads were added. Then, the tissue was homogenized (300 Hz, 30 s). After standing on ice for 30 min, the mixture was centrifuged at 4 °C (10000 g, 10 min), and the supernatant was taken as the test sample. The IL-4 and IL-1β levels in the supernatant were measured according to the instructions of the kit (Thermo Fisher Scientific, China).

Network pharmacology methods

Screening for drug targets and related diseases: First, multiple data platforms, such as the PDB (https://www.rcsb.org) and TTD (http://db.idrblab.net/tt), were used to construct the target protein library of CAM. Docking software, such as INVDOCK and tarfisdock, was used to connect CAM with all targets in the protein library to predict its target. Additionally, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed. Combining these data with those from GeneCard (https://www.genecards.org), UniProt (https://www.UniProt.org), TCMSP (https://old.tcmsp-e.com/tcmsp.php) and other databases, the predicted target proteins were analyzed one by one, and a relationship diagram was drawn with Cytoscape. This established the target protein-disease type network (degree > 3) to determine the most relevant diseases, which were consistent with the results from enrichment analysis.

Screening for disease targets and drug-disease intersection targets: The most relevant disease from the above analyses was liver cancer, which was selected for further study. Using the GEO database (https://www.ncbi.nlm.nih.gov/geo/) with 'liver cancer' as the search term, the GSE121248 dataset and GPL570 platform were downloaded. logFC ≥ 1 and adj *P* value < 0.05 were the screening conditions to identify the differentially expressed genes. Moreover, the liver cancer-related targets were integrated with the search term 'liver cancer' in the GeneCards database (https://www.genecards.org/), and the genes differentially expressed in liver cancer were combined to obtain a comprehensive list of liver cancer targets. The target gene of CAM in liver cancer was determined by intersection of the target of CAM and the target of liver cancer.

Construction of the protein-protein interaction network and enrichment analysis: The intersecting target gene of CAM and liver cancer was introduced into the STRING database (https://string-db.org) to obtain protein interaction information, and the research species was set as *Homo sapiens*. The lowest interaction score used to obtain the protein-protein interaction (PPI) network was 0.009. Then, Cytoscape 3.7.2 topology analysis of the PPI network was performed, and the core target of CAM in liver cancer was obtained. Then, GO and KEGG enrichment analyses were performed by R software ($P < 0.05$).

Molecular docking verification and visual analysis: ChemOffice software was used to generate the 3D structure of CAM, which was loaded into AutoDock Tools 1.5.6 as the docking ligand. The crystal structures of albumin (ALB) (PDB ID: 1YSX), estrogen receptor 1 (ESR1) (PDB ID: 3HA8), epidermal growth factor receptor (EGFR) (PDB ID: 5Y9T), caspase 3 (CASP3) (PDB ID: 4JJE), and androgen receptor (AR) (PDB ID: 2PIT) were downloaded from the PDB database (http://www.rcsb.org). AutoDock Tools 1.5.6 software was used to remove the water and hydrogen atoms and to perform ligand separation, and the resulting protein structures were stored as the acceptors. AutoDock Vina

1.1.2 was used for molecular docking. The conformation with the lowest binding energy from all docking runs was selected as the docking conformation, and Discovery Studio 2016 Client software was used to visualize the docking conformation.

RESULTS

Distribution and metabolism of CAM in various tissues of mice

There was a good linear relationship between isovaleryl SPM III in the approximate concentration range of 0.1-200.0 μg/mL. The regression equations of the correlation curves in the liver, intestine, kidney, lung, spleen and brain were Y = 0.1977C-0.1072 (R^2 = 0.9953), Y = 0.1929C-0.3317 (R^2 = 0.9970), Y = 0.1447C-0.3909 (R^2 = 0.9982), Y = 0.2512C-0.7149 (R^2 = 0.9942), Y = 0.1748C-0.7313 (R^2 = 0.9966) and Y = 0.2100C-0.9697 (R^2 = 0.9957), respectively, each with an RSD value less than 2%. Thus, the results are reliable and reproducible. The contents of CAM in different tissues and organs 24 h after intragastric administration to mice is shown in Figure 2. The results showed that 2.5 h after CAM treatment, the maximum drug concentration was reached in the liver, kidney, lung and spleen. In the intestine, the maximum drug concentration was reached 0.5 h after administration, and CAM was not detected in the brain.

Anti-inflammatory effect of CAM in mice

IL-4 and IL-1β are common inflammatory cytokines; thus, ELISA was used to analyze the IL-4 and IL-1β levels in different mouse organs and tissues to determine the anti-inflammatory effect of CAM (Figure 3). CAM significantly reduced IL-4 levels in the lung and kidney and especially the liver and spleen. CAM also significantly reduced IL-1β levels in the spleen, liver, and kidney and particularly the small intestine and lung.

Screening of CAM targets and related diseases

From the conformation analysis and scoring results, the targets of CAM were selected. After excluding the ineffective and repeated data, 293 targets were obtained. The 293 predicted target proteins were analyzed by GO and KEGG enrichment analyses. The output GO enrichment analysis results included three pathways: Biological processes (BP) (Figure 4A), cellular components (CC) (Figure 4B), and molecular functions (MF) (Figure 4C). The core targets of CAM are mainly involved in the response to neutrophil degranulation and the immune response and in neutrophil activation, purine-containing compound metabolism, glycosyl compound metabolism and protein autophosphorylation. The molecular functions mainly involve carboxylic acid binding, organic acid binding, protein tyrosine kinase activity and serine endopeptidase activity. In terms of cell composition, the main targets involved vesicle cavity, cytoplasmic vesicle cavity, and collagen-containing extracellular matrix (ECM) pathways. The KEGG enrichment pathway analysis results in Figure 4D demonstrate that the pathways in which CAM participates in disease treatment mainly include the Ras, FoxO, and insulin signaling pathways.

The 293 predicted target proteins were analyzed one by one, and the data from multiple databases were combined to screen 77 main targets (circles) and 22 main diseases (rectangles) (Figure 5). The targets with the highest degrees were EGFR (degree = 7), mesenchymal epithelial cell transforming (MET) (degree = 7), matrix metalloproteinas (MMP)-2 (degree = 7), and ESR1 (degree = 6). The main diseases involved were liver cancer (degree = 18), breast cancer (degree = 13), lung cancer (degree = 12), and prostate cancer (degree = 10).

Screening liver cancer targets and their intersection with those of CAM

First, 580 differentially expressed genes in liver cancer were screened using the GEO database to construct a volcano plot (Figure 6A). Genes with significant upregulation and downregulation were selected to draw a heatmap (Figure 6B). Among them, 167 differentially expressed genes were upregulated in hepatocellular carcinoma, such as AKR1B10, SPINK1, ROBO1, CCL20, and RRM2, and 413 differentially expressed genes were downregulated in hepatocellular carcinoma, such as SRPX, CLEC4 M, CLEC4G, CLEC1B, OIT3, and CRHBP. After integration with the liver cancer-related targets from the GeneCards database, a total of 943 liver cancer-related targets were retrieved, and duplicate values were removed from the combined dataset to obtain 1523 liver cancer disease targets.

R software was used to match and map 294 CAM target genes with the 1523 liver cancer disease targets, resulting in 114 intersecting target genes, including transthyretin, bone morphogenetic protein 2, ALB, Glutathione S-transferase P1, PNP, and PLG. These results are shown in the Venn diagram in Figure 6C.

Establishment of the PPI network and enrichment analysis

The obtained PPI network is shown in Figure 6D. The PPI network was used with Network Analyzer in Cytoscape v3.7.2 for degree of freedom, mediation and centrality algorithm topology analysis to select the greater than the average targets as the key targets. Only the top 15 targets were retained (Table 1).

Table 1 Key target genes of carrimycin in the treatment of liver cancer

Gene name	Degree	Betweenness centrality	Closeness
ALB	110	0.04074405	0.97413793
ESR1	102	0.02219136	0.91129032
EGFR	97	0.01545146	0.87596899
IGF1	95	0.01457077	0.86259542
HSP90AA1	93	0.02011442	0.84962406
CASP3	93	0.01307351	0.84962406
AR	91	0.01215915	0.83703704
SRC	90	0.01137002	0.83088235
PPARG	89	0.01582869	0.82481752
MAPK1	88	0.00943128	0.81884058
IL2	86	0.01154328	0.80714286
SOD2	84	0.01534439	0.79577465
MAPK14	84	0.00918383	0.79577465
NOS3	83	0.0095422	0.79020979
MMP9	83	0.00820256	0.79020979

ALB: Albumin; ESR1: Estrogen receptor 1; EGFR: Epidermal growth factor receptor; IGF: Insulin-like growth factor; HSP: Heat shock protein; CASP3: Recombinant caspase 3; AR: Androgen receptor; SRC: Sarcoma gene; PPARG: Peroxisome proliferator activated receptor gamma; MAPK1: Mitogen-activated protein kinase 1; IL: Interleukin; SOD: Superoxide dismutase; NOS: Nitric oxide synthase; MMP: Matrix metallo proteinase.

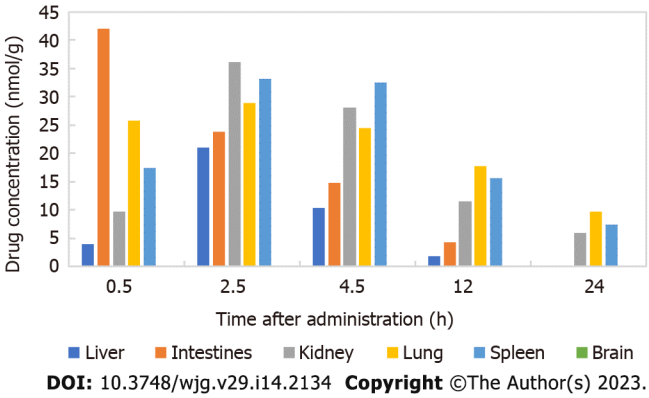

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Figure 2 Concentrations of carrimycin in different tissues and organs after intragastric administration to mice (nmol/g). 2.5 h after carrimycin treatment, the maximum drug concentration was reached in the liver, kidney, lung and spleen. In the intestine, the maximum drug concentration was reached 0.5 h after administration, and carrimycin was not detected in the brain.

Finally, we screened the core target proteins of CAM in liver cancer: ALB, Insulin-like growth factor 1 (IGF1) (somatomedin C), ESR1, EGFR, AR, mitogen-activated protein kinase 1 (MAPK1), MAP2K1 (gene encoding mitogen-activated protein kinase 1), CASP3, *etc.*

The GO enrichment analysis results included the terms BP (Figure 7A), MF (Figure 7B) and CC (Figure 7C). The core targets of the active components of CAM were mainly enriched in processes including reproductive structure development, reproductive system development, muscle cell proliferation, response to reactive oxygen species, cell response to reactive oxygen species, molecular functions, nuclear receptor activity, transcription factor activity, and ligand activation, while the cellular components included vesicle cavity, secretion granules, cytoplasm vesicle cavity, membrane microregion, membrane region and ECM.

KEGG pathway enrichment analysis of the key targets of CAM in liver cancer performed in R resulted in 140 signaling pathways ($P < 0.05$). The top 20 pathways were selected to create a bubble map (Figure 7D). The main pathways involved in CAM treatment of liver cancer include the PI3K-Akt, MAPK, Ras, FoxO, estrogen, and hepatitis B pathways and others. CAM acts on multiple targets in the

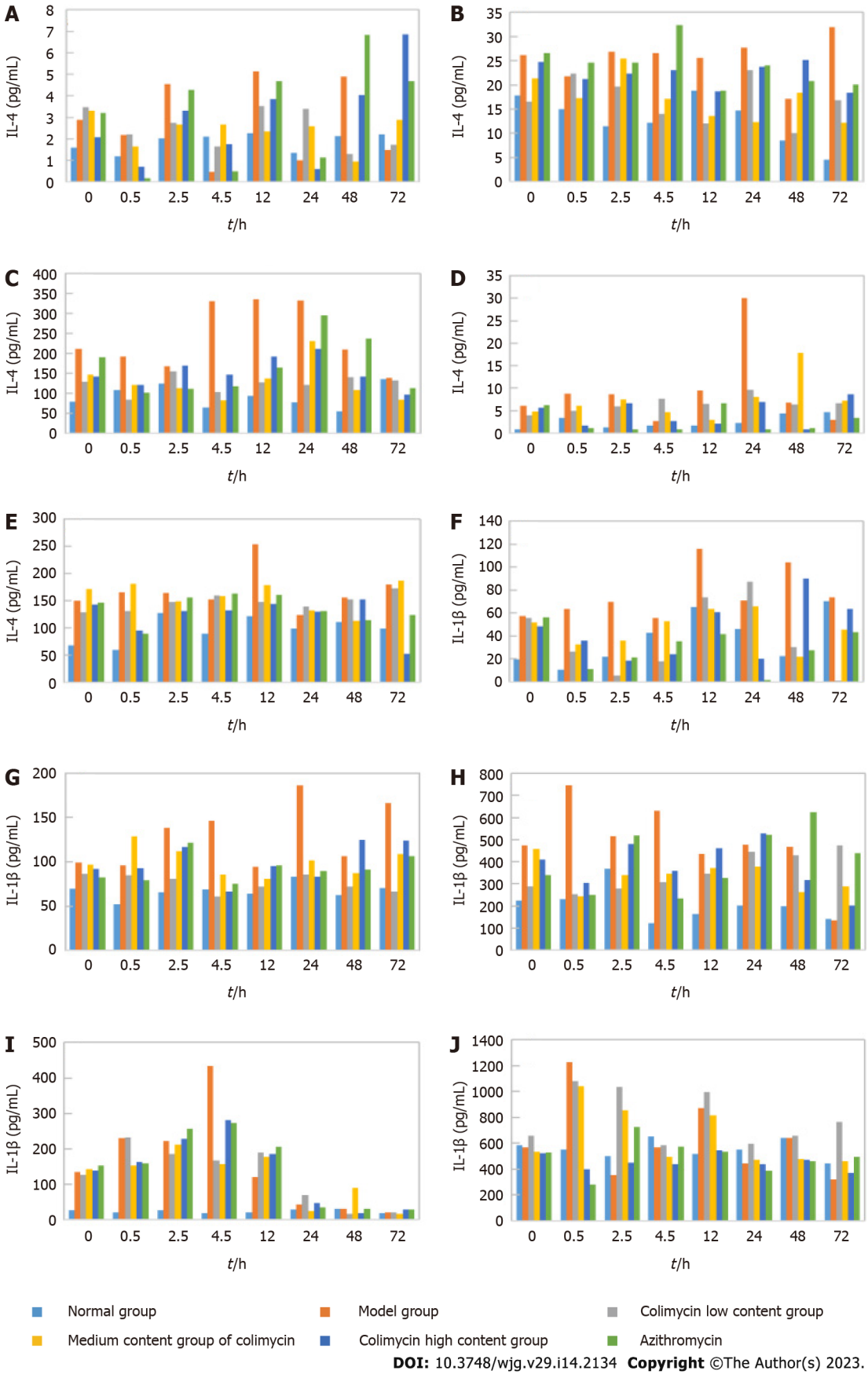

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Figure 3 Anti-inflammatory effect of carrimycin in mice detected by enzyme-linked immunosorbent assay. A: Interleukin-4 (IL-4) expression in the intestine; B: IL-4 expression in the lung; C: IL-4 expression in the liver; D: IL-4 expression in the spleen; E: IL-4 expression in the kidney; F: IL-1β expression in the intestine; G: IL-1β expression in the lung; H: IL-1β expression in the liver; I: IL-1β expression in the spleen; J: IL-1β expression in the kidney. IL: Interleukin.

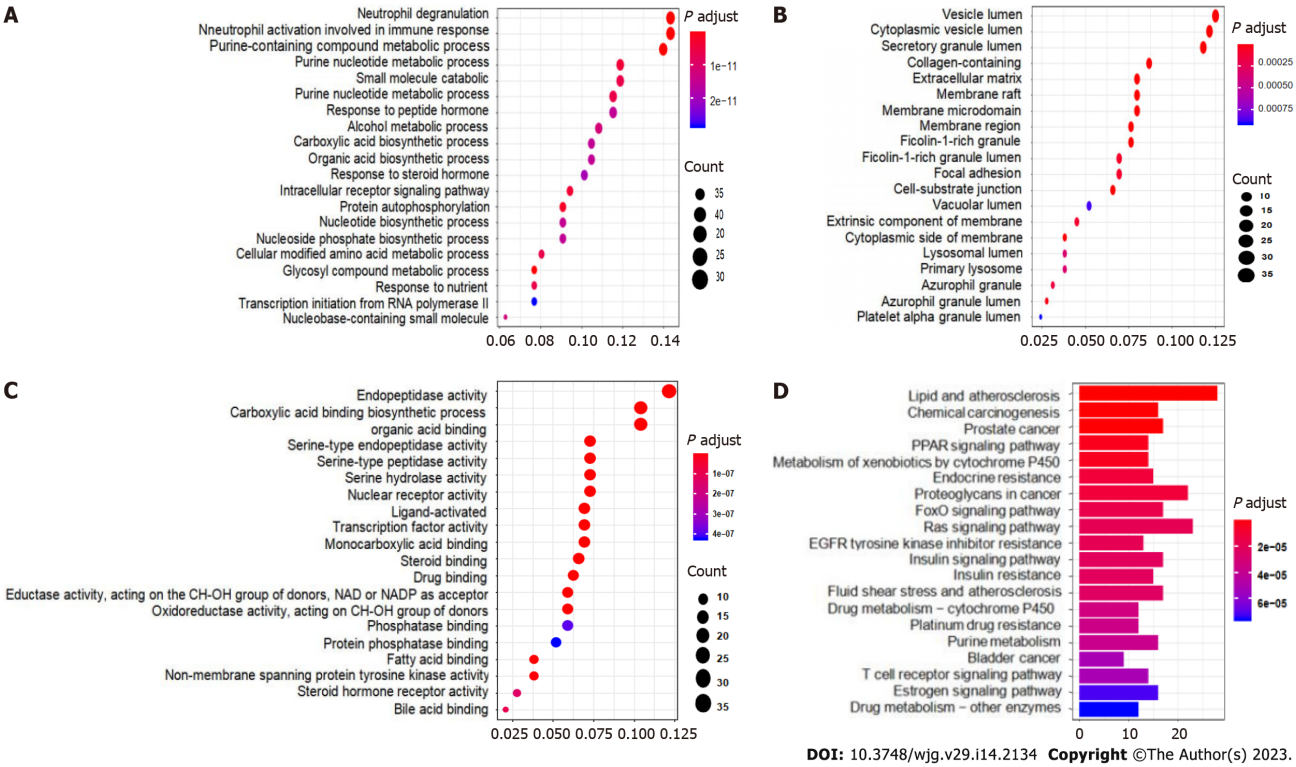

Figure 4 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analyses of the targets of carrimycin. A: Biological processes; B: Cellular components; C: Molecular functions; D: Kyoto Encyclopedia of Genes and Genomes enrichment analysis of carrimycin showing that the main pathways in which carrimycin is involved in the treatment of diseases include the Ras, FoxO, and insulin signaling pathways. PPAR: Peroxisome proliferator-activated receptor.

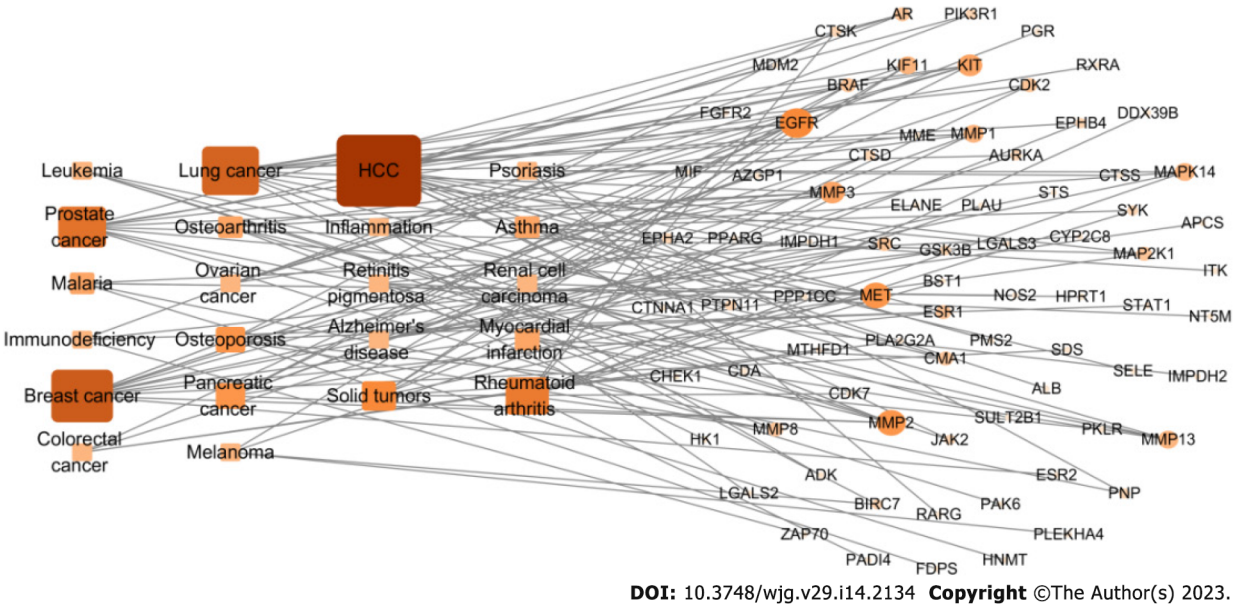

Figure 5 Target-disease network of carrimycin. The 293 predicted target proteins were analyzed one by one by combining multiple databases to screen 77 main targets (circles) and 22 main diseases (rectangles). The main diseases involved were liver cancer (degree = 18), breast cancer (degree = 13), lung cancer (degree = 12), and prostate cancer (degree = 10). HCC: Hepatocellular carcinoma.

PI3K-Akt signaling pathway to produce effects against liver cancer by activating or inhibiting certain proteins in this pathway (Figure 8A).

According to the relationships between the key targets of action and the signaling pathways, a key target-pathway network map was constructed using R language and Cytoscape v3.7.2 (Figure 9). The graph has 80 nodes and 307 edges. The nodes include 60 related targets indicated by yellow rectangles and 20 signaling pathways indicated in red type V. The node size corresponds to the degree value; the

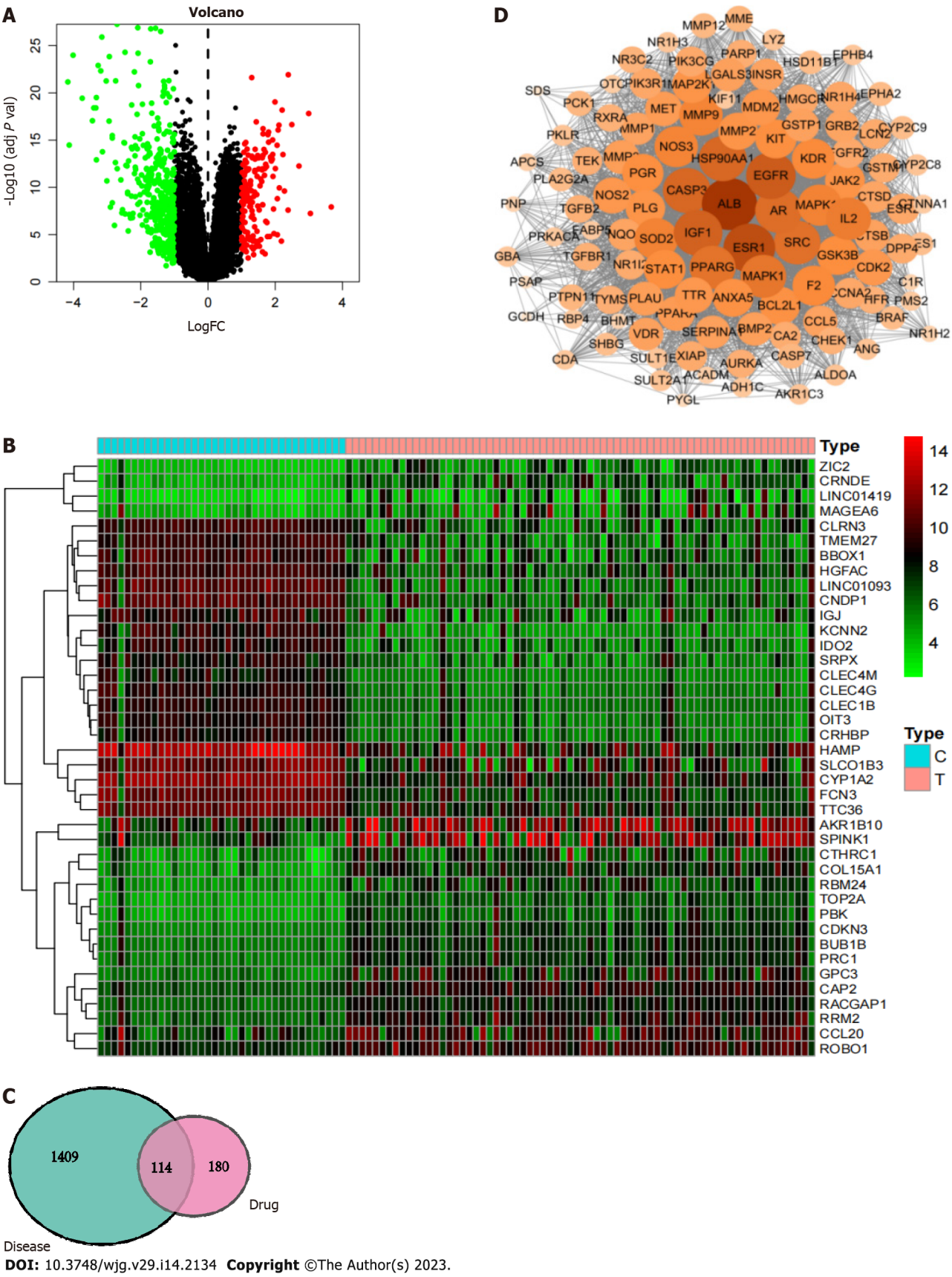

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Figure 6 Network pharmacology illustration. A: Differential gene volcano plot; B: Differential gene heatmap. Up- and downregulated differentially expressed genes can be derived from volcano plots and heatmaps; C: Venn diagram of the common targets of carrimycin and liver cancer. The common disease-drug targets were determined from this map; D: Protein-protein interaction network of carrimycin and acquisition of the core targets of carrimycin in liver cancer.

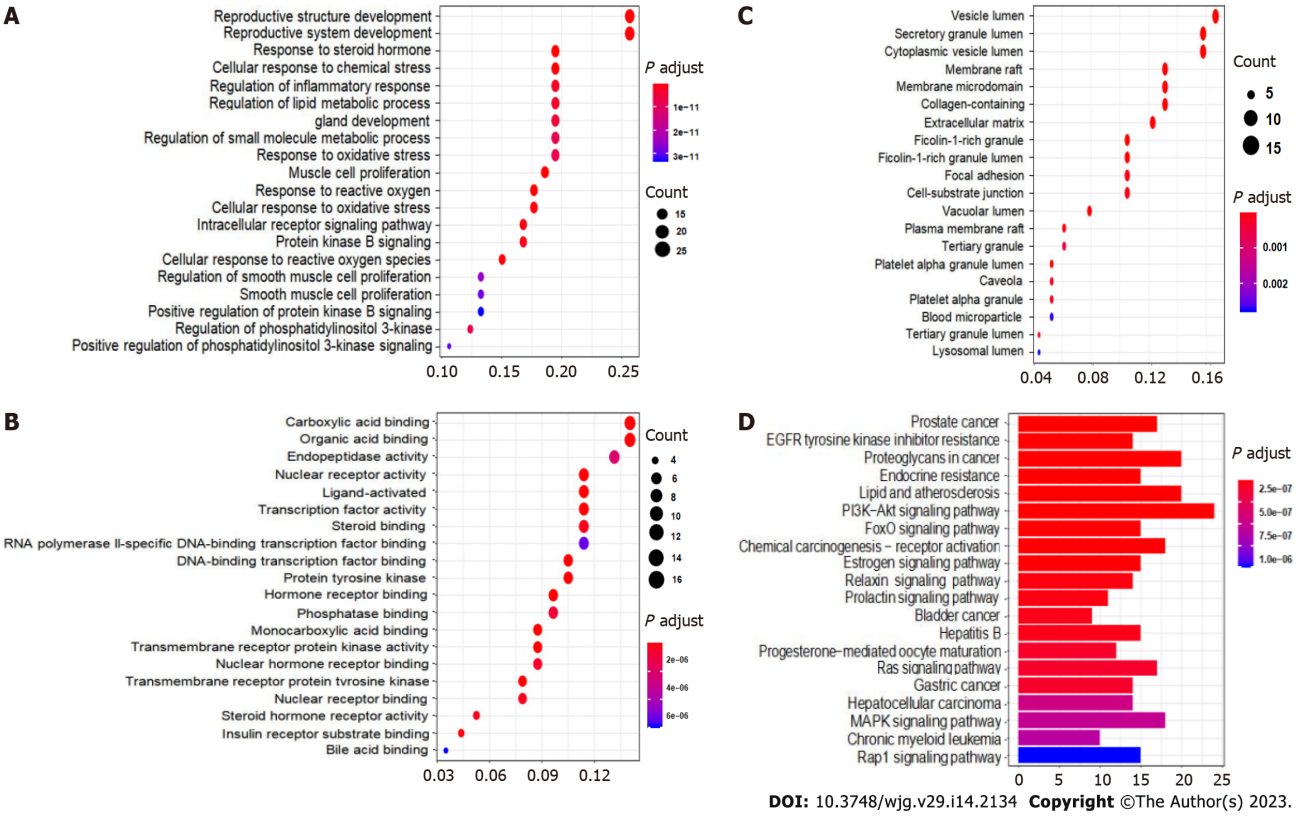

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Figure 7 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analysis results of the intersecting targets. A: Biological processes; B: Molecular functions; C: Cellular components; D: Kyoto Encyclopedia of Genes and Genomes enrichment analysis of the key targets of carrimycin in the treatment of liver cancer. The main pathways involved include the PI3K-Akt, MAPK, Ras, and FoxO signaling pathways.

larger the node is, the more important this factor is the network. Therefore, CAM can regulate multiple biological pathways and treat liver cancer by acting on multiple targets, such as MAPK1, MAP2K1, phosphoinositide-3-kinase, regulatory subunit 1, EGFR and MET.

Molecular docking of CAM with key targets

In molecular docking, it is generally thought that a stronger affinity between a ligand and a receptor gives a lower binding energy, and hence, a greater probability of interaction. When the obtained binding energy is less than zero, the receptor and ligand can bind spontaneously. The binding energies for each receptor-CAM interaction are shown in Table 2. All of the binding energies are lower than -5.0 kcal/mol, indicating that CAM has a good ability to bind to these targets. To further study these interactions, the relationships between CAM and these targets were plotted and analyzed (Figure 10).

DISCUSSION

In this study, reverse molecular docking and network pharmacology were used to screen the targets of action and related diseases of CAM, analyze the function and pathway enrichment of the targets of action, and establish a network of the key target proteins in disease to determine the most relevant disease types from the enrichment analysis results. In addition, the distribution and metabolism of CAM and its effect on inflammatory factors in various tissues and organs were examined, and the mechanism by which CAM can treat disease was systematically explored.

According to the HPLC analysis of the distribution and metabolism of CAM in mice, the maximum concentration of CAM in the intestine was reached 0.5 h after administration and that in the liver, kidney, lung and spleen occurred 2.5 h after administration. CAM was eliminated faster from the liver and intestine than from the kidney, lung and spleen. CAM was not detected at any time point in the brain, indicating that this compounds does not easily cross the blood-brain barrier.

IL-4 is a common inflammatory cytokine that is involved in the occurrence of inflammation, and the inflammatory microenvironment is closely related to the deterioration of tumor cells[5]. IL-4 inhibits the inflammatory response in advanced liver cancer, mainly because an increase in its levels can weaken the extent of the inflammatory response and viral replication in the liver[6]. IL-1β is a proinflammatory cytokine derived from monocytes and macrophages that can participate in the processes of infection,

Table 2 Binding energy between carrimycin and the key target

Key target	Binding energy (kcal/mol)
ALB	-9.3
ESR1	-5.8
EGFR	-6.5
CASP3	-6.1
AR	-5.6
HSP90AA1	-5.0
IGF1	-5.1
MAPK1	-6.7
MAP2K1	-5.2
IL2	-5.8

ALB: Albumin; ESR1: Estrogen receptor 1; EGFR: Epidermal growth factor receptor; IGF: Insulin-like growth factor; HSP: Heat shock protein; CASP3: Recombinant caspase 3; AR: Androgen receptor; MAPK1: Mitogen-activated protein kinase 1; IL: Interleukin.

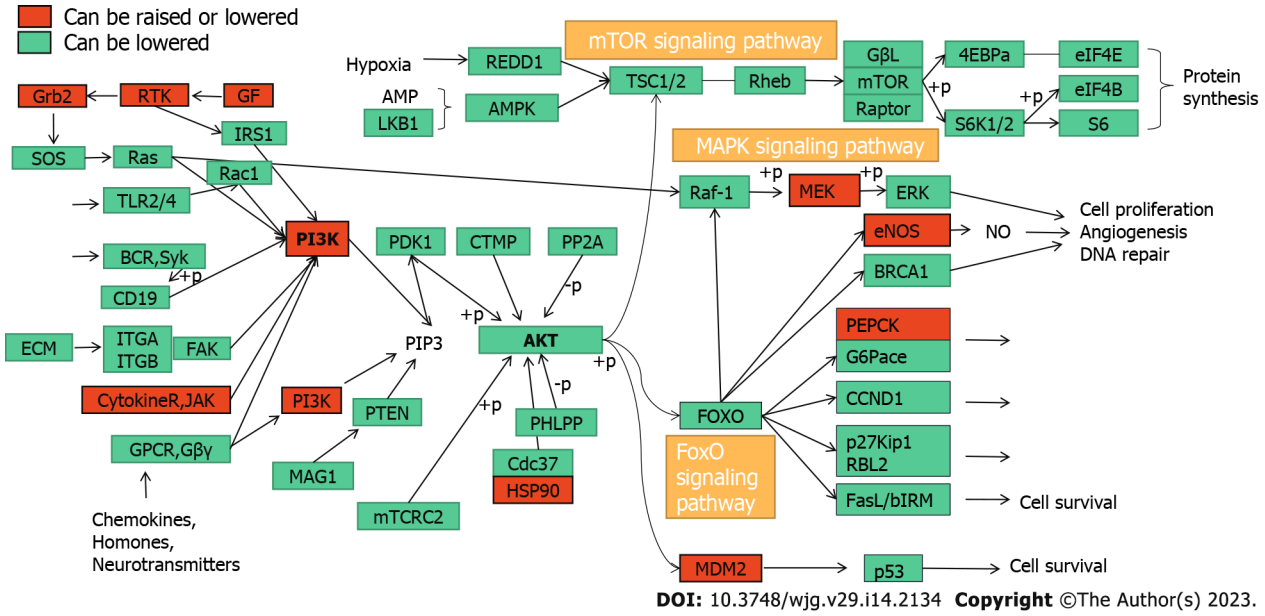

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Figure 8 PI3K-Akt signaling pathway regulation. This plot shows the genes that are upregulated or downregulated (red) and downregulated (green) by carrimycin in the PI3K-Akt pathway. Grb2: Growth factor receptor bound protein 2; RTK: Receptor tyrosine kinase; GF: Growth factor; IRS1: Insulin receptor substrate 1; TLR: Toll-like reCEPT; BCR: Breakpoint cluster region; ECM: Extracellular matrix; ITGA: Integrin subunit alpha; FAK: Focal Adhesion Kinase; JAK: Janus kinase; GPCR: G protein-coupled receptor; AMP: Adenosine Mono Phosphate; LKB1: Liver kinase B1; REDD1: regulated in development and DNA damage responses-1; AMPK: AMP-activated protein kinase; TSC: tuberous sclerosis; Rheb: Right Hand Equipment Bay; PDK1: Pyruvate dehydrogenase kinase, isozyme 1; CTMP: Carboxy terminal modulator protein; PP2A: Protein phosphatase 2A regulatory subunit; PIP: Phosphoinositide-binding protein; PTEN: Phosphatase and tensin homolog; PHLPP: PH domain leucine-rich repeat protein phosphatase; CDC: Cell division cycle gene; HSP: Heat shock protein; RAF: Rheumatoid Arthritis Factor; MEK: Mitogen-activated protein; ERK: Extracellular regulated protein kinases; ENOS: Endothelial nitric oxide synthase; BRCA: Breast cancer susceptibility genes; PEPCK: Phosphoenolpyruvate carboxykinase; FOXO: Forkhead Box O; MDM2: Murine double minute 2; CCND1: Recombinant Cyclin D1; RBL2: Retinoblastoma-like 2; MDM2: Murine double minute 2; mTOR: Mammalian target of rapamycin (a protein).

inflammation, immune injury, tissue destruction and so on[7]. Moreover, relevant studies have shown that IL-1β may be involved in the pathogenesis and evolution of tumors[8]. Therefore, the effects of CAM on inflammatory factors in various tissues and organs were examined. CAM was found in the lung, kidney, liver and spleen and had particularly affected the IL-1β levels in the spleen, liver, and kidney and especially the small intestine and lung.

The network pharmacology results showed that CAM is involved in disease treatment through processes including neutrophil degranulation, neutrophil activation, immune response participation, purine-containing compound metabolism, glycosyl compound metabolism and protein autophos-

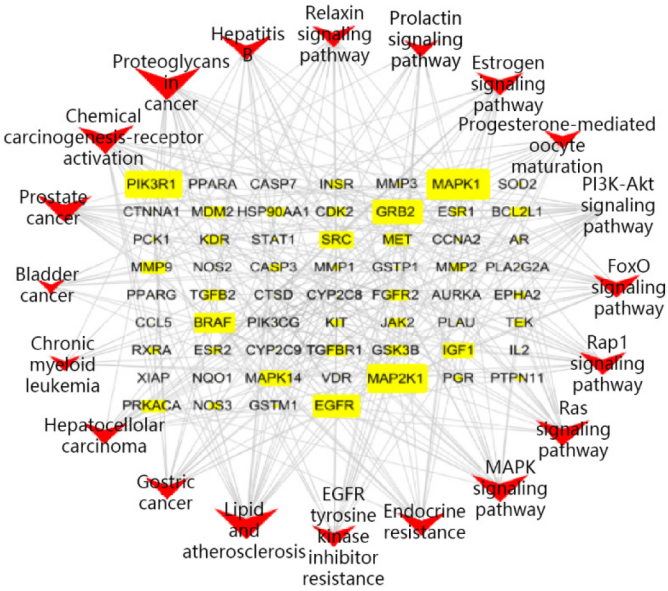

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Figure 9 Target-pathway network of carrimycin. The nodes include 60 related targets displayed as yellow rectangles and 20 signaling pathways indicated in red type V. The main targets acting on the pathway include MAPK1, MAP2K1, and EGFR, *etc.*

phorylation. Its molecular functions mainly entail carboxylic acid binding, organic acid binding, protein tyrosine kinase activity, endopeptidase activity and serine endopeptidase activity. In terms of cell composition, CAM is mainly involved in vesicle cavity, cytoplasmic vesicle cavity, secretory granule cavity and collagen-containing ECM.

Neutrophils are a kind of natural immune cell, and with the deepening of research, the relationship between neutrophils and tumors has attracted attention. Many studies have shown that neutrophils have potential antitumor functions[9] and cytotoxic effects on tumor cells[10]. Neutrophils are also an important member of the human immune system, playing an important role in the defense mechanisms of the human body[11]. Neutrophils release proteolytic substances such as myeloperoxidase (MPO), neutrophil elastase (NE), and tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) and active substances with biological effects related to their degranulation functions[12]. MPO and NE are present in the celestial green granules of neutrophils, and their extracellular release *via* degranulation produces biological effects[13]. MPO can catalyze the formation of HOCl *in vivo*, and HOCl and NE can inhibit tumor growth at certain concentrations[14]. TRAIL is a member of the TNF superfamily, whose receptors are expressed and distributed on the surfaces of many tumor cells. Therefore, for some tumor cells, TRAIL can not only induce tumor cell apoptosis but also inhibit tumor angiogenesis through selective killing effects[8].

Purines are heterocyclic aromatic compounds. Since Noell and Robins[15] discovered 2-amino-6-alkyl thiopurine derivatives as antitumor drugs in 1962, considerable efforts have been made to develop purine derivatives with antitumor activity over the last few decades[16]. Purine compounds, such as mercaptopurine, which was discovered earlier, and nelarabine and clofarabine, which were discovered later, have been used as antitumor drugs for many years[17]. Therefore, the metabolic processes of purine-containing compounds have inhibitory effects on tumors.

Protein phosphorylation and dephosphorylation regulate almost all life activities, such as cell signal transduction, differentiation, growth, and apoptosis. Approximately 30% of the proteins in the body can undergo different types of phosphorylation[18]. Abnormal protein phosphorylation often leads to abnormal cellular activities and even cell damage or carcinogenesis[19]. Abnormalities in cellular activities, such as those involved in tumor growth, differentiation, apoptosis and movement, are all accompanied by abnormalities in protein phosphorylation in their corresponding cellular signal transduction pathways.

KEGG analysis of the enriched pathways of the CAM targets showed that the pathways involved in the treatment of diseases mainly included the Ras, FoxO, insulin, estrogen, and peroxisome proliferator-activated receptor (PPAR) signaling pathways. The Ras signaling pathway was named because this protein is a common mediator of the signal transduction processes of many growth factors. It has been reported that the Ras family diabetes-related gene RRAD can inhibit the proliferation, aerobic glycolysis and apoptosis of hepatocellular carcinoma cells by downregulating ACTG1 levels[20]. FoxO transcription factors shuttle through and outside the nucleus to regulate pathways involved in cell differentiation, proliferation, and stress, cell cycle metabolism, and tumor inhibition. The pathogenesis of hepatitis B-related liver cancer is closely related to activation of the FOXM1/FoxO3a signaling pathway[21]. Insulin signaling affects energy metabolism and growth, and this signal is transmitted

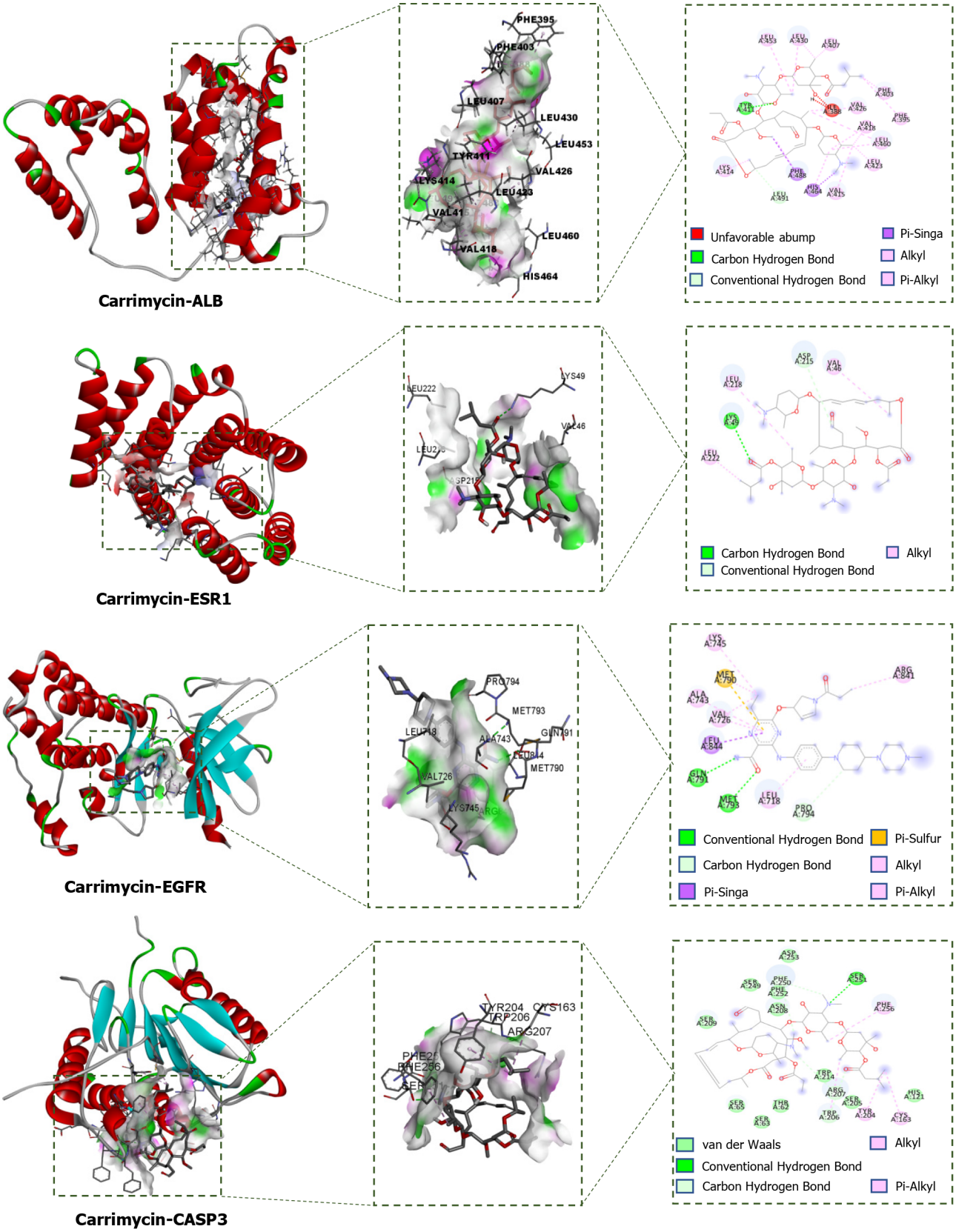

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Figure 10 Molecular docking poses of the core targets in liver cancer. In each of the docking simulations, hydrogen bonds were found between carrimycin and the four targets. The binding energies were all under -5, which suggested that the docking results were good and that the drug could bind to the target. ALB: Albumin; ESR1: Estrogen receptor 1; EGFR: Epidermal growth factor receptor; CASP3: Recombinant caspase 3.

through the Akt branch. The Akt signaling pathway can inhibit tumor cell proliferation, induce apoptosis and downregulate the expression of Bcl-2[22]. Akt is a regulatory molecule downstream of PI3K. Akt is activated after being phosphorylated and can affect the expression of the downstream

metastasis-related gene MMP-9, the apoptosis-related gene Bcl-2 and the proliferation-related gene cyclin D1[23]. Akt plays an important role in regulating cell proliferation, metastasis and apoptotic processes, and its hyperactivation is closely related to the development of various tumors, including liver cancer[24]. Dysregulation of the estrogen signaling pathway is an important factor in the development of many malignant tumors, such as those found in breast cancer, liver cancer and lung cancer. Estrogen exerts its physiological and pathological functions by binding to the estrogen receptor (ESR). ESR-α36 expression is upregulated in hepatocellular carcinoma, so ESR-α36 may be involved in the rapid estrogen signal transduction that mediates hepatocarcinogenesis[25]. Abnormal expression of ESR-α36 in the liver is related to hepatocyte proliferation. Estrogen passes through the ESR-α36 and EGFR/SRC axes to induce MAPK/ESR-K phosphorylation and promote the expression of cyclin D1. ESR-α36 interacts with the EGFR/SRC/SHC complex through the EGFR/ESR-α36/ERK signaling axis and mediates rapid estrogen signal transduction in hepatoma cells. Thus, the ESR-α36/EGFR signaling loop is a potential target for the development of a new approach to treat liver cancer, as the ESR-α36/EGFR positive regulatory loop plays an important role in the maintenance and positive regulation of hepatoma cells[26]. PPAR-γ is an extremely important regulator of fat and glucose metabolism and plays an important role in autoimmune responses and inflammatory processes[27]. PPAR-γ is expressed in bronchial airway epithelial cells in situ and can inhibit the activity of these cells, activate endogenous PPAR-γ, and antagonize the inflammatory response. Zhu *et al*[28] suggested that PPAR-γ can affect the transport of macrophages and increase their phagocytosis, promoting the occurrence of a practical immune response. PPAR-γ ligand stimulation in tumor cells and tissues can have a major antitumor effect[29].

According to the analysis of CAM targets, EGFR intersects with multiple signaling pathways, including those in healthy tissues and liver cancer, breast cancer, kidney cancer, colon cancer and other tumor cells[30]. When EGFR binds to its ligand, the receptor can dimerize specific tyrosine residues can be phosphorylated. These phosphorylated tyrosine residues can recruit other signaling molecules to initiate the downstream signal transduction pathway that mediates the proliferation, metastasis and drug resistance of tumor cells. In liver cancer tissues, the expression of EGFR is significantly upregulated. For example, when EGFR is activated by its ligand, it can mediate the growth, proliferation and metastasis of liver cancer cells through multiple signaling pathways, such as the PI3K-Akt, JAK/STAT3, and Ras/MEK/ERK pathways[31]. In organisms, a variety of proteases participate in the degradation of the basement membrane and ECM, among which MMPs play the most prominent role. MMPs are the main targets of drugs against tumor invasion and metastasis. MMP-2 can specifically degrade the main components of the basement membrane and intercellular matrix (type IV collagen) and improve tumor cell invasiveness and metastasis. Therefore, MMP-2 is considered one of the most important MMPs in these processes[32]. It has been reported that a low level of MMP-2 expression can promote both the activation of nuclear factor-kappa B protein signaling and the invasion and metastasis of lung cancer[33]. MMP-3 is one of the key enzymes involved in ECM synthesis and the regulation of catabolic activity. MMP-3 is also closely related to the invasion and metastasis of tumor cells[34].

After analysis of the PPI network constructed with the intersecting targets of CAM and liver cancer, it was found that ALB, IGF1, ESR1, EGFR, AR, MAPK1, SRC, MAPK14, KIT, MMP-2, and CASP3 are both involved in the pathological process of liver cancer and also key targets of CAM. Molecular docking showed that CAM could bind well to ALB, ESR1, EGFR, CASP3 and other targets through hydrogen, conjugated and other bonding interactions, which further confirmed the therapeutic effect of CAM on liver cancer. Studies have shown that ALB is a serum protein that is almost exclusively expressed in liver tissues and that ALB genes have a closed conformation in other tissues[35]. Dai *et al*[36] showed that ESR1 expression is a candidate tumor suppressor gene in hepatocellular carcinoma (HCC). Further, promoter hypermethylation may be a mechanism by which expression of ESR1 is repressed, and the extent of hypermethylation of ESR1 may be a marker for HCC status and progression. EGFR is at the intersection of multiple signaling pathways and also an important mediator of physiological activities such as cell growth, proliferation, differentiation and adhesion. EGFR expression levels were found to be low in healthy tissues but significantly high in liver, breast, kidney, colon and other tumor cells[30]. When EGFR binds to its ligand, the receptor can dimerize specific tyrosine residues can be phosphorylated. These phosphorylated tyrosine residues can recruit other signaling molecules to initiate the downstream signal transduction pathway that mediates the proliferation, metastasis and drug resistance of tumor cells. The expression of EGFR was found to be significantly upregulated in hepatocellular carcinoma, and high EGFR expression is also closely related to the malignant transformation of hepatocellular carcinoma[37]. A large number of studies have reported the role of EGFR in the occurrence and development of liver cancer. For example, when EGFR is activated by its ligand, it can mediate the growth, proliferation and metastasis of liver cancer cells through multiple signaling pathways, such as the PI3K-Akt, JAK/STAT3, and Ras/MEK/ERK pathways[31]. CASP3 is a protease that can specifically cleave poly ADP ribose polymerase and acetyl-devd-7-amino-4-methyl-coumarin, resulting in DNA cleavage and the promotion of apoptosis, both of which play a core roles in the executive stage of apoptosis. Xie *et al*[38] have confirmed activation of macrophages plays a critical role in liver injury and fibrogenesis during hepatitis B virus (HBV) infection. And hepatitis Be antigen (HBeAg) can obviously induce the production of macrophage inflammatory cytokines. During HBV-induced macrophage activation, the ESR-K/CREB signaling pathway promotes miR-212-3p expression.

MiR-212-3p can inhibit the production of inflammatory cytokines induced by HBeAg by regulating the target gene MAPK1.

According to GO analysis of the intersecting genes of CAM and liver cancer targets, CAM was found to be involved in the regulation of the inflammatory response and protein kinase B signaling associated with cancer. KEGG analysis of the effects of CAM on the key targets of liver cancer showed that many signaling pathways, such as the FoxO, PI3K-Akt, estrogen, Ras, and MAPK signaling pathways, coincide with the pathways of the predicted targets of CAM, which proves that CAM can indeed affect liver cancer cells through the activities of multiple targets and multiple pathways.

CONCLUSION

In this study, we examined the distribution and metabolism of CAM and its effects on inflammatory factors in various tissues and organs of mice. Using reverse molecular docking technology, we screened for diseases related to CAM activity in liver cancer. Moreover, we systematically analyzed the targets and biological pathways of CAM in the treatment of liver cancer by network pharmacology and molecular docking to further explore the mechanism by which CAM is involved in disease treatment. However, network pharmacology methods are based on data research, so there are some remaining limitations, such as incomplete database data, imperfect screening criteria for components and targets and diverse pathway enrichment methods, which will affect the accuracy of the research results. Therefore, follow-up experiments need to be carried out on the material basis of the pharmacodynamic pathways to verify the research results here, which can provide a theoretical and experimental basis for treating liver cancer or inflammation with CAM.

ARTICLE HIGHLIGHTS

Research background

As a new national first-class drug, carrimycin (CAM) has much potential medicinal value. The clinical results show that its antibacterial activity and pharmacodynamic activity are significantly greater than those of similar antibiotics. Through the combination of animal experiments and network pharmacology, this study demonstrates that CAM plays a positive role in the treatment of inflammation and some cancers.

Research motivation

At present, the mechanism of CAM in the treatment of liver cancer is not clear, so the mechanism can be speculated by network pharmacology.

Research objectives

To gain a deeper understanding of CAM, the distribution, metabolism and anti-inflammatory effects of CAM in organs were assessed, and the anti-liver cancer mechanism of CAM was analyzed.

Research methods

Taking the content of isovaleryl spiramycin III as the index, the distribution and metabolism of CAM in various tissues and organs of mice and its effect on inflammatory factors in various tissues and organs of mice were detected. The target of CAM was determined by reverse molecular docking technology, the disease type corresponding to each target protein was selected, the target protein disease type network was established, and the key targets of CAM in liver cancer were screened by network pharmacological methods. The core target was verified by molecular docking and visual analysis.

Research results

The maximum CAM concentration was reached in the liver, kidney, lung and spleen 2.5 h after intragastric administration. In the intestine, the maximum drug concentration was reached 0.5 h after administration. CAM is predicted to regulate related pathways by acting on many targets, such as albumin, estrogen receptor 1, epidermal growth factor receptor and caspase 3, to treat cancer, inflammation and other diseases.

Research conclusions

CAM had an inhibitory effect on inflammation. It also predicts the multitarget, complexity of CAM involving multiple pathways and the diversity of CAM effects in the treatment of liver cancer, which provides a basis and direction for further clinical research.

Research perspectives

We examined the distribution and metabolism of CAM in murine tissues and organs and the effect of CAM on inflammatory factors and performed a systematic analysis of the targets and biological pathways involved in the treatment of liver cancer with CAM by network pharmacology, which led to further insights into the mechanism of treatment of the disease with CAM.

FOOTNOTES

Author contributions: Li XY, Luo YT, Guan QX, Wang YH, Yang ZX, and Shang YZ performed the experiments and acquired and analyzed the data; Li XY and Luo YT wrote the manuscript; and all authors approved the final version of the article.

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ORIGINAL ARTICLE

Basic Study

Effect and mechanism of reactive oxygen species-mediated NOD-like receptor family pyrin domain-containing 3 inflammasome activation in hepatic alveolar echinococcosis

Cai-Song Chen, Yao-Gang Zhang, Hai-Jiu Wang, Hai-Ning Fan

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Abstract

BACKGROUND

The NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome is a significant component of the innate immune system that plays a vital role in the development of various parasitic diseases. However, its role in hepatic alveolar echinococcosis (HAE) remains unclear.

AIM

To investigate the NLRP3 inflammasome and its mechanism of activation in HAE.

METHODS

We assessed the expression of NLRP3, caspase-1, interleukin (IL)-1β, and IL-18 in the marginal zone and corresponding normal liver of 60 patients with HAE. A rat model of HAE was employed to investigate the role of the NLRP3 inflammasome in the marginal zone of HAE. Transwell experiments were conducted to investigate the effect of *Echinococcus multilocularis* (*E. multilocularis*) in stimulating Kupffer cells and hepatocytes. Furthermore, immunohistochemistry, Western blotting, and enzyme-linked immunosorbent assay were used to evaluate NLRP3, caspase-1, IL-1β, and IL-18 expression; flow cytometry was used to detect apoptosis and reactive oxygen species (ROS).

RESULTS
NLRP3 inflammasome activation was significantly associated with ROS. Inhibition of ROS production decreased NLRP3-caspase-1-IL-1β pathway activation and mitigated hepatocyte damage and inflammation.

CONCLUSION
E. multilocularis induces hepatocyte damage and inflammation by activating the ROS-mediated NLRP3-caspase-1-IL-1β pathway in Kupffer cells, indicating that ROS may serve as a potential target for the treatment of HAE.

Key Words: Hepatic alveolar echinococcosis; Inflammasome; Inflammation; Kupffer cell; NLR family pyrin domain-containing 3 protein; Reactive oxygen species

Core Tip: In recent years, the role of the NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome in parasitic diseases has attracted widespread attention. However, the role and clinical significance of the NLRP3 inflammasome in Hepatic alveolar echinococcosis (HAE) remain unclear. Herein, we explored the mode of NLRP3 inflammasome activation in the tissues of patients with HAE, a rat model of HAE, rat Kupffer cells, and hepatocytes. Our experiments showed that inhibiting reactive oxygen species (ROS) production reduces NLRP3-caspase-1-IL-1β pathway activation. Decreased IL-1β expression alleviated inflammation and apoptosis rates in hepatocytes in HAE. We conclude that ROS-mediated NLRP3 inflammasome activation was a key factor leading to hepatocyte injury and triggering a cascade of inflammatory reactions. Therefore, ROS production may be a promising target for the treatment of HAE.

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INTRODUCTION

Echinococcosis is a global zoonotic parasitic disease categorised into two main subtypes, namely cystic echinococcosis and alveolar echinococcosis, which are caused by the larvae of *Echinococcus granulosus* and *Echinococcus multilocularis* (*E. multilocularis*), respectively. Alveolar echinococcosis, also termed "parasite cancer"[1], is a helminthiasis that progresses slowly and mainly invades the liver. However, if left untreated, it can lead to high morbidity and mortality[2]. It is estimated that nearly 600000 people worldwide have been infected[3,4]. Cases of hepatic alveolar echinococcosis (HAE) have risen sharply in recent years. Qinghai Province in China is a region with a high and increasing incidence of echinococcosis[5]. Moreover, epidemiological evidence suggests that the medical and economic burden of HAE will increase significantly in the next decade[6].

Inflammasomes were first proposed by Martinon *et al*[7] in 2002 as multimeric complexes that form in response to various physiological and pathological stimuli. Inflammasomes are important components of the innate immune system, and inflammasome activation is essential for pathogen clearance[8]. The main components include nucleotide-binding oligomerization domain-like receptors (NLRs), leucine-rich repeats, and a protein-protein interaction domain, which could be a pyrin domain, a caspase recruitment domain, or a baculovirus inhibitor of an apoptosis protein repeat domain. NLRs are cytosolic pattern-recognition receptors that act as sensors of the innate immune system. They can recognize microbial structures or pathogenic components, called pathogen-associated molecular patterns or damage-associated molecular patterns, which are generated by endogenous stress, and trigger downstream inflammatory pathways, to eliminate infection and repair damaged tissues[9]. An inflammasome is defined by its sensor protein. Five pattern-recognition receptors have been shown to form inflammasomes: Absent in melanoma 2 (AIM2), NLR family caspase recruitment domain-containing 4 (NLRC4), NOD-like receptor family pyrin domain-containing 1 (NLRP1), NLRP3, and pyrin[10]. Among them, the NLRP3 inflammasome is the most studied.

The NLRP3 inflammasome, a signaling molecule in the innate immune system, can convert inactive cysteine aspartate proteolytic enzyme 1 precursor (pro-caspase-1) into active caspase-1, which in turn

converts inactive pro-interleukin (IL)-1β and pro-IL-18 into mature IL-1β and IL-18, respectively. NLRP3 inflammasome expression is transcriptionally regulated by nuclear factor kappa B (NF-κB), and is very low in non-activated macrophages[11]. NLRP3-caspase-1-IL-1β pathway activation exerts effects on processes, such as stress, inflammation, and injury repair[12], which have been extensively studied in tumour development[13-15] and metastasis[16]. In recent years, the role of the NLRP3 inflammasome in parasitic diseases has attracted widespread attention. The NLRP3 inflammasome is upregulated in malaria[17]. Furthermore, in amoebic diseases, macrophage α5β1 integrin expression is associated with NLRP3 inflammasome activation[18]. The NLRP3 inflammasome and caspase-1/11 pathway protect against acute *Trypanosoma cruzi* (*T. cruzi*) infection[19]. The NLRP3 inflammasome controls *T. cruzi* infection *via* a caspase-1-dependent, IL-1R-independent, nitric oxide mechanism[20]. Inflammasome-derived IL-1β production induces nitric oxide-mediated resistance to *Leishmania*[21]. The development of Leishmaniasis occurs *via* NLRP3 inflammasome-mediated IL-1β production[22]. *Schistosoma mansoni* activates the NLRP3 inflammasome and alters adaptive immune responses through Dectin-2[23]. NLRP3 inflammasome activation in mice results in fibrosis of hepatic stellate cells in schistosomiasis [24]. Although the NLRP3 inflammasome is activated differently in different parasitic diseases, it plays an important role in these diseases. Assessing inflammasomes may aid in the rapid identification and elimination of these pathogenic factors, and provide potential means for the treatment and prevention of infection.

NLRP3 inflammasome activation is related to mitochondrial autophagy and reactive oxygen species (ROS) production[25,26]. The mechanism of ROS-mediated NLRP3 inflammasome activation is closely related to Kupffer cells[27,28]. Inhibiting ROS production or using nicotinamide adenine dinucleotide phosphate, an oxidase inhibitor, can block NLRP3 inflammasome activation[29,30]. As an important mediator of oxidative stress, ROS is closely related to inflammation. *T. cruzi* infection promotes the synthesis and release of ROS, with NF-κB pathway activation and decreased expression of inflammatory cytokines in NLRP3 knock-out mice, while alleviating acute-phase symptoms[31]. Protein kinase C/ROS-mediated NLRP3 inflammasome activation correlates with pathological changes in leishmaniasis [32]. ROS inhibition and prevention of potassium channel opening inhibits NLRP3 inflammasome expression, implying that ROS production and potassium channel opening are pivotal for NLRP3 inflammasome activation in human prostate epithelial cells[33].

The occurrence and development of parasitic diseases is often accompanied by inflammation[34,35]. In a previous study, we determined that HAE has a clear marginal zone[36]. However, the role and clinical significance of the NLRP3 inflammasome in HAE remain unclear. Herein, we explored NLRP3 inflammasome activation in the tissues of patients with HAE, a rat model of HAE, rat Kupffer cells, and hepatocytes. We investigated the mechanism of NLRP3 inflammasome activation in HAE and the effect of its downstream products. We hypothesised that *E. multilocularis* aggravates hepatic damage and inflammation by activating the NLRP3 inflammasome in HAE.

MATERIALS AND METHODS

Patient tissues

Tissue specimens were collected from 60 patients with HAE who underwent surgery at the Department of Hepatobiliary and Pancreatic Surgery of the Affiliated Hospital of Qinghai University from January to December 2020. Patients did not receive adjuvant therapy before surgery. HAE was confirmed by pathological examination. The pathological stage and degree of differentiation of the lesions were based on the PNM classification, where P refers to a parasitic mass in the liver, N refers to the involvement of neighbouring organs, and M refers to metastasis[37]. The marginal zone of the lesion (< 0.5 cm away from the lesion) and the tissue adjacent to the lesion (> 3 cm away from the lesion) were rapidly frozen in liquid nitrogen, within 15 min of surgical resection, and stored at -80 °C.

Parasites and animal experiments

E. multilocularis specimens were obtained from the Key Laboratory of Echinococcosis, Qinghai Province, China, and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Burlington, Ontario, Canada) supplemented with 10% foetal bovine serum (FBS) (Gibco) at 37 °C in 5% carbon dioxide. Sprague-Dawley rats were purchased from the Nanjing Qinglongshan Laboratory Animal Breeding Centre, China. Feeding and housing were performed under specific pathogen-free conditions. Eight- to 10-wk-old male Sprague-Dawley rats were used as hosts. Rats were fixed in the dorsal position and anaesthetised with 1%-3% isoflurane. The abdominal cavity was opened, and the skin, superficial fascia, deep fascia, muscle, and peritoneum were dissected to expose the liver. A syringe needle was inserted into the liver obliquely at a 45-degree angle (penetration depth, 0.5 cm). Each rat was injected with a suspension of approximately 1200-1500 *E. multilocularis*. The method of counting the number of *E. multilocularis* was similar to the cell counting plate method. After injecting the suspension, the liver was covered with absorbable hemostat gauze to stop bleeding, and the abdominal incision was closed. For *in vivo* experiments, N-tert-Butyl-α-phenylnitrone (PBN; Sigma, Saint Louis, MO, United States) was used as a ROS scavenger. Nine 6-mo-old HAE rats were divided into three groups (three rats per group), to

test the efficacy of PBN[38,39]. The three groups were treated with different concentrations-20, 50, and 100 mg/kg/d-intraperitoneally for 30 d. ROS production in the three groups was used to determine the relative efficient concentration of PBN in HAE rats. Subsequently, 30 HAE rats were randomly divided into three groups (10 rats per group), namely the 50 mg/kg/d PBN group, normal saline group, and no-treatment group (control). Intraperitoneal injection was performed at the same time and the same dose (50 mg/kg/d) in the PBN and normal saline groups for 30 d.

Immunohistochemistry and haematoxylin eosin staining

Paraffin sections were incubated in 3% hydrogen peroxide at 25 °C for 5-10 min to inhibit endogenous peroxidase activity. Primary antibodies (NLRP3: Abcam, Shanghai, China; caspase-1: Thermo Scientific, Waltham, MA, United States; and IL-1β and IL-18: Boster Biotechnology, Wuhan, China) were added and incubated at 4 °C overnight, washed with phosphate-buffered saline (PBS), and incubated with the appropriate secondary antibody at 20-37 °C for 10-30 min. Haematoxylin and eosin staining was performed, as described previously[40]. The optical density and area of each image were measured using an Image-Pro Plus 6.0 Image Analysis System (Media Cybernetics Inc., Bethesda, MD, United States), and the mean density was calculated.

Immunofluorescence staining for cell localisation

Normal goat serum blocking solution was added to reduce background staining. Primary antibodies (CD68: Proteintech, Wuhan, China; NLRP3: Abcam; and caspase-1: Thermo Scientific) were added. The sections were incubated at 4 °C overnight, washed with PBS, and incubated with fluorescent secondary antibodies for 2 h, followed by a 30-min incubation in the dark with 4′,6-diamidino-2-phenylindole (DAPI; Beyotime Biotechnology, Shanghai, China). Cells were observed and images captured under a fluorescence microscope (Olympus BX33; Olympus Corp., Tokyo, Japan).

Analysis of ROS production and apoptosis by flow cytometry

To detect ROS, Kupffer cells were resuspended in 2′-7′-dichlorofluorescin diacetate from a ROS Assay Kit (Nanjin Jiancheng Bioengineering Institute, Nanjing, China) and incubated at 25 °C for 30 min. The liver was cut into 2-3 mm^3 sections with scissors and single-cell suspensions were harvested for ROS detection. Cells were washed twice with PBS. Relative ROS production was detected through the fluorescein isothiocyanate (FITC) channel (500 nm). For the apoptosis assay, cells were stained with Annexin V-FITC (MultiSciences, Hangzhou, China) and propidium iodide, according to the manufacturer's instructions. Briefly, 1-10 × 10^5 cells were washed with PBS and resuspended in 500 μL of 1 × binding buffer. Next, 5 μL Annexin V-FITC and 10 μL propidium iodide were added to each tube. The cells were incubated at 25 °C in the dark for 5 min. Flow cytometry was performed on a FACSCalibur (Becton and Dickinson, Rutherford, NJ, United States). FlowJo software version 10.5 (Becton and Dickinson) was used to analyse the data.

Co-cultivation experiments

For the co-cultivation experiments, normal Buffalo rat liver cells were purchased from the Institute of Cell Biology of the Chinese Academy of Sciences, Shanghai, China. Buffalo rat liver cells (3 × 10^5) were plated in the top chamber of a 6-well plate (0.4-μm Pore Polycarbonate Membrane Insert; Corning Life Sciences, Shanghai, China). *E. multilocularis* (1 × 10^3) and rat liver macrophages (1 × 10^6) were cultured in the lower chamber. Buffalo rat liver hepatocytes, liver macrophages, and *E. multilocularis* were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco). N-acetyl-L-cysteine (NAC; Macklin Biochemical, Shanghai, China) was chosen as a ROS scavenger[41] in a co-culture system, by treating cells with 5, 10, or 20 mmol/L NAC and detecting ROS production in Kupffer cells at 24, 48, and 72 h, to test its efficacy. The supernatant was collected and stored at -80 °C. IL-1β and IL-18 were detected, along with NLRP3, caspase-1, and ROS.

Western blotting

Protein samples were quantified using a BCA Protein Assay Kit (Bio-Rad, Mississauga, Ontario, Canada). Western blotting was performed, as described previously[42]. Briefly, samples were subjected to polyacrylamide gel electrophoresis under reducing conditions, transferred to polyvinylidene fluoride membranes, incubated with primary antibodies (NLRP3: Abcam; and caspase-1: Thermo Scientific) overnight at 4 °C, washed, and blotted with the Goat Anti-Rabbit IgG H&L (HRP) secondary antibody (Abcam). The blots were developed with ECL reagent (Sangon Biotech, Shanghai, China) using an Image Quant LAS 4000 Imaging System (GE Healthcare, Chicago, IL, United States) and analysed using Image-Pro Plus 6.0 (Media Cybernetics Inc.). Band intensity was normalised to the intensity of b-actin (Abcam).

Enzyme-linked immunosorbent assay

The Rat IL-1b ELISA Kit (Abcam) and Rat IL-18 enzyme-linked immunosorbent assay (ELISA) Kit (MultiSciences) were used, according to the manufacturers' instructions, to determine IL-1b and IL-18 levels in serum and the co-cultured cell supernatant. Each sample was assayed in duplicate and the

optical density of each well was measured immediately using a microplate reader (iMark; Bio-Rad, Hercules, CA, United States).

Extraction and identification of Sprague-Dawley rat Kupffer cells

Kupffer cells were isolated from Sprague-Dawley rats by collagenase perfusion and density gradient centrifugation[43,44]. Briefly, Sprague-Dawley rat liver tissue was perfused with type IV collagenase (Beyotime Biotechnology) and cut into 2-3 mm^3. The cell suspension was separated by centrifugation at 500 × g for 5 min, followed by the addition of 30% Percoll density gradient separation solution (Macklin Biochemical) and centrifugation at 900 × g for 15 min. Kupffer cells were collected from the interface between the PBS and 30% Percoll layers and cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin at 37 °C in 5% carbon dioxide. For identification, cells were adhered to a glass coverslip, fixed with 4% paraformaldehyde for 15 min, and blocked with normal goat serum. Diluted (1:100) primary antibody (CD68: Proteintech) was added and incubated at 4 °C overnight, followed by incubation with fluorescent secondary antibody at 20-37 °C for 1 h. DAPI (Beyotime Biotechnology) was added, with a further incubation in the dark for 5 min. Cells were observed and images captured under a fluorescence microscope (Olympus BX33; Olympus Corp.) at 200 × and 400 × magnification.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, United States). The Pearson test was used for correlation analysis and the chi-squared test was applied to analyze the relationship between NLRP3 expression and the clinicopathological characteristics of patients with HAE. Quantitative data are presented as the mean ± SD. Data were analyzed using student's *t*-test or one-way analysis of variance, as appropriate. All *P* values were two-sided; as $P < 0.05$ was considered statistically significant.

RESULTS

NLRP3, Caspase-1, and IL-1β were upregulated in the marginal zone and NLRP3 expression was associated with HAE

Hematoxylin and eosin-stained tissues from patients with HAE exhibited a clear marginal zone with inflammation (Figure 1A). Immunohistochemical staining of NLRP3, caspase-1, IL-1β, and IL-18 in the marginal zone and corresponding normal liver of 60 patients with HAE (Figure 1B) showed that the expression of NLRP3, caspase-1, and IL-1β was higher in the marginal zone than in the corresponding normal liver, with no significant difference in IL-18 expression (Figure 1C). Using the median expression level of NLRP3 (0.45) as the cut-off, the 60 patients with HAE were divided into a high expression group ($n = 30$) and a low expression group ($n = 30$). Clinicopathological analysis showed that NLRP3 expression in the marginal zone of patients with HAE was associated with jaundice symptoms and Child-Pugh class, but not age, sex, alpha-fetoprotein, or primary lesion (Table 1).

Western blotting (Figure 1D and E) also showed that the expression of NLRP3 (Figure 1F) and caspase-1 (Figure 1G) was higher in the marginal zone than in the corresponding normal liver. These results suggest that the NLRP3-caspase-1-IL-1β pathway may be activated in HAE and that this activation may play a role in the marginal zone.

ROS production increased in the marginal zone and was associated with NLRP3 activation

Lesion growth in HAE rats is shown in Figure 2A. Higher ROS production was observed in the marginal zone compared to the corresponding normal liver. Similarly, cells in the marginal zone of HAE liver showed higher ROS production (Figure 2B and C). ROS production correlated with the relative levels of NLRP3 (linear correlation co-efficient, $r = 0.9489$) (Figure 2D). Thus, we speculated that ROS production may play a role in NLRP3-caspase-1-IL-1β pathway activation in the marginal zone of HAE.

ROS-mediated NLRP3 inflammasome activation in HAE rats

PBN (50 mg/kg/d) significantly reduced ROS production (Figure 3A) and was used in subsequent experiments. After 30 d of intervention, ROS production was detected in the marginal zone of HAE rats in each group (Figure 3B). The relative values of the marginal zone are shown in Figure 3C. Immunohistochemical staining of NLRP3, caspase-1, IL-1β, and IL-18 in the HAE rat marginal zone and corresponding normal liver, after 30-days of treatment, is shown in Figure 3D. The expression of NLRP3, caspase-1), and IL-1β was higher in the marginal zone than in the corresponding normal liver, with no significant difference in IL-18 expression (Figure 3E). NLRP3 and caspase-1 protein expression in the marginal zone and corresponding normal liver is shown in Figure 3F; their relative protein levels are shown in Figure 3G and H, respectively. The Pearson test showed a correlation between ROS production and NLRP3 expression (Figure 3I), and between NLRP3 expression and inflammation in the marginal zone (Figure 3J).

Table 1 Correlation between NOD-like receptor family pyrin domain-containing 3 expression and clinicopathological parameters

Characteristics	*n*	NLRP3		*P* value
		Low expression, *n* = 30	High expression, *n* = 30	
Age (yr)				0.787
≥ 50	21	11	10	
< 50	39	19	20	
Sex				0.195
Male	33	14	19	
Female	27	16	11	
Serum AFP (ng/mL)				0.796
< 20	32	16	15	
≥ 20	28	14	15	
Jaundice symptoms				< 0.001
Yes	34	8	26	
No	26	22	4	
Primary lesion size (cm)				0.071
≥ 5	51	23	28	
< 5	9	7	2	
Child-Pugh class				0.012
A	42	26	16	
B	18	4	12	

AFP: Alpha-fetoprotein; NLRP3: NOD-like receptor family pyrin domain-containing 3.

E. multilocularis activated the NLRP3-caspase-1-IL-1β pathway in Kupffer cells

Cells expressing NLRP3 and caspase-1 were localized in the marginal zone of HAE rats (Figure 4A and B). This suggests that NLRP3 and caspase-1 are highly expressed in macrophages (Kupffer cells), with no obvious expression in hepatocytes. To test whether *E. multilocularis* activates the NLRP3-caspase-1-IL-1β pathway in rat Kupffer cells by enhancing ROS production, which promotes IL-1β synthesis and release, leading to hepatocyte damage and triggering an acute inflammatory response, Kupffer cells were isolated (Figure 4C and D). *E. multilocularis* was also isolated from HAE lesions (Figure 4E). In 24-h co-cultures of *E. multilocularis* and Kupffer cells, ROS production was significantly increased in Kupffer cells (Figure 4F).

ROS-mediated NLRP3-Caspase-1-IL-1β pathway activation in Kupffer cells

NAC (5 mmol/L) effectively abrogated ROS production in co-cultured cells (Figure 5A). Co-cultures of *E. multilocularis*, Kupffer cells, and hepatocytes were prepared (Figure 5B). ROS production in Kupffer cells at 24 h, 48 h, and 72 h with or without NAC is shown in Figure 5C. Significant suppression of ROS production was evident in NAC-treated co-cultures (Figure 5D). The effects on IL-1β and IL-18 expression are shown in Figure 5E and F. Hepatocyte apoptosis at 24 h, 48 h, and 72 h is shown in Figure 5G, with relative levels shown in Figure 5H. Western blotting analysis of NLRP3 and caspase-1 is shown in Figure 5I, NLRP3 IntDen/β-actin IntDen protein expression in Figure 5J, and caspase-1 IntDen/β-actin IntDen protein expression in Figure 5K. The apoptosis rate and expression of NLRP3 and caspase-1 were significantly reduced after inhibiting ROS production.

DISCUSSION

As a global zoonotic parasitic disease, the occurrence of HAE is rare; however, if left untreated, it can lead to high morbidity and mortality, with significant economic burden. Inflammation is crucial in the pathogenesis and progression of serious liver diseases[45-47]. Activation of the inflammatory response is inextricably linked to the activation of innate immunity[12]. The NLRP3 inflammasome is a critical component of the innate immune system that mediates the secretion of pro-inflammatory cytokines in

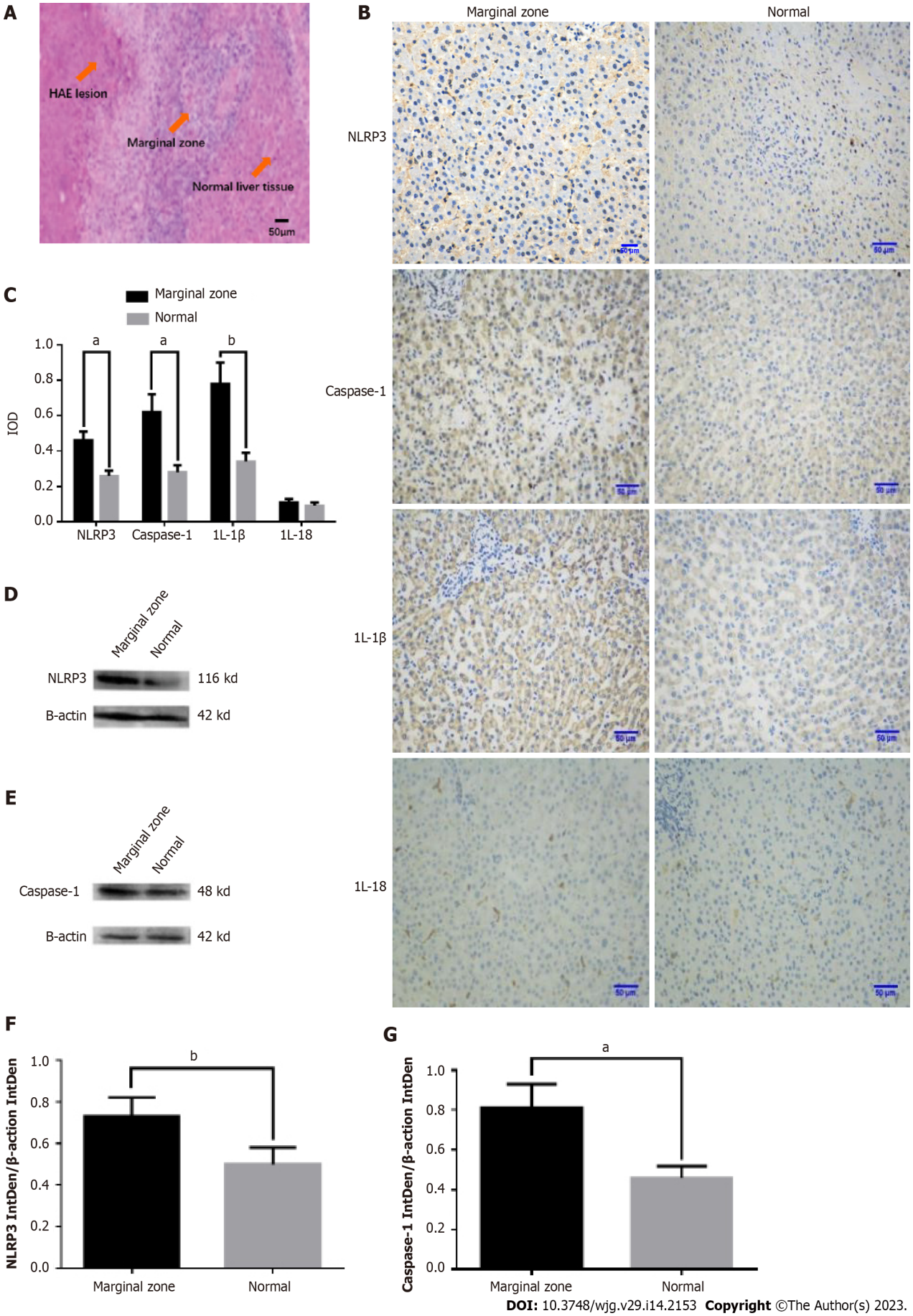

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Figure 1 NOD-like receptor family pyrin domain-containing 3, caspase-1, and interleukin-1β were upregulated in the marginal zone and

NOD-like receptor family pyrin domain-containing 3 expression was associated with hepatic alveolar echinococcosis. A: Hematoxylin and eosin staining of the lesion, marginal zone, and corresponding normal liver in hepatic alveolar echinococcosis; B: Immunohistochemical staining of NOD-like receptor family pyrin domain-containing 3 (NLRP3), caspase-1, interleukin (IL)-1β, and IL-18 in the marginal zone and corresponding normal liver; C: Relative expression of NLRP3, caspase-1, IL-1β, and IL-18; D: NLRP3 protein expression evaluated by western blotting; E: Caspase-1 protein expression evaluated by Western blotting; F: Relative protein expression levels of NLRP3 IntDen/β-actin IntDen; G: Relative protein expression levels of caspase-1 IntDen/β-actin IntDen. C, n = 60. Scale bar: 50 μm. Western blotting was performed in triplicate (mean ± SD). $^{a}P < 0.05$; $^{b}P < 0.01$. HAE: Hepatic alveolar echinococcosis; IOD: Integral Optical Density; NLRP3: NOD-like receptor family pyrin domain-containing 3. IL-1β: Interleukin-1β; IL-18: Interleukin-18.

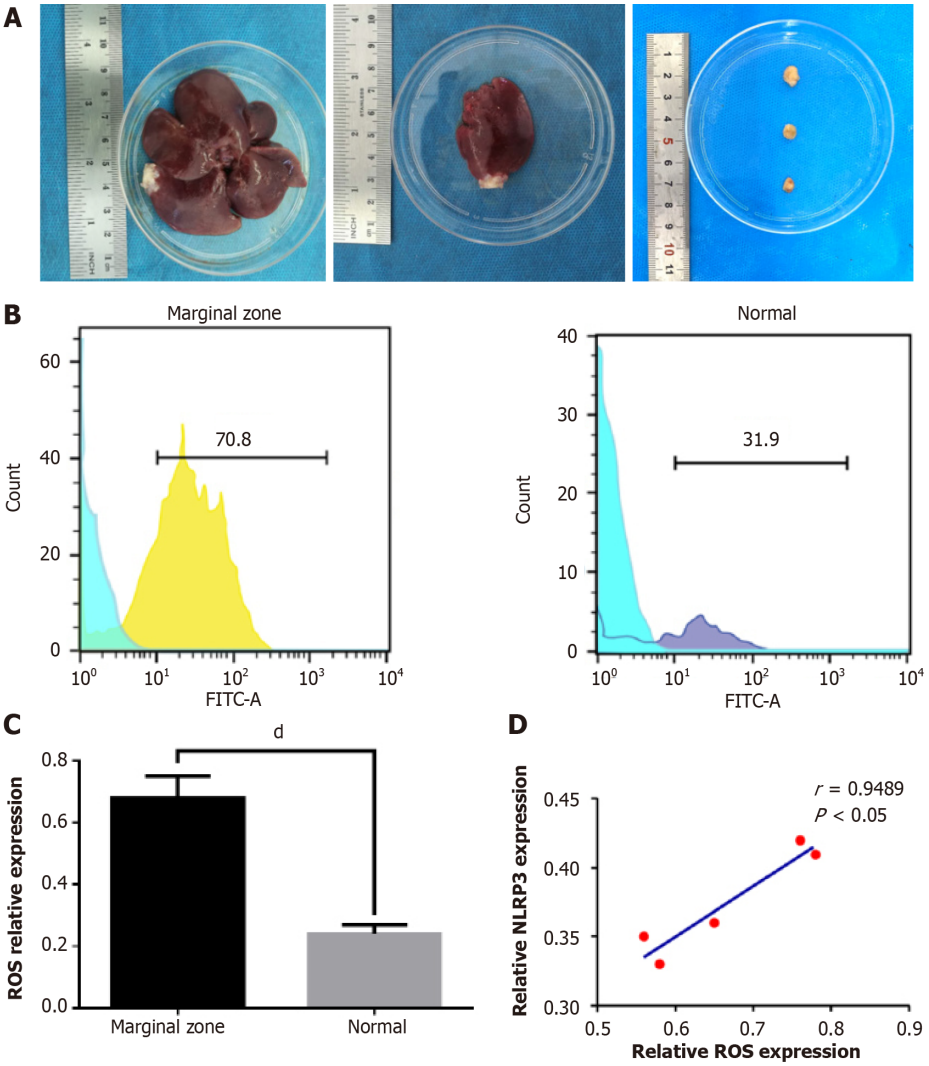

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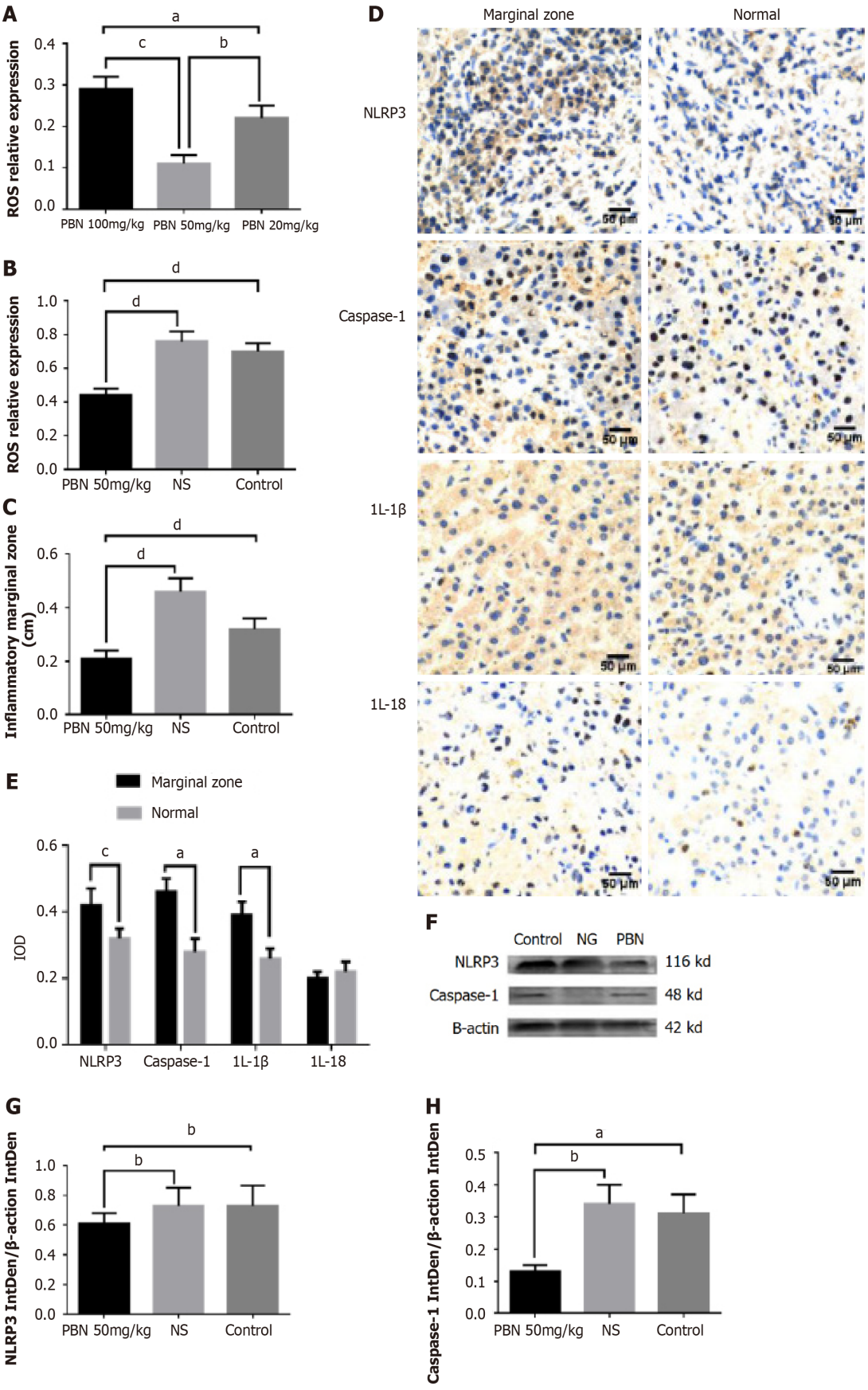
Figure 2 Reactive oxygen species were highly produced in the hepatic alveolar echinococcosis marginal zone and were intimately associated with the activation of NOD-like receptor family pyrin domain-containing 3. A: Lesion growth in hepatic alveolar echinococcosis rats; B: Reactive oxygen species (ROS) production in the marginal zone and corresponding normal liver; C: Relative levels of ROS production; D: Relationship between ROS and NLRP3. C and D, n = 5 rats per group. $^{d}P < 0.0001$. NLRP3: NOD-like receptor family pyrin domain-containing 3; ROS: Reactive oxygen species; FITC-A: Fluorescein isothiocyanate isomer I-A.

response to infection and cellular damage. Multiple cellular events, including ionic flux, mitochondrial dysfunction, and ROS production, trigger its activation[48]. The aberrant activation of the NLRP3 inflammasome is closely related to various liver diseases, including liver cancer[45,46], viral hepatitis [49], non-alcoholic fatty liver disease[47,50], and parasitic diseases, such as schistosomiasis[51,52], leishmaniasis[53], malaria[54], and trypanosomiasis[55]. Our study confirms that the NLRP3 inflammasome plays a pivotal role in HAE *via* the NLRP3-caspase-1-IL-1β pathway involving inflammation in the marginal zone.

As important participants in oxidative stress, ROS are also closely related to inflammation[56]. The balance of ROS can regulate apoptosis and cell proliferation, activating a series of signal transduction pathways. Excessive ROS production damages cell integrity, resulting in tissue dysfunction[57]. ROS inhibitors significantly reduce this damage and alleviate acute liver injury. Consistent with a previous


A
ROS relative expression
PBN 100mg/kg
PBN 50mg/kg
PBN 20mg/kg
B
ROS relative expression
PBN 50mg/kg
NS
Control
C
Inflammatory marginal zone (cm)
PBN 50mg/kg
NS
Control
D
Marginal zone
Normal
NLRP3
Caspase-1
1L-1β
1L-18
50 μm
E
Marginal zone
Normal
IOD
NLRP3
Caspase-1
1L-1β
1L-18
F
Control
NG
PBN
NLRP3
116 kd
Caspase-1
48 kd
B-actin
42 kd
G
NLRP3 IntDen/β-action IntDen
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Control

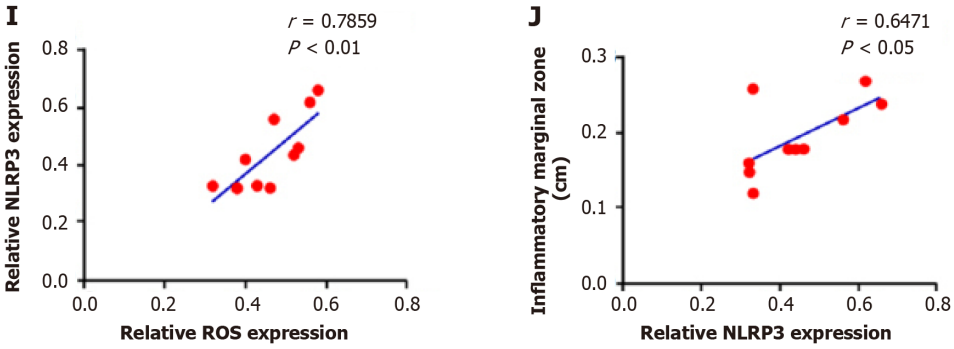

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Figure 3 Reactive oxygen species-mediated NOD-like receptor family pyrin domain-containing 3 inflammasome activation in hepatic alveolar echinococcosis rats. A: Reactive oxygen species (ROS) production in the 20, 50, and 100 mg/kg/d N-tert-Butyl-α-phenylnitrone (PBN) groups; B: ROS production in the 50 mg/kg/d PBN, normal saline (NS), and control groups; C: Analysis of inflammation; D: Immunohistochemical staining of NLRP3, caspase-1, interleukin (IL)-1β, and IL-18; E: Relative expression of NLRP3, caspase-1, IL-1β, and IL-18 in the marginal zone compared with the corresponding normal liver; F: Western blotting analysis of the PBN, NS, and control groups; G: NLRP3 IntDen/β-actin IntDen protein expression; H: Caspase-1 IntDen/β-actin IntDen protein expression; I: Relationship between ROS production and the relative expression of NLRP3; J: The relative expression of NLRP3 and inflammation observed in the marginal zone. Western blotting was performed in triplicate (mean ± SD). Scale bar, 50 μm. A, n = 3 rats per group; B, C, and E, n = 10 rats per group; I and J, n = 10. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P < 0.0001$. NLRP3: NOD-like receptor family pyrin domain-containing 3; ROS: Reactive oxygen species; PBN: N-tert-Butyl-α-phenylnitrone; NS: Normal saline; IOD: Integral optical density; IL-1β: Interleukin-1β; IL-18: Interleukin-18.

study[45], our results showed that decreased ROS production in HAE inhibits NLRP3-caspase-1-IL-1β pathway activation and alleviates hepatocyte damage. ROS act as a critical regulator of various inflammatory processes and have received much attention[58]. In inflammatory liver disease, ROS induce fatty liver and ischemia/reperfusion injury by promoting inflammation and cell death[59]. In kidney disease, ROS is closely related to acute kidney injury in rats *via* ROS-mediated NLRP3 inflammasome activation [60]. In cardiovascular disease, ROS mediate several aspects of the stress-response signalling network [61]. In parasitic diseases, *Neospora caninum* evades immunity by inducing mitophagy and inhibiting pro-inflammatory cytokine production in a ROS-dependent manner[62]. Studies on parasitic diseases, inflammatory liver disease, cardiovascular disease, kidney disease, intestinal disease, and ischaemia/reperfusion injury also suggest that ROS production contributes to the activation of the NLRP3 inflammasome[63,64]. Although the specific mechanism of ROS-mediated NLRP3 inflammasome activation is unknown, the effect of ROS-mediated NLRP3 inflammasome activation under disease conditions has been confirmed[65]. Consistent with previous studies[61,64], our results indicated that NLRP3 inflammasome activation is intimately associated with ROS production. In addition, ROS-mediated NLRP3 inflammasome activation plays a vital role in the progression of inflammation in HAE. If the balance of ROS can be maintained by internal ROS scavengers and external antioxidants, ROS-mediated progression of inflammation and pathological processes can be alleviated or inhibited.

As NLRP3 inflammasome expression is transcriptionally regulated by NF-κB, it is very low in non-activated macrophages[11]. We localised cells expressing NLRP3 and caspase-1 in the marginal zone of HAE rats. The results showed that NLRP3 and caspase-1 were highly expressed in Kupffer cells (resident macrophages of the liver that play a leading role in the regulation of liver homeostasis). The marginal zone in HAE is a consequence of self-healing caused by continuous stimulation of *E. multilocularis*, and the inflammatory response is important in the progression of HAE. In the early stages of HAE, there are few abnormalities in liver function and liver enzyme levels. As the disease progresses, the imbalance of the internal environment of the liver and severe liver injury manifest in patients with mid-to-late-stage HAE as jaundice, abdominal pain, and even liver failure. Kupffer cells are associated with acute liver injury and secrete various cytokines under continuous stimulation by *E. multilocularis* [66]. IL-1β secreted by Kupffer cells is an important factor causing liver damage[64]. In a previous study [36], we observed typical chronic granulomatous changes around the lesion, accompanied by lymphocyte infiltration, hepatocyte degeneration and necrosis, and Kupffer cell proliferation and differentiation in tissues from patients with HAE. In this study, we used CD68 to identify Kupffer cells, and confirmed that they play an important role in the marginal zone. Kupffer cells are activated in response to liver injury. Activated Kupffer cells express markers of M1-like or M2-like macrophages, depending on external signals. Liver inflammation is regulated by the balance between pro-inflammatory M1 Kupffer cells and anti-inflammatory M2 Kupffer cells, which are partially self-renewal in a stable state [67]. We observed chronic granulomatous changes in the marginal zone of lesions in patients with HAE. Therefore, pro-inflammatory M1 Kupffer cells may be more mobile than anti-inflammatory M2 Kupffer cells in HAE. Activated Kupffer cells secrete IL-1β, a common pro-inflammatory cytokine, suggesting that pro-inflammatory M1 Kupffer cells play a key role in liver inflammation, although further research is needed.

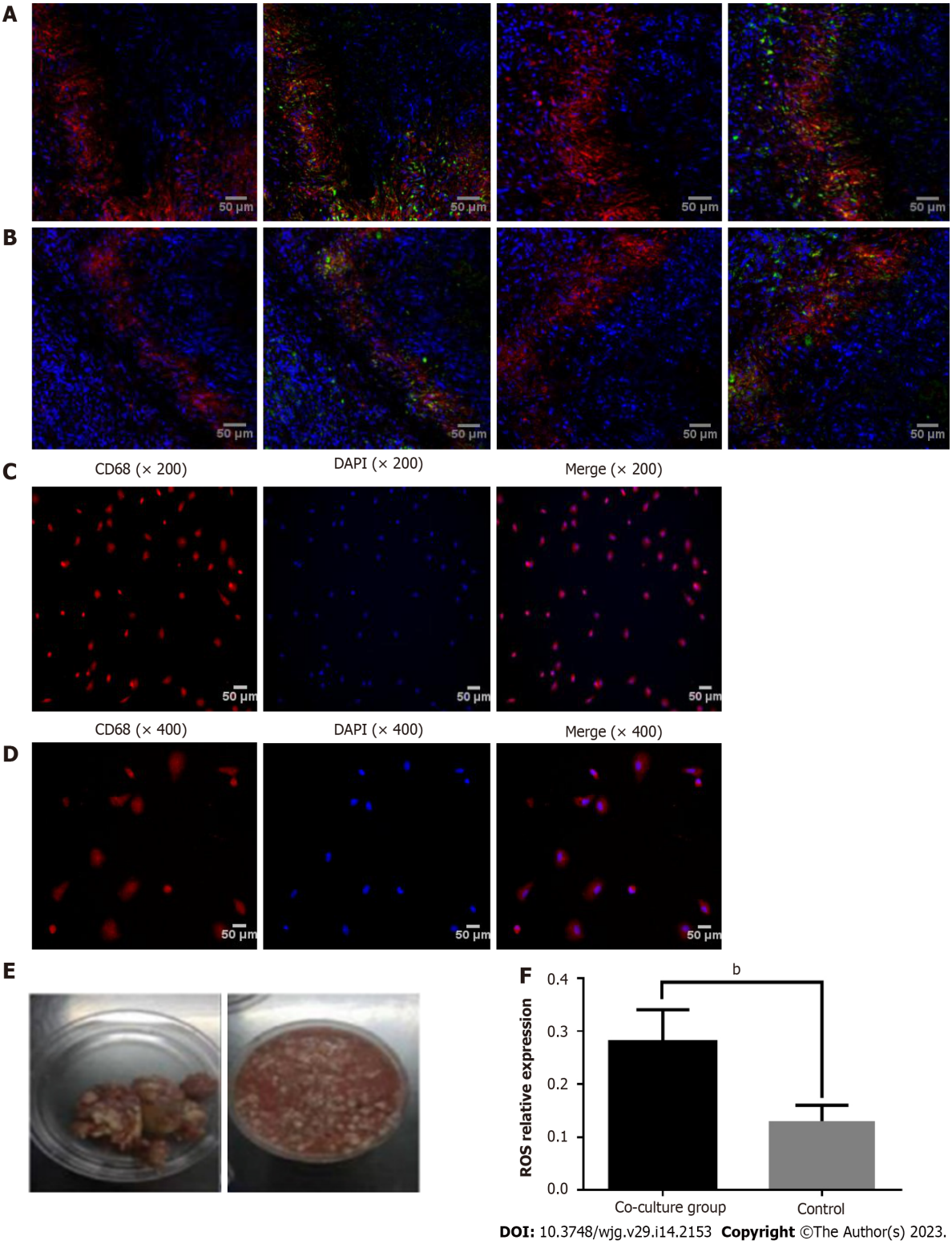

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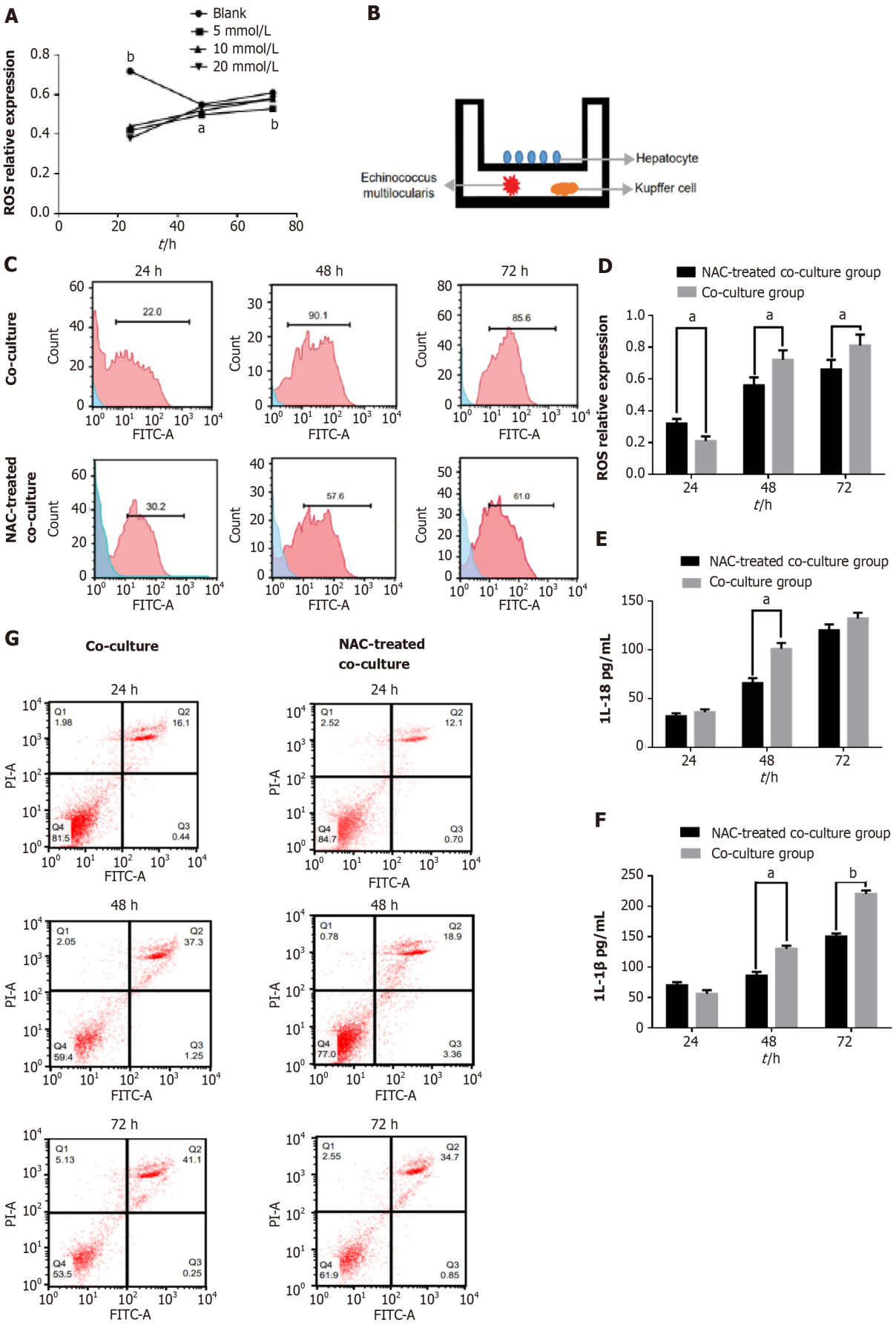
Figure 4 *Echinococcus multilocularis* activation of the NOD-like receptor family pyrin domain-containing 3-caspase-1-interleukin-1β pathways in Kupffer cells. A: Cellular localisation of NOD-like receptor family pyrin domain-containing 3 (NLRP3) in the marginal zone (red denotes NLRP3 inflammasomes, blue denotes 4',6-diamidino-2-phenylindole (DAPI) stained nuclei, and green denotes the macrophage marker, CD68); B: Cellular localisation of caspase-1 in the marginal zone (red denotes caspase-1, blue denotes DAPI stained nuclei, and green denotes CD68); C: The identification of Kupffer cells at 200 × magnification and; D: The identification of Kupffer cells at 400× magnification (red denotes CD68 and blue denotes DAPI stained nuclei; the final image is a fusion image); E: Isolation of *E. multilocularis*; F: Relative expression in the co-culture and control groups. Scale bar, 50 μm. F, $n = 3$. $^{b}P < 0.01$. NLRP3: NOD-like receptor family pyrin domain-containing 3; DAPI: 4',6-diamidino-2-phenylindole.

Among the members of the inflammasome family, NLRP3 is the most well-characterised. The NLRP3 inflammasome is associated with several autoimmune and inflammatory diseases[68]. NLRP3 inflammasome activation leads to pro-inflammatory programmed cell death, known as pyroptosis[69]. NLRP3


A
Blank
5 mmol/L
10 mmol/L
20 mmol/L
ROS relative expression
t/h
B
Echinococcus multilocularis
Hepatocyte
Kupffer cell
C
24 h
48 h
72 h
Co-culture
NAC-treated co-culture
Count
FITC-A
22.0
90.1
85.6
30.2
57.6
61.0
D
NAC-treated co-culture group
Co-culture group
ROS relative expression
t/h
E
NAC-treated co-culture group
Co-culture group
1L-18 pg/mL
t/h
F
NAC-treated co-culture group
Co-culture group
1L-1β pg/mL
t/h
G
Co-culture
NAC-treated co-culture
24 h
48 h
72 h
PI-A
FITC-A
Q1 1.98
Q2 16.1
Q4 81.5
Q3 0.44
Q1 2.52
Q2 12.1
Q4 84.7
Q3 0.70
Q1 2.05
Q2 37.3
Q4 59.4
Q3 1.25
Q1 0.78
Q2 18.9
Q4 77.0
Q3 3.36
Q1 5.13
Q2 41.1
Q4 53.5
Q3 0.25
Q1 2.55
Q2 34.7
Q4 61.9
Q3 0.85

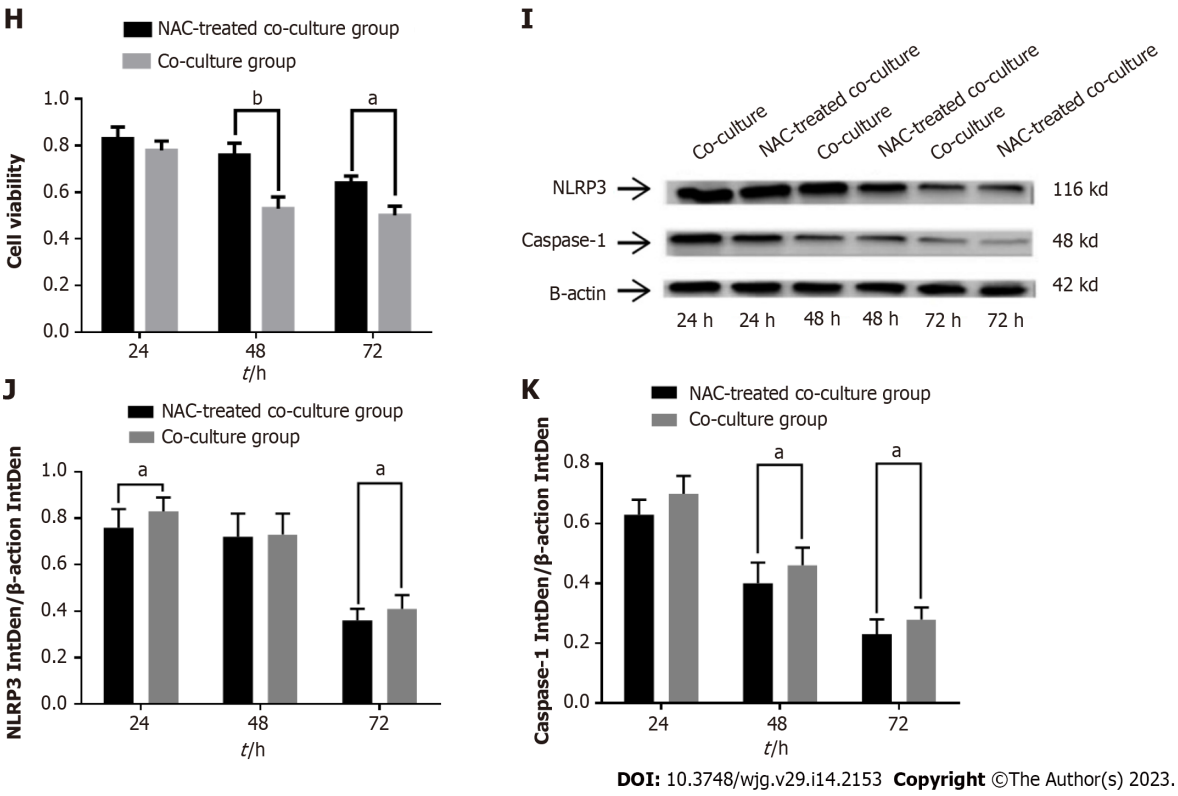


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Figure 5 Reactive oxygen species-mediated NOD-like receptor family pyrin domain-containing 3-caspase-1-interleukin-18 pathway activation in Kupffer cells. A: Reactive oxygen species (ROS) production with the indicated N-acetyl-L-cysteine (NAC) dose at the specified time points. $^{b}P <$ 0.01, blank *vs* 5 mmol/L; $^{a}P < 0.05$, 10 mmol/L *vs* 5 mmol/L. $^{b}P < 0.0120$ mmol/L *vs* 5 mmol/L; B: Representation of the Transwell model used in this study; C: ROS production in the co-culture groups treated with or without NAC at the indicated time points; D: Relative production of ROS; E: Interleukin (IL)-18 expression; F: IL-1β expression; G: Apoptosis of hepatocytes in the co-culture groups treated with or without NAC at 24, 48, and 72 h; H: Cell viability; I: Western blotting analysis of the indicated proteins; J: NLRP3 IntDen/β-actin IntDen protein expression; K: Caspase-1 IntDen/β-actin IntDen protein expression. $^{a}P < 0.05$; $^{b}P < 0.01$. NLRP3: NOD-like receptor family pyrin domain-containing 3; ROS: Reactive oxygen species; FITC-A: Fluorescein isothiocyanate isomer I-A; PI: Propidium Iodide; NAC: N-acetyl-L-cysteine; IL-1β: Interleukin-1β; IL-18: Interleukin-18.

inflammasome activation results in caspase-1 activation, which triggers the release of pro-inflammatory cytokines, IL-1β and IL-18. NLRP3 inflammasome activation has received widespread attention, and significant progress has been made in understanding the molecular mechanisms underlying the priming step of NLRP3 inflammasome activation. NLRP3 inflammasome activation is triggered by several cellular signals: Mitochondrial dysfunction, ROS production, potassium and calcium ion signalling, and lysosomal rupture[48]. As HAE is a parasitic disease that mainly affects the liver, we considered the modes of NLRP3 inflammasome activation in liver and parasitic diseases. In non-alcoholic fatty liver disease, free fatty acids induce ROS production, which has been proposed as a common mechanism of NLRP3 inflammasome activation, resulting in hepatocyte injury and steatosis[68,69]. In viral hepatitis, NLRP3 inflammasome activation is mediated by elevated ROS production, resulting in liver inflammation and hepatocellular pyroptosis under hydrogen peroxide stress[70]. Wei *et al*[46] showed that 17β-estradiol-induced NLRP3 inflammasome activation inhibited liver cancer by triggering apoptosis and inhibiting protective autophagy. Additionally, Ma *et al*[71] showed that NLRP3 inflammasomes play an important role in host defence against *Talaromyces marneffei* (*T. marneffei*) infection *via* the Dectin-1/Syk signalling pathway, *T. marneffei* yeast triggers NLRP3-ASC-caspase-1 inflammasome assembly to facilitate IL-1β maturation. While NLRP3 inflammasome activation plays different roles at various stages of leishmaniasis, ROS-mediated NLRP3 inflammasome activation has been confirmed[32]. Schistosomiasis attracted our attention as a parasitic disease that mainly affects the liver. In a study of *Schistosoma japonicum* (*S. japonicum*), Zhang *et al*[72] showed that *S. japonicum* induces liver fibrosis *via* NF-κB signaling and NLRP3 inflammasome activation in Kupffer cells, promoting cytokine production and the mechanism of NLRP3 inflammasome activation during *S. japonicum* infection was found to be dependent on ROS production and lysosomal activity. NLRP3 expression is closely related to an increase in ROS production and the degree of inflammation in the marginal zone. Therefore, we hypothesised that *E. multilocularis* activates the ROS-mediated NLRP3-caspase-1-IL-1β pathway in Kupffer cells, promoting IL-1β synthesis and release, leading to hepatocyte damage. We showed that *E. multilocularis* alone triggers ROS production in Kupffer cells, and reduces the impact of ROS production in a co-culture system, effectively inhibiting IL-1β production and reducing the apoptosis of rat hepatocytes.

IL-1β, mainly synthesised by macrophages, is an important pro-inflammatory cytokine that activates lymphocytes, macrophages, and natural killer cells, and is involved in various pathological processes [73]. IL-1β is activated in neurological diseases (especially Parkinson's disease and Alzheimer's disease), intestinal diseases, and cancer[74,75]. Previous studies have led to the development of IL-1β-targeted therapies, which have achieved considerable success[76]. IL-1β is also associated with parasitic infections[20,21]. IL-1β may eliminate parasites by associating with other components of the immune system[77]. IL-1β can also co-ordinate innate and adaptive immune responses to eliminate pathogens. However, excess IL-1β leads to inflammatory diseases[78]. As mentioned above, IL-1β expression may be critical in inflammatory diseases, and blocking IL-1β may be a useful strategy for treating inflammatory diseases. We confirmed that IL-1β levels were increased in HAE and may be responsible for the development of HAE infection. We did not observe a corresponding increase in IL-18 Levels, possibly because the ROS-mediated NLRP3-caspase-1-IL-18 pathway was not activated in HAE.

Patients do not show obvious clinical symptoms in the early stages of HAE. Patients begin treatment in the mid-to-late stages of the disease, which are often accompanied by jaundice, abdominal pain, abnormal liver function, and declining immune system function. Radical surgery is the first choice for the treatment of alveolar echinococcosis. There is no doubt that choosing the correct treatment is key to improving the outcome of HAE. From previous studies[79,80], we have accumulated experience in the surgical treatment of HAE, and patients with blood vessel invasion (hepatic vein, portal vein, or inferior abdominal vein) and lymph node metastasis were still given the opportunity to undergo surgery. Similar to liver cancer lesions, HAE lesions are usually characterised by milky white surfaces. However, the specimens are harder and denser than cancer. Most lesions have clear boundaries. Some small lesions are distributed around the large lesions. Liquefaction necrosis is observed in the center of the larger lesions, with a distinct marginal zone around the lesion. However, unlike patients with liver cancer who often benefit from immunotherapeutic drugs, few drugs are available for the treatment of HAE. Drugs that protect the liver, maintain stable liver function, and inhibit parasites (*e.g.*, albendazole) are routine choices, owing to the lack of evidence from pathophysiological research on the occurrence and development of HAE. An obvious pathological change in the development of HAE is inflammation. We begin with the NLRP3 inflammasome, an important factor in the inflammatory signaling pathway. We explored the mode of activation and the effect of downstream products on HAE. ROS-mediated NLRP3 inflammasome activation was a key factor leading to hepatocyte injury and triggering a cascade of inflammatory reactions. There are a number of challenges in translating findings into the clinic. However, the use of dietary antioxidants to inhibit oxidative stress and reduce ROS production may be a possible treatment option. Therefore, ROS production may be a promising target for the treatment of HAE.

Herein, the expression of NLRP3, caspase-1, and IL-1β was significantly elevated in the marginal zone in patients with HAE. To the best of our knowledge, this is the first study to explore the effect and mechanism of ROS-mediated NLRP3 inflammasome activation in HAE. *In vivo* experiments showed that inhibiting ROS production reduces NLRP3-caspase-1-IL-1β pathway activation. Decreased IL-1β expression alleviated inflammation in the HAE marginal zone. *In vitro* data revealed significantly decreased apoptosis rates in hepatocytes, corresponding to reduced ROS-mediated NLRP3-caspase-1-IL-1β pathway activation in Kupffer cells. However, our study has a few limitations. We did not verify whether M1 Kupffer cells or M2 Kupffer cells were more mobile in the regulation of inflammatory response, nor did we further explore the activation modes of NLRP3 inflammasome. Additionally, although NLRP3 inflammasome is the most well-characterised inflammasome, further studies are warranted to determine whether other inflammasomes are activated in the HAE marginal zone. We conclude that *E. multilocularis* induces hepatocyte damage and inflammation by activating the ROS-mediated NLRP3-caspase-1-IL-1β pathway in Kupffer cells. The increase in ROS production caused by *E. multilocularis* may be a potential mechanism of NLRP3 inflammasome activation. In addition, crosstalk between signaling pathways may be involved in the regulatory network in the marginal zone. Future studies should clarify the function and mechanisms of the immune responses in the progression of HAE. This will enhance our understanding of the occurrence and development of infection and facilitate the development of diagnostics and therapy.

CONCLUSION

Our *in vivo* experiments showed that inhibiting ROS production reduces NLRP3-caspase-1-IL-1β pathway activation. Decreased IL-1β expression alleviated inflammation in the HAE marginal zone. *In vitro* data revealed significantly decreased apoptosis rates in hepatocytes, corresponding to reduced ROS-mediated NLRP3-caspase-1-IL-1β pathway activation in Kupffer cells. We conclude that *E. multilocularis* induces hepatocyte damage and inflammation by activating the ROS-mediated NLRP3-caspase-1-IL-1β pathway in Kupffer cells.

ARTICLE HIGHLIGHTS

Research background

In recent years, the role of the NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome in parasitic diseases has attracted widespread attention. However, the role and clinical significance of the NLRP3 inflammasome in Hepatic alveolar echinococcosis (HAE) remain unclear.

Research motivation

To investigate the mechanism of NLRP3 inflammasome activation in HAE and the effect of its downstream products may enhance our understanding of the occurrence and development of infection.

Research objectives

To investigate the NLRP3 inflammasome and its mechanism of activation in HAE.

Research methods

We assessed the expression of NLRP3, caspase-1, interleukin (IL)-1β, and IL-18 in the marginal zone and corresponding normal liver of 60 patients with HAE. A rat model of HAE was employed to investigate the role of the NLRP3 inflammasome in the marginal zone of HAE. Transwell experiments were conducted to investigate the effect of *Echinococcus multilocularis* (*E. multilocularis*) in stimulating Kupffer cells and hepatocytes. Furthermore, immunohistochemistry, Western blotting, and enzyme-linked immunosorbent assay were used to evaluate NLRP3, caspase-1, IL-1β, and IL-18 expression; flow cytometry was used to detect apoptosis and reactive oxygen species (ROS).

Research results

NLRP3 inflammasome activation was significantly associated with ROS. Inhibition of ROS production decreased NLRP3-caspase-1-IL-1β pathway activation and mitigated hepatocyte damage and inflammation.

Research conclusion

E. multilocularis induces hepatocyte damage and inflammation by activating the ROS-mediated NLRP3-caspase-1-IL-1β pathway in Kupffer cells, indicating that ROS may serve as a potential target for the treatment of HAE.

Research perspectives

Although NLRP3 inflammasome is the most well-characterised inflammasome, further studies are warranted to determine whether other inflammasomes are activated in the HAE marginal zone and clarify the function and mechanisms of the immune responses in the progression of HAE.

FOOTNOTES

Author contributions: Chen CS and Fan HN conceived and designed the experiments; Chen CS, Zhang YG, and Wang HJ collected the samples; Chen CS and Zhang YG performed the experiments and data analyses; Chen CS wrote the first draft of the manuscript; Zhang YG and Fan HN provided comments for the revisions; all authors read and approved the final manuscript.

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Institutional review board statement: The study was reviewed and approved by the Institutional Review Board at Affiliated Hospital of Qinghai University (approval No. P-SL-2019054).

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Ethics Committee of the Affiliated Hospital of Qinghai University (approval No. 2019-SF-131).

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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

Data sharing statement: No additional data are available.

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ORIGINAL ARTICLE

Basic Study

Changes in the gut mycobiome in pediatric patients in relation to the clinical activity of Crohn's disease

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Abstract

BACKGROUND

Numerous studies have shown that in Crohn's disease (CD), the gut microbiota is of great importance in the induction and maintenance of inflammation in the gastrointestinal tract. Until recently, studies have focused almost exclusively on bacteria in the gut. Lately, more attention has been paid to the role of intestinal fungi.

AIM

To study the gut mycobiome analysis of pediatric patients with CD (in different stages of disease activity) compared to healthy children.

METHODS

Fecal samples were collected from patients: With active, newly diagnosed CD (n = 50); active but previously diagnosed and treated CD (n = 16); non-active CD and who were in clinical remission (n = 39) and from healthy volunteers (n = 40).

Fungal DNA was isolated from the samples. Next, next generation sequencing (MiSeq, Illumina) was performed. The composition of mycobiota was correlated with clinical and blood parameters.

RESULTS

Candida spp. were overrepresented in CD patients, while in the control group, the most abundant genus was *Saccharomyces*. In CD patients, the percentage of *Malassezia* was almost twice that of the control ($P < 0.05$). In active CD patients, we documented a higher abundance of *Debaryomyces hansenii* (*D. hansenii*) compared to the non-active CD and control ($P < 0.05$) groups. Moreover, statistically significant changes in the abundance of *Mycosphaerella*, *Rhodotorula*, and *Microidium* were observed. The analyses at the species level and linear discriminant analysis showed that in each group it was possible to distinguish a specific species characteristic of a given patient population. Moreover, we have documented statistically significant correlations between: *D. hansenii* and patient age (negative); *C. zeylanoides* and patient age (positive); *C. dubliniensis* and calprotectin (positive); *C. sake* and calprotectin (positive); and *C. tropicalis* and pediatric CD activity index (PCDAI) (positive).

CONCLUSION

Mycobiome changes in CD patients, and the positive correlation of some species with calprotectin or PCDAI, give strong evidence that fungi may be of key importance in the development of CD.

Key Words: Intestinal mycobiome; Fungi; Crohn's disease; Inflammatory bowel disease; Next generation sequencing; Molecular microbiology

Core Tip: There is growing evidence that intestinal microorganisms are associated with pathogenesis of Crohn's disease (CD). Until recently, studies have focused almost exclusively on bacteria. In this study we showed alterations in the fungal composition in pediatric patients with CD. Changes within the specific species of fungi depending on disease activity, and the positive correlation of some species with calprotectin or pediatric CD activity index, give strong evidence that these microorganisms may be of key importance in the development and course of CD. Some fungal species can be helpful in predicting an exacerbation of the disease or even predicting the diagnosis of CD.

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INTRODUCTION

Crohn's disease (CD) belongs to the group of chronic gastrointestinal tract disorders known as inflammatory bowel diseases (IBD), which are characterized by a relapsing and remitting course. The pathogenesis of CD is not fully understood, but it is supposed that its development and severity are influenced by a combination of genetic, immune and microbiological disorders[1,2]. Intestinal microorganisms are increasingly indicated as one of the key factors in the etiology of IBD[1,3-5].

The human gut is colonized by a stable and abundant community of microorganisms, collectively referred to as the gut microbiota, consisting of bacteria, archaea, fungi, protozoans, and viruses[6]. These microbes have physiological functions associated with nutrition, regulation of immune homeostasis, and protection of the host against pathogenic bacteria[3,6]. Recent advances in molecular techniques have recognized alterations in the composition, abundance, and function of the microbiota in CD, which is known as dysbiosis[1,6]. To date, it has not been established whether the changes in the microbiota are a direct cause of the disease or only a secondary effect of the chronic inflammatory process and the treatment applied. However, numerous studies in animal and human models[7-10] have shown that the gut microbiota in CD is of great importance in the induction and maintenance of inflammation in the gastrointestinal tract.

Until recently, studies have focused almost exclusively on bacteria in the gut[4,11-13]. Lately, more attention has been paid to the role of intestinal fungi. These microorganisms, their metabolites, and their interaction with bacterial populations may directly or indirectly influence the host's immune response

[1]. Fungi interact with the immune system among others *via* Dectin-1, which is one of the most important pattern recognition receptors[7,14]. This receptor has an influence on the activation of macrophages, neutrophils, and dendritic cells. Dectin-1 recognizes β-1.3 glucans that are components of the fungal cell wall and thus activates the intracellular signal that leads to the production of inflammatory cytokines[15]. It has been suggested that Dectin-1 polymorphisms are strongly associated with an excessive immune response to commensal fungi and thus may contribute to the development of CD [7]. In addition, the induction of anti-*Saccharomyces cerevisiae* antibodies (ASCA) that recognize yeast cell wall mannans is a serological biomarker frequently present in patients with CD, which may suggest that the disease may be associated with an unusual interaction of the immune system with fungi.

Taking into account the likely relationship between the mycobiota and CD, the aim of this study was a detailed taxonomic analysis of the fungal composition in children with CD. In our study, we analyzed the differences between the mycobiomes of CD patients and healthy children, as well as the changes that occur in patients' mycobiomes depending on disease activity.

MATERIALS AND METHODS

Study population

Pediatric patients with CD, aged 2 to 18 years, hospitalized at the University Children's Hospital in Krakow in the years 2016–2019, were recruited for the study. Diagnosis was made on the basis of the clinical picture, as well as endoscopic, histopathological, and radiological examinations, according to the revised Porto criteria[16]. The study protocol was approved by the Jagiellonian University Bioethics Committee (No. 1072.6120.21.2020). Disease phenotype was evaluated according to the Paris criteria [17]. The patients were recruited into two research groups on the basis of clinical disease activity according to the pediatric CD activity index (PCDAI).

Patients with active CD, PCDAI > 10 points.

In this group, two subgroups were distinguished: Ia–patients with newly diagnosed CD (before the implementation of any treatment); and Ib–patients previously diagnosed and treated with aminosalicylates (5-ASAs) and/or azathioprine.

Patients with non-active CD, PCDAI ≤ 10 points.

This group (II) included patients who were previously diagnosed with CD and treated with 5-ASAs and/or azathioprine and were in clinical remission at the time of inclusion in the study.

The exclusion criteria were: Lack of consent to participate in the study; patients under 2 or over 18 years of age; use of antibiotics and/or probiotics and/or antifungal drugs within 30 d before collecting stool samples; confirmed infections of the gastrointestinal tract; isolated perianal fistula.

The control group included healthy non-hospitalized children and adolescents without antibiotic, antifungal, or probiotic treatment during the 30-d period before stool sample collection.

Samples

At inclusion, routine laboratory tests evaluating biochemical parameters and calprotectin were performed and the PCDAI score was evaluated in all patients. Stool samples from all participants were obtained at the University Children's Hospital in Krakow and immediately frozen at -80 °C, then delivered under deep freeze conditions to the Department of Microbiology of Jagiellonian University Medical College in Krakow, where DNA was isolated and further analyses were performed.

Isolation of DNA from stool samples

The stool samples were thawed and 150 mg of each material was used for fungal DNA isolation using the Genomic Mini AX Stool Kit (A&A Biotechnology, Gdansk, Poland), according to our previously described modification[18,19].

The isolates were measured with a NanoDrop spectrophotometer (Thermo Fisher, Waltham, MA, United States) in the A260 nm and A260 nm/280 nm ratios to determine the purity and concentration of DNA.

Preparation of genomic library

The DNA extracted was used to perform polymerase chain reaction (PCR) amplification (T100 Thermal Cycler, BioRad, California, United States), with primers targeting the ITS-1 regions of the fungal rDNA gene[20] to prepare ITS libraries.

The primer sequences were the following: [Adapter sequences for MiSeq sequencer (Illumina, San Diego, United States) are written in bold][21]:

Forward primer:

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTAAAAGTCGTAACAAGGTTTC;

Reverse primer:

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTTCAAAGAyTCGATGATTCAC.

The composition of the reaction mixture was as follows: 5 μL of DNA, 12.5 μL of Kapa Biosystems (Roche, Basel, Switzerland), 0.5 μL of each primer (20 mmol/L, Genomed, Warsaw, Poland), 6.5 μL of water (A&A Biotechnology, Gdansk, Poland). Thermal cycling conditions included an initial denaturation at 95 °C for 5 min followed by 50 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s.

The volume of 5 μL of each amplicon was subjected to electrophoretic separation on 1.5% agarose gel (Prona, Basica Le, Burgos, Spain) diluted 10 times with TBE buffer (Sigma-Aldrich, Saint Louis, United States). The PCR products were visualized in the FastGene FAS–Digi Pro (Nippon Genetics, Duren, Germany) in the presence of UV light, to check library quality. Subsequent steps (purification, sample indexing, sample quantification, and pooling) were prepared according to the Illumina library preparation protocols[21].

Next generation sequencing

The library concentration was measured using PicoGreen fluorescent dye (Thermo Fisher, Waltham, MA, United States) and pooled with 30% spike-in PhiX control DNA (Illumina, San Diego, United States). Next, the pooled libraries were applied to the Reagent Kit V3 cartridge (600 cycles) (Illumina, San Diego, United States) and the sequencing was carried out using MiSeq (Illumina, San Diego, United States) at the Department of Clinical Immunology of the University Children's Hospital in Krakow.

Negative control (containing water instead of DNA) and positive control (MSA 1010 Mycobiome Genomic DNA mix, ATCC, Manassas, United States) were included throughout the genomic library procedure and next generation sequencing sequencing.

Bioinformatics analysis

The ITS-targeted amplicon pair reads were demultiplexed on the basis of the unique molecular barcodes. The short read of raw data was collected as FASTQ files. Then, the per-sample raw taxonomic classification was performed using the Illumina 16S Metagenomics workflow, on the basis of the UNITE Fungal ITS Database v7.2.

Sequences were clustered into operational taxonomic units (OTUs) at a similarity cutoff value of 97%-98%. This classification was performed on the basis of the algorithm described by Wang *et al*[20]. Next, the metagenomic reads were aligned to the UNITE Fungal ITS Database v7.2 reference taxonomy database. During this step, the candidate fungal reads were identified, and the sequences which did not match the reference fungal ITS database were filtered out (these were features with counts below 4 counts, 20% of prevalence filter). In the normalization step, sequences were trimmed for low-quality scores (less than 3). Finally, filtered and normalized reads were matched again to the fungi, and a definitive taxonomic assignment was performed. Abundance profiling was characterized on the basis of the OTU data, which were compared at different taxonomic levels based on the annotations. The diversity within samples was measured on the basis of alpha and beta diversity indices. To calculate the diversity within samples (richness and evenness), Observed, ACE, Chao1, Shannon, Simpson, and Fisher matrices were applied, and analysis of variance (ANOVA) statistics were performed. Beta diversity analysis was performed on the basis of Principal Coordinate Analysis using Bray–Curtis and the Jaccard index. The statistical significance of sample grouping was tested using the Permutational MANOVA (PERMANOVA) statistical method. The linear discriminant analysis (LDA) Effect Size (LEfSe) algorithm was used to discover statistically significant biomarkers. The Kruskal–Wallis rank sum test was performed to describe features with significant abundance at different taxonomic levels. A 0.05 *P* value cutoff and 2.0 LDA score cutoff were established to determine the most significant features.

RESULTS

Characteristics of the study population

One hundred and five pediatric patients were included in the study. The control group consisted of 40 children. The characteristics of the study groups are presented in Table 1.

DNA quality

A DNA purity of ≥ 1.7 was assumed. All samples examined met this criterion.

Analysis of the biodiversity of the gut mycobiota

The data obtained by sequencing consisted of 4859784 reads (1680 minimum reads per sample, 79543 maximum) with an average number of reads of 33748. All samples were included in the analysis.

Alpha diversity at the phylum level (L2) was different and statistically significant between: Ia *vs* control (expressed as the Shannon, Chao, and Fisher indices; Ia *vs* II (Fisher index); and II *vs* control (Chao index) (Table 2). Moreover, we observed statistically significant differences in alpha diversity at the species level (L7) between groups: Ia *vs* II (expressed as both Chao and Fisher indices); Ia *vs* control (Chao and Fisher indices); Ib *vs* II (Fisher index); Ib *vs* control (Fisher index) (Table 2).

Table 1 Clinical data from patients with Crohn's disease and healthy subjects

	Group Ia (*n* = 50)	Group Ib (*n* = 16)	Group II (*n* = 39)	Control (*n* = 40)
General characteristics				
Male:Female (ratio)	29:21 (1.38)	8:8 (1)	22:17 (1.29)	15:25 (0.6)
Age (years), mean ± SD	13 ± 2.9	15 ± 2.8	12.5 ± 4.3	11 ± 4.1
BMI (kg/m^2), mean ± SD	17.0 ± 3.1	18.1 ± 2.7	18.1 ± 3.2	18.3 ± 3.8
PCDAI, mean ± SD	34.4 ± 12.7	38.2 ± 18.6	4.1 ± 3.9	N/A
Disease distribution by Paris classification (number of patients)				
A1a	4	2	7	
A1b	46	14	30	
A2	0	2	2	
L1	2	1	2	
L2	6	2	7	
L3	16	4	12	
L4aL1	8	2	6	
L4aL2	8	3	5	
L4aL3	10	4	7	
B1	39	11	27	
B2	11	5	12	

Group Ia: Active/newly diagnosed Crohn's disease (CD); Group Ib: Active/previously diagnosed and treated CD; Group II: Non-active CD; control: Healthy subjects; Paris classification: Age: A1a (0–10 yr), A1b (10–17 yr), A2 (17–40 yr); Location: L1 (distal 1/3 ileum and/or limited cecal disease), L2 (colonic), L3 (ileocolonic), L4a (upper disease proximal to ligament of Treitz), L4b (upper disease distal to ligament of Treitz and proximal to distal 1/3 ileum); Behaviour: B1 (non-structuring non-penetrating), B2 (structuring), B3 (penetrating), B2B3 (both penetrating and structuring disease either at the same or different times); BMI: Body mass index; PCDAI: Pediatric Crohn's disease activity index; N/A: Not applicable.

Table 2 Alpha diversity metrics *P* values at the phylum (L2) and species (L7) level, expressed as Shannon, Chao and Fisher indices

Index/group	Shannon		Chao		Fisher	
	L2 level	L7 level	L2 level	L7 level	L2 level	L7 level
Ia *vs* Ib	0.713	0.251	0.836	0.121	0.533	0.312
Ia *vs* II	0.606	0.584	0.228	0.001[a]	0.000[a]	0.011[a]
Ia *vs* control	0.000[a]	0.313	0.006[a]	0.000[a]	0.000[a]	0.000[a]
Ib *vs* II	0.492	0.128	0.356	0.185	0.132	0.041[a]
Ib *vs* control	0.072	0.053	0.103	0.077	0.072	0.027[a]
II *vs* control	0.086	0.710	0.044[a]	0.631	0.998	0.938

[a]$P < 0.05$, statistically significant value.
Group Ia: Active/newly diagnosed Crohn's disease (CD); Group Ib: Active/previously diagnosed and treated CD; Group II: Non-active CD; control: Healthy subjects.

Furthermore, statistically significant differences were observed at the phylum and species level for beta diversity between groups Ia *vs* control for three indices. Additionally, differences at the species level were observed between groups II and control (expressed as the Jensen–Shannon index) (Table 3).

Analysis of the composition of the gut mycobiota

The gut mycobiota was evaluated at six taxonomic levels (phylum, class, order, family, genus, species). Three of these, L2 (phylum), L6 (genus), and L7 (species) were described in this study to include the most representative taxa. At the phylum level (L2), all OTUs obtained were assigned to two main phyla:

Table 3 Beta diversity metrics *P* values at the phylum (L2) and species (L7) level, expressed as Bray-Curtis, Jensen-Shanon and Jaccard indices

Index/group	Bray-Curtis		Jensen-Shannon		Jaccard	
	L2 level	L7 level	L2 level	L7 level	L2 level	L7 level
Ia *vs* Ib	0.753	0.727	0.746	0.92	0.813	0.632
Ia *vs* II	0.409	0.291	0.404	0.176	0.469	0.389
Ia *vs* control	0.038[a]	0.006[a]	0.009[a]	0.001[a]	0.035[a]	0.008[a]
Ib *vs* II	0.532	0.374	0.543	0.468	0.517	0.332
Ib *vs* control	0.172	0.086	0.125	0.064	0.15	0.094
II *vs* control	0.313	0.057	0.206	0.026[a]	0.393	0.087

[a]$P < 0.05$, statistically significant value.

Group Ia: Active/newly diagnosed Crohn's disease (CD); Group Ib: Active/previously diagnosed and treated CD; Group II: Non-active CD; control: Healthy subjects.

Ascomycota and *Basidiomycota*. The others were non-fungal and belonged mainly to plant taxa. The *Ascomycota* phylum was clearly predominant in all groups, respectively: 83% in group Ia; 86% in group Ib; 78% in group II; 80% in the control group (Figure 1). In groups Ia and Ib, the percentage of these fungi was significantly higher compared to the control group ($P = 0.039$; $P = 0.046$, respectively).

At the genus level (L6) (Figure 2), *Candida* dominated in all groups including CD patients (Ia–46%, Ib–45%, II–44%), and in groups Ia and II the abundance was statistically higher compared to the control group (26%; respectively: $P < 0.001$; $P = 0.035$). On the other hand, in the control group, the dominant genus was *Saccharomyces* (38%), and the abundance of this fungus was significantly higher in comparison to Ia (27%; $P < 0.001$). Interestingly, in all groups that included CD patients, the prevalence of *Malassezia* was almost twice as high (11%–14%) compared to the control group (7%), although statistically significant differences were only documented between group Ia and control (14% *vs* 7%, $P = 0.015$). Furthermore, in the group of patients with active CD (Ia and Ib), *Debaryomyces* constituted a higher percentage (respectively: 5%; 6%) compared to the other groups (2%), but significant differences were noted only between group Ia and the control ($P = 0.024$). Additionally, a significantly higher level of *Rhodotorula* was observed in the control group (9%) compared to the groups Ia (1%, $P < 0.001$), Ib (0.3%, $P = 0.001$), and II (2%, $P = 0.039$), and in group group Ib compared to group II (0.3% *vs* 2%, $P = 0.014$). Moreover, a significantly lower level of *Mycosphaerella* was observed in group Ib (0.3%) compared to groups II (5%, $P = 0.015$) and control (1%, $P = 0.016$). Statistical differences were also observed between group II (5%) and control (1%, $P = 0.027$) and for group Ia and II (2% *vs* 5%, $P = 0.029$). Statistical differences for *Microidium* were also found between groups Ib and control (2% *vs* 4%, $P < 0.001$).

At the species level (L7), *S. cerevisiae* and *Candida albicans* (*C. albicans*) were dominant in all investigated groups and quantitatively constituted nearly 50% of all fungi. In the control group, *S. cerevisiae* was the most abundant yeast (38%), in contrast to the group of patients with CD (23% in Ia; 28% in Ib; 20% in II), in whom an increased percentage of individual *Candida* species was observed (Figure 3). In groups Ia, II, and control, the dominant species of the genus *Candida* was *C. albicans* (respectively 23%; 32%; 17%), and within this species, statistically significant differences were observed between groups Ia *vs* Ib (23% *vs* 11%, $P = 0.008$) and Ib *vs* control (11% *vs* 17%, $P = 0.008$). Differences close to statistical significance were documented between group II and the control (32% *vs* 17%, $P = 0.054$). The mycobiome of the patients in group Ib was overrepresented by *Candida dubliniensis* (19%) and *Candida zeylanoides* (12%) and the percentage of these fungi was significantly higher than in group II (respectively: 6%, $P = 0.001$; 1%, $P = 0.003$). A higher percentage of *Candida tropicalis* (10%) was observed in group Ia compared to the other groups (0.1%–4%), although statistically significant changes were found between groups Ia and Ib (10% *vs* 0.1%, $P = 0.008$) and Ib and control (0.1% *vs* 2%, $P = 0.021$). Furthermore, among the control group, a statistically higher percentage of *Candida krusei* (*C. krusei*) was observed compared to group II (2% *vs* 1%, $P = 0.005$).

Patients with CD (especially those newly diagnosed with active disease) were characterized by a higher prevalence of *Malassezia restricta* compared to the control group (11% *vs* 6%, Figure 3); however, these changes were not statistically significant. Statistical differences were observed between groups Ib and II (10% *vs* 9%, $P = 0.001$). Among controls, a significantly higher percentage (9%) of *Rhodotorula mucilaginosa* (*R. mucilaginosa*) was found in comparison to Ia (0.7%, $P = 0.001$); Ib (0.3%, $P = 0.002$) and II (2%, $P = 0.027$). Statistically significant differences were also found for groups Ib and II ($P = 0.015$). In the control group, a slight increase in *Saccharomyces cariocanus* was observed in comparison to group II (3% *vs* 2%, $P = 0.046$) and for *Microidium phyllanthi* (*M. phyllanthi*) compared to all CD groups (4% *vs* 2%, $P < 0.05$). There were also statistically significant differences in *Mycosphaerella tassiana* (*M. tassiana*)

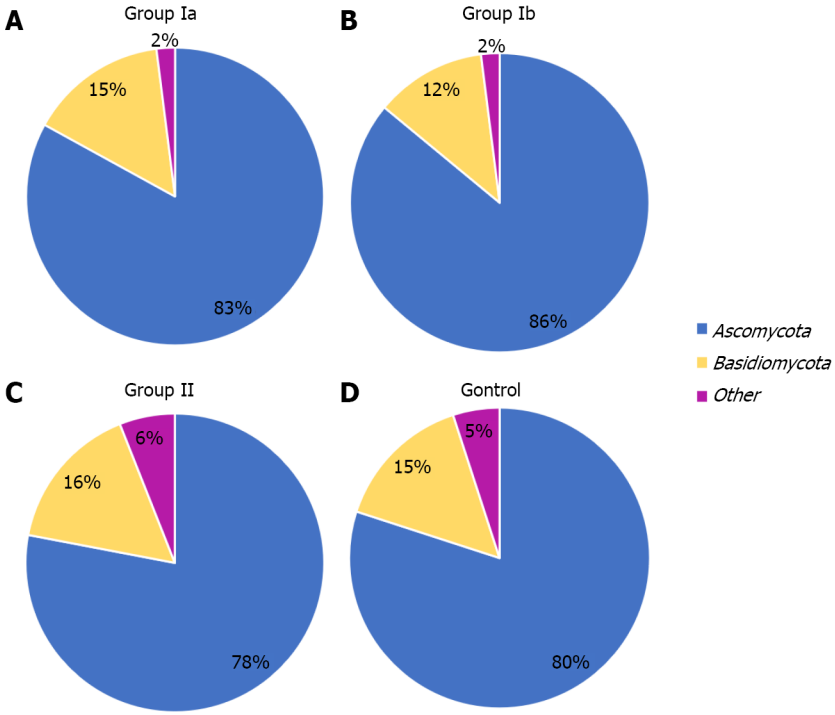

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Figure 1 Fungal profiles at the phylum level (L2). A: Active/newly-diagnosed Crohn's disease (CD); B: Active/previously diagnosed and treated CD; C: Non-active CD; D: Healthy subjects.

abundance between groups Ia *vs* II (2% *vs* 5%, $P = 0.029$) and Ib *vs* control (0.3% *vs* 0.6%, $P = 0.016$). In addition, the changes in abundance of *Debaryomyces hansenii* (*D. hansenii*) (3% *vs* 1%, $P = 0.020$) and *Malassezia globosa* (*M. globosa*) (2% *vs* 5%, $P = 0.037$) were documented between groups Ia and II, and in *M. globosa* between groups II *vs* control (5% *vs* 1%, $P = 0.030$), and also in *Epicoccum nigrum* (*E. nigrum*) between Ia *vs* control (2% *vs* 0.7%, $P = 0.015$).

Linear discriminant analysis-determination of biomarkers

Owing to LDA we have selected species of fungi that could be biomarkers characterizing and distinguishing a given group of patients (Figure 4). We have shown that *C. tropicalis* was overrepresented in group Ia compared to the other groups ($P = 0.026$). Whereas increased richness of *C. albicans* ($P = 0.005$), *M. globosa* ($P = 0.011$) and *M. tassiana* ($P = 0.024$) was documented in group II compared to the other groups. The fungal biomarkers that differentiated the control group from the CD patient groups were *R. mucilaginosa* ($P < 0.001$), *C. krusei* ($P = 0.013$), and *M. phyllanthi* ($P < 0.001$). Group Ib was characterized by a higher abundance of *E. nigrum* compared to the other groups ($P = 0.011$).

Correlation between the abundance of fungi and patients' clinical parameters

The analysis of the abundance of fungal species showed significant correlations between: *D. hansenii* and *C. zeylanoides* and patient age (negative and positive, respectively); *C. dubliniensis* and *C. sake* and calprotectin (both positive) and a positive correlation between *C. tropicalis* and PCDAI (Table 4).

DISCUSSION

Numerous studies suggest that changes in the intestinal microbiota and the interaction between microorganisms and the host may lead to the development of IBD. The main limitation of the available studies[12,22-24] is the predominance of experiments in adult patients who have been treated for a long time and who have been sick for many years, which could significantly affect the results obtained. Moreover, the studies of the intestinal microbiota in IBD conducted so far have focused primarily on disorders within the bacteriobiome. There is a lack of more comprehensive research, including fungi, which constitute a very important component of the intestinal microbiome[25]. In our study, we recruited children and adolescents with both newly and previously diagnosed CD and we compared their mycobiome in the exacerbation and remission periods. In addition, we recruited healthy children into a control group. Such a study allowed a more reliable determination of the relationship between fungi in the digestive tract and the course of CD. Moreover, the main exclusion criterion in all groups

Table 4 Correlations of fungal species with patient parameters

Variables/age		Variables/calprotectin		Variables/PCDAI	
Debaryomyces hansenii	$P < 0.008$	*Candida dubliniensis*	$P < 0.003$	*Candida tropicalis*	$P < 0.006$
	$r = -0.08$		$r = 0.29$		$r = 0.58$
Candida zeylanoides	$P < 0.004$	*Candida sake*	$P < 0.04$		
	$r = 0.08$		$r = 0.19$		

PCDAI: Pediatric Crohn's disease activity index.

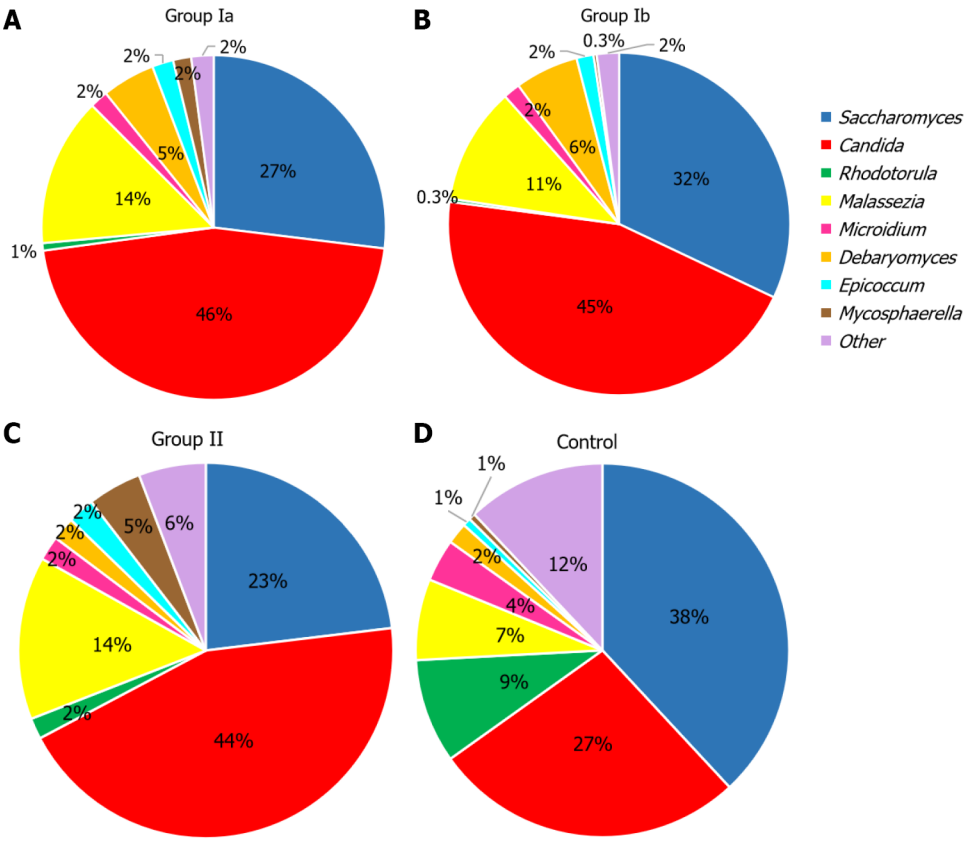

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Figure 2 Fungal profiles at the genus level (L6). A: Active/newly-diagnosed Crohn's disease (CD); B: Active/previously diagnosed and treated CD; C: Non-active CD; D: Healthy subjects.

was the use of antibiotics, probiotics, and antifungal drugs that could significantly change the composition of the mycobiome. Thus, the impact of confounding factors associated with antimicrobial therapy was eliminated. Our data have shown that the mycobiota of pediatric CD patients was different in comparison to healthy controls, and also depended on the stage of disease activity.

The alpha diversity (which is a measure of biodiversity within the study groups) of the fungal microbiota was significantly different (both at the phylum and species levels) in group Ia compared to the control expressed as the Chao and Fisher indices (Table 2). Additionally, biodiversity was higher in control compared to the Ia group for the three analyzed indices. Lower alpha diversity in the samples from patients with active, newly diagnosed CD indicated a reduction in fungal biodiversity, *i.e.*, lower number of taxons in a single sample. These data are consistent with previous reports[24,26,27]. Interestingly, we have shown that patients in the non-active phase of CD (II) were characterized by significantly higher alpha biodiversity indices at the level L7 (species) compared to patients with newly diagnosed, active disease (Ia). This provides the basis for the conclusion that while patients achieve remission, normalization of mycobiome occurs, an indicator of which is the increase in the measure of biodiversity[28].

The analysis of beta diversity (which is a measure of differences between the study groups) has shown that there were statistically significant differences between group Ia and the control, both at the

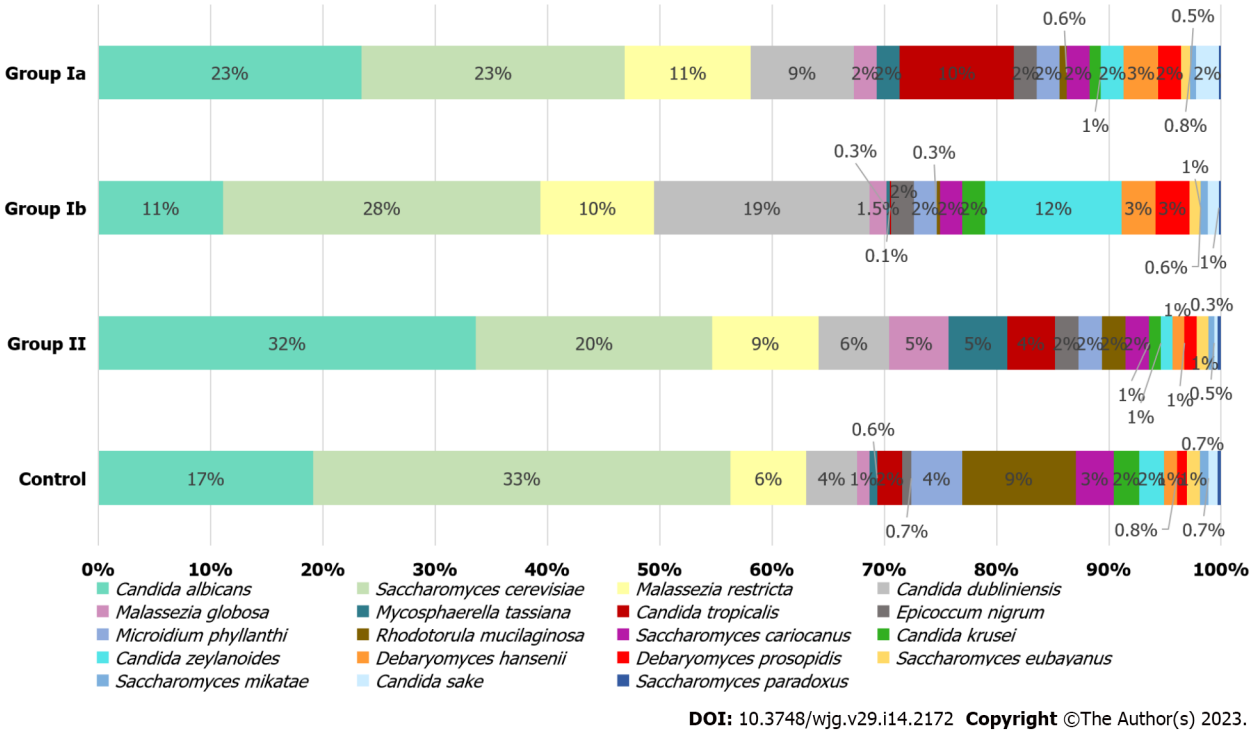

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Figure 3 Fungal profiles at the species level (L7). Group Ia: Active/newly-diagnosed Crohn's disease (CD); Group Ib: Previously diagnosed and treated CD; Group II: Non-active CD; Control: Healthy subjects.

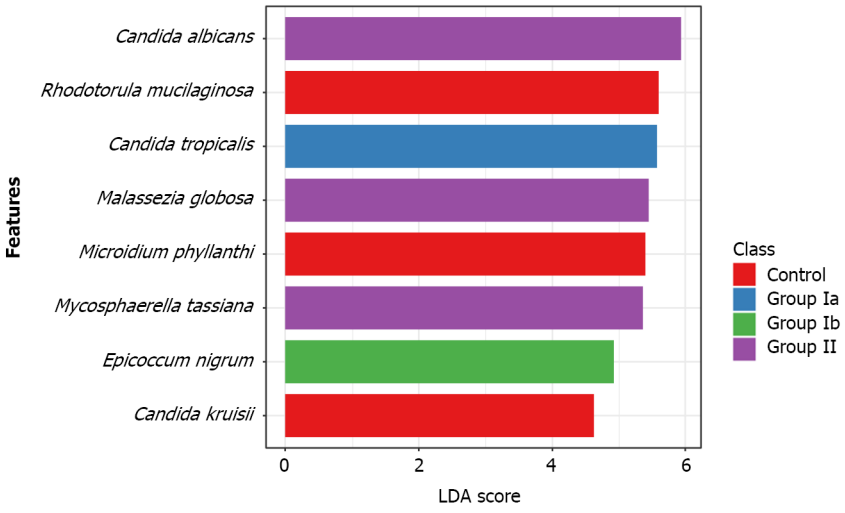

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Figure 4 Histogram of the linear discriminant analysis logarithmic scores for statistically different fungi between study and control groups. Positive linear discriminant analysis scores represent fungi that were over-abundant in Group Ia–active/newly diagnosed Crohn's disease (CD) (blue bars), Group Ib–active/previously diagnosed and treated CD (green bars), Group II–non-active CD (purple bars), and control–healthy subjects (red bars) groups.

L2 and L7 levels (expressed by the three indices) (Table 3). These results indicate a significantly different taxonomic composition at the phylum and species levels between these groups. The different composition of mycobiota seen in the patients of group Ia compared to healthy children indicates that dysbiosis appears at the very beginning of the disease. However, the treatment (groups Ib and II) likely reduces the differences in mycobiome composition compared to controls.

At the phylum level, *Ascomycota* was predominant in all groups (Figure 1), but in patients with active CD (irrespective of whether it was a newly diagnosed or previously diagnosed disease), the percentage of this phylum was significantly higher (83%–86%) compared to the control group (80%). These observations are consistent with studies by other researchers showing an increased percentage of *Ascomycota* in adult patients with active CD[22,29].

At the genus level (Figure 2), we observed that among patients with CD (irrespective of disease activity), *Candida* was the predominant genus in contrast to the control group (significant differences between Ia *vs* control and II *vs* control; $P < 0.05$). Analyses at the species level (Figure 3), showed that in each of the studied groups it was possible to distinguish a specific species within the genus *Candida*, characteristic of a given patient population. Thus, an excessive abundance of *C. tropicalis* has been observed in newly diagnosed patients in active phase of CD (group Ia) (Figure 3). Interestingly, in patients in remission (group II), the abundance of this species decreased more than double. Moreover, in the control group, *C. tropicalis* was 5 times lower than in group Ia. This may suggest that *C. tropicalis* may be a biomarker of inflammation in the gut in the course of CD, which was supported by LDA analysis (Figure 4). It has previously been shown that overrepresentation of *C. tropicalis* is commonly found among patients with active CD[12,23,30] and possibly influences the initiation and maintenance of inflammation[23]. Hoarau *et al*[23] showed that a higher prevalence of *C. tropicalis* fungi was positively correlated with levels of ASCA and with the abundance of *Serratia marcescens* and *Escherichia coli*. Researchers demonstrated that these microorganisms interact with each other in the gut, forming a biofilm that can promote an excessive immune response and cause intestinal mucosal barrier dysfunction, contributing to the formation of inflammatory lesions[23]. Additionally, studies in animal models of IBD confirmed that *C. tropicalis* has strong immunogenic potential. Supplementation in mice with *C. tropicalis* resulted in excessive secretion of pro-inflammatory cytokines, such as tumor necrosis factor alpha, interferon gamma and, interleukin-17, and then intensification of inflammation in the gut [7]. In addition, in other independent studies, *C. tropicalis* has induced dysbiosis that involved changes in the presence of mucin-degrading bacteria, leading to altered tight junction protein expression with increased intestinal permeability. Then, it induced a strong Th1/Th17 response, leading to an accelerated pro-inflammatory phenotype in experimental colitis mice[31]. Interestingly, in our research, a positive correlation between *C. tropicalis* and PCDAI was observed, which strengthens the above suggestions that these fungi may be associated with the exacerbation of the disease.

Analysis at the species level has shown that *C. albicans* constituted the highest percentage in the group of patients in remission (II) compared to the other groups (Figure 3), and LDA analysis has documented that this species may be a fungal biomarker for patients in non-active CD ($P = 0.005$, Figure 4). Previous studies showed that these yeasts were more abundant in CD patients, and it was suggested that *C. albicans* may promote IBD by increasing the inflammatory response[27,32,33]. However, it should be noted that these studies did not take into account patients in remission. Patients in the active phase or all CD patients together (undifferentiated according to the phase of disease activity) were compared with healthy people. In our research, an additional comparison of the exacerbation and remission groups has provided evidence that there are more *C. albicans* in non-active CD compared to active disease. It is likely that the treatment used to lead the patient into remission somehow promotes the growth of *C. albicans*; however, the effect of anti-inflammatory treatment commonly used in CD (like 5-ASAs; azathioprine) on the mycobiome has not been proven so far. Moreover, the growth of fungi in the gut is closely related to changes in the bacterial microbiota, so it is also possible that changes in the bacterial community that occur during remission contribute to the increased abundance of *C. albicans*[12,23,34-36].

The results of our study show an increased number of *C. dubliniensis* and *C. zeylanoides* in active but already treated patients (Ib) compared to other groups (Figure 3). To our knowledge, this is the first such report. Perhaps the increase in the colonization by these species of fungi was related to the treatment or a long-term inflammatory process. However, it should be noted that among treated patients in remission (II), the abundance of *C. dubliniensis* and *C. zeylanoides* significantly decreased ($P = 0.001$, $P = 0.003$; respectively) compared to treated patients in the active phase (Ib). It is possible that colonization by these fungi may depend on the disease activity and long-term inflammatory process that promotes their multiplication. Although *C. dubliniensis* is not an epidemiologically significant species, some reports indicate that it may cause opportunistic infections that may be associated with a higher degree of intestinal colonization[37,38]. Thus, its involvement in the maintenance of inflammation in CD cannot be ruled out, especially since a positive significant correlation of this species with calprotectin (which is a marker of intestinal inflammation) was observed (Table 4).

C. zeylanoides are fungi found in food products[39-41], so their presence may be associated with different diets of patients. On the other hand, we documented that these fungi were positively significantly correlated with age (Table 4). Perhaps, the higher abundance of *C. zeylanoides* in group Ib was related to the fact that we had slightly elderly patients in this group compared to the other one (Table 1). There are several reported cases in which *C. zeylanoides* caused fungemia[42-44]; however, there is no evidence that this microorganism has a connection with chronic or autoimmune diseases.

An interesting subject of study is *D. hansenii*. The abundance of these fungi was higher among patients with active CD, however, statistically significant changes were only observed between groups Ia and II ($P = 0.020$). The fact that the prevalence of this species was three times higher in patients with active CD compared to patients in remission and control (Figure 3) indicates that this fungus may be involved in maintaining intestinal inflammation or even inducing disease. Jain *et al*[45] came to similar conclusions. The researchers showed that damaged intestinal tissue (in a mouse model of CD) was over-colonized with *D. hansenii*, which consequently impeded the healing and regeneration process of intestinal crypts. Interestingly, when mice received an antifungal drug (amphotericin), the wounds

began to heal[45]. In the same study, the authors conducted an analysis with the participation of adult people with CD and showed that *D. hansenii* was detected in all samples taken from patients (100%), but only in one healthy person (10%). Further detailed observation of tissue biopsies showed that this fungus was present only in the inflamed mucosa, whereas it was absent from the non-inflamed mucosa sampled from the same patient[45]. Our study is the first report documenting the presence of *D. hansenii* in children and adolescents with CD. Furthermore, our data indicate that the abundance of these fungi depends on the phase of disease activity. These observations lead to the conclusion that *D. hansenii* may be involved in maintaining inflammation.

In this study, we have documented that CD patients were over-colonized with *Malassezia* species compared to the control group (Figures 2 and 3). This is consistent with the observations of other researchers[12,27,46]. Patients with CD often suffer from malabsorption syndrome, manifested by fatty diarrhea. *Malassezia* requires lipids to multiply; therefore, it is possible that increased detectability of these yeasts in CD patients is related to the fact that excess fat in the stool creates an optimal environment for *Malassezia* over-colonization. However, the participation of these yeasts in maintaining inflammation cannot be excluded because there are a few studies that demonstrate the immunogenic properties of *Malassezia* in the context of IBD[46,47]. The species analysis provided interesting observations regarding the changes in individual species of *Malassezia* depending on the activity of the disease (Figure 3). Thus, among children in remission (II), *M. globosa* colonization increased and these changes were statistically significant compared to group Ia ($P = 0.037$) and the control group ($P = 0.030$). On the other hand, patients in the exacerbation phase had a higher percentage of *M. restricta* ($P = 0.001$). The participation of *M. restricta* was previously reported in individual studies. Limon *et al*[46] demonstrated that these fungi were significantly more abundant in CD patients than in healthy subjects, and in addition, their presence was associated with exacerbation in a mouse model of IBD[46]. Furthermore, the team showed that these yeasts were especially present in patients with IBD caspase recruitment domain-containing protein 9 (*CARD9*) risk. CARD9 is a signaling adapter protein that is essential for antifungal innate immunity in mice and humans. Single nucleotide polymorphism in the gene for CARD9 is the strongest genetic risk factor linked to IBD. A certain variant of this gene caused human immune cells to produce more potent inflammatory cytokines in response to *M. restricta*[46]. Additionally, Li *et al*[12] showed that the biopsies collected from inflamed mucosa were over-colonized by *M. restricta* in relation to tissue without inflammation from the same patient[12]. A study on the immunogenicity of fungi of the genus *Malassezia* in the context of IBD confirmed that both *M. globosa* and *M. restricta* have strong pro-inflammatory properties. Both yeast species induced the production of inflammatory cytokines by dendritic cells in large part mediated through Dectin2 and CARD9 signaling [47]. Furthermore, *M. restricta* exacerbated colitis in germ-free mouse models of IBD triggered by inflammatory reactions mediated by CARD9[46]. The above data indicate that fungi of the genus *Malassezia* significantly influence the host's immune response, so their role in the pathogenesis of IBD cannot be ruled out. Understanding the species changes that occur in the various phases of disease activity, which were documented in our analysis, forms the basis for further research and gives an insight into the role of these yeasts in the course of CD.

Another species whose participation in CD is worth considering, is *Epicoccum nigrum*. These fungi have been overlooked so far, but this is probably due to the difficulties in culturing and detecting these yeasts. In this study, we have shown that the abundance of these fungi was significantly higher in CD patients (Ia) compared to healthy children ($P = 0.015$, Figure 3). Wheeler *et al*[48] documented the first reports on *E. nigrum* in CD and suggested its association with the treatment applied. The researchers showed that extended pretreatment with antifungal drugs restructured the intestinal fungal population. Increased colonization by the *E. nigrum* fungi, among others, has been observed. It is unknown whether the observed changes were due to alterations in the microbial community that promoted *E. nigrum* overgrowth, or whether these yeasts were resistant to the applied treatment. However, these changes were correlated with exacerbated disease in chemically induced models of experimental colitis[48]. In our study, the exclusion criterion was the use of antifungal drugs, so the presence of *E. nigrum* was not related to treatment. However, it cannot be ruled out that the alterations observed in the microbial community in CD patients promote the overgrowth of *E. nigrum*. Fungal populations of the intestinal tract directly or indirectly help to maintain healthy intestinal homeostasis. Any changes in the composition of the mycobiome may cause serious disruption, especially in genetically susceptible people to diseases (*e.g.* predisposed to CD). On the other hand, the LDA analysis (Figure 4) has shown that *E. nigrum* may be a fungal biomarker for patients with active, previously diagnosed disease (Ib). Perhaps the presence of these fungi is associated with the active long-term inflammation. It cannot be ruled out that chronic and long-term inflammatory lesions in the intestine favor the growth of this species. The overrepresentation of *E. nigrum* found in this report in CD patients requires further research.

In this study, we also demonstrated an increase in colonization level by *R. mucilaginosa* and *M. phyllanthi* among healthy subjects compared to CD patients ($P < 0.05$, Figure 3). LDA analysis has shown that these species may be a fungal biomarker for healthy children (Figure 4). *R. mucilaginosa* are fungi commonly found in soil, plants, and animals; however, they are also food contaminants. They are often isolated from fruit juices and dairy products[49]. The increase in the occurrence of these fungi in healthy children could be related to a varied and rich diet used by healthy people. In contrast, patients with CD

usually are on a dairy-free diet and exclude many products from their meals, so they carry fewer yeasts associated with foods in the digestive tract. On the other hand, *M. phyllanthi* are plant pathogens that can be delivered to the gastrointestinal tract through the consumption of vegetables and fruits. Patients with CD significantly reduce their consumption of legumes, and stone fruits, and thus, they have a lower abundance of plant-related fungi than healthy people following a varied diet[50,51].

CONCLUSION

In conclusion, this study confirms alterations in intestinal fungal composition in pediatric patients with CD and shows that some species of fungi may be a microbiological marker related to the activity of the disease. In patients with CD, we have documented an increased load of fungi with potential pro-inflammatory effects (*e.g.*, *Candida* spp., *Malassezia* spp.)[7,14,23,46], while fungi with potential anti-inflammatory effects (such as *Saccharomyces*)[23,52-55] were found in a lower percentage. Moreover, changes within the specific species of fungi depending on disease activity, and the positive correlation of some species with calprotectin or PCDAI, give strong evidence that these microorganisms may be of key importance in the development and course of CD.

Furthermore, we have shown that some fungal species can be helpful in predicting an exacerbation of the disease or even predicting the diagnosis of CD. Further research should focus on selecting fungal species that could be biomarkers to help predict disease exacerbation. Moreover, future research should assess whether the mycobiota could be a therapeutic target.

ARTICLE HIGHLIGHTS

Research background

The recurrent and chronic course of Crohn's disease (CD), its systemic after effects, and intestinal complications constitute a serious clinical problem, and since the pathogenesis of the disease is unknown, causal treatment is currently not used. As CD incidence age keeps falling and there is a growing number of cases, we are led to undertake intensive studies to determine the possible causes of this disease. Recently, due to the growing interest in the topic of intestinal microbiome, a hypothesis has emerged that the initiation of CD is associated with dysbiosis within the gut microbiota. And while the importance of bacteria in the pathogenesis has been, up to now, a common subject of research, the involvement of fungi has usually been overlooked. The few available studies including mycobiome analysis concern adults and not children, previously treated patients, or those with long-term disease. These shortcomings distort the results due to the impact of confounding variables (such as treatment, age, or the long-term disease process) on changes in the fungal composition.

Research motivation

Undoubtedly, the composition of the microbiome has a significant influence on maintaining internal balance and health and microbial changes constitute an important factor inducing pathological processes. Due to the fact that fungi are an important component of the gut microbiota, it is possible that alterations in the composition of the gut mycobiome may have an impact on the induction of CD.

Research objectives

Taking into account the likely relationship between the mycobiota and the host, the aim of this study was to perform a detailed taxonomic analysis of the fungal composition in pediatric patients with CD. In our study, we recruited children and adolescents with newly and previously diagnosed CD and compared their mycobiome in the exacerbation and remission periods. Additionally, we recruited healthy children into a control group. Such a study allowed for a more reliable determination of the relationship between fungi in the digestive tract and the course of CD.

Research methods

DNA was isolated from stool samples from patients: With active, newly diagnosed CD (n = 50); active but previously diagnosed and treated CD (n = 16); non-active CD who were in clinical remission (n = 39) and healthy volunteers (n = 40). The next step was to prepare genomic libraries for next generation sequencing (NGS). NGS was performed using a MiSeq sequencer (Illumina). The composition of the gut mycobiota was analyzed using UNITE Fungal ITS Database v7.2, and then correlated with clinical and blood parameters.

Research results

Our study confirms alterations in fungal composition in pediatric CD patients and shows that some species of fungi may be a kind of microbiological marker related to the activity of the disease. In CD

patients, we have documented an increased load of fungi with potential pro-inflammatory effects (*e.g.* *Candida* spp., *Malassezia* spp.), while fungi with potential anti-inflammatory effects (such as *Saccharomyces*) were found in a lower percentage. Interestingly, the greatest alterations in mycobiome composition (compared to the control group) were observed among newly diagnosed patients, before implementing any therapeutic approaches. This is strong evidence that fungi may play an important role in the development of CD. This thesis is supported by the fact that a positive correlation of some species with calprotectin or pediatric CD activity index was documented. Furthermore, owing to linear discriminant analysis, we have shown that some fungal species could be biomarkers characterizing and distinguishing a given group of patients (depending on the disease activity) which in the future may be helpful in predicting an exacerbation of the disease or even predicting the diagnosis of CD.

Research conclusions

Changes in the composition of the intestinal mycobiome occur already at the beginning of the disease (in newly diagnosed and untreated patients). Furthermore, the composition of fungi changes depending on the activity of CD.

Research perspectives

Further research should focus on selecting fungal species that could be biomarkers to help predict disease exacerbation. Furthermore, next research should assess whether the fungal mycobiota could be a therapeutic target.

FOOTNOTES

Author contributions: Krawczyk A performed the molecular investigations, interpreted the data, prepared the tables and figures, and wrote the manuscript; Kowalska-Duplaga K recruited patients, and revised the paper; Zapała B analyzed data, and revised the paper; Książek T performed the investigations; Drażniuk-Warchoł M recruited patients; Salamon D, and Gosiewski T coordinated the study, interpreted data, and revised the article; all authors approved the final version of the article.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Acoustic radiation force impulse predicts long-term outcomes in a large-scale cohort: High liver cancer, low comorbidity in hepatitis B virus

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Abstract

BACKGROUND

Acoustic radiation force impulse (ARFI) is used to measure liver fibrosis and predict outcomes. The performance of elastography in assessment of fibrosis is poorer in hepatitis B virus (HBV) than in other etiologies of chronic liver disease.

AIM

To evaluate the performance of ARFI in long-term outcome prediction among different etiologies of chronic liver disease.

METHODS

Consecutive patients who received an ARFI study between 2011 and 2018 were enrolled. After excluding dual infection, alcoholism, autoimmune hepatitis, and others with incomplete data, this retrospective cohort were divided into hepatitis B (HBV, n = 1064), hepatitis C (HCV, n = 507), and non-HBV, non-HCV (NBNC, n = 391) groups. The indexed cases were linked to cancer registration (1987-2020) and national mortality databases. The differences in morbidity and mortality among the groups were analyzed.

RESULTS

At the enrollment, the HBV group showed more males (77.5%), a higher prevalence of pre-diagnosed hepatocellular carcinoma (HCC), and a lower prevalence of comorbidities than the other groups ($P < 0.001$). The HCV group was older and had a lower platelet count and higher ARFI score than the other groups ($P < 0.001$). The NBNC group showed a higher body mass index and platelet count, a higher prevalence of pre-diagnosed non-HCC cancers ($P < 0.001$), especially breast cancer, and a lower prevalence of cirrhosis. Male gender, ARFI score, and HBV were independent predictors of HCC. The 5-year risk of HCC was 5.9% and 9.8% for those ARFI-graded with severe fibrosis and cirrhosis. ARFI alone had an area under the receiver operating characteristic curve (AUROC) of 0.742 for prediction of HCC in 5 years. AUROC increased to 0.828 after adding etiology, gender, age, and platelet score. No difference was found in mortality rate among the groups.

CONCLUSION

The HBV group showed a higher prevalence of HCC but lower comorbidity that made mortality similar among the groups. Those patients with ARFI-graded severe fibrosis or cirrhosis should receive regular surveillance.

Key Words: Non-alcoholic fatty liver disease; Hepatitis B; Hepatocellular carcinoma; Acoustic radiation force impulse; Mortality; Comorbidity

Core Tip: Among 1962 patients who received an acoustic radiation force impulse (ARFI) study, the 5-year risk of hepatocellular carcinoma (HCC) was 5.9% and 9.8% for those ARFI-graded with severe fibrosis and cirrhosis, respectively. The prevalence of HCC was highest in the hepatitis B virus (HBV) group. However, the HBV group showed the lowest comorbidities among the groups after adjusting for age, gender, and body mass index. This made the mortality rate similar among the groups. ARFI alone had an area under the receiver operating characteristic curve (AUROC) of 0.742 for prediction of HCC in 5 years. The AUROC increased to 0.828 after adding etiology, gender, age, and platelet score. Those patients with ARFI-estimated severe fibrosis or cirrhosis should receive active surveillance of HCC in all etiologies.

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INTRODUCTION

Chronic liver diseases are major risk factors for hepatocellular carcinoma (HCC)[1,2]. Regular screening of high-risk groups to detect HCC in the early stage can increase the chance to eradicate HCC and improve survival rates[3,4]. Liver cirrhosis is a major HCC risk factor[1-4]. In the last two decades, Fibroscan has been used to assess the HCC risk for patients with chronic liver diseases[5-9]. An alternative is the acoustic radiation force impulse (ARFI) imaging which uses ultrasound as a push pulse to measure the liver stiffness[10-12]. Both modalities are quite good in patients with chronic hepatitis C virus (HCV) infection and non-alcoholic liver diseases[13]. However, the correlation was relatively poor in patients with chronic hepatitis B virus (HBV) infection. We have been using ARFI to assess liver stiffness since 2011[12,13], which allowed the informatics department in our institute to produce a uniquely available large-scale cohort of patient data for research purposes. This would facilitate us to evaluate the ability of ARFI to assess the HCC risk using a consecutive cohort that is much larger than what has been found in prior work. The short-term correlation between ARFI and liver fibrosis is relatively poor in HBV, but the long-term effect is still not fully investigated. As well, studies into the predictive ability of ARFI for HCC among patients with different etiologies of chronic liver disease are still rare[14]. Finally, the chronic HBV infection is characterized by an initial immune tolerance phase[15]. This could be a survival strategy[16] despite an increased risk of HCC caused by chronic persistent HBV infection[1,2,17,18]. Therefore, we examined the differences in morbidities and mortalities between HBV and other etiologies in this cohort to help shed some light on this question.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board (IRB) of the Chang Gung Medical Foundation (CGMH IRB No. 201801283B0 and No. 202200758B0).

ARFI measurement

ARFI imaging was done with an Acuson S2000 system (Siemens Medical Solutions). Liver stiffness was measured with a standardized protocol at two locations of the right hepatic lobe[12,13]. The mean of the two locations was used as the final measurement. Most of the studies were done by one senior technician (HTW). Because it is not covered by the Taiwanese National Health Insurance Administration, the charge of ARFI (around 50 United States dollars) was mostly paid by the patients. The exceptions were patients undergoing a liver biopsy study or those participating in clinical trials (around 25%), where the cost was paid for by research grants. The first ARFI study was used in this analysis. The ARFI-estimated fibrosis grades was according to the cutoff values in our previous histology proven study[13].

Patients

The data of this study were retrieved from the Chang Gung Research Database of the Chang Gung Memorial Hospital, Linkou and Taipei branches. All patients who received ARFI imaging between January 2011 and September 2018 represented as the index patients. A total population of 2405 patients were included. The Chang Gung Research Database includes the original electronic medical records, comprising health care facility information, patient demographics, diagnosis information, drug information, procedure information, and other health digital information. All the personal identifiers were replaced by a code. We organized a team that included researchers from both hepatology and informatics departments of Chang Gung Memorial Hospital, along with outside technical consultants, to deal with this project[19]. We excluded patients with incomplete viral markers and clinical data, dual viral hepatitis, alcoholic liver diseases, autoimmune liver diseases, toxic hepatitis, genetic liver diseases, and those whose ARFI studies were with an interquartile range over a median ratio > 30%, which is a recommended quality assurance criterion (Figure 1)[20]. According to the viral markers, patients were classified into three groups: Hepatitis B, hepatitis C, and non-hepatitis B, non-hepatitis C (NBNC) patients. The NBNC group consisted mostly of patients with non-alcoholic fatty liver disease[19]. All the patients were followed for liver biochemistry, alpha-fetoprotein, and liver ultrasound at 3-12-mo intervals.

Comorbidity

The comorbidities under investigation included hypertension [I10-I15], type 2 diabetes mellitus [E8-13], dyslipidemia [E78], myocardial infarction [I21-I23, I1252], atrial fibrillation [I48], heart failure [I50], and ischemic stroke [I63-I66]. The ascertainment of these comorbidities was based on three diagnoses from the outpatient department or one diagnosis from the inpatient department.

Antiviral therapy

Patients could have undergone any of several different antiviral regimens in the study period. Similar interferon regimens had been used in both HBV and HCV groups, and such immune modulatory regimens were not uniform. Therefore, we simply recorded whether interferon therapy was given pre-enrollment or post-enrollment.

Similarly, multiple nucleot(s)ide analogue (NA) regimens for HBV and direct antiviral agent (DAA) regimens for HCV were possibly taken in the study period. Their mechanisms are suppression or elimination of viral replication. We recorded such oral therapies in pre-enrollment and/or post-enrollment periods as one category.

Cancer registration data link

In addition to their medical records, study subjects were linked with the database of Cancer Registration, Chang Gung Memorial Hospital, which records information on all cancers diagnosed in this hospital since 1987. We linked this database up to June 30, 2020.

Mortality data link

This study used the national citizen identification numbers of patients to search the mortality data bank established by the Statistics Office, Department of Health, Taiwan. The mortality data bank stored death certificate data, which includes patient demographic data such as the time, place, and cause of death, and the name of the official who issued the document. Causes of death were classified using the International Classifications of Diseases, Injuries and Causes of Death (ICD-10, World Health Organization, 2015). The data was linked up to December 31, 2020.

This mortality data link will collect data for those patients lost to follow-up in this hospital.

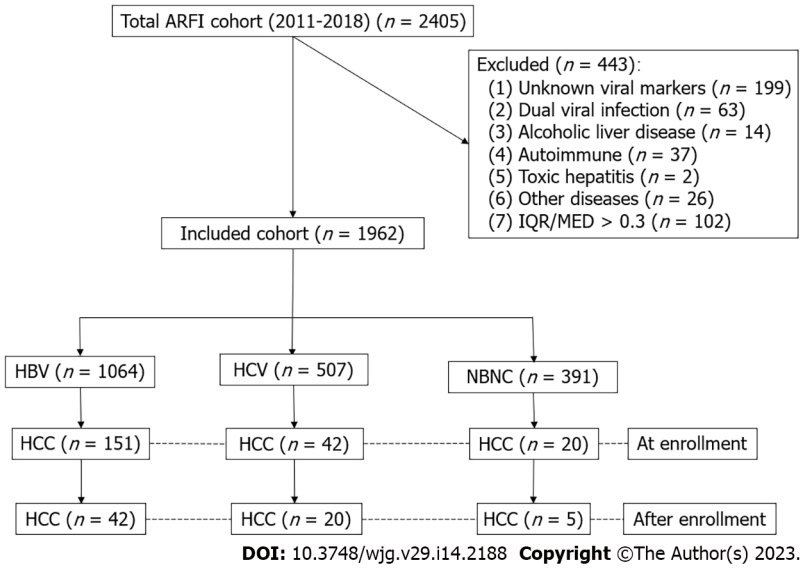

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Figure 1 Patient flow chart. HCC: Hepatocellular carcinoma; ARFI: Acoustic radiation force impulse; HBV: Hepatitis B; HCV: Hepatitis C; NBNC: Non-hepatitis B, hepatitis C.

Statistical analysis

Patient characteristics are represented as the number and percentage, or the mean ± SD, as appropriate. Continuous variables of the three independent groups were compared using one-way analysis of variance (ANOVA) with Bonferroni correction. Categorical variables were tested using the chi-square test, or the chi-square test for trend, as applicable. Logistic regression was conducted to identify independent risk factors for HCC for records before the ARFI study. Cox proportional hazards model was conducted to identify independent risk factors of HCC for records after the ARFI study. The Mantel-Cox procedure (Log Rank test) was applied to compare risk factors and the cumulative risk of HCC among different groups after the ARFI study. The area under the receiver operating characteristic curve (AUROC) was calculated using a scoring system for HCC risk prediction. The scoring system was based on the hazard ratio in the multivariate analysis. Briefly, ARFI and FIB4 were according to its values and modified by timing 0.5 or 2. Other factors were calculated as: Gender (G) (male = 2, female = 0); etiology score (E) (HBV = 3, HCV = 2, NBNC = 1); age (year) score (A) (0-35 = 0, 35-40 = 1, 40-45 = 2, 45-50 = 3, 50-55 = 4, 55-60 = 5; > 60 = 6; platelet (10^9/L) score (A) (0-100 = 3, 100-150 = 2, > 150 = 1). Statistical analyses were performed using the SPSS software (version 22; SPSS Inc., Chicago, IL, United States), and a P value of < 0.05 was judged as statistically significant.

RESULTS

Baseline demographic features

A total of 1962 patients met our inclusion criteria. Among them, 1064, 507, and 391 patients were in the HBV, HCV, and NBNC groups, respectively (Table 1). The HBV group had more men and a lower prevalence of hypertension, diabetes mellitus, heart failure, ischemic stroke, and dyslipidemia. Patients in the HCV group were older and had a lower male ratio, lower platelet counts, higher fibrosis index based on four factors (FIB4), and mean ARFI score. The NBNC group had a higher body mass index (BMI), lower prothrombin time and international normalized ratios (INR), lower FIB4, higher platelet count, lower rate of estimated cirrhosis, and shorter duration of follow-up.

Alanine aminotransferase level grades at the enrollment

ARFI is strongly influenced by liver inflammation. This especially happens when alanine aminotransferase (ALT) levels are greater than 5 × (180 U/L) the upper limit of normal (0-36). So, we list the ALT level grades in the Supplementary Table 1. In total, 7.3% of patients had ALT level greater than 180 U/L.

Comorbidity

There were significant differences in the prevalence of hypertension, diabetes mellitus, heart failure, ischemic stroke, and dyslipidemia between the HBV group and the other two groups (Table 1). After adjusting for age, gender, and BMI, the prevalence of hypertension, diabetes mellitus, ischemic stroke, and dyslipidemia was significantly lower in the HBV group than in the non-HBV groups (Supplementary Table 2).

Table 1 Baseline demographic features of the cohort

	HBV (*n* = 1064)	HCV (*n* = 507)	NBNC (*n* = 391)	*P* value	Missing rate
Demographics					
Age (yr)	52.05 ± 10.90	58.69 ± 10.84	51.97 ± 13.10	< 0.0001 (2 & 3, 1 & 2)	0.00%
Male sex, *n* (%)	825 (77.5)	273 (53.8)	233 (59.6)	< 0.0001	0.00%
Weight (kg)	68.36 ± 12.30	64.53 ± 12.17	70.94 ± 14.09	< 0.0001 (2 & 3, 1 & 2, 1 & 3)	0.97%
Height (cm)	166.34 ± 7.62	161.67 ± 8.57	164.18 ± 8.75	< 0.0001 (2 & 3, 1 & 2, 1 & 3)	2.80%
BMI (kg/m^2)	24.66 ± 3.62	24.58 ± 3.75	26.25 ± 4.23	< 0.0001 (2 & 3, 1 & 3)	3.47%
Lab data at ARFI study					
Spleen index (cm^2)	31.97 ± 14.47	33.63 ± 16.72	34.07 ± 16.05	0.0640 (1 & 3)	0.03%
Albumin (mg/dL)	4.345 ± 0.51	4.29 ± 0.51	4.43 ± 0.48	0.0040 (2 & 3)	42.15%
AST (U/L)	57.50 ± 102.16	62.46 ± 58.08	58.40 ± 50.72		1.12%
ALT (U/L)	75.05 ± 162.88	71.52 ± 69.61	83.46 ± 78.32		1.33%
Bilirubin (mg/dL)	0.93 ± 1.23	0.91 ± 1.19	0.92 ± 1.21		12.79%
Prothrombin time (INR)	1.10 ± 0.13	1.11 ± 0.21	1.06 ± 0.13	0.0040 (2 & 3, 1 & 3)	42.15%
Platelets (10^9/L)	177.86 ± 61.86	170.13 ± 60.19	218.96 ± 76.08	< 0.0001 (2 & 3, 1 & 3)	13.25%
FIB4	2.454 ± 2.720	3.31 ± 3.04	2.06 ± 2.13	< 0.0001 (2 & 3, 1 & 2)	23.35%
Mean ARFI (m/s)[1]	1.40 ± 0.46	1.60 ± 0.61	1.42 ± 0.62	< 0.0001 (2 & 3, 1 & 2)	0.00%
Stiffness status[1]				< 0.0001	
Cirrhosis	331 (31.1)	163 (32.1)	78 (19.9)		
Severe fibrosis	138 (13.0)	110 (21.7)	61 (15.6)		
Moderate fibrosis	114 (10.7)	68 (13.4)	29 (7.4)		
Mild or non-fibrosis	481(45.2)	166(32.7)	223(57.0)		
Comorbidities					
Hypertension	181 (17.0)	147 (29.0)	115 (29.4)	< 0.0001	
Diabetes	119 (11.2)	103 (20.3)	76 (19.4)	< 0.0001	
Heart failure	14 (1.3)	17 (3.4)	7 (1.8)	0.023	
Atrial fibrillation	9 (0.8)	11 (2.2)	5 (1.3)	0.091	
Myocardial infraction	12 (1.1)	5 (1.0)	2 (0.5)	0.567	
Ischemic stroke	6 (0.6)	17 (3.4)	8 (2.0)	< 0.0001	
Dyslipidemia	170 (16.0)	95 (18.7)	125 (32.0)	< 0.0001	
Follow-up (year)					
mean ± SD	4.59 ± 2.22	4.34 ± 2.35	3.13 ± 2.15	< 0.0001 (1 & 3, 2 & 3)	
Median/IQR	4.54/3.59	3.97/3.88	2.61/3.23		

[1]Cutoff values according to reference 11.

ARFI: Acoustic radiation force impulse; FIB4: Fibrosis-4 index; BMI: Body mass index; HBV: Hepatitis B; HCV: Hepatitis C; NBNC: Non-hepatitis B, non-hepatitis C; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Cancers diagnosed before ARFI study

We linked our index cases with cancer registration databases between 1987 and 2020. There were many cancer diagnoses prior to enrollment. These cancers were either well-treated or receiving active therapy. The HBV group had the highest rate of pre-enrollment HCC diagnosis (14.1%, $P < 0.001$, Table 2, upper panel). On the other hand, the NBNC group had the highest rate of non-HCC cancer (10%, $P < 0.001$), with breast cancer having the highest rate (4.3% for total or 10.8% for females alone, $P < 0.001$). Factors associated with a high risk of HCC as identified by logistic regression were male sex, high mean ARFI

Table 2 Cancers and mortality in different groups

	HBV	HCV	NBNC	*P* value
Total cases	1064	507	391	
Female	239 (22.5)	234 (46.2)	158 (40.4)	< 0.027
Follow years before enrollment	-2.65 ± 3.98	-2.78 ± 5.56	-3.30 ± 6.25	< 0.001 (3 & 1, 3 & 2)
Cancers, pre-enrollment				
HCC	150 (14.1)	42 (8.3)	20 (5.1)	< 0.001
Non-HCC cancer	36 (3.4)	30 (5.9)	39 (10.0)	< 0.001
Colon cancer	9 (0.8)	3 (0.6)	6 (1.5)	NS
Breast cancer	1 (0.1)	9 (1.8)	17 (4.3)	< 0.001[1]
Hematology cancers	3 (0.3)	4 (0.8)	2 (0.5)	NS
Follow years before enrollment	-2.76 ± 4.55	-3.57 ± 5.18	-3.22 ± 4.40	NS
Cancers, post-enrollment				
HCC	43 (4.0)	19 (3.7)	5 (1.3)	0.033
Non-HCC tumor	21 (2.2)	14 (2.8)	12 (3.1)	NS
Colon cancer	2 (0.1)	3 (0.6)	1 (0.3)	NS
Breast cancer	2 (0.2)	0 (0.0)	1 (0.3)	NS
Hematology cancers	6 (0.6)	0 (0.0)	1 (0.5)	NS
Prostate cancer	2 (0.1)	1 (0.2)	1 (0.3)	NS
Follow years after enrollment	2.43 ± 1.86	2.51 ± 1.86	2.25 ± 2.00	NS
Mortality total	72 (6.8)	41 (8.1)	21 (5.4)	NS
HCC	35 (3.3)	13 (2.6)	4 (1.0)[2]	0.057
Non-HCC cancers	16 (1.5)	8 (1.6)	7 (1.8)[2]	NS
Liver disease	6 (0.6)	7 (1.4)	6 (1.5)	NS
Non-liver disease	15 (1.4)	13 (2.6)	4 (1.0)	NS
Follow years after enrollment	2.59 ± 1.96	2.69 ± 1.99	2.00 ± 1.87	NS

[1]Female only.
[2]$P = 0.027$.
Remark: Different cancers on the same patient were counted separately. HCC: Hepatocellular carcinoma; HBV: Hepatitis B; HCV: Hepatitis C; NBNC: Non-hepatitis B, non-hepatitis C; NS: Not significant.

score, HBV, older age, higher aspartate aminotransferase (AST) level, lower ALT level, and lower platelet count (Table 3). Hypertension and diabetes mellitus were associated with a higher risk of pre-diagnosed HCC while dyslipidemia was associated with a lower risk of pre-diagnosed HCC.

Cancers diagnosed after ARFI

After excluding patients with cancer diagnosed before enrollment, the HBV (4.0%) and NBNC (1.3%) groups exhibited the highest and lowest rates of HCC occurrence, respectively ($P = 0.033$, Table 2, middle panel). Factors associated with HCC diagnosis were male sex ($P = 0.013$), high ARFI score (< 0.001), and HBV ($P = 0.019$, Table 4). In Cox's regression analysis, male gender, HBV, platelet count, and ARFI score were associated with a higher risk of HCC.

HCC development after enrollment in subjects with different fibrosis stages

According to the cutoff values of mean ARFI determined in our previous histology proven cases[13], we divided the study population into cirrhosis, severe fibrosis, moderate fibrosis, and mild to non-fibrosis groups. The cumulative risk of HCC development was highest in the cirrhosis group, followed by the severe fibrosis group, and lowest in the none to moderate fibrosis groups ($P < 0.001$, Figure 2). The 5-year risk of HCC was 5.9% and 9.8% for those patients ARFI-graded with severe fibrosis and cirrhosis, respectively. When those patients with ALT greater than 5 × upper limit normal were removed, the 5-year risk of HCC was 6.1% and 10.4% for those with severe fibrosis and cirrhosis, respectively

Table 3 Logistic regression for hepatocellular carcinoma diagnosed before enrollment

	Univariate analysis	Multivariate analysis		95%CI	
	P value	*P* value	Hazard ratio	Lower	Upper
Male	< 0.001	0.000	3.399	2.150	5.373
Etiology	< 0.001	0.000	-	-	-
HBV		0.000	4.009	2.219	7.241
HCV		0.169	1.644	0.810	3.335
Age, yr	< 0.001	0.000	1.047	1.029	1.066
ALT	0.243	0.001	0.993	0.989	0.997
AST	0.010	0.000	1.012	1.007	1.017
Bilirubin	0.170	0.729	0.980	0.875	1.098
Platelet	0.138	0.035	1.003	1.000	1.005
Spleen index	0.060	0.811	1.001	0.990	1.013
BMI	0.015	0.076	0.956	0.910	1.005
ARFI	< 0.001	0.010	1.556	1.110	2.181
Interferon therapy	0.011	0.012			
Pre-enrollment		0.618	1.204	0.581	2.496
Post-enrollment		0.004	0.211	0.073	0.617
Oral anti-virus agents	0.041	0.181			
Pre-enrollment		0.168	1.721	0.795	3.728
Post-enrollment		0.156	0.667	0.381	1.167
Pre- and post-enrollment		0.527	1.534	0.408	5.773
Hypertension	< 0.001	0.000	2.551	1.702	3.824
Diabetes mellitus	< 0.001	0.031	1.618	1.044	2.508
Dyslipidemia	< 0.001	0.000	0.358	0.207	0.620
Ischemic stroke	0.341	0.884	1.097	0.317	3.794

1481/1962 (75.5%) with complete data; cases (hepatocellular carcinoma), n = 189. HBV: Hepatitis B; HCV: Hepatitis C; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; ARFI: Acoustic radiation force impulse.

(Supplementary Figure 1). There was no difference in predictive abilities across different etiologies (Supplementary Figure 2), suggesting that high ARFI score is a good predictor of HCC diagnosis for HBV patients, along with HCV and NBNC patients.

AUROCs of different non-invasive scoring models for HCC prediction

The AUROCs showed that ARFI or FIB4 alone gave similar 3- or 5-year predictions of HCC (AUROCs around 0.739-0.756) (Figure 3). After adding gender, etiology, age, and platelet score, the AUROCs increased to 0.772-0.840.

Mortality during study period

No significant difference in total mortality was found across the three groups. The HBV group had a marginally higher HCC related mortality (3.3%, P = 0.057) (Table 2, lower panel). However, the non-HCC related mortality was the lowest in the HBV group (P < 0.027). Male sex, age, lower ALT level, higher AST level, lower BMI, higher ARFI score, and ischemic stroke were associated with a higher risk of mortality in Cox's regression analysis (Supplementary Table 3).

DISCUSSION

In our patient cohort, 212 patients were diagnosed with HCC prior to enrollment and 67 developed

Table 4 Cox's regression analysis for hepatocellular carcinoma occurring after enrollment

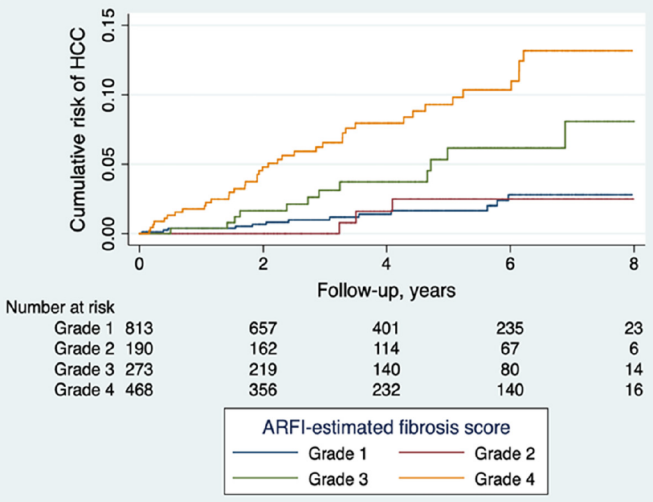
	Univariate analysis	Multivariate analysis		95%CI	
	P value	*P* value	Hazard ratio	Lower	Upper
Male	0.005	0.009	20.796	1.293	6.045
Etiology	0.019	0.057			
HBV		0.023	40.473	1.232	16.245
HCV		0.150	20.670	0.701	10.173
Age (yr)	< 0.001	0.094	10.028	0.995	1.061
ALT (U/L)	0.188	0.211	0.994	0.985	1.003
AST (U/L)	0.605	0.449	10.005	0.993	1.017
Bilirubin (mg/dL)	0.962	0.326	0.772	0.460	1.295
Platelet (10^9/L)	< 0.001	0.041	0.993	0.986	1.000
Spleen index (cm^2)	0.019	0.169	10.012	0.995	1.030
BMI	0.334	0.524	0.973	0.894	1.059
ARFI (m/s)	< 0.001	0.000	20.775	10.624	4.742
Interferon therapy	0.814	0.943			
Pre-enrollment		0.734	0.772	0.174	3.435
Post-enrollment		0.914	0.949	0.366	2.459
Oral anti-virus therapy	0.0174	0.086			
Pre-enrollment		0.977	10.031	0.134	7.948
Post-enrollment		0.029	20.105	10.080	4.102
Pre- and post-enrollment		0.110	30.362	0.758	14.903
Hypertension	0.001	0.211	10.554	0.778	3.104
Diabetes mellitus	0.002	0.304	10.460	0.710	3.006
Ischemic stroke	0.305	0.971	00.000	0.000	1.46E+269
Dyslipidemia	0.233	0.065	10.920	0.960	3.840

1274/1749 (72.8%) with complete data, 49 cases (hepatocellular carcinoma). HBV: Hepatitis B; HCV: Hepatitis C; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; ARFI: Acoustic radiation force impulse.

HCC after enrollment (Table 2). High ARFI scores were associated with pre-diagnosed HCC occurrences in a multivariate analysis ($P < 0.001$, Table 3) and were a good predictor of HCC after enrollment according to Cox's regression analysis ($P < 0.001$, Table 4). The ARFI-estimated fibrosis grades showed a severity-dependent increased risk of HCC in the post-enrollment period (Figure 2). The 5-year risk of HCC was 5.9% and 9.8% for those patients who were ARFI-graded with severe fibrosis and cirrhosis, respectively. When age, gender, and platelet count were added, there was only a small rise of AUROC from 0.753 to 0.84 within 3 years and 0.742 to 0.828 within 5 years in the prediction of HCC. These results suggest that liver fibrosis is the main risk factor for HCC (Figure 3). Our finding is consistent with others in a recent study review[21] using non-invasive fibrosis diagnosis models. Patients with grades 3 and 4 fibrosis should receive active surveillance of HCC.

ARFI-graded fibrosis predicts the HCC occurrence well among different etiologies (Supplementary Figure 2). Although the correlation between ARFI and liver histology fibrosis was poorer in the HBV group[13] than in the other two etiologies, HCC risk prediction was as good as that in the other groups. This question was not well-addressed in prior work where such investigations had much smaller clinical datasets or single etiology cohorts. Additional investigations should further pursue this link.

In terms of prior work, ARFI studies on non-HCC patients typically focused on its utility of fibrosis staging[10-12,22]. Exceptions include the work by Sun *et al*[23], who correlated ARFI with the indocyanine green test and found a positive correlation between the two. ARFI was also correlated to Child-Pugh scores in that study. They suggested that ARFI imaging is a useful tool for assessing liver functional reserve. As another example, a recent meta-analysis reported that ARFI scores may be a good



DOI: 10.3748/wjg.v29.i14.2188

Figure 2 Cumulative risk of hepatocellular carcinoma after enrollment in different acoustic radiation force impulse-fibrosis grades. Higher risk of hepatocellular carcinoma (HCC) was found in acoustic radiation force impulse (ARFI)-severe fibrosis and -cirrhosis grades than in none to moderate fibrosis grades. The 5-year risk of HCC was 9.8 % for ARFI fibrosis graded as cirrhosis; 5.9% for that graded as severe fibrosis; and only 1.7%-2.0% for that lower or equal to moderate fibrosis. Purple: Cirrhosis; Yellow: Severe fibrosis; Green: Moderate fibrosis, Blue: None or mild fibrosis [Log rank test: $P = 0.026$ (3 *vs* 4); $P = 0.015$ (3 *vs* 1 + 2); $P < 0.001$ (4 *vs* 1 + 2)]. HCC: Hepatocellular carcinoma; ARFI: Acoustic radiation force impulse.

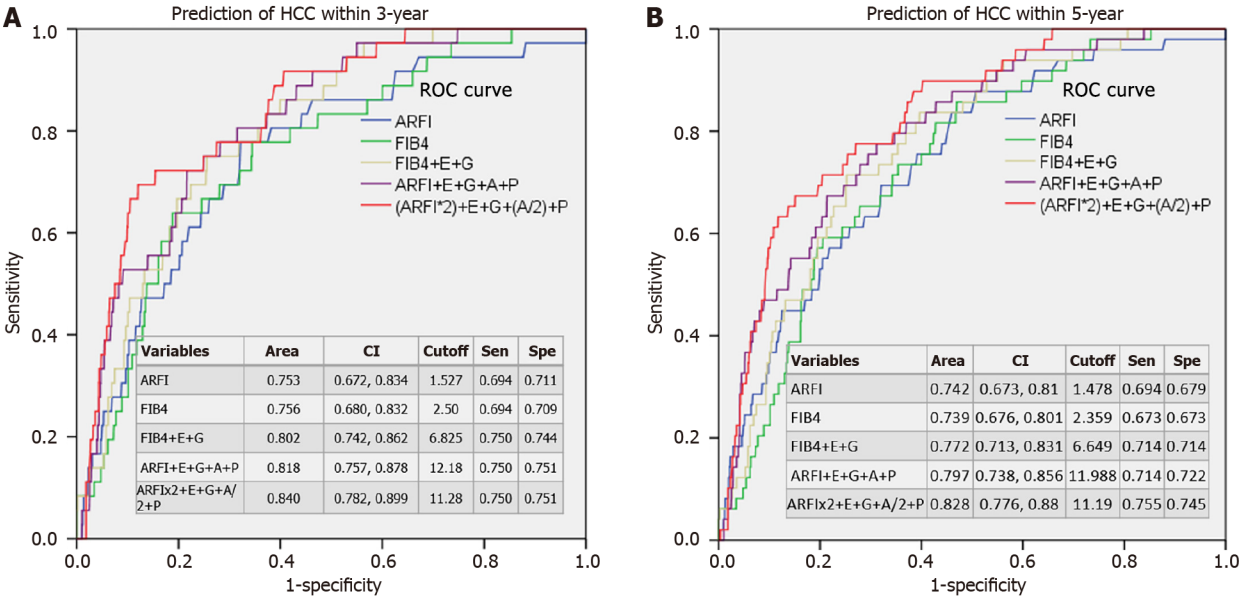


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Figure 3 Area under the receiver operating characteristic curve of hepatocellular carcinoma prediction in different non-invasive scoring models. A: Within 3-year period after enrollment; B: Within 5-year period after enrollment. The area under the receiver operating characteristic curve (AUROC) shows that acoustic radiation force impulse (ARFI) or FIB4 alone give similar 3- or 5-year predictions of hepatocellular carcinoma (HCC) (AUROC around 0.739-0.756). After adding G (gender score), E (etiology score), A (age score), and P (platelet score), the AUROC may increase to 0.772-0.840. Both ARFI and FIB4 models predict 3- or 5-year HCC quite satisfactorily, suggesting that fibrosis is the main risk factor for HCC. ARFI: Acoustic radiation force impulse; FIB4: Fibrosis index based on four factors; G: Gender score, male = 2, female = 0; E: Etiology score, hepatitis B virus (HBV) = 3, HCV = 2, NBNC = 1; A: Age score (year), 0-35 = 0, 35-40 = 1, 40-45 = 2, 45-50 = 3, 50-55 = 4, 55-60 = 5; > 60 = 6; P: Platelet score (10^9), 0-100 = 3, 100-150 = 2, > 150 = 1; Sen: Sensitivity; Spe: specificity; HCC: Hepatocellular carcinoma; ARFI: Acoustic radiation force impulse; FIB4: Fibrosis-4 index.

predictor of HCC recurrence-free survival in patients receiving radiofrequency ablation[24]. In a series of 1808 patients who received ARFI, those patients with an ARFI score > 1.33 cm/s showed a higher probability of HCC development than those with an ARFI score ≤ 1.33 cm/s[25]. Therefore, there is good evidence that ARFI can measure liver fibrosis, reflex the liver functional reserve, and predict HCC recurrence. Our study adds to these conclusions, as Cox's regression analysis confirmed that high ARFI score was a risk factor for HCC and mortality ($P < 0.001$, Table 3 and Supplementary Table 3).

In aspects of HCC diagnosis, the HBV group had higher occurrences than the NBNC group (Table 2). This aligns with previous investigations, *e.g.*, the study by Chen *et al*[26] who reported higher occurrences of HCC in their HBV (4.8%) and HCV (4.7%) groups compared to their NBNC group (0.3%). Even so, the prevalence of HCC in our NBNC group was much higher than their value, but this is because Chen *et al*[26] included healthy subjects in their NBNC group, while our NBNC cohort was a disease group. Even though NBNC patients had a lower incidence of HCC ($P < 0.001$), this was offset by higher incidences of non-HCC cancers. Consequently, the total mortality rate was similar among different groups (Table 2). This is consistent with a recent meta-analysis by Mantovani *et al*[27] that concluded that extra-hepatic cancers were increased in non-alcoholic fatty liver disease. It should be noted that such trends were only identified in the pre-enrollment period because it had a longer past history. We can record many cancers in the pre-enrollment period because the diagnosis of all cancer types has been recorded in our cancer registration database since 1987. We found that breast cancer ($P < 0.001$), colon cancer, and hematologic cancers were the main cancers in the pre-enrollment period. These types of cancers respond to treatment well, allowing us to include such survivors in our cohort.

There were lower rates of comorbidities in the HBV group than in the other groups (Table 1). This seems to be related to the high prevalence of metabolic syndrome in the HCV or NBNC group. However, after adjusting for gender, age, and BMI, such a phenomenon was still present (Supplementary Table 2). As early as 2006, Jan *et al*[28] had reported a lower prevalence of diabetes, hypertension, obesity, hyperlipidemia, and obesity in HBV than HCV or NBNC in a population-based study in northern Taiwan. Another nationwide study by Kuo *et al*[29] that examined 1376344 diabetes patients between 2000 and 2012 also confirms these results. Before enrollment, they excluded patients with a history of myocardial infarction (2.28% in HBV group and 4.19% in non-HBV group, $P < 0.001$) and cerebrovascular diseases (15.6% in HBV group and 24.3% in non-HBV group, $P < 0.001$). The percentage of excluded patients was lower in their HBV group than the non-HBV groups for both diseases. After enrollment and propensity matching, the risk of all-cause mortalities, myocardial infarction, ischemic stroke, and heart failure was higher in the non-HBV group than in the HBV group ($P < 0.001$) in a mean follow-up period of 5.3 year[29]. A study by Sung *et al*[30] from Korea showed similar findings. They pointed out that the difference was more profound in HBV with liver dysfunction than in those without liver dysfunction. They suggested that the HBV-related proinflammatory effect may be the reason for the decreased risk of comorbidity.

Previous studies did not discuss the reason for these low comorbidities in the HBV patients. A recent review suggested that chronic HBV infection may protect infected subjects from the development of metabolic syndrome and hepatic steatosis[31]. The immune system is a double-edged sword. Its efforts against microorganisms may induce host tissue damage[32]. Chronic persistent HBV infection is characterized by an initial immune tolerance phase that allows active HBV replication without immune-mediated inflammation to liver tissue[33]. Liver inflammation occurs only when the immune system is triggered to attack HBV carrying hepatocytes[34]. This contrasts with HCV and NBNC groups with persistently mild inflammation in the liver.

There is a longer HBV-related immune tolerance phase in East Asian than in African, which could be related to genetic polymorphism in human leukocyte antigen (HLA)-DP and -DQ loci[16,35]. Such HBV-related gene variants not only decrease antigen presentation to avoid fatal immune response, but also establish an environment that is suitable for chronic persistent HBV infection[16]. Our recent study indicated that those HBV-related single nucleotide polymorphisms (SNPs) in HLA-DP and -DQ loci were associated with high viral load in the HCC family[36]. Such patients would be more likely to be associated with liver dysfunction as those mentioned in Sung's series[30]. From the above clues, we suspect that the low comorbidity trend in the HBV group may be partly associated with a low antigen presentation ability.

One of our limitations is that life-long disease consequences are not easy to examine. The mean follow-up duration after enrollment could be considered as relatively short (3.13 to 4.59 years). We may need longer and more specific studies to explore the link between HBV infection and comorbidity. It should be noted that HBV-related SNPs in HLA-DP and -DQ loci in East Asians are quite different from other ethnicities[16]. The low comorbidity in the HBV group may be limited to East Asian. Another limitation is that the contribution of therapy to morbidity and mortality was difficult to evaluate. We noticed that the post-enrollment interferon therapy was associated with a lower prevalence of pretreatment HCC and post-enrollment oral antivirus therapy was associated with a lower risk of post-enrollment HCC. However, these findings may be due to a relatively better condition of chronic liver disease, which could have made the pre-enrollment therapy unnecessary. Similar situations concerning about therapeutic response in HCC-related mortality could be presented. With the success of checkpoint inhibitors in HCC therapy[37], future predictive biomarker study will be needed to clarify the difference in mortality among groups[38].

CONCLUSION

We conclude that even ARFI-based fibrosis prediction in the HBV group is poorer than that in other

groups, its performance or clinical significance in predicting HCC or mortality is as good as that in other etiologies. The HBV group had the highest risk of HCC and the NBNC group had the highest risk of non-HCC tumors, especially breast cancer. Low prevalence of comorbidities in the HBV group was found, which may be a consequence of a low prevalence of metabolic syndrome and low antigen presentation ability.

ARTICLE HIGHLIGHTS

Research background

Acoustic radiation force impulse is used to measure liver fibrosis and predict outcomes. The performance of elastography in assessment of fibrosis is poorer in hepatitis B virus (HBV) than in other etiologies of chronic liver disease.

Research motivation

Whether there are differences in performance of acoustic radiation force impulse (ARFI) in long term outcome prediction among different etiologiesof chronic liver disease remains to be studied.

Research objectives

We collected a cohort of patients who received ARFI studies. After excluding unsuitable cases, 1962 patients were included as the indexed cases. They were classified into HBV, HCV, and non-HBV, non-HCV (NBNC) groups. We examined the differences in demographics, comorbidity, carcinogenesis, and mortality among these groups at and after enrollment.

Research methods

These indexed cases were linked to the hospital's cancer registration and national mortality databases to obtain complete outcome data. The data at enrollment were analyzed for differences among three groups and logistic regression was performed to search for predictors associated with cancers. Cox regression analysis and area under the receiver operating characteristic curve (AUROC) were used to assess the performance of ARFI in predicting hepatocellular carcinoma (HCC) and mortality.

Research results

At enrollment, the HBV group showed more males (77.5%), a higher prevalence of pre-diagnosed HCC, and a lower prevalence of comorbidities than the other groups ($P < 0.001$). The HCV group was older and had a lower platelet count and higher ARFI score than the other groups ($P < 0.001$). The NBNC group showed a higher body mass index, platelet count, and prevalence of pre-diagnosed non-HCC cancers ($P < 0.001$), especially breast cancer, and a lower prevalence of cirrhosis. After enrollment, male gender, ARFI score, and HBV were independent predictors of HCC. The 5-year risk of HCC was 5.9% and 9.8% for those ARFI-graded with severe fibrosis and cirrhosis, respectively. ARFI alone had an AUROC of 0.742 for prediction of HCC in 5 years. AUROC increased to 0.828 after adding etiology, gender, age, and platelet score. No difference in mortality rate was noted among the groups.

Research conclusions

The HBV group showed a higher prevalence of HCC but a lower prevalence of comorbidity that made mortality similar among the groups. Those ARFI-graded as severe fibrosis or cirrhosis should receive regular surveillance.

Research perspectives

The immune tolerance is a hallmark of HBV which could be related to poor antigen presentation of human leukocyte antigen-DP and -DQ molecules in HBV surface antigen carriers. Whether such behavior is associated with a low prevalence of comorbidity requires future study.

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FOOTNOTES

Author contributions: Tai DI contributed to study concept and design; Tai J, Chen HM, Hsu CY, Chen CJ, Jeng WJ, Chang ML, and Tai DI contributed to data acquisition; Tai J, Harrison AP, Chen HM, Hsu CY, Jeng WJ, Chang ML, and Tai DI contributed to data analysis and interpretation; Tai J, Harrison AP, and Tai DI contributed to drafting of the manuscript; Harrison AP, Jeng WJ, Chang ML, and Lu L contributed to critical review of the manuscript for important intellectual content; Tai J, Chen HM, and Hsu CY contributed to statistical analysis; Tai DI and Lu L contributed to obtaining funding; Chen HM and Hsu CY contributed to technical or material support; Tai DI contributed to study supervision.

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SYSTEMATIC REVIEWS

Effectiveness of *Helicobacter pylori* eradication in the treatment of early-stage gastric mucosa-associated lymphoid tissue lymphoma: An up-to-date meta-analysis

Fabian Fellipe Bueno Lemos, Caroline Tianeze de Castro, Mariana Santos Calmon, Marcel Silva Luz, Samuel Luca Rocha Pinheiro, Clara Faria Souza Mendes dos Santos, Gabriel Lima Correa Santos, Hanna Santos Marques, Henrique Affonso Delgado, Kádima Nayara Teixeira, Cláudio Lima Souza, Márcio Vasconcelos Oliveira, Fabrício Freire de Melo

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Abstract

BACKGROUND

Gastric mucosa-associated lymphoid tissue (MALT) lymphoma (GML) is usually a low-grade B-cell neoplasia strongly associated with *Helicobacter pylori* (*H. pylori*)-induced chronic gastritis. Clinical practice guidelines currently recommend *H. pylori* eradication as the preferred initial treatment for early-stage GML. To determine the practical effect of bacterial eradication as the sole initial therapy for early-stage GML, an updated analysis and review of available evidence is imperative.

AIM

To perform a meta-analysis to assess the rate of complete remission (CR) of *H. pylori*-positive early-stage GML following bacterial eradication.

METHODS

We performed independent, computer-assisted literature searches using the PubMed/MEDLINE, Embase, and Cochrane Central databases through September 2022. Prospective and retrospective observational studies evaluating the CR of early-stage GML following bacterial eradication in *H. pylori*-positive patients. The risk of bias was assessed using Joanna Briggs Institute (JBI) Critical Appraisal Tools. The pooled estimate of the complete histopathological remission rate and respective confidence intervals (95%CI) were calculated following the random-effects model. Heterogeneity and inconsistency were assessed using Cochran's *Q* test and *I2* statistic, and heterogeneity was defined as $P < 0.01$ and $I^2 > 50\%$, respectively. Subgroup and meta-regression analyses were conducted to explore potential sources of heterogeneity.

RESULTS

The titles and abstracts of 1576 studies were screened; 96 articles were retrieved and selected for full-text reading. Finally, 61 studies were included in the proportional meta-analysis (P-MA). Forty-six were prospective and fifteen were retrospective uncontrolled, single-arm, observational studies. The overall risk of bias was low to moderate in all but a single report, with an average critical appraisal score across all studies of 79.02%. A total of 2936 *H. pylori*-positive early-stage GML patients, in whom *H. pylori* was successfully eradicated, were included in the analysis. The pooled CR of *H. pylori*-positive early-stage GML after bacterial eradication was 75.18% (95%CI: 70.45%-79.91%). P-MA indicated the substantial heterogeneity in CR reported across studies (I^2 = 92%; $P < 0.01$). Meta-regression analysis identified statistically significant effect modifiers, including the proportion of patients with t(11;18)(q21;q21)-positive GML and the risk of bias in each study.

CONCLUSION

Comprehensive synthesis of available evidence suggests that *H. pylori* eradication is effective as the sole initial therapy for early-stage GML. Although the substantial heterogeneity observed across studies limits the interpretation of the pooled overall CR, the present study is a relevant to informing clinical practice.

Key Words: Lymphoma; B-cell; Marginal zone; Gastric mucosa-associated lymphoid tissue lymphoma; Stomach lymphoma; *Helicobacter pylori*; Therapeutics; Eradication therapy

Core Tip: Gastric mucosa-associated lymphoid tissue (MALT) lymphoma (GML) is usually a low-grade B-cell neoplasia strongly associated with *Helicobacter pylori* (*H. pylori*)-induced chronic gastritis. Clinical practice guidelines currently recommend *H. pylori* eradication as the preferred initial treatment for early-stage GML. Despite advances in determining the practical effect of bacterial eradication as sole initial therapy for early-stage GML, an updated meta-analysis of available evidence is imperative. We performed a systematic review with proportional meta-analysis to assess the complete remission rate of *H. pylori*-positive early-stage GML after eradication therapy.

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INTRODUCTION

Marginal zone lymphomas (MZLs) are the third most common type of non-Hodgkin B-cell lymphoma following diffuse large B-cell lymphoma and follicular lymphoma[1]. The 5th edition of the World Health Organization Classification of Hematolymphoid Tumors - Lymphoid Neoplasms subdivides MZL into 4 subtypes: Extranodal MZL of mucosa-associated lymphoid tissue (MALT), primary cutaneous MZL, nodal MZL, and pediatric MZL[2].

Gastric MALT lymphoma (GML) is a low-grade B-cell neoplasia commonly associated with *Helicobacter pylori* (*H. pylori*)-induced chronic gastritis[3]. GML provides the best-characterized model of the antigen-induced transition from normal to malignant marginal-zone B-cells[4]. Despite the lack of lymphoid follicles in the normal gastric mucosa, MALT may appear as a result of inflammation. *H. pylori* chronic gastritis induces specific T helper cells and the subsequent expansion of polyclonal B cells, which can undergo malignant transformation[4,5]. Similar to that of gastric cancer, in advanced-stage GML, inflammatory signaling pathway and pro-oncogenic genetic changes allow a microenvironment-independent progression of the tumor, characterizing a "hit-and-run" mechanism[5,6]. The overwhelming evidence suggesting a causal relationship between *H. pylori* infection and GML is also supported by epidemiological data[7].

Although robust comparative studies such as randomized clinical trials have not been carried out, clinical practice guidelines currently recommend *H. pylori* eradication as the sole initial treatment for early-stage GML[8]. Triple-therapy, which comprises a proton pump inhibitor (PPI) for 4 wk and clarithromycin with either amoxicillin or metronidazole for 10-14 d, remains standard. However, given the increasing rate of bacterial clarithromycin resistance in many countries, international guidelines also recommend bismuth quadruple therapy (BQT) or concomitant non-BQT as possible alternatives[9-11]. Accordingly, a previous systematic review with pooled data analyses highlighted that, after a long-term follow-up period, lymphoma disappeared in more than 75% of low-grade, stage I or II_1 gastric lymphoma patients treated with bacterial eradication[12]. This study also identified that when the neoplastic lesion is confined to the submucosa, the main lesion is localized in the distal stomach, and t(11;18)(q21;q21) translocation is absent, the effectiveness of *H. pylori* eradication is even greater.

Given the low incidence of GML and the small sample sizes and heterogeneity of available studies [12], there is a need for an updated statistical analysis of the current evidence regarding *H. pylori* eradication as the sole initial therapy. Here, we performed a systematic literature review with meta-analysis to assess the complete histopathologic remission rate of *H. pylori*-positive early-stage GML after bacterial eradication therapy.

MATERIALS AND METHODS

This study followed the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guideline, which consists of a 27-item checklist and a 3-phase flowchart. The checklist includes items considered critical to the transparent reporting of a systematic review[13].

Literature search

The search strategy was designed following the Joanna Briggs Institute (JBI) Manual for Evidence Synthesis (https://synthesismanual.jbi.global). We performed independent, computer-assisted searches of the PubMed/MEDLINE, Embase, and Cochrane Central databases for studies published before September 2022. Medical Subject Headings and Embase Subject Headings (Emtree) index terms and free-text words were combined. Search terms included "Lymphoma, B-Cell, Marginal Zone," "Mucosa-Associated Lymphoid Tissue Lymphoma," "Marginal Zone B-Cell Lymphoma," "MALT lymphoma," "Stomach lymphoma," "Helicobacter pylori," "Therapeutics," and "Eradication therapy." Boolean operators (AND, OR) were also used to narrow or broaden the search as required. All citations were exported to the Rayyan (https://www.rayyan.ai/) tool and all duplicates were removed.

Study selection

Two researchers independently assessed the articles according to predefined eligibility criteria. In the case of disagreement, a 3rd researcher was consulted. The titles and abstracts of the articles were analyzed and studies that did not fit the inclusion criteria were excluded. The full texts were then revised to select eligible studies for meta-analysis.

Studies that met the following criteria were included: (1) Prospective and retrospective observational studies (cohort, case-control, and case series) evaluating the complete remission (CR) rate of early-stage GML after bacterial eradication therapy in *H. pylori*-positive patients; and (2) Studies including *H. pylori*-positive patients exclusively treated with antibiotic eradication therapy. Also, only trials enrolling patients with either stage I or II_1 GML according to Lugano classification were included[14].

Exclusion criteria were as follows: (1) Studies that did not report the CR rate of *H. pylori*-positive early-stage GML after bacterial eradication; (2) Studies investigating high-grade or diffuse large B cell lymphomas, except for those where it is possible to extrapolate data from a subgroup with early-stage GML; (3) Studies that included patients with non-gastric sites of MALT lymphoma or ineligible study subjects, such as animals or children; (4) Full-text article not available or article not available in English; (5) Case reports, reviews, meta-analyses, systematic reviews, editorials, conference abstracts; and (6) Studies with insufficient data regarding treatment outcome.

Risk of bias assessment

Two researchers independently assessed the risk of bias using the JBI checklists for cohort, case-control, and case series studies[15]. In cases of disagreement, a 3rd researcher was consulted. These tools include multiple questions to assess the methodological quality of a study and determine the extent to which a study has addressed the possibility of bias in its design, conduct, and analysis. The bias percentage risk was calculated by the number of "yes" (Y) answers selected in the checklist. Questions with "not applicable" (N/A) answers were not considered in the calculation. The risk of bias was classified using the following categories: High (scores up to 49.0%), moderate (scores between 50.0% and 70.0%), and low (scores above 70.0%).

Data extraction

Two investigators extracted data from the selected studies using a predefined data extraction worksheet. Any discrepancies were resolved by a 3rd reviewer. The primary outcome was the complete histopathologic remission of the lymphoma after bacterial eradication in *H. pylori*-positive early-stage GML patients. Data were extracted with respect to the following: (1) Included study-related information (1st author, year of publication, country of origin, study design, and study size); (2) Clinical characteristics of the study population (disease stage, diagnostic methods for *H. pylori* infection, and eradication schemes); (3) Number of *H. pylori*-positive early-stage GML patients treated only with bacterial eradication; (4) Number of patients in whom *H. pylori* was successfully eradicated (either provided or calculated); and (5) Number of patients who finally achieved complete remission of the lymphoma (either provided or calculated). The stage of the lymphoma was assessed using the Lugano classification system[13].

Statistical analysis

The pooled estimate of the complete histopathological remission rate and respective confidence intervals (95%CI) were calculated following the random-effects model. Forest plots were used to summarize the results. Heterogeneity and inconsistency were assessed using Cochran's Q test and I^2 statistic[16]; heterogeneity was defined as $P < 0.01$ and $I^2 > 50\%$, respectively. A subgroup analysis by study design (prospective; retrospective) was conducted to create more homogenous groups. Furthermore, a meta-regression analysis was conducted to explore potential sources of heterogeneity, such as publication year (≤ 2015; > 2015), geographic region of the study (Asian; Western), the prevalence of the translocation t(11;18)(q21; q21), and risk of bias (low; moderate; high). Analysis of publication bias was not performed as this measure is inappropriate for proportional meta-analysis (P-MA)[17]. All analyses were performed using R software version 4.2.1 (R: A Language and Environment for Statistical Computing, Vienna, Austria), using the 'Meta' package, version 5.2-0.

RESULTS

Literature search and study selection

Figure 1 depicts the flow of information through the different phases of the systematic review. Database searches identified 2375 reports, and duplicates were removed. The titles and abstracts of 1576 studies were screened and 96 articles were retrieved and selected for full-text reading. Finally, 61 studies were included in the meta-analysis. Reasons for exclusion were as follows: (1) 10 reports did not consider different stages in CR calculation; (2) 8 had insufficient data on *H. pylori* infection status; (3) 6 were conference abstracts; (4) 5 were publications of the same investigator or group; (5) 4 had insufficient data on the outcome; and (6) 2 included ineligible study subjects.

Study characteristics

Table 1 summarizes the characteristics of the studies included in the P-MA. The included reports were prospective and retrospective observational studies published between 1993 and 2021. A sample of 3315 patients with early-stage GML was obtained, of which 3003 were *H. pylori*-positive. A total of 2936 patients in whom *H. pylori* was successfully eradicated were included in the analysis. Twenty-nine of the included studies were conducted in Asian countries and 32 in Western countries. Concerning study design, 46 were prospective and 15 were retrospective uncontrolled, single-arm, observational studies. The median number of *H. pylori*-eradicated early-stage GML patients was 38 (ranging from 6-193). Multiple diagnostic tests for *H. pylori* infection and eradication were used, including histologic examination, *H. pylori* culture, rapid urease testing, 13C- or 14C-urea breath testing, serology, and *H. pylori* antigen stool testing. In most studies, at least 2 diagnostic tools were used to determine *H. pylori* infection status. Also in most studies eradication therapy consisted of a combination of 2 antibiotics, such as amoxicillin and clarithromycin, with a PPI. However, dual and quadruple therapies (2 antibiotics + PPI + bismuth or 3 antibiotics + PPI, respectively) were also used. Treatment duration ranged from 7 d to 21 d (Table 2).

Table 1 Characteristics of the included studies

Ref.	Country	Design	Study period	Study population				
				Early-stage gastric MALT lymphoma, *n*	Lugano stage	Median follow-up in mo	*H. pylori*-positive early-stage GML, *n*	Diagnosis of *H. pylori* infection
Yang *et al*[18], 2021	China	Retrospective	2003-2015	70	70	30	52	UBT; HE
Schmelz *et al*[19], 2019	Germany	Prospective	2001-2010	109	109	12	99	HE
Sugizaki *et al*[20], 2018	Japan	Prospective	2010-2016	97	97	37.4	97	HE; HpC; RUT; UBT; HpSA; S
Song *et al*[21], 2018	China	Prospective	2000-2013	122	122	38	47	RUT; HE; UBT
Li *et al*[22], 2016	China	Retrospective	2001-2013	75	75	62.9	69	HE; UBT
Kim *et al*[24], 2016	Korea	Retrospective	2001-2014	49	49	51	40	HE; UBT; RUT
Moleiro *et al*[23], *2016*	Korea	Retrospective	2005-2014	103	103	50.5	82	RUT; UBT; HE
Park *et al*[25], 2016	Portugal	Retrospective	1993-2013	103	103	105	87	HE; HpC; S; UBT
Grgov *et al*[26], 2015	Serbia	Prospective	2002-2012	20	20	NR	20	RUT; HE
Nonaka *et al*[27], 2014	Japan	Retrospective	2007-2012	16	16	NR	12	HE; S; UBT
Lima *et al*[28], 2014	Brazil	Prospective	2009-2010	8	8	24	7	RUT; HE; UBT
Wündisch *et al*[29], 2012	Germany	Prospective	1993-1999	120	120	122	120	HE
Choi *et al*[30], 2011	Korea	Retrospective	2003-2010	35	35	21.5	26	HE; RUT; UBT
Ono *et al*[31], 2011	Japan	Retrospective	2003-2009	21	21	1	13	RUT; UBT; HpC; HE; S
Andriani *et al*[32], 2009	Italy	Retrospective	1993-2006	60	60	65	60	HE
Sumida *et al*[33], 2009	Japan	Prospective	1997-2007	66	66	40	57	HE; S; UBT
Stathis *et al*[34], 2009	Switzerland	Retrospective	1990-2006	105	105	75.6	85	HE; S; UBT
Terai *et al*[35], 2008	Japan	Prospective	1995-2006	74	74	46	70	RUT; HE; S; UBT
Fischbach *et al*[36], 2007	Germany	Retrospective	NR	108	108	42.2	108	HE; UBT
Kim *et al*[37], 2007	Korea	Prospective	1996-2006	99	99	41	99	HE; RUT
El-Zahabi *et al*[38], 2007	Lebanon	Retrospective	1999-2005	22	22	12	19	HE; S
Hong *et al*[39], 2006	Korea	Prospective	1996-2003	90	90	45	90	HE; RUT; UBT
Wündisch *et al*[40], 2006	Germany	Retrospective	1993-2003	196	196	27	196	HE
Akamatsu *et al*[41], 2006	Japan	Prospective	1993-2006	55	55	37.3	38	HpC; HE
Wündisch *et al*[42], 2005	Germany	Prospective	NR	120	120	75	120	HE
Montalban *et al*[43], 2005	Spain	Prospective	1993-2002	24	24	64	24	HE; UBT
Chen *et al*[44], 2005	Taiwan	Prospective	1996-1999	34	34	70	31	HE; RUT; S

Taji *et al*[45], 2005	Japan	Prospective	1995-2001	13	13	32.5	12	HE; HpC; S; UBT; RUT
Takenaka *et al*[46], 2004	Japan	Prospective	1995-2002	33	33	5	33	HpC; RUT
Fischbach *et al*[47], 2004	Germany	Prospective	NR	90	90	44.6	80	RUT; HE; UBT
Sheu *et al*[48], 2003	Taiwan	Prospective	NR	15	15	NR	15	RUT; HE
Caletti *et al*[49], 2002	Italy	Prospective	1997-1999	51	51	24	51	HE; RUT; S
Nakamura *et al* [50], 2002	Japan	Prospective	1994-2001	21	21	14.5	17	HpC; S
Liu *et al*[51], 2002	France; Netherlands; Italy; Germany; England	Retrospective	NR	111	111	NR	111	HE; HpC
Bertoni *et al*[52], 2002	England; Italy; Switzerland	Prospective	NR	62	62	24	62	HE; S
Ohashi *et al*[53], 2002	Japan	Prospective	NR	13	13	NR	13	RUT; HE; HpC
Kim *et al*[54], 2002	Korea	Prospective	NR	20	20	18.3	20	RUT; HE
Matsushima *et al* [55], 2002	Japan	Prospective	1995-1997	14	14	27.5	14	RUT; HE; HpC; UBT
Kanda *et al*[56], 2001	Japan	Prospective	1994-1999	13	13	7	13	HE
Raderer *et al*[57], 2001	Austria	Retrospective	1997-1999	22	22	25	22	HE
Nakamura *et al* [58], 2001	Japan	Prospective	1994-1998	41	41	20.5	41	HpC; S; HE
Ruskoné-Fourmestraux *et al* [59], 2001	France	Prospective	1995-1998	44	44	35	34	HE; HpC; S; PCR
Thiede *et al*[60], 2001	Germany	Prospective	NR	97	97	20.8	97	NR
de Jong *et al*[61], 2001	Netherlands	Prospective	NR	23	23	37	23	HE; HpC
Urakami *et al*[62], 2000	Japan	Prospective	NR	47	47	20	47	RUT; HE; HpC
Papa *et al*[63], 2000	Italy	Prospective	1995-1999	7	7	48	7	HE; UBT
Yamashita *et al* [64], 2000	Japan	Prospective	NR	21	21	NR	21	HE; RUT; HpC
Ohashi *et al*[65], 2000	Japan	Prospective	NR	11	11	NR	11	RUT; HE; HpC
Nakamura *et al* [66], 2000	Japan	Prospective	1993-1998	30	30	NR	26	HpC
Savio *et al*[67], 2000	Italy	Prospective	1991-1997	76	76	NR	76	HE
Weston *et al*[68], 1999	United States	Prospective	NR	68	68	NR	65	HE
Steinbach *et al*[69], 1999	United States	Prospective	NR	34	34	NR	28	HE; RUT; S
Nobre-Leitão *et al* [70], 1998	Portugal	Prospective	NR	17	17	12	17	HE; HpC
Thiede *et al*[71], 1997	Germany	Prospective	NR	84	84	NR	84	NR
Sackmann *et al* [72], 1997	Germany	Prospective	NR	22	22	10	22	HE; HpC
Neubauer *et al*	Germany	Prospective	NR	50	50	24	50	HE

[73], 1997								
Pinotti *et al*[74], 1997	Italy; Switzerland	Prospective	1986-1995	86	86	23.3	45	HE; S
Savio *et al*[75], 1996	Italy; England	Prospective	1991-1993	13	13	NR	13	HE
Bayerdörffer *et al* [76], 1995	Germany	Prospective	NR	33	33	12.5	33	HE
Roggero *et al*[77], 1995	Switzerland; Italy	Prospective	NR	26	26	12	26	HE
Wotherspoon *et al* [78], 1993	England; Italy	Prospective	NR	6	6	NR	6	HE

CR: Complete remission rate; GML: Gastric mucosa-associated lymphoid tissue lymphoma; *H. pylori*: *Helicobacter pylori*; HE: Histologic examination; HpC: *Helicobacter pylori* culture; HpSA: *Helicobacter pylori* stool antigen; MALT: Mucosa-associated lymphoid tissue; NR: Not reported; PPI: Proton pump inhibitor; RUT: Rapid urease test; S: Serology; UBT: 13C- or 14C-urea breath test.

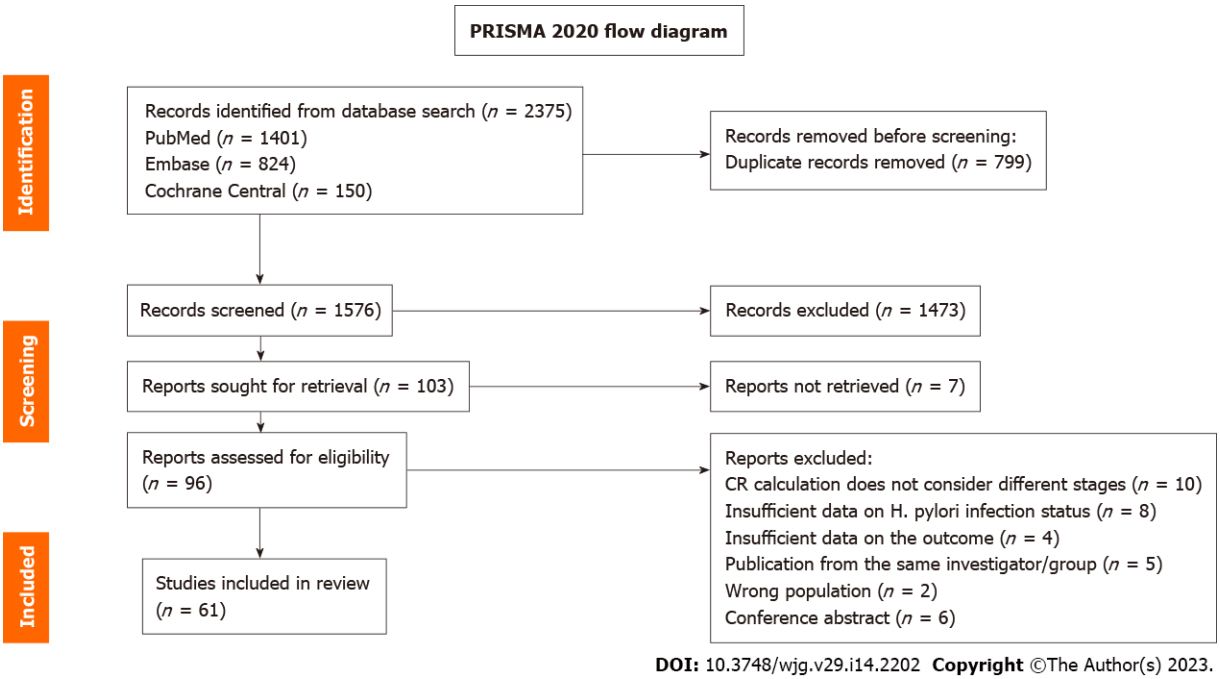

Figure 1 Preferred Reporting Items for Systematic Reviews and Meta-analyses 2020 flow diagram. The flow chart describes the flow of information through the different stages of the systematic review and maps the number of records identified, included, and excluded, and the reasons for study exclusion. *H. pylori*: *Helicobacter pylori*.

Risk of bias in studies

Risk of bias was assessed using JBI checklists (Figure 2). The included single-arm uncontrolled observational studies were classifiable and assessed as case series. The overall risk of bias was low to moderate in all but 1 study[36], with an average critical appraisal score across all studies of 79.02% (Figure 2A).

An increased risk of bias was due to "No" or "Unclear" answers to the following questions: (1) Was there clear reporting of the presenting site(s)/clinic(s) demographic information? (54/61 studies); (2) Did the case series have consecutive inclusion of participants (24/61 studies); (3) Did the case series have complete inclusion of participants? (22/61 studies); (4) Was there clear reporting of the demographics of the participants in the study? (6/61 studies); (5) Was statistical analysis appropriate? (6/61 studies); (6) Was the condition measured in a standard, reliable way for all participants included in the case series? (4/61 studies); (7) Was there clear reporting of clinical information of the participants? (4/61 studies); (8) Was there clear reporting of clinical information of the participants? (3/61 studies); (9) Were the outcomes or follow-up results of cases clearly reported? (8/61 studies); and (10) Were there clear criteria for inclusion in the case series? (2/61 studies). Figure 2B shows the discriminated assessments for each question across all studies.

Table 2 Characteristics of studies reporting the complete remission rate of *Helicobacter pylori*-positive early-stage gastric mucosa-associated lymphoid tissue lymphoma after bacterial eradication

Ref.	Region	*H. pylori*-positive early-stage gastric MALT lymphoma, *n*	*H. pylori*-eradicated gastric MALT lymphoma patients, *n*	CR, *n*	t(11;18)(q21;q21)-investigated gastric MALT lymphoma, *n*	t(11;18)(q21;q21)-positive gastric MALT lymphoma, *n*	Eradication regimen
Yang *et al*[18]	Asian	52	48	38	NR	NR	7-d-14-d triple therapy or 10-d quadruple therapy
Schmelz *et al*[19]	Western	99	99	66	69	7	7-d triple therapy
Sugizaki *et al*[20]	Asian	97	86	84	73	1	7-d triple therapy
Song *et al*[21]	Asian	47	47	35	NR	NR	14-d triple therapy
Li *et al*[22]	Asian	69	69	54	NR	NR	ND-day quadruple therapy
Kim *et al*[24]	Asian	40	35	35	NR	NR	7-d-14-d triple therapy or 7-d-14-d bismuth quadruple therapy
Moleiro *et al*[23]	Western	82	81	77	NR	NR	7-d triple therapy or 7-d bismuth quadruple therapy
Park *et al*[25]	Asian	87	81	73	NR	NR	7-d-14-d triple therapy
Grgov *et al*[26]	Western	20	20	17	NR	NR	10-d triple therapy
Nonaka *et al*[27]	Asian	12	12	9	NR	NR	7-d triple therapy
Lima *et al*[28]	Western	7	7	5	8	4	7-d triple therapy or 10-d triple therapy
Wündisch *et al*[29]	Western	120	120	96	66	10	14-d dual therapy or 10-d triple therapy
Choi *et al*[30]	Asian	26	26	22	NR	NR	ND-day triple therapy or ND-day bismuth quadruple therapy
Ono *et al*[31]	Asian	13	13	13	NR	NR	7-d-triple therapy
Andriani *et al*[32]	Western	60	53	42	NR	NR	7-d-14-d triple therapy or 10-d bismuth quadruple therapy
Sumida *et al*[33]	Asian	57	57	47	66	7	7-d triple therapy
Stathis *et al*[34]	Western	85	85	66	NR	NR	ND-day triple therapy
Terai *et al*[35]	Asian	70	70	56	22	0	7-d triple therapy
Fischbach *et al*[36]	Western	108	108	35	NR	NR	NR
Kim *et al*[37]	Asian	99	99	84	NR	NR	7-d triple therapy or 7-d bismuth quadruple therapy
El-Zahabi *et al*[38]	Asian	19	19	8	NR	NR	ND-day quadruple therapy
Hong *et al*[39]	Asian	90	90	85	NR	NR	14-d triple therapy or 14-d bismuth quadruple therapy
Wündisch *et al*[40]	Western	196	193	146	NR	NR	NR

Akamatsu *et al* [41]	Asian	38	38	29	8	6	7-d triple therapy or ND-day quadruple therapy
Wündisch *et al* [42]	Western	120	120	96	65	10	14-d dual therapy or 10-d triple therapy
Montalban *et al* [43]	Western	24	24	22	NR	NR	14-d triple therapy
Chen *et al*[44]	Asian	31	30	24	NR	NR	14-d triple therapy
Taji *et al*[45]	Asian	12	12	7	13	4	14-d triple therapy
Takenaka *et al* [46]	Asian	33	31	26	NR	NR	ND-day triple therapy
Fischbach *et al* [47]	Western	80	80	56	NR	NR	7-d triple therapy
Sheu *et al*[48]	Asian	15	15	11	NR	NR	14-d triple therapy
Caletti *et al*[49]	Western	51	45	25	NR	NR	7-d triple therapy
Nakamura *et al* [50]	Asian	17	17	2	23	7	14-d triple therapy
Liu *et al*[51]	Western	111	111	48	111	44	14-d dual therapy
Bertoni *et al*[52]	Western	62	62	46	NR	NR	7-d triple therapy; 14-d triple therapy or 14-d bismuth quadruple therapy
Ohashi *et al*[53]	Asian	13	13	11	NR	NR	14-d triple therapy
Kim *et al*[54]	Asian	20	20	18	NR	NR	7-d triple therapy or 7-d bismuth quadruple therapy
Matsushima *et al*[55]	Asian	14	14	10	NR	NR	ND-day triple therapy
Kanda *et al*[56]	Asian	13	12	9	NR	NR	ND-day dual therapy or ND-day triple therapy
Raderer *et al*[57]	Western	22	21	15	NR	NR	ND-day dual therapy or ND-day triple therapy
Nakamura *et al* [58]	Asian	41	41	29	NR	NR	ND-day triple or ND-day quadruple therapy
Ruskoné-Fourmestraux *et al*[59]	Western	34	34	19	NR	NR	14-d triple therapy
Thiede *et al*[60]	Western	97	97	77	NR	NR	14-d dual therapy or 7-d triple therapy
de Jong *et al*[61]	Western	23	23	13	NR	NR	ND-day dual therapy; ND-day triple therapy or ND-day quadruple therapy
Urakami *et al* [62]	Asian	47	44	42	NR	NR	7-d-14-d triple therapy
Papa *et al*[63]	Western	7	7	7	NR	NR	7-d triple therapy
Yamashita *et al* [64]	Asian	21	21	14	NR	NR	14-d triple therapy
Ohashi *et al*[65]	Asian	11	11	9	NR	NR	14-d triple therapy
Nakamura *et al* [66]	Asian	26	25	13	NR	NR	14-d dual therapy; 7-d triple therapy (14-d PPI); 14-d triple therapy
Savio *et al*[67]	Western	76	76	71	NR	NR	NR

Weston *et al*[68]	Western	65	58	38	NR	NR	ND-day triple or ND-day quadruple therapy
Steinbach *et al*[69]	Western	28	28	14	NR	NR	21-d bismuth quadruple therapy
Nobre-Leitão *et al*[70]	Western	17	17	17	NR	NR	14-d triple therapy
Thiede *et al*[71]	Western	84	79	68	NR	NR	NR-day-dual or 7-d-triple therapy
Sackmann *et al*[72]	Western	22	22	12	NR	NR	14-d-dual therapy
Neubauer *et al*[73]	Western	50	50	40	NR	NR	14-d-dual therapy or 7-d-triple therapy
Pinotti *et al*[74]	Western	45	44	30	NR	NR	14-d-triple or quadruple therapy
Savio *et al*[75]	Western	13	12	11	NR	NR	NR-day-triple or quadruple therapy
Bayerdörffer *et al*[76]	Western	33	33	23	NR	NR	14-d-dual therapy
Roggero *et al*[77]	Western	26	25	5	NR	NR	14-d-triple therapy
Wotherspoon *et al*[78]	Western	6	6	5	NR	NR	NR-day-dual or triple therapy

CR: Complete remission rate; *H. pylori*: *Helicobacter pylori*; MALT: Mucosa-associated lymphoid tissue; NR: Not reported; PPI: Proton pump inhibitor.

P-MA of the CR

The overall CR of *H. pylori*-positive early-stage GML after bacterial eradication was 75.18% (95%CI: 70.45%-79.91%). P-MA highlighted substantial heterogeneity in CR rate reported across studies (I^2 = 92%; P < 0.01) (Figure 3A).

Exploring heterogeneity - subgroup and meta-regression analysis

Considering the high heterogeneity across studies (I^2 = 92%; P < 0.01), a subgroup analysis by study design was conducted. The subgroup analysis revealed that retrospective and prospective studies presented similar overall CR rate after eradication therapy: 75.51% (95%CI: 64.96%-86.07%; I^2 = 96%; P < 0.01) and 75.08% (95%CI: 69.80-80.36; I^2 = 89%; P < 0.01), respectively (Figure 3B). The meta-regression analysis indicated that the proportion of patients with t(11;18)(q21;q21)-positive GML and study risk of bias were sources of heterogeneity. More precisely, studies with greater than 30% of patients with t(11;18)(q21;q21)-positive GML and high risk of bias showed the pooled estimate of the CR rate decreased to 0.40 (95%CI: -0.59 to -0.22; P < 0.0001) and 0.43 (95%CI: -0.77 to -0.09; P = 0.0139), respectively. There was no significant difference in outcomes with respect to geographic region (Table 3).

DISCUSSION

GML is rare and typically comprises a low-grade neoplasm[18]. *H. pylori* infection is predominant pathogenic mechanism underlying the development of GML[19], and international guidelines strongly recommend *H. pylori* eradication therapy for all patients irrespective of stage. In localized *H. pylori*-positive GML, bacterial eradication is the preferred initial treatment[79,80].

This study aimed to provide an up-to-date, comprehensive synthesis of evidence regarding *H. pylori* eradication as the sole initial therapy for early-stage GML. We identified prospective and retrospective uncontrolled, single-arm observational studies with a total of 3315 patients with early-stage GML, of which 3003 were *H. pylori*-positive. A total of 2936 patients in whom *H. pylori* was successfully eradicated were included in the analysis. The unavailability of robust comparative studies (*e.g.*, prospective cohort studies) precluded pairwise meta-analysis (PW-MA); instead, a P-MA was conducted. In contrast to comparative PW-MA, which calculates a pooled estimate of effect over 2 groups, P-MA enables the calculation of a grouped overall proportion[81,82]. Though single-group analysis may not produce measures of relative association, it can be useful for estimating the impact of a treatment on a given condition in the absence of higher-quality evidence. This represents an alternative for informed decision making, especially in our field where robust comparative studies are scarce.

Table 3 Meta-regression according to selected covariates

Subgroup	Studies, *n*	Estimate	95%CI	*P* value	I^2, %
Year					
≤ 2015	54	-	-	-	92.5
> 2015	7	0.11	-0.03 to 0.25	0.1188	
Region					92.8
Asian	29	-	-	-	
Western	32	-0.06	-0.15 to 0.03	0.2145	
Proportion of patients with t(11;18)(q21;q21)-positive gastric MALT lymphoma					
≤ 30%	7				
> 30%	4	-0.40	-0.59 to -0.22	< 0.0001	88.6
Risk of bias					92.3
Low	39	-	-	-	
Moderate	21	0.02	-0.07 to 0.12	0.6190	
High	1	-0.43	-0.77 to -0.09	0.0139	

MALT: Mucosa-associated lymphoid tissue.

P-MA highlighted that the overall CR rate of *H. pylori*-positive early-stage GML after bacterial eradication was 75.18% (95%CI: 70.45%-79.91%), suggesting that *H. pylori* eradication as the sole initial therapy for early-stage GML is effective. These results are similar to those found in a pooled data analysis published in 2010 by Zullo *et al*[12] [77.5% (95%CI: 75.3%-79.7%)]. On the other hand, the substantial heterogeneity observed across studies (I^2 = 92%; P < 0.01) limits, though does not preclude, the interpretation of the pooled overall CR rate. Subgroup analysis revealed that retrospective and prospective studies estimated similar overall CR rates after eradication therapy [75.51% (95%CI: 64.96%-86.07%; I^2 = 96%; P < 0.01) and 75.08% (95%CI: 69.80%-80.36%; I^2 = 89%; P < 0.01), respectively]. Nevertheless, meta-regression analysis indicated that the proportion of patients with t(11;18)(q21;q21)-positive GML and the studies' risk of bias were sources of heterogeneity. More precisely, studies with greater than 30% of patients with t(11;18)(q21;q21)-positive GML or high risk of bias decrease in 0.40 (95%CI: -0.59 to -0.22; P < 0.0001) and 0.43 (95%CI: -0.77 to -0.09; P = 0.0139) the pooled estimate of the CR rate, respectively. In this sense, we reiterate the results of Zullo *et al*[12] which highlight the presence of the t(11;18)(q21; q21) translocation as a predictor of lymphoma remission after bacterial eradication. In contrast to the previous pooled analysis[12], our study did not observe significant differences in lymphoma remission between Western and Asian countries.

Hence, our results reaffirm that *H. pylori* eradication should be given as the first-line treatment for localized low-grade GML[8]. The anti-*H. pylori* regimen should be chosen based on regional microbial susceptibility; in many regions, BQT or high-dose PPI clarithromycin-containing triple therapy may be recommended as first-line empirical treatment[83]. In case of eradication failure, second-line treatment should be attempted following the currently recommended algorithm for empirical *H. pylori* eradication or as guided by individual antibiotic susceptibility testing. For patients with GML refractory to *H. pylori* eradication, irradiation and systemic oncological therapies should be used, depending on the stage of the disease. Radiotherapy (RT) is the first-line choice for the treatment of localized GML. Chemotherapy, immunotherapy, or combination chemoimmunotherapy are mainly considered if RT is not feasible or otherwise not indicated[84,85].

To our knowledge, our study is the first systematic review with meta-analysis to assess the CR rate of *H. pylori*-positive early-stage GML after *H. pylori* eradication. Our work has strengths in its design and execution, such as the use of random-effects meta-analysis to address heterogeneity between included studies, subgroup analyses by study design, and meta-regression to explore possible sources of heterogeneity. Nonetheless, the present analysis has several limitations inherent to the included studies and study design. Due to the unavailability of language resources (*e.g.*, professional translators), we could not include studies in languages other than English. Although limiting study inclusion based on the language of publication is a common practice in systematic reviews, it introduces the risk of ignoring key data, referred to as language bias, which may limit the interpretation of our findings[86].

Moreover, discriminated assessments for each JBI Critical Appraisal Tool question across all reports showed that the included series had serious gaps in clinical and demographic information reporting. Thus, exploring possible sources of heterogeneity and identifying predictors of lymphoma remission was difficult. Furthermore, incomplete and non-consecutive inclusion of patients in several studies

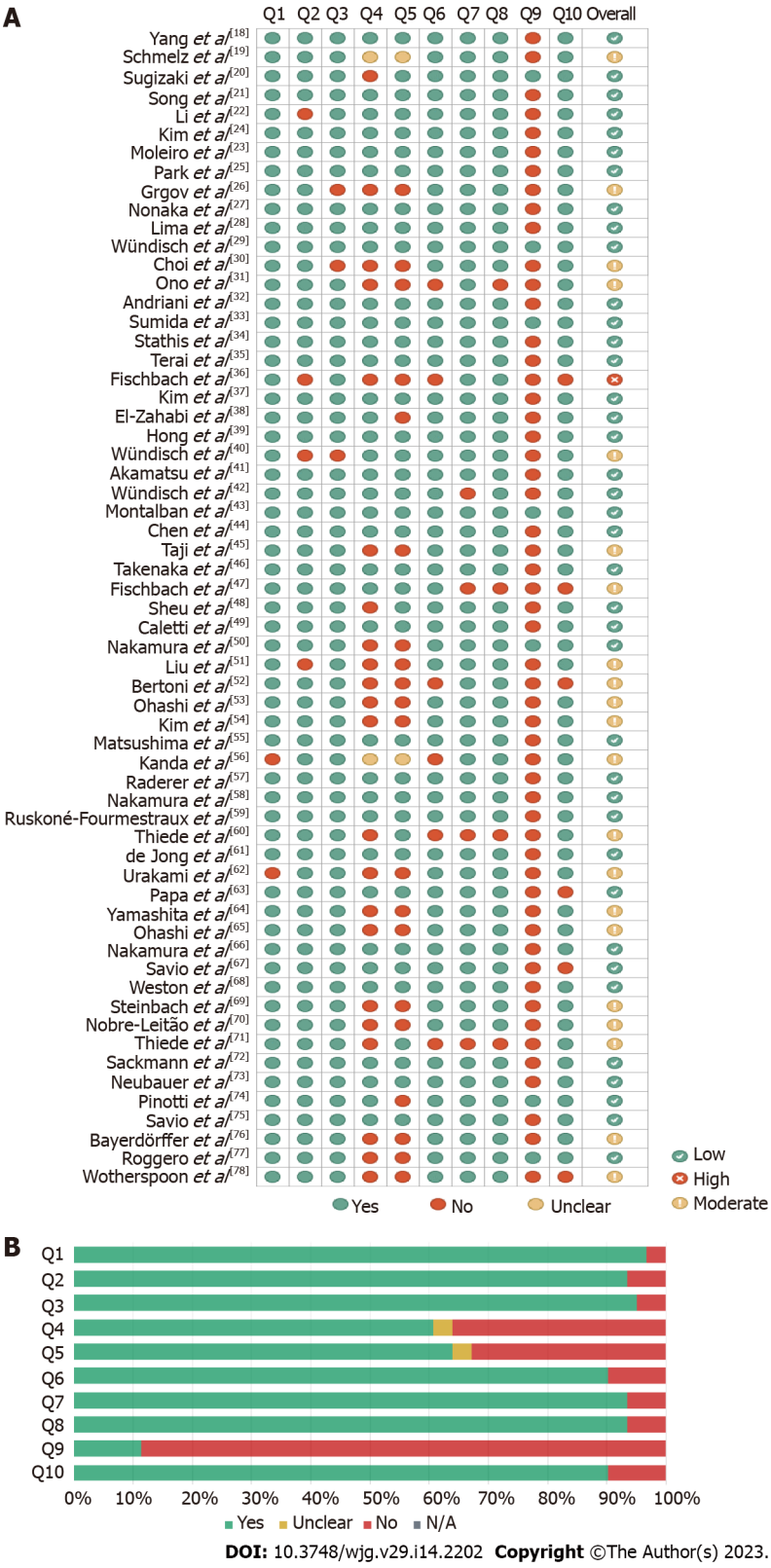

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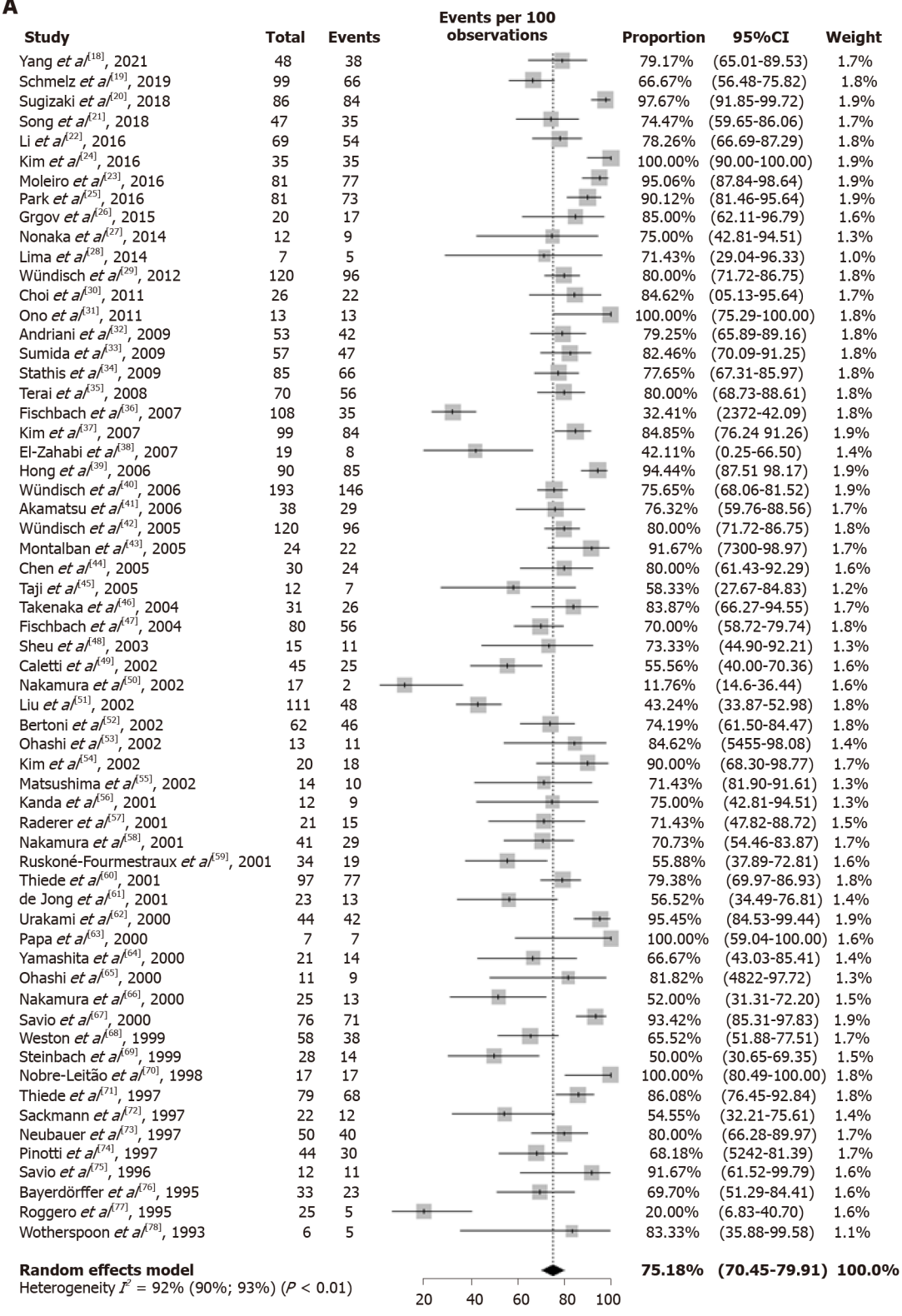
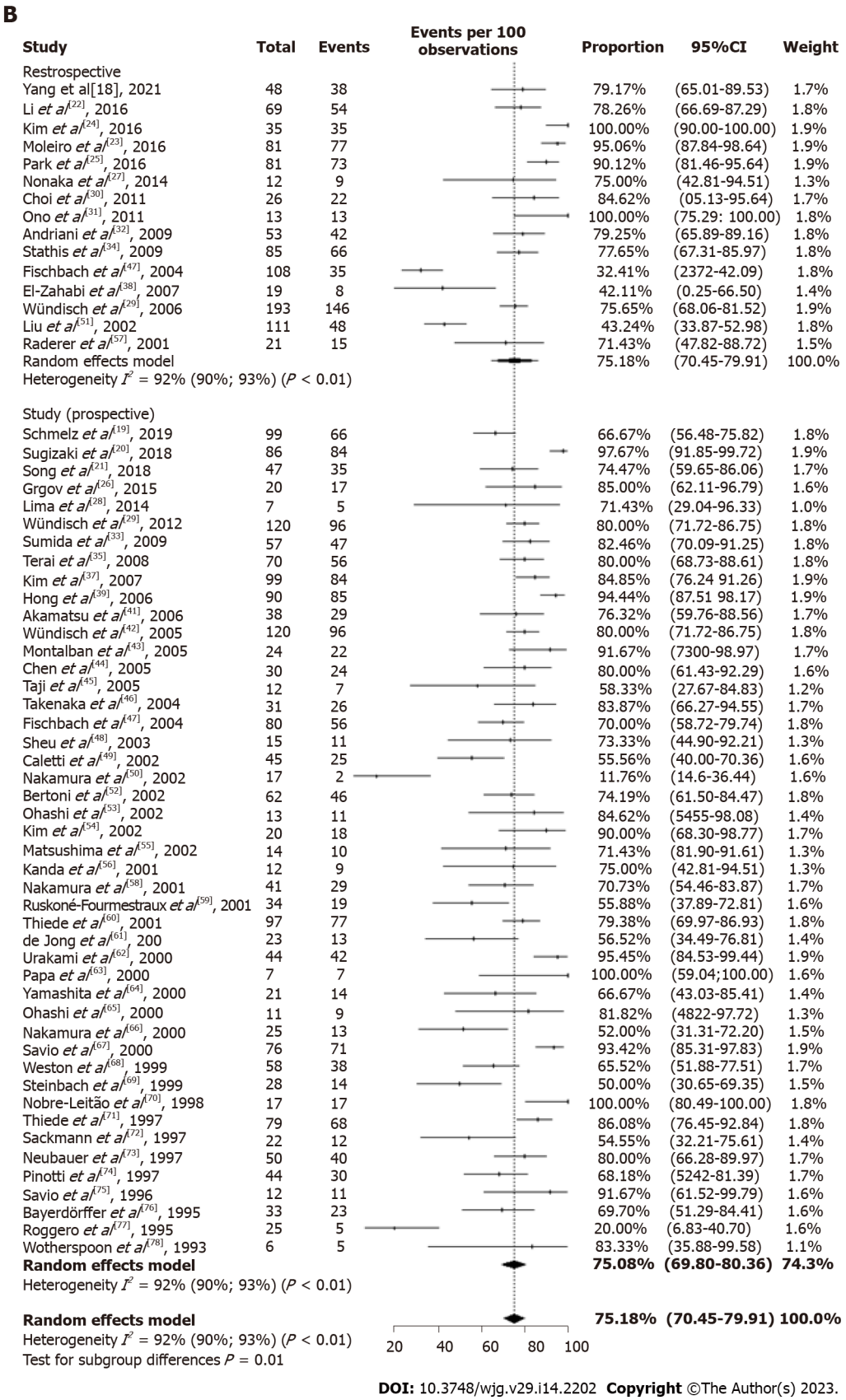
Figure 2 Risk of bias assessment by the Joanna Briggs Institute Critical Appraisal Tool. The critical appraisal checklist for case series consists of 10 questions: Q1: Were there clear criteria for inclusion in the case series? Q2: Was the condition measured in a standard, reliable way for all participants included in the case series? Q3: Were valid methods used for identification of the condition for all participants included in the case series? Q4: Did the case series have consecutive inclusion of participants? Q5: Did the case series have complete inclusion of participants? Q6: Was there clear reporting of the demographics of the participants in the study? Q7: Was there clear reporting of clinical information of the participants? Q8: Were the outcomes or follow-up results of cases clearly reported? Q9: Was there clear reporting of the presenting site(s)/clinic(s) demographic information? Q10: Was statistical analysis appropriate? The percentage of risk of bias was calculated by the number of "yes" answers selected in the checklist. Questions with "not applicable" answers were not considered in the calculation. The risk of bias was classified using the following categories: High (scores up to 49.0%), moderate (scores between 50.0% and 70.0%), and low (scores above 70.0%). A: Overall risk of bias; B: Risk of bias summary: Discriminated assessments for each question across all studies. N/A: Not applicable.

A


Study
Total
Events
Events per 100 observations
Proportion
95%CI
Weight
Yang et al[18], 2021 48 38 79.17% (65.01-89.53) 1.7%
Schmelz et al[19], 2019 99 66 66.67% (56.48-75.82) 1.8%
Sugizaki et al[20], 2018 86 84 97.67% (91.85-99.72) 1.9%
Song et al[21], 2018 47 35 74.47% (59.65-86.06) 1.7%
Li et al[22], 2016 69 54 78.26% (66.69-87.29) 1.8%
Kim et al[24], 2016 35 35 100.00% (90.00-100.00) 1.9%
Moleiro et al[23], 2016 81 77 95.06% (87.84-98.64) 1.9%
Park et al[25], 2016 81 73 90.12% (81.46-95.64) 1.9%
Grgov et al[26], 2015 20 17 85.00% (62.11-96.79) 1.6%
Nonaka et al[27], 2014 12 9 75.00% (42.81-94.51) 1.3%
Lima et al[28], 2014 7 5 71.43% (29.04-96.33) 1.0%
Wündisch et al[29], 2012 120 96 80.00% (71.72-86.75) 1.8%
Choi et al[30], 2011 26 22 84.62% (05.13-95.64) 1.7%
Ono et al[31], 2011 13 13 100.00% (75.29-100.00) 1.8%
Andriani et al[32], 2009 53 42 79.25% (65.89-89.16) 1.8%
Sumida et al[33], 2009 57 47 82.46% (70.09-91.25) 1.8%
Stathis et al[34], 2009 85 66 77.65% (67.31-85.97) 1.8%
Terai et al[35], 2008 70 56 80.00% (68.73-88.61) 1.8%
Fischbach et al[36], 2007 108 35 32.41% (2372-42.09) 1.8%
Kim et al[37], 2007 99 84 84.85% (76.24 91.26) 1.9%
El-Zahabi et al[38], 2007 19 8 42.11% (0.25-66.50) 1.4%
Hong et al[39], 2006 90 85 94.44% (87.51 98.17) 1.9%
Wündisch et al[40], 2006 193 146 75.65% (68.06-81.52) 1.9%
Akamatsu et al[41], 2006 38 29 76.32% (59.76-88.56) 1.7%
Wündisch et al[42], 2005 120 96 80.00% (71.72-86.75) 1.8%
Montalban et al[43], 2005 24 22 91.67% (7300-98.97) 1.7%
Chen et al[44], 2005 30 24 80.00% (61.43-92.29) 1.6%
Taji et al[45], 2005 12 7 58.33% (27.67-84.83) 1.2%
Takenaka et al[46], 2004 31 26 83.87% (66.27-94.55) 1.7%
Fischbach et al[47], 2004 80 56 70.00% (58.72-79.74) 1.8%
Sheu et al[48], 2003 15 11 73.33% (44.90-92.21) 1.3%
Caletti et al[49], 2002 45 25 55.56% (40.00-70.36) 1.6%
Nakamura et al[50], 2002 17 2 11.76% (14.6-36.44) 1.6%
Liu et al[51], 2002 111 48 43.24% (33.87-52.98) 1.8%
Bertoni et al[52], 2002 62 46 74.19% (61.50-84.47) 1.8%
Ohashi et al[53], 2002 13 11 84.62% (5455-98.08) 1.4%
Kim et al[54], 2002 20 18 90.00% (68.30-98.77) 1.7%
Matsushima et al[55], 2002 14 10 71.43% (81.90-91.61) 1.3%
Kanda et al[56], 2001 12 9 75.00% (42.81-94.51) 1.3%
Raderer et al[57], 2001 21 15 71.43% (47.82-88.72) 1.5%
Nakamura et al[58], 2001 41 29 70.73% (54.46-83.87) 1.7%
Ruskoné-Fourmestraux et al[59], 2001 34 19 55.88% (37.89-72.81) 1.6%
Thiede et al[60], 2001 97 77 79.38% (69.97-86.93) 1.8%
de Jong et al[61], 2001 23 13 56.52% (34.49-76.81) 1.4%
Urakami et al[62], 2000 44 42 95.45% (84.53-99.44) 1.9%
Papa et al[63], 2000 7 7 100.00% (59.04-100.00) 1.6%
Yamashita et al[64], 2000 21 14 66.67% (43.03-85.41) 1.4%
Ohashi et al[65], 2000 11 9 81.82% (4822-97.72) 1.3%
Nakamura et al[66], 2000 25 13 52.00% (31.31-72.20) 1.5%
Savio et al[67], 2000 76 71 93.42% (85.31-97.83) 1.9%
Weston et al[68], 1999 58 38 65.52% (51.88-77.51) 1.7%
Steinbach et al[69], 1999 28 14 50.00% (30.65-69.35) 1.5%
Nobre-Leitão et al[70], 1998 17 17 100.00% (80.49-100.00) 1.8%
Thiede et al[71], 1997 79 68 86.08% (76.45-92.84) 1.8%
Sackmann et al[72], 1997 22 12 54.55% (32.21-75.61) 1.4%
Neubauer et al[73], 1997 50 40 80.00% (66.28-89.97) 1.7%
Pinotti et al[74], 1997 44 30 68.18% (5242-81.39) 1.7%
Savio et al[75], 1996 12 11 91.67% (61.52-99.79) 1.6%
Bayerdörffer et al[76], 1995 33 23 69.70% (51.29-84.41) 1.6%
Roggero et al[77], 1995 25 5 20.00% (6.83-40.70) 1.6%
Wotherspoon et al[78], 1993 6 5 83.33% (35.88-99.58) 1.1%
Random effects model 75.18% (70.45-79.91) 100.0%
Heterogeneity I^2 = 92% (90%; 93%) (P < 0.01)
20
40
60
80
100



DOI: 10.3748/wjg.v29.i14.2202

Figure 3 Overall complete remission rate of *Helicobacter pylori*-positive early-stage gastric mucosa-associated lymphoid tissue lymphoma. A: After eradication therapy; B: After eradication therapy by study design. CI: Confidence interval.

compromises the reliability of their results and increases the risk of bias. Another limitation was the failure to report the confirmation method for *H. pylori* eradication, which could be a covariate explaining the heterogeneity between studies. Inadequate reporting was an important reason for the exclusion of

studies during screening and a complicating factor for data extraction. Observational studies evaluating the CR of GML after bacterial eradication should stratify the observed outcome according to *H. pylori* infection status. Furthermore, it is necessary to discriminate the lymphoma stage in *H. pylori*-positive patients undergoing treatment. In fields in which reliable and robust studies are scarce, proper reporting of the available evidence is vital to inform clinical practice. Therefore, this meta-analysis should be interpreted in the context of these limitations.

CONCLUSION

This comprehensive evidence synthesis suggests the effectiveness of *H. pylori* eradication as the sole initial therapy for early-stage GML. Although the substantial heterogeneity observed across studies limits the interpretation of the pooled overall CR rate, our study is a relevant alternative for informing clinical practice. Further robust comparative observational studies are needed to identify predictive factors for GML remission following *H. pylori* eradication and to provide more reliable evidence in our field.

ARTICLE HIGHLIGHTS

Research background

Gastric mucosa-associated lymphoid tissue (MALT) lymphoma (GML) is usually a low-grade, B-cell neoplasia strongly associated with *Helicobacter pylori* (*H. pylori*)-induced chronic gastritis. As such, clinical practice guidelines currently recommend *H. pylori* eradication as the preferred initial treatment for early-stage GML.

Research motivation

Studies that aim to evaluate the effects of *H. pylori* eradication on early-stage GML are generally small and heterogenous single-arm uncontrolled observational studies. Hence, we recognized the need for an updated powerful statistical synthesis of the available evidence regarding the practical effect of *H. pylori* eradication as sole initial therapy for early-stage GML.

Research objectives

We aimed to perform a systematic review with an up-to-date proportional meta-analysis (P-MA) to assess the complete remission (CR) rate of *H. pylori*-positive early-stage GML after bacterial eradication therapy.

Research methods

We performed independent computer-assisted searches of PubMed/MEDLINE, Embase and Cochrane Central databases culling reports published before September 2022. Prospective and retrospective observational studies evaluating the CR rate of early-stage GML after bacterial eradication therapy in *H. pylori*-positive patients were eligible for inclusion. The risk of bias was assessed using the JBI Critical Appraisal Tools. We followed the random-effects model to calculate the pooled estimate of the complete histopathological remission rate and respective confidence intervals (95%CI). We used Cochran's Q test and I^2 statistic to assess the heterogeneity and inconsistency, and we set the threshold for heterogeneity as $P < 0.01$ and $I^2 > 50\%$, respectively. Subgroup and meta-regression analyses were conducted to explore potential sources of heterogeneity.

Research results

P-MA highlighted that the overall CR of *H. pylori*-positive early-stage GML after bacterial eradication was 75.18% (95%CI: 70.45%-79.91%). On the other hand, the substantial heterogeneity observed across studies ($I^2 = 92\%$; $P < 0.01$) limits, but does not preclude, the interpretation of the pooled overall CR rate. Subgroup analysis revealed that retrospective and prospective studies presented similar overall CR rate estimates after eradication therapy: 75.51% (95%CI: 64.96%-86.07%; $I^2 = 96\%$; $P < 0.01$) and 75.08% (95%CI: 69.80%-80.36%; $I^2 = 89\%$; $P < 0.01$), respectively. Nevertheless, meta-regression analysis indicated that the proportion of patients with t(11;18)(q21;q21)-positive GML and the studies' risk of bias were sources of heterogeneity. More precisely, studies with greater than 30% of patients with t(11;18)(q21;q21)-positive GML and high risk of bias decrease in 0.40 (95%CI: -0.59 to -0.22; $P < 0.0001$) and 0.43 (95%CI: -0.77 to -0.09; $P = 0.0139$) the pooled estimate of the CR rate, respectively.

Research conclusions

Comprehensive evidence synthesis suggests the effectiveness of *H. pylori* eradication as the sole initial therapy for early-stage GML. Although the substantial heterogeneity observed across studies limits the

interpretation of the pooled overall CR rate, the present study is a relevant alternative for informing clinical practice.

Research perspectives

Inadequate reporting was an important reason for the exclusion of studies during screening and a complicating factor for data extraction. As reliable and robust studies are scarce in our field, we emphasize that proper reporting of the available evidence is vital to inform clinical practice. Further robust comparative observational studies are needed to identify predictive factors for GML remission following *H. pylori* eradication and to provide more reliable evidence in our field.

FOOTNOTES

Author contributions: Lemos FFB, Castro CT, Teixeira KN, Souza CL, Oliveira MV, and Freire de Melo F contributed to the conceptualization of the manuscript; Lemos FFB, Castro CT, Teixeira KN, Souza CL, Oliveira MV, and Freire de Melo F designed the study methodology; Lemos FFB, Castro CT, Calmon MS, Silva Luz M, Pinheiro SLR, Faria Souza Mendes dos Santos C, Santos GLC, Marques HS and Delgado HA were responsible for manuscript visualization; Lemos FFB, Calmon MS, Silva Luz M, Pinheiro SLR, Faria Souza Mendes dos Santos C, Santos GLC, Marques HS, Delgado HA, Teixeira KN, Souza CL, Oliveira MV, and Freire de Melo F contributed to the investigation; Lemos FFB, Calmon MS, Silva Luz M, Pinheiro SLR, Faria Souza Mendes dos Santos C, Santos GLC, Marques HS, Delgado HA performed formal analysis; Lemos FFB wrote the original draft; Castro CT and Silva Luz M were responsible for manuscript editing; Castro CT, Silva Luz M, Teixeira KN, Souza CL, and Oliveira MV were responsible for manuscript writing and review; and Freire de Melo F supervised the writing of the original draft.

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P-Editor: Wang JJ

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